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RESEARCH ARTICLE

# Integrated *In Silico* and *In Vivo* Analysis of Vitamin $D_3$ Supplementation in Obesity and Diabetes

**Alfreds Roosevelt[1,2], Rina Agustina[3], Filmaharani Filmaharani[1], Anggun Nurhidayah[1], Muhammad Rayza Azmin[1], Jumriani Jumriani[1], Ratnawati Ratnawati[1], Muh Arfandy Gunawan[1], Nadila Pratiwi Latada[4], Mukarram Mudjahid[3,4], Widya Hardiyanti[4,5], Asbah Asbah[4] and Firzan Nainu[3,4,*]**

*[1]Postgraduate Program in Pharmacy, Faculty of Pharmacy, Hasanuddin University, Makassar 90245, Indonesia*
*[2]Departement of Pharmacy, Sandi Karsa Polytechnic, Sulawesi Selatan 90245, Indonesia*
*[3]Department of Pharmacy, Faculty of Pharmacy, Hasanuddin University, Makassar 90245, Indonesia*
*[4]Unhas Fly Research Group, Faculty of Pharmacy, Hasanuddin University, Makassar 90245, Indonesia*
*[5]Study Program of Pharmacy, Faculty of Medicine and Health Sciences, Universitas Muhammadiyah Makassar, Makassar 90221, Indonesia*

**([*]Corresponding author's e-mail: firzannainu@unhas.ac.id)**

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## Abstract

Obesity and diabetes are interconnected metabolic disorders with a rising prevalence worldwide. Although vitamin $D_3$ is known to regulate glucose metabolism and oxidative stress, its ability to inhibit digestive enzymes linked to adipogenesis has not been demonstrated. This study proposed that vitamin $D_3$ possesses dual anti-obesity and antidiabetic activities by targeting pancreatic lipase, α-amylase, and α-glucosidase, and confirmed this hypothesis through in silico docking and *in vivo* experiments using *Drosophila melanogaster*. Docking simulations using AutoDock Vina showed that vitamin $D_3$ had a stronger affinity for pancreatic lipase (−7.5 kcal/mol) than orlistat (−6.8 kcal/mol), with stable hydrophobic interactions at the catalytic sites. The *D. melanogaster* $w^{1118}$ strain was reared on a high-fat diet (2% virgin coconut oil) supplemented with vitamin $D_3$ (10 or 100 mM). The experimental groups (n = 20 flies per group, 5 replicates) were assessed for hemolymph glucose, locomotor activity, oxidative stress (NBT assay), and gene expression (*trbl* and *sod1*). Data were analyzed using 1-way ANOVA with Tukey's post-hoc test ($p < 0.05$). Vitamin $D_3$ supplementation significantly reduced hemolymph glucose and ROS levels, improved crawling performance, and restored *trbl* and *sod1* expression in HFD-fed flies. These effects were consistent with docking predictions, indicating that enzyme inhibition may underlie the observed metabolic benefits. In conclusion, vitamin $D_3$ shows promising antiobesity and antidiabetic effects in a *D. melanogaster* model, supporting its role as a potential modulatory supplement. However, limitations such as the use of an invertebrate model, high compound concentrations, and the absence of mammalian or clinical validation highlight the need for further studies in higher organisms.

**Keywords:** Pancreatic lipase, α-amylase, α-glucosidase, *trbl*, *sod1*, *Drosophila melanogaster*

## Introduction

Obesity is characterized by abnormal or excessive fat accumulation and is commonly defined by a Body Mass Index (BMI) exceeding 30.0 kg/m$^2$ [1, 2]. According to the World Health Organization (WHO), approximately 150 million adults aged ≥ 18 years are affected by obesity in 2023 [3, 4]. In Indonesia, data from the National Basic Health Survey (RISKESDAS) showed a progressive rise in the prevalence of overweight individuals from 10.5% in 2007 to 14.8% in 2013 and 21.8% in 2018 [5]. The 2023 Indonesian Health Survey further indicated that the prevalence of obesity has increased to 23.4% [6]. Obesity is a major risk factor for chronic diseases, particularly diabetes mellitus, which is characterized by persistent hyperglycemia. The coexistence of obesity and diabetes (obesity-diabetes comorbidity) is associated with insulin resistance, chronic low-grade inflammation and excessive visceral fat accumulation [7]. Evidence indicates that obese individuals have an eightfold higher risk of developing diabetes than that of individuals with normal body weight. Consequently, the regulation of cholesterol and blood glucose levels is critical for preventing associated complications. Several molecular targets have been implicated in the management of obesity and diabetes comorbidity, including human pancreatic lipase (1LPB), human pancreatic α-amylase (2QV4), human pancreatic α-glucosidase (3L4Y), and FTO protein (3LFM) [8, 9].

Currently, several pharmacological agents are employed in clinical practice, such as pancreatic lipase inhibitors (e.g., orlistat) to reduce lipid absorption, and α-amylase and α-glucosidase inhibitors (e.g., acarbose) to delay carbohydrate digestion. These approaches effectively attenuate postprandial hyperglycemia and hyperlipidemia [10, 11]. However, their long-term administration is often limited by adverse effects, including steatorrhea, cholelithiasis, hepatic dysfunction, congestive heart failure, and osteoporosis, as well as high treatment costs [12, 13]. Despite these advances, there remains an urgent need for therapeutic strategies that can simultaneously address lipid dysregulation, insulin resistance, and oxidative stress while maintaining safety and tolerability, particularly in resource-limited settings. This unmet need underscores the importance of exploring multifunctional bioactive candidates that are safer and more affordable [14].

Vitamin $D_3$ has emerged as a promising candidate. Several studies have demonstrated that vitamin $D_3$ supplementation reduces plasma HbA1c levels and enhances pancreatic β-cell function by regulating energy homeostasis and suppressing inflammation [15, 16]. Vitamin $D_3$ also attenuates reactive oxygen species (ROS) production in adipocytes by regulating NADPH oxidase expression [17]. The vitamin D receptor (VDR), expressed in key metabolic tissues, including adipose tissue, the liver, and the pancreas, plays a pivotal role in regulating genes involved in lipid storage and insulin sensitivity. This provides a strong biological rationale for vitamin $D_3$ as a potential modulator of glucose metabolism, adipogenesis, and lipolytic activity. In obesity-diabetes comorbidity, increased ROS production activates Nuclear Factor κB (NF-κB), altering the expression of genes involved in antioxidant defense and metabolic regulation, ultimately driving pro-inflammatory cytokine production [18, 19]. Genes such as *trbl* and *sod1*, which contribute to lipid and protein metabolism, have been identified as key regulators in this context, although their investigation remains challenging owing to methodological complexities [20-22].

Animal models are essential for elucidating these mechanisms; however, mammalian models often face limitations in terms of cost, duration, and ethical concerns. Consequently, alternative approaches, such as *in silico* studies and simple model organisms, are increasingly being employed. Molecular docking provides insights into the binding affinity of bioactive candidates with metabolic targets, while *Drosophila melanogaster* serves as a cost-effective and genetically tractable *in vivo* model. The selection of pancreatic lipase, α-amylase, α-glucosidase, and FTO protein as docking targets is justified by their well-documented involvement in gastrointestinal lipid-glucose metabolism and epigenetic regulation of adiposity, rendering them relevant for assessing the multitarget effects of vitamin $D_3$ [23, 24]. *D. melanogaster* has long been utilized in biomedical research because of its high degree of genetic conservation, with over 75% of human disease-related genes having orthologs in the fly genome [25, 26]. Its energy metabolism system, including insulin-like peptides (*Dilps*), the midgut, and the fat body, shares functional similarities with the pancreas, liver, and adipose tissues of mammals [27, 28]. Therefore, *D. melanogaster* provides a powerful and efficient platform for investigating metabolic phenotypes in obesity-diabetes comorbidity while minimizing ethical and financial constraints. Previous studies have successfully employed *D. melanogaster* to model either diabetes or obesity, and these models have been used to screen potential therapeutic agents [29-34]. A combined *D. melanogaster* model of obesity and diabetes comorbidity has also been reported [35], providing a relevant platform for evaluating potential therapeutic agents. Building on this framework, the present study examined whether vitamin $D_3$ exerts anti-obesity and antidiabetic effects in this comorbidity model and whether these effects correspond to its predicted binding affinity to key metabolic targets, as assessed through molecular docking. The findings are anticipated to provide mechanistic insights and preclinical evidence supporting the potential of vitamin $D_3$ as an adjunctive supplement for managing obesity–diabetes comorbidity.

## Materials and methods

### Materials

Vitamin $D_3$ (CAS No.: 67-97-0) was sourced from Wellgreen Technologies Co., Ltd. Tween 80 (CAS No.: 9005-65-6) was supplied by Merck, and PEG 40 (CAS No.: 61788-85-0) was obtained from Hefei TNJ Chemical Industry Co. Ltd.

### *Drosophila* stocks

In this study, *the D. melanogaster* $w^{1118}$ strain, obtained from the Host Defense and Responses Laboratory, Kanazawa University (Japan), was used as an *in vivo* model. Flies were reared on standard cornmeal medium under controlled conditions (25 °C, 60% RH, 12 h light/dark cycle) to maintain physiological consistency and ensure experimental reproducibility.

### Preparation of vitamin $D_3$ solution

Tween 80 (1%) and PEG 40 (1%) were used as solvents to prepare the vitamin $D_3$ solution. Subsequently, a 1:1 solvent ratio was used to dilute vitamin $D_3$ to concentrations of 10 and 100 mM [36].

### Molecular docking

Molecular docking simulations were performed on a Windows 11 (64-bit) system using an Asus TUF A15 workstation (Intel Core i7-12700H @ 3.20 GHz, 16 GB DDR5 RAM, Nvidia GeForce RTX 3050 GPU). Ligand-protein docking was conducted using UCSF Chimera coupled with AutoDock Vina, employing pre-processed structures of cholecalciferol and selected target enzymes: Human Pancreatic Lipase (PDB: 1LPB), α-amylase (PDB: 2QV4), and α-glucosidase (PDB: 3L4Y). The grid parameters were confined to the active sites to ensure specificity in the binding predictions. Docking outputs, including binding affinities and interaction profiles (e.g., hydrogen bonds and hydrophobic interactions), were assessed and visualized using BIOVIA Discovery Studio to validate the conformational plausibility and molecular compatibility at target sites [8].

### Establishment of obesity model

Adult $w^{1118}$ *D. melanogaster* (5 - 7 days) were randomly assigned to 4 groups, each consisting of 5 males and 15 females [37]. One group was fed a standard diet and served as the untreated control, while the second group was fed a high-fat diet (HFD). The remaining 2 groups received HFD supplemented with vitamin $D_3$ at concentrations of 10 and 100 mM. The flies were allowed to mate for 3 - 5 days to generate larvae, which were subsequently used to establish an obesity model [38]. Third-instar larvae from each group were selected for further analysis. The compositions of the diets used in this study are detailed in **Table 1**. Each group included replicates that were used for hemolymph glucose level testing, crawling assays, nitro blue tetrazolium (NBT) reduction assays, and gene expression analysis (**Figure 1**).

**Table 1** Experimental diet composition.

| Ingredient | Control food | HFD | HFD + Vit.$D_3$ 10 | HFD + Vit.$D_3$ 100 |
|---|---|---|---|---|
| Cornmeal (g) | 7.5 | 7.5 | 7.5 | 7.5 |
| Yeast (g) | 3 | 3 | 3 | 3 |
| Agar (g) | 0.9 | 0.9 | 0.9 | 0.9 |
| Sucrose (g) | 4.5 | 4.5 | 4.5 | 4.5 |
| VCO (%) | - | 2 | 2 | 2 |
| Propionic acid (μL) | 400 | 400 | 400 | 400 |

| Ingredient | Control food | HFD | HFD + Vit.D$_3$ 10 | HFD + Vit.D$_3$ 100 |
|---|---|---|---|---|
| Methylparaben (µL) | 450 | 450 | 450 | 450 |
| Vitamin D$_3$ (mM) | - | - | 10 | 100 |
| Distilled water (mL) | 100 | 100 | 100 | 100 |

HFD, high fat diet; VCO, virgin coconut oil

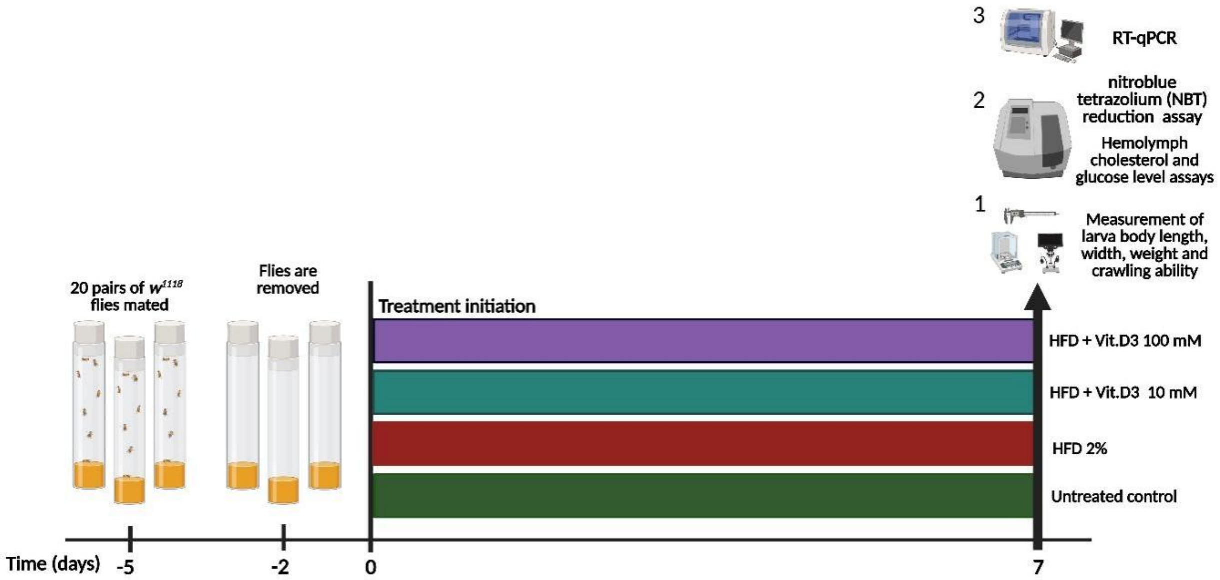

**Figure 1** Experimental design used in this study. Four groups of 3rd instar larvae of *D. melanogaster* larvae were used to assess the effects of HFD with or without Vitamin D$_3$ treatment at varying concentrations (10 and 100 mM). One group was reared without any treatment and served as the untreated control. Created with BioRender.com

### Body weight and crawling assays

Body weight and locomotor activity assessments were performed on third-instar larvae following gentle rinsing with NaCl solution to eliminate residual food particles. The larvae were air-dried before analysis. For body weight measurement, groups of ten third-instar larvae from each treatment group were weighed using an analytical balance to calculate the average weight per larva [37]. Locomotor activity was evaluated using a larval-crawling assay. An agar-coated plate was placed on a sheet of graph paper to serve as the testing arena. Second-instar larvae were collected and rinsed with NaCl solution to remove any food debris and then individually transferred to the center of the agar plate using a soft brush. The plate was covered to prevent escape, and the larvae were allowed to crawl freely for 1 min. As the larvae moved, they left visible trails on the agar surface. Locomotor activity was quantified by counting the number of 0.1 cm$^2$ grid squares each larva traversed within the 1-minute observation period. The treatment effects were determined by comparing the crawling distance and locomotor performance of the treated larvae with those of the control group [39].

### Length and width measurements

To eliminate residual food particles, 4 larvae from each treatment group were gently rinsed with sterile sodium chloride (NaCl) solution and subsequently air-dried at room temperature. Morphometric analysis was performed by measuring the body length and width of each larva using a digital caliper of high precision. Individual measurements were recorded for all 4 larvae in each treatment group. The obtained values were used to calculate the mean body length and width for each group, enabling a comparative evaluation of the morphological changes induced by the treatments [39].

### Cholesterol and glucose testing

Hemolymph samples from *D. melanogaster* larvae were analyzed to determine the cholesterol and glucose concentrations using a previously established protocol [37] with minor modifications. Seventy third-instar larvae were surface-cleaned and homogenized using a sterile micropestle. The resulting homogenate was diluted with 20 µL of sterile 0.9% sodium chloride (NaCl) solution. Quantification of glucose and cholesterol levels was performed using the Glucose Oxidase-Peroxidase Aminoantipyrine (GOD-PAP) reagent (Glory Diagnostics, Barcelona, Spain) and Stanbio Cholesterol Liquicolor kit (Stanbio Laboratory, Texas, USA), respectively, following the manufacturers' standard protocols. Absorbance was measured at 500 nm using a Shimadzu UV-Vis 1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). These measurements provided quantitative biochemical profiles for assessing the metabolic effects of the treatments.

### Nitroblue tetrazolium reduction assay

The amount of ROS in the hemolymph of third-instar larvae was measured using a nitroblue tetrazolium (NBT) reduction test, in compliance with a standard procedure [38, 40]. The ROS in the samples caused the yellow dye NBT (Merck, Frankfurter, Germany) to be reduced to insoluble formazan particles, giving the test a blue hue. The absorbance at 595 nm was measured to determine the ROS concentration. Fifty third-instar larvae were gathered, cleaned of any remaining food particles using phosphate-buffered saline (PBS), and then treated on ice to avoid melanization. After extracting the hemolymph, 100 µL of hemolymph was combined with 200 µL of 1× PBS, and an equal proportion of NBT solution was added to obtain a final volume of 300 µL. The reaction mixture was allowed to stand at room temperature for an hour in the dark. The addition of 300 µL of 100% glacial acetic acid stopped this process. The samples were centrifuged for 1 min at maximum speed, and after being diluted with 50% acetic acid, the absorbance of the supernatant was measured at 595 nm.

### Gene expression analysis

Total RNA was extracted from ten third-instar *D. melanogaster* larvae previously treated with the respective compounds using the PureLink™ RNA Mini Kit (Invitrogen, Thermo Fisher Scientific, MA, USA), following the manufacturer's protocol. Quantitative assessment of gene expression was performed via reverse transcription quantitative PCR (RT-qPCR) using the Luna® Universal 1-Step RT-qPCR Kit (New England Biolabs, MA, USA) according to the manufacturer's protocol. Each reaction was conducted in a 10 µL volume, with the following thermal cycling conditions: Reverse transcription at 50 °C for 10 min, initial denaturation at 95 °C for 2 min, followed by 40 amplification cycles at 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s. To ensure the specificity of the amplification, a melt curve analysis was performed at the end of each run. Gene expression levels were normalized to those of the internal reference gene, *rp49*, using the same amplification conditions. Primer sequences used in this analysis are provided in **Table 2**.

**Table 2** Primers used in the RT-qPCR assay.

| Genes | Forward primer (5'→3') | Reverse primer (5'→3') |
|---|---|---|
| *sod1* | AGGTCAACATCACCGACTCC | GTTGACTTGCTCAGCTCGTG |
| *cat* | TTCCTGGATGAGATGTCGCACT | TTCTGGGTGTGAATGAAGCTGG |
| *trbl* | AAATCGCCGCATTTCGTCAG | TTAGCCTGGCTGTACTTGGC |
| *rp49* | CGCTTCAAGGGACAGTATCTG | AAACGCGGTTCTGCATGAG |

### Data analysis

All phenotypic and molecular data were analyzed using GraphPad Prism 9 software (GraphPad Software, Boston, USA). Results are presented as bar graphs, and statistical significance was determined using 1-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test.

## Results and discussion

### In silico study or molecular docking of vitamin $D_3$ activity based on the human pancreatic lipase receptor (1LPB) and human pancreatic α-amylase (2QV4)

Lipase is a key enzyme that facilitates the breakdown of triacylglycerol (TG) reserves, releasing fatty acids (FAs) essential for cellular lipolysis [41]. In the digestive tract, dietary TGs undergo partial hydrolysis by lipase enzymes to produce free FAs and diacylglycerols. These lipid components are then emulsified by bile salts into fine droplets. The subsequent digestion process results in the formation of free FAs, mono- and diacylglycerols, bile salts, fat-soluble vitamins, and lysophosphatidic acid, which assemble into mixed micelles that are readily absorbed by enterocytes. As shown in **Table 3**, the target receptor for the molecular docking study was human pancreatic, which was validated based on an RMSD value of less than 2 Å, as shown in **Figure 2**. Following the validation process, molecular docking simulations were performed on the Human Pancreatic Lipase receptor using compounds identified from the spectral database, along with orlistat and acarbose, which served as reference inhibitors for lipase and α-amylase [42, 43].

**Table 3** Validation of molecular docking simulation.

| Drug target receptor | PDB ID | Docking site (x; y; z) | Docking Area (x; y; z) SD (Å) | RMSD (Å) |
|---|---|---|---|---|
| Human Pancreatic lipase | 1LPB | −0.423, 16.723, 26.546 | 42×40×40 | 0.755 |
| Human Pancreatic α-Amylase | 2QV4 | 12.942, 47.17, 26.2 | 42×40×40 | 0.188 |

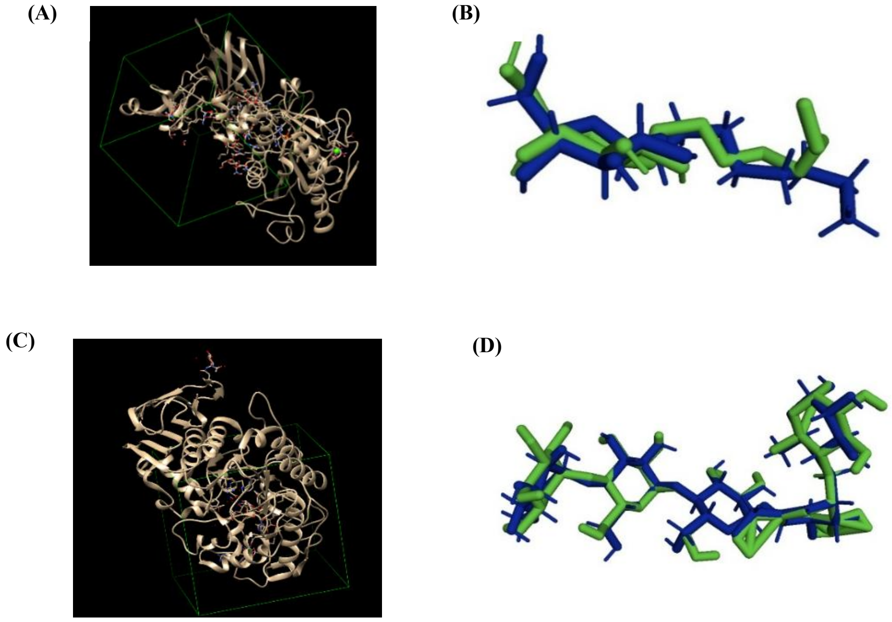

**Figure 2** The grid box was positioned around the binding site of the human pancreatic lipase receptor (1LPB) (A), highlighting the redocked ligand (green) and the native ligand (blue) (B), with an RMSD value of 0.755 Å. Human Pancreatic α-amylase and (2QV4) (C) highlighting the redocked ligand (green) and the native ligand (blue) (D), with an RMSD value of 0.188 Å.

**Table 4** Molecular docking parameter.

| Receptor | Compound | ΔG (kcal/mol) | Ki (nM) |
|---|---|---|---|
| Human Pancreatic lipase (1LPB) | Vitamin $D_3$ | −7.5 | 3.16 |
| | Orlistat | −5.5 | 92.47 |
| Human Pancreatic α-Amylase (2QV4) | Vitamin $D_3$ | −9.0 | 0.25 |
| | Acarbose | −7.9 | 1.01 |

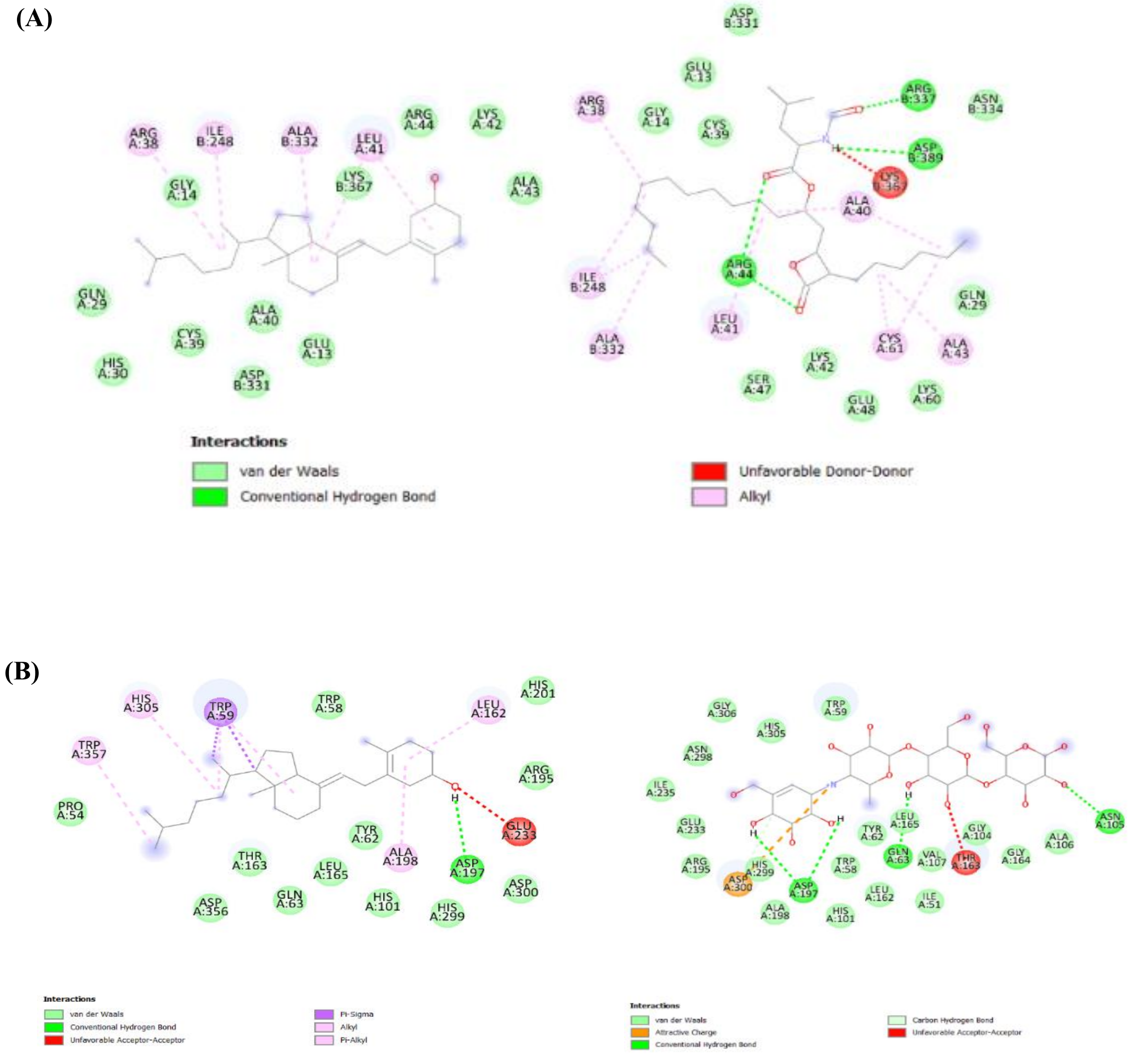

**Figure 3** Molecular interaction visualization between the ligand and target protein based on molecular docking analysis. Interaction of Vitamin $D_3$ and orlistat with 1LPB protein (A). Interaction of Vitamin $D_3$ and acarbose with the 2QV4 protein (B).

In this study, molecular docking validation was conducted by redocking native ligands into human pancreatic lipase (PDB ID: 1LPB) and α-amylase (PDB ID: 2QV4), resulting in RMSD values of 0.755 and 0.188 Å, respectively (**Table 3**). These values fell within the acceptable threshold (RMSD < 2.0 Å), indicating that the docking protocol employed was reliable and accurate. Structural superimposition of the docked and crystallographic ligand conformations (**Figures 2(B)** and **2(D)**) further confirmed the precision of the grid box parameters used in the docking simulations. The docking results revealed that Vitamin $D_3$ exhibited a higher binding affinity than the reference inhibitors for both enzymatic targets (**Table 4**). For pancreatic lipase, Vitamin $D_3$ displayed a binding energy of –7.5 kcal/mol with an inhibition constant ($K_i$) of 3.16 nM, surpassing orlistat (–5.5 kcal/mol; $K_i$: 92.47 nM). Similarly, Vitamin $D_3$ demonstrated superior affinity toward α-amylase, with a binding energy of –9.0 kcal/mol and $K_i$ of 0.25 nM, compared to acarbose (–7.9 kcal/mol; $K_i$: 1.01 nM). Interaction analysis (**Figures 3(A)** and **3(B)**) revealed the presence of conventional hydrogen bonds and hydrophobic interactions within the active sites of the respective enzymes. These findings are consistent with previous reports indicating the role of Vitamin $D_3$ in enhancing insulin sensitivity and regulating lipid metabolism [44]. Moreover, this aligns with the significant reduction in total cholesterol and glucose levels observed in third-instar *Drosophila* larvae following Vitamin $D_3$ administration. The dual inhibitory potential demonstrated in silico underscores the promise of Vitamin $D_3$ as a natural therapeutic candidate for the management of obesity and associated metabolic disorders.

### Vitamin $D_3$ improves body weight and crawling ability of obesity model

The sensitivity of *D. melanogaster* to food modifications makes it an excellent model for metabolic studies. The body weight and movement of larvae are influenced by high-fat diets, making them a useful model for studying the physiological and metabolic changes associated with obesity [38]. Here, we investigated the effects of vitamin $D_3$ in a *D. melanogaster* obesity model, specifically focusing on the increases in larval body weight and crawling abilities after being fed a high-fat diet. HFD induction was successful in establishing an obese model, as shown in **Figure 4**. When fed HFD, the body weight of *D. melanogaster* increased (**Figure 4(A)**), and their capacity to crawl decreased (**Figure 4(B)**). These results are consistent with those observed in other models, including mammals, showing lower mobility and increased body weight. Notably, in the obesity model, vitamin $D_3$ administration dramatically increased body weight and crawling ability, returning these parameters to normal.

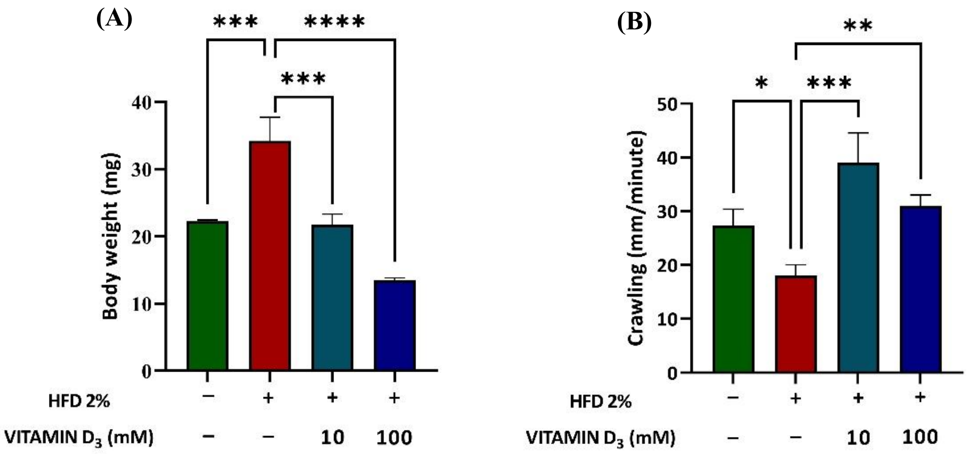

**Figure 4** Improvement in body weight (A) and crawling ability (B) in third-instar *Drosophila* larvae treated with vitamin $D_3$ following HFD consumption. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

Metabolic dysfunction is a key factor underlying excessive weight gain associated with obesity, primarily due to imbalances in glucose and lipid metabolism. Adenosine triphosphate (ATP) is the main energy source for cellular functions and physical movement. Disruptions in metabolic pathways can compromise ATP production and, consequently, locomotor performance. Recent studies have indicated that vitamin $D_3$ supplementation may help correct these metabolic impairments in obese models. The observed enhancements in body weight management and motor abilities, such as crawling, support the hypothesis that vitamin $D_3$ plays a role in re-establishing metabolic homeostasis through its regulatory effects on carbohydrate and lipid metabolic pathways.

### Vitamin $D_3$ improves length and width and tribble gene expression

HFD exposure has been demonstrated to alters larval morphology, establishing *D. melanogaster* as a valuable model organism for examining the physiological adaptations associated with obesity. In this study, we investigated the effects of vitamin $D_3$ supplementation in a *D. melanogaster* model of obesity compounded by diabetic features. Specifically, we assessed alterations in larval body length, body width, and trbl gene expression following HFD exposure. As shown in **Figure 5**, the induction of an obesity model via HFD was successful, resulting in significant increases in body body width (**Figure 5(A)**), length (**Figure 5(B)**), and *trbl* expression (**Figure 5(C)**). These outcomes align with findings from prior research in both mammalian models [40, 45] and *D. melanogaster* [46], which reported comparable morphological changes and elevated *trbl* expression under obesity-inducing conditions. Importantly, vitamin $D_3$ supplementation markedly attenuated the effects of HFD, significantly restoring body length, body width, and *trbl* expression to levels comparable to those observed in the control group.'

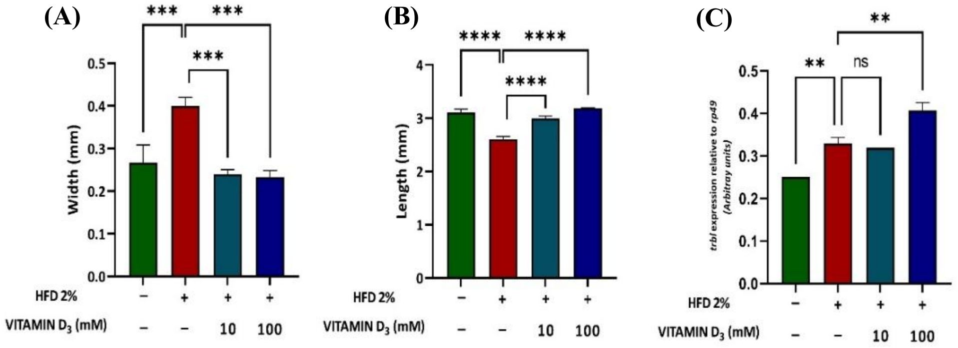

**Figure 5** Body width, length, and *trbl* gene expression changes in third-instar larvae fed a high-fat diet with or without vitamin $D_3$. When supplemented with 10 and 100 mM vitamin $D_3$, larvae fed an HFD exhibited increased body width (A) and decreased body length (B), whereas supplementation with vitamin $D_3$ lowered the width and increased the length. In response to HFD, third-instar larvae showed upregulation of *trbl* expression (C), independent of vitamin $D_3$ therapy. The high-fat diet group was compared with the data from each group. *** $p < 0.001$; **** $p < 0.0001$; breadth and length, *trbl* expression; HFD, high-fat diet; ns: Not significant.

Obesity-induced increases in body length and width are often associated with disruptions in lipid and glucose metabolism [35, 38], which affect both body size and the function of Tribble, a protein expressed by the *trbl* gene that is a key regulator of lipid and glucose homeostasis [47]. Vitamin $D_3$ supplementation appears to alleviate these metabolic disturbances, as evidenced by the restoration of normal body dimensions and *trbl* gene expression. This suggests that vitamin $D_3$ may help regulate lipid and glucose homeostasis by modulating the metabolic pathways that control *trbl* gene function.

### Vitamin $D_3$ improves cholesterol and glucose levels

Metabolic disorders, such as obesity and diabetes, are commonly characterized by elevated glucose and cholesterol levels, which may be associated with changes in body size following HFD exposure. To explore this connection, *D. melanogaster* larvae were treated with HFD, and subsequent measurements of glucose and cholesterol levels were measured. **Figures 6(A)** and **6(B)** illustrate that third-instar larvae exposed to HFD exhibited significantly increased glucose and cholesterol concentrations compared to the control group. Treatment with 10 mM vitamin $D_3$ led to a notable reduction in both glucose and cholesterol levels, indicating a potential corrective effect on metabolic imbalance. Despite this, cholesterol levels in the vitamin treated group remained slightly elevated relative to controls, although they were still significantly lower than those in the HFD group. Conversely, larvae receiving a higher dose of vitamin $D_3$ (100 mM) under HFD conditions showed a pronounced increase in glucose levels, suggesting a possible adverse effect at higher concentrations.

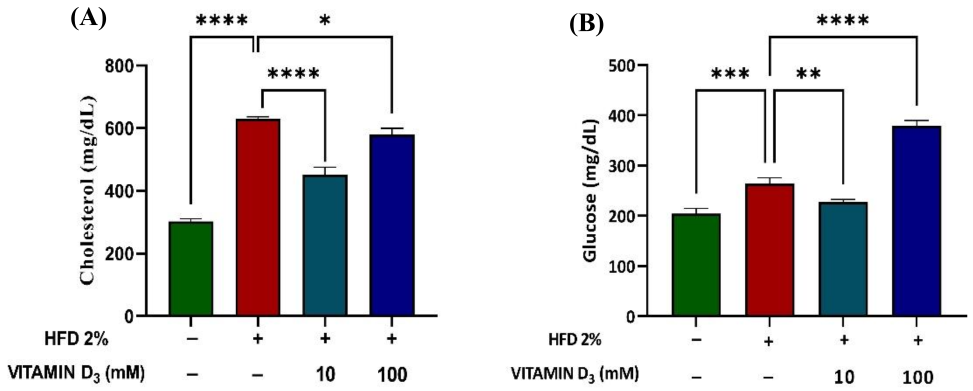

**Figure 6** Vitamin $D_3$ treatment affects the levels of cholesterol (A) and glucose (B) in *D. melanogaster* larvae fed a high-fat diet. *, $p < 0.01$; *, $p < 0.001$; ***, $p < 0.0001$; HFD, high-fat diet.

Our findings also showed that a high-fat diet significantly elevated cholesterol levels in third-instar larvae, indicating disruptions in lipid metabolism, consistent with a previous report [35]. Nevertheless, supplementation with 10 mM vitamin $D_3$ effectively reduced cholesterol levels compared to that in the control group. Interestingly, at a concentration of 100 mM, the cholesterol level rebounded, although it remained lower than that in the HFD-only group, suggesting that its effectiveness was diminished compared to the 10 mM dose. In this study, we observed an increase in glucose levels following HFD feeding. The elevated blood glucose levels in obese larvae were primarily due to excess fat accumulation, where surplus calories were stored as fat, impairing insulin sensitivity and leading to insulin resistance, which disrupted glucose metabolism [48]. These metabolic disturbances are commonly associated with conditions such as insulin resistance and diabetes [49, 50]. Supplementation with 10 mM vitamin $D_3$, but not 100 mM, significantly reduced glucose levels compared to the control group, indicating that vitamin $D_3$ can help lower glucose levels in *D. melanogaster* larvae fed a high-fat diet.

Our data demonstrated that HFD treatment significantly elevated cholesterol and glucose levels in third-instar larvae, suggesting detrimental effects on lipid and glucose metabolism. One notable observation from our study is the dose-dependent effect of vitamin $D_3$. While 10 mM vitamin $D_3$ was effective in reducing glucose and cholesterol levels, higher doses (100 mM) did not exhibit the same level of efficacy. This suggests that there may be a threshold beyond which vitamin $D_3$ supplementation becomes less effective or potentially harmful, possibly due to metabolic limitations or saturation of vitamin D receptors. These findings highlight the need for careful optimization of vitamin $D_3$ dosage in future studies and clinical settings.

### Vitamin $D_3$ treatment resulted in the upregulation of *sod1* and *cat* gene expression

A high-fat diet has been linked to the oxidative stress response [19, 51], and higher glucose and cholesterol levels are frequently associated with increased ROS production [52]. A possible modulatory effect on the oxidative stress pathway may be suggested by the observed increases in glucose and cholesterol levels following vitamin $D_3$ treatment, as illustrated in **Figures 6(A)** and **6(B)**. After exposure to HFD, we measured the ROS levels in *Drosophila* larvae treated with and without vitamin $D_3$ using an NBT test to learn more about this. Our findings showed that larvae fed a high-fat diet had higher ROS levels, which increased further when treated with 100 mM vitamin $D_3$ (**Figure 7(A)**). Alternatively, ROS levels were significantly reduced after treatment with 10 mM vitamin D3. According to these results, vitamin $D_3$ may be crucial in reducing the negative effects of oxidative stress, possibly by increasing the production of antioxidant *enzymes*, such as catalase and superoxide dismutase (SOD), which neutralize ROS and maintain cellular redox balance [17, 53-55]. A simultaneous decrease in ROS levels may be linked to the reduction in cholesterol and glucose levels observed with 10 mM vitamin $D_3$, suggesting that vitamin $D_3$ may control endogenous antioxidant defenses. We investigated the expression levels of *sod1* and cat using RT-qPCR to experimentally validate this hypothesis. The information shown in **Figures 7(B)** and **7(C)** shows how vitamin $D_3$ and HFD supplementation affect the *transcriptional activity of sod1 and cat genes*. The expression of these genes was considerably higher in the HFD and vitamin $D_3$ supplement groups than that in the control group. Notably, the group supplemented with vitamin $D_3$ showed a greater increase in cat gene expression.

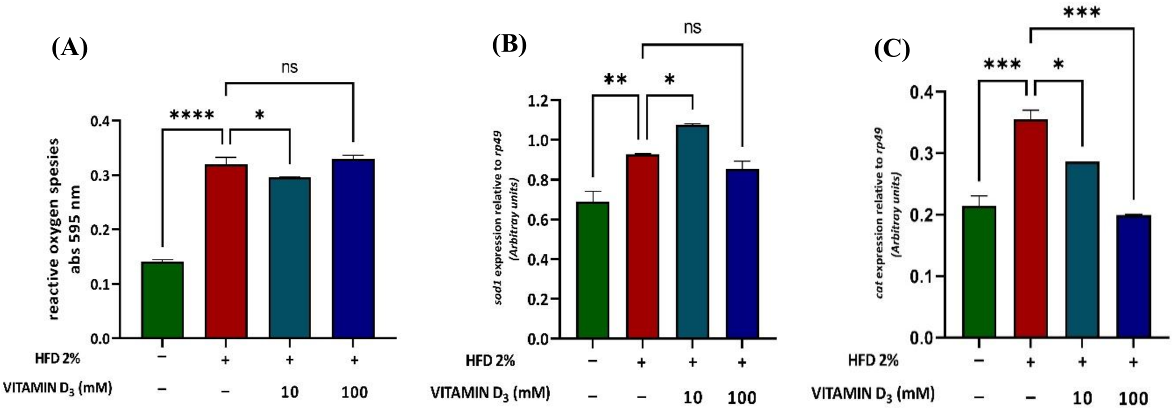

**Figure 7** Phenotypic and molecular alterations associated with oxidative stress in *D. melanogaster* exposed to a high-fat diet (HFD) supplemented with Vitamin $D_3$. Low-dose supplementation of Vitamin D3, but not high-dose supplementation, effectively reduced ROS levels induced by an HFD (A), which correlated with the upregulation of *sod1* (B) and *cat* (C) gene expression. NS-not significant; ** $p < 0.001$; ** $p < 0.0001$; HFD, high-fat diet; ROS-reactive, reactive.

The data presented in **Figures 7(A) - 7(C)** revealed a notable increase in ROS levels and upregulation of *sod1* and *cat* gene expression in third-instar larvae exposed to an HFD, indicating heightened oxidative stress. Interestingly, the addition of 10 mM vitamin $D_3$ to the HFD further stimulated the expression of both genes, despite the ROS levels remaining comparable to those in the HFD-only group. Although vitamin $D_3$ is commonly recognized for its antioxidant effects, these results suggest a more complex interaction between vitamin $D_3$ and oxidative stress in this model. The specific mechanisms responsible for the observed increase in *cat* gene expression in response to vitamin $D_3$ require further investigation.

## Conclusions

In conclusion, vitamin $D_3$ demonstrates significant potential as an anti-obesity agent, exhibiting strong inhibitory activity against 1LPB and 2QV4 *in silico* and effectively reducing cholesterol levels in an obese *D. melanogaster* model. The observed improvements in physiological outcomes, including survival, body size, and movement, as well as the regulation of metabolism-related genes, suggest that vitamin $D_3$ may reduce obesity via multiple mechanisms. These findings highlight the potential of this strategy as a therapeutic supplement for managing obesity and its associated complications and support the need for further investigation into its molecular mechanisms in advanced preclinical and clinical models.

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## Declaration of generative AI in scientific writing

The authors declare that AI tools (ChatGPT and Paperpal) were used to assist in language editing and translation during the preparation of this manuscript. The content, ideas, interpretations, and conclusions remain entirely the authors' original work and responsibility.

## CRediT author statement

**Alfreds Roosevelt**: Conceptualization, Methodology, Software, Data curation, Writing - Original draft preparation, Visualization; **Rina Agustina**: Software, Validation, Supervision, Funding acquisition; **Filmaharani Filmaharani**, **Anggun Nurhidayah**, **Muhammad Rayza Azmin**, **Ratnawati Ratnawati**, **Jumriani Jumriani**: Investigation, Writing - Review & Editing; **Muh Arfandy Gunawan**, **Widya Hardiyanti**, **Asbah Asbah**, **Nadila Pratiwi Latada**, **Mukarram Mudjahid**: Investigation, Resources, Writing - Review & Editing; **Firzan Nainu**: Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition

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