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Dietary propolis transiently delays autoimmune diabetes onset and modulates immune responses in NOD mice

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ABSTRACT

Ethnopharmacological relevance: Propolis is a honey bee-derived resinous substance used in traditional medicine across many cultures to treat inflammatory and immune-related ailments. Its documented anti-inflammatory and immunomodulatory actions suggest potential value in autoimmune disease, yet its effects in type 1 diabetes (T1D) have not been examined.

Aim of the study: To investigate whether dietary propolis modulates immune responses and alters the progression of autoimmune diabetes in non-obese diabetic (NOD) mice.

Materials and methods: Female NOD mice (n = 66) received standard chow or chow supplemented with 1.8% or 3.6% (w/w) raw Danish propolis from 4 to 30 weeks of age. Diabetes incidence was monitored longitudinally. At 14 weeks, pancreatic insulitis, T cell subsets in pancreatic lymph nodes and spleen, serum cytokines, and gut microbiota composition were assessed.

Results: Propolis delayed diabetes onset, significantly reducing incidence at 17 weeks, although cumulative incidence converged by 30 weeks. High-dose propolis reduced pancreatic immune infiltration and decreased $CD4^+$ T helper cells in pancreatic lymph nodes, with a non-significant trend toward increased splenic T cell counts. Serum IL-10 and IFN-γ were elevated, while IL-4 was reduced. *Akkermansia muciniphila* abundance was increased.

Conclusions: Dietary propolis delayed autoimmune diabetes onset in NOD mice, although cumulative incidence converged by the end of the study, and was associated with changes in immune parameters and gut microbiota composition. These findings support further investigation of propolis as a candidate for modulating disease progression in autoimmune T1D, though the underlying mechanisms remain to be established.

1. Background

Propolis is a resinous substance produced by honey bees, rich in polyphenols such as flavonoids and phenolic acids (Zhu et al., 2023), which contribute to its well-documented anti-inflammatory properties (Hossain et al., 2022; Bhatti et al., 2024). Propolis has a long history of use in traditional medicine across many cultures, with documented applications dating back to ancient Greek, Roman, and Egyptian civilizations (Kuropatnicki et al., 2013). In Eastern European folk medicine in particular, propolis has traditionally been prepared as an ethanolic tincture and applied topically to treat wounds, burns, and skin infections, as well as used as an oral rinse for mouth infections, reflecting its long-standing use for inflammatory and immune-related conditions (Kuropatnicki et al., 2013). This ethnomedicinal heritage has motivated sustained scientific interest in its pharmacological properties, particularly its effects on the immune system. These effects have primarily been attributed to propolis' inhibition of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Búfalo et al., 2013), a central transcription factor involved in regulating immune and inflammatory gene expression (Liu et al., 2017). Polyphenols can

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interfere with NF-κB signaling at several levels, including inhibition of inhibitor of κB kinase activity, stabilization of inhibitor of κB alpha, and prevention of NF-κB nuclear translocation and DNA binding, thereby reducing the transcription of pro-inflammatory mediators (Yu et al., 2022). Propolis flavonoids have also been demonstrated to cause decreased pro-inflammatory cytokine production through selective elimination of monocytes and macrophages (Hougee et al., 2005).

Interestingly, propolis may also exert immunostimulatory effects and has been reported to increase pro-inflammatory cytokine levels in mice (H. Zhu et al., 2024; Rifa'i and Widodo, 2014), suggesting that its immunological actions are context-dependent, and thus capable of both dampening excessive inflammation and enhancing immune responsiveness. While the immunosuppressive effects are linked to NF-κB inhibition, the immune-enhancing effects are thought to involve pattern recognition receptors, through the upregulation of Toll-like receptor 4 (TLR4), which is primarily expressed on cells of myeloid origin, including monocytes, macrophages, and dendritic cells (Vaure and Liu, 2014; H. Zhu et al., 2024; Orsatti and Sforcin, 2012). TLR4 activation triggers downstream NF-κB signaling, leading to the production of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and IL-12p40 (Liu et al., 2017). Upon TLR4 activation, IL-12p40 combines with the p35 subunit of IL-12 to form the bioactive heterodimer IL-12p70 (Gilmour et al., 2024). This cytokine promotes IFN-γ secretion by T cells and natural killer (NK) cells through activation of STAT4 (Wang et al., 1999), thereby supporting Th1 differentiation and activation. Thus, propolis may modulate immune responsiveness in both directions through the TLR4-NF-κB pathway, acting either suppressively or stimulatory depending on context. However, due to its complex and variable chemical composition (Šturm and Ulrih, 2020), delineating the precise cellular mechanisms remains challenging, and its dual immunomodulatory effects likely reflect this biochemical diversity.

Nonetheless, given these properties, propolis may hold therapeutic potential for autoimmune diseases such as type 1 diabetes (T1D), a chronic T cell-mediated condition characterized by the progressive inactivation and destruction of insulin-producing pancreatic β-cells (Burrack et al., 2017). This immune-driven β-cell loss results in insulin deficiency and necessitates lifelong exogenous insulin therapy. A characteristic feature of T1D is insulitis, defined as the infiltration of autoreactive immune cells into the islets of Langerhans (Pugliese, 2016). In humans, insulitis is observed primarily at or near disease onset, whereas it is more consistently present and progressive in animal models such as Non-Obese Diabetic (NOD) mice (In't Veld, 2014). This local immune response is typically accompanied by dysregulated cytokine signaling that promotes pro-inflammatory, Th1-skewed immune activity within the pancreas (Pilström et al., 1995).

The NOD mouse is a widely used and well-characterized preclinical model of spontaneous autoimmune diabetes. Female NOD mice develop disease in a manner that resembles human T1D, both immunologically and pathologically, whereas the male are more resistant to disease (Chen et al., 2018). Disease onset is marked by early lymphocytic infiltration of the pancreatic islets, followed by gradual β-cell destruction and eventual hyperglycemia (Chen et al., 2018). Given these parallels, the NOD mouse provides a robust and relevant system for exploring the immunopathogenesis of autoimmune diabetes and assessing the therapeutic potential of immunomodulatory interventions such as propolis.

In this study, it is investigated whether dietary administration of propolis modulates immune responses and alters the progression of autoimmune diabetes in female NOD mice. Specifically, immune infiltration in pancreatic islets, systemic cytokine profiles, and T cell subset distribution in lymphoid organs are evaluated. To our knowledge, this is the first study to evaluate the immunological and disease-associated effects of propolis in the NOD mouse model.

2. Methods

2.1. Collection of propolis

Raw propolis was obtained from two Danish organic beekeepers located in North Jutland and North Zealand. Propolis from these beekeepers has been extensively characterized in previous studies (J. P. Vind et al., 2026; J. Vind et al., 2026; Steintún et al., 2026). The chemical composition was investigated by spectroscopy for overall composition and by HPLC for polyphenol content consistent with the compositional criteria outlined in the ISO propolis guideline (ISO 24381:2023). However, propolis composition can vary between individual batches even from the same beekeeper sources, and the specific batch used in the present study was not independently subjected to compositional analysis. To ensure sample freshness, propolis was harvested within the same year the study started.

2.2. Dose selection

Propolis doses were determined using an interspecies dose-conversion approach based on the principle that dose is proportional to metabolic body size across species, where metabolic body size is derived from body weight (W) via an allometric scaling relationship ($W^{-0.25}$, or $W^{-0.5}$ for species under 100 g body weight, such as an NOD mouse). This approach was used to convert a known dose in one species to an equivalent dose in another, based on the ratio of their respective metabolic body sizes. Using a reference human oral propolis dose of 1 g/day, based on doses reported in clinical studies (Zhang et al., 2025), and an assumed average human body weight of 70 kg, the corresponding mouse-equivalent dose was calculated as approximately 0.09 g/day for a mouse of 25 g body weight. Assuming an average daily feed intake of 5 g/mouse, this corresponds to a dietary concentration of 1.8% (w/w). The high-dose group was formulated at twice this concentration (3.6% w/w) to evaluate a potential dose-response relationship.

2.3. Study design

2.3.1. Housing and assignment to experimental groups

Sixty-six female NOD mice were housed in a rodent barrier facility at the Department of Veterinary and Animal Sciences, University of Copenhagen, with the sample size determined based on feasibility and ethical considerations for this exploratory study. The environment was maintained at a temperature of 20-24 °C with a relative humidity of 55% ± 10%, and a 12-h light-dark cycle (lights on from 6 a.m. to 6 p.m.). Mice were kept in open cages with a maximum of six animals per cage (grouped as 6, 5, and 5 animals per cage, respectively). Housing and health monitoring followed the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) (Nicklas et al., 2010). The mice were four weeks old upon arrival. Immediately after arrival, mice were randomly allocated to cages by an animal technician, with each cage assigned to one of the three dietary groups (n = 22 per group). The control group received standard Altromin 1324 pellets, while the low-dose and high-dose groups received modified Altromin 1324 pellets containing 1.8% and 3.6% (w/w) raw propolis, respectively. All diets were prepared by Brogaarden (Lage, Germany). At the start of the dietary intervention, six mice from each group were randomly selected for sacrifice at 14 weeks of age to assess early diabetes progression. These animals were housed separately in cages of three per group prior to sacrifice. This time point was selected to capture the prediabetic stage, as NOD mice are typically in the early phases of autoimmune development at this age, but with immune infiltrations in most islets (Chen et al., 2018). The pancreas was harvested for histological evaluation of insulitis, while the spleen and pancreatic lymph nodes (PLNs) were collected for analysis of T cell subsets distribution. Blood samples were obtained via retro-orbital sinus puncture under anesthesia for cytokine profiling. Feces were also collected for gut microbiota analysis. All samples were

immediately placed on ice. One mouse in the high-dose group was diagnosed with diabetes on the day of sacrifice and was therefore excluded from data analysis. However, its fecal sample was retained for gut microbiota analysis, as this outcome was assessed independently of immunological status. A timeline of the experimental design is presented in Fig. 1.

2.3.2. Diabetes onset monitoring

Diabetes monitoring of the remaining mice (n = 16 per group) began when the mice reached 10 weeks of age, with blood glucose levels measured weekly by tail vein puncture using a handheld glucometer. If a mouse exhibited blood glucose levels exceeding 12.0 mmol/L, a confirmatory measurement was taken the following day. Mice with confirmed hyperglycemia (>12.0 mmol/L on two consecutive days) were classified as diabetic and euthanized. Monitoring continued until 30 weeks of age. To ensure nutritional adequacy, feed and water intake were recorded weekly on a per-cage basis from 11 to 14 weeks of age. Feed and water intake were monitored throughout to confirm comparable consumption across dietary groups; no differences were observed at the cage or individual level, based on weekly body weight monitoring (Supplementary Fig. 1, Supplementary Fig. 2)

2.4. Histological assessment of insulitis

Insulitis was assessed on twenty islets per 14-week-old mouse from hematoxylin and eosin (H&E)-stained pancreatic sections (5 μm thick), cut at 100 μm intervals. Islets were classified based on the degree of immune cell infiltration into the following categories: no insulitis, peri-insulitis, mild insulitis (<25% infiltration), moderate insulitis (25-75% infiltration), and severe insulitis (>75% infiltration or complete islet destruction). An insulitis index was then calculated for each mouse by assigning numerical values to these categories (from 0 for no insulitis to 4 for severe insulitis) and averaging the scores across the 20 islets, providing a single quantitative measure of insulitis severity. Scoring was performed blindly, and islets were evaluated in random order.

2.5. Cell isolation and flow cytometry analysis

Single-cell suspensions were prepared from spleen and PLNs by mechanically dissociating freshly isolated organs through a 100 μm nylon mesh into cold PBS. Cells were then washed and resuspended in PBS with 1% FC-block CD16/CD32 (eBioscience, San Diego, USA).

For flow cytometric analysis, cells were stained with fluorophore-conjugated antibodies targeting surface and intracellular markers according to the manufacturer's instructions (eBioscience). Following standard gating on lymphocytes and singlets, T cells were defined as $CD3^+CD45^+$, helper T cells (Th) as $CD3^+CD4^+$, and regulatory T cells (Tregs) as $CD3^+CD4^+FoxP3^+$ (representative gating strategy shown in Supplementary Fig. 3). Intracellular FoxP3 staining was performed following fixation and permeabilization as per the manufacturer's protocol (eBioscience).

Samples were acquired using an Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and data were analyzed in the BD Accuri C6 software to determine the percentage of T cell subsets per organ. The total number cells were counted using the TC20 Automated Cell Counter (Bio-Rad, Hercules, CA, USA). One control sample was lost during processing and excluded from flow cytometric analysis of the pancreatic lymph nodes (control, n = 5 for this endpoint). Blinding was not performed for flow cytometric analysis, consistent with common practice in exploratory studies of this scale.

2.6. Cytokine measurement

Serum levels of IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, C-X-C motif ligand 1 (CXCL1), IL-10, IL-12p70, and TNF-α were measured using the V-PLEX Proinflammatory Panel 1 Mouse Kit (Meso Scale Diagnostics, Rockville, MD, USA) on a MESO QuickPlex SQ 120 MM platform and analyzed using Discovery Workbench software (MSD). Samples were processed on this automated platform, which quantifies cytokine concentrations directly from measured signal intensities without investigator input, limiting the potential for measurement bias despite the absence of formal blinding during sample handling. One low-dose and one high-dose sample were excluded from analysis of IL-4 due to unreliable readings (low-dose, n = 5; high-dose, n = 4, for IL-4 only). IL-2, IL-5, and CXCL1 were also measured but are not reported, as no significant or trend-level differences were observed between groups for these analytes; IL-12p70 concentrations fell below the assay's lower limit of quantification and were therefore excluded from statistical analysis.

2.7. Gut microbiota analysis

Genomic DNA was extracted from fecal samples using the DNeasy PowerSoil Pro Kit (Qiagen, Venlo, The Netherlands) on a QIAcube automated platform, following mechanical lysis with a TissueLyser II. All procedures were carried out according to the manufacturer's protocols. DNA libraries were prepared using a custom two-step PCR protocol to amplify near full-length 16S rRNA genes, with both positive and negative controls included throughout the process. The pooled library was sequenced on a PromethION 2 Solo platform (Oxford Nanopore Technologies, Oxford, UK) using an R10.4.1 flow cell.

Sequencing data were collected and basecalled in super-accurate mode using the MinKNOW GUI (version 24.06.10; Oxford Nanopore Technologies, Oxford, UK). Raw data processing, including filtering, trimming, chimera removal, and taxonomic assignment, was performed using the LACA pipeline (Hui et al., 2025). The average number of raw reads per sample, including sequencing artefacts, was 154,955 (range: 113,499–215,386; SD = 36,189). Following quality filtering and chimera removal, an average of 24,205 classified reads per sample remained (range: 20,910–26,497; SD = 1626). The resulting feature table was further analyzed using QIIME2 (Bolyen et al., 2019). Beta diversity was assessed using Bray-Curtis and Jaccard distance metrics, calculated from rarefied feature tables (20,000 reads per sample).

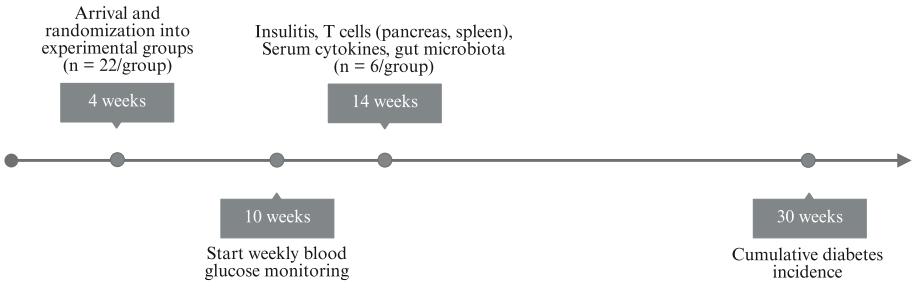

Fig. 1. Timeline of experimental design. Female NOD mice were randomized into dietary groups at 4 weeks of age (n = 22 per group). From 10 weeks of age, diabetes monitoring was initiated, and at 14 weeks of age, six mice per group were sacrificed for immunological and microbiota analyses. Cumulative diabetes incidence was recorded up to 30 weeks of age.

Differences in beta diversity were tested using Permutational Multivariate Analysis of Variance (PERMANOVA). The differences in bacterial relative distribution was evaluated using Analysis of Compositions of Microbiomes (Lin and Peddada, 2020) implemented in QIIME2. Sequencing and bioinformatic processing were performed by an investigator without knowledge of group identity or study hypotheses.

2.8. Statistical analysis

GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA) and MATLAB version R2023b (MathWorks Inc., Natick, MA, USA) were used for statistical analyses. A p-value < 0.05 was considered statistically significant.

Cumulative diabetes incidence curves were analyzed using the Kaplan-Meier method, and group differences were assessed using the log-rank test for trend. Insulitis scores were compared across groups using the χ^2 test, with pairwise comparisons between individual score categories performed using Fisher's exact test. Holm correction was applied to adjust for multiple testing.

Differences in age of onset, T cell subset distributions, serum cytokine concentrations and insulitis indexes were analyzed using either one-way ANOVA for normally distributed data or the Kruskal-Wallis test for non-normally distributed data. Normality was assessed using the Shapiro-Wilk test. For multiple comparisons following ANOVA, Tukey's test was applied; in cases of unequal variances, the Brown-Forsythe test was used. For post hoc comparisons following the Kruskal-Wallis test, Dunn's test was employed.

2.9. Ethical approval

The experiment complied with the Danish Act on Animal Experimentation which implements the Directive 2010/63/EU on the Protection of Animals used for Scientific Purposes. It was approved by the Danish Animal Experiments Inspectorate at Ministry of Food, Agriculture, and Fisheries of Denmark (License number: 2021-15-0201-00847).

2.10. Data and resource availability

All datasets and critical resources generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

3. Results

3.1. Orally administered propolis delays diabetes onset in NOD mice

To investigate the effect of orally administered propolis on the development of autoimmune diabetes, female NOD mice were fed either standard chow (control) or chow supplemented with a low (1.8% w/w) or high (3.6% w/w) dose of propolis starting at 4 weeks of age. Cumulative diabetes incidence was monitored until 30 weeks and is presented in Fig. 2a.

The earliest onset of diabetes was observed in the control group (13 weeks), with slightly later onsets in the low-dose (14 weeks) and high-dose (16 weeks) groups. At 17 weeks, a log-rank test for trend indicated a statistically significant difference in diabetes onset across treatment groups, with more incidents observed in the control ($n = 7$) and low-dose ($n = 6$) groups compared to the high-dose group ($n = 2$). The control group reached a cumulative diabetes incidence of 50% at 18 weeks of age, whereas the low and high-dose groups reached this threshold at 22 weeks. However, by the end of the study, cumulative diabetes incidence was similar across all groups.

Fig. 2b shows the onset age of diabetes for each mouse. Although the mean age of onset was higher in the high-dose group compared with the control and low-dose groups, the onset age did not differ significantly between groups (one-way ANOVA, $p = 0.081$, $R^2 = 0.159$).

3.2. Higher dose of propolis reduced immune infiltrations of islets of langerhans

To examine the protective effects of propolis on immune infiltration in the pancreatic islets of Langerhans, insulitis was assessed in H&E-stained pancreatic sections from NOD mice at 14 weeks of age. Each islet was scored on a 5-point scale ranging from no infiltration to severe insulitis (>75% infiltration or complete islet destruction). Representative images of each insulitis score are shown in Fig. 3a, and the distribution of insulitis scores is presented in Fig. 3b. In the control group, 17.5% of islets showed no infiltration, while approximately 43% exhibited moderate or severe insulitis. In contrast, 43.0% of islets in the high-dose group demonstrated no insulitis, and only 29% were scored as moderate or severe. The low-dose group displayed a relatively even distribution across all scoring categories. A χ^2 test confirmed a significant difference in the overall distribution of insulitis scores between groups ($p = 0.001$). Subsequent pairwise comparisons revealed that the

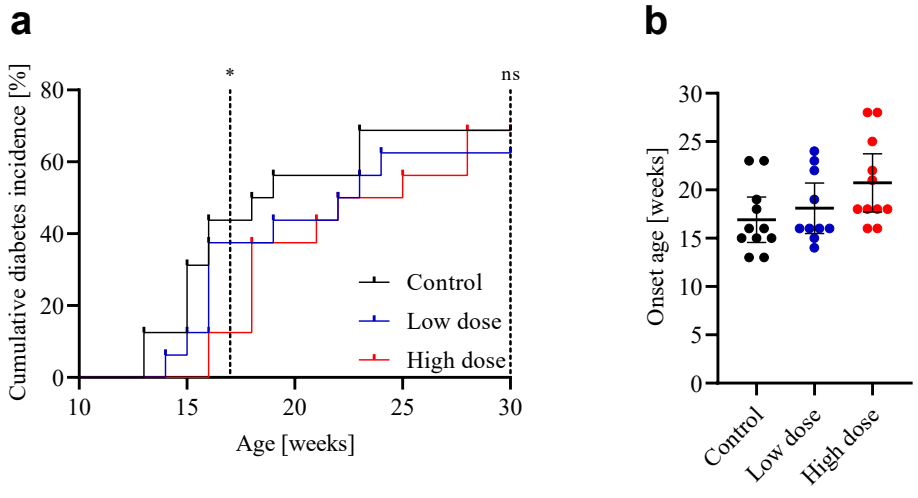

Fig. 2. (a) Cumulative diabetes incidence in female non-obese diabetic (NOD) mice ($n = 16$/group). Diabetes incidence was monitored over time. Groups were compared using the log-rank test for trend. Statistical significance was last observed at 17 weeks of age, with no differences at the study endpoint (30 weeks). Statistically significant pairwise comparisons are shown. $p < 0.05$*, *ns*: not significant. (b) Age of diabetes onset in NOD mice. Symbols represent individual mice; lines and error bars indicate mean ± 95% confidence interval for each group.

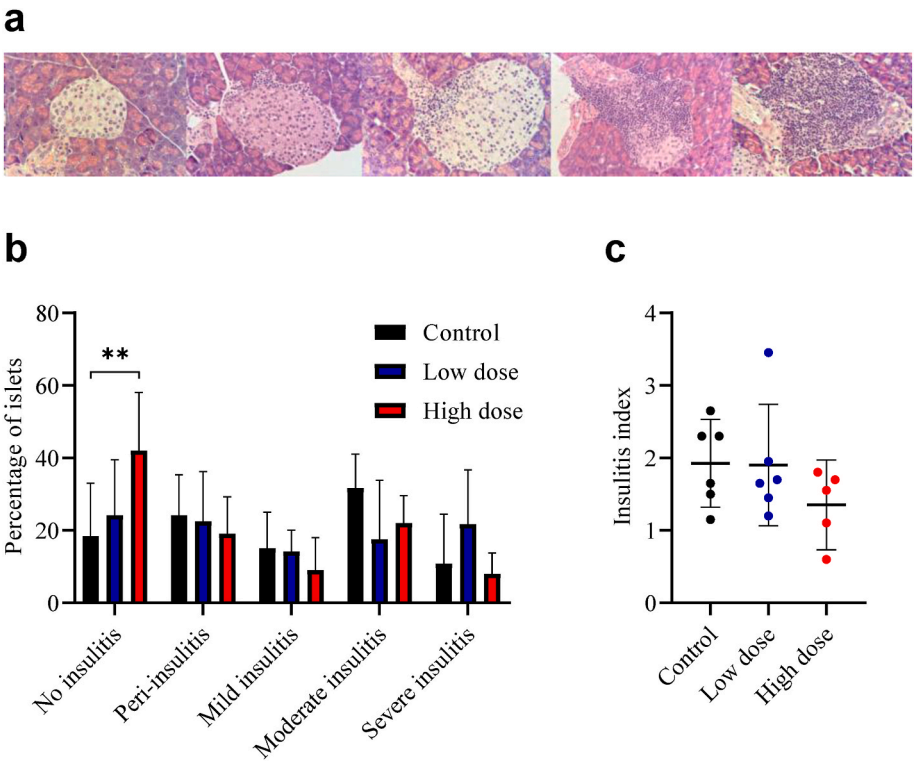

Fig. 3. Insulitis scoring in pancreatic islets of female non-obese diabetic (NOD) mice. Insulitis was assessed in hematoxylin and eosin (H&E)-stained pancreatic sections from NOD mice in the control, low-dose, and high-dose propolis groups at 14 weeks of age (n = 6 control, 6 low-dose, 5 high-dose). Each islet was graded on a 5-point scale according to the extent of immune cell infiltration: no insulitis, peri-insulitis, mild insulitis (<25% infiltration), moderate insulitis (25-75% infiltration), or severe insulitis (>75% infiltration or complete islet destruction). (a) Representative images illustrating increasing insulitis severity from left to right. (b) Percentage of islets exhibiting each insulitis grade in the different treatment groups. Bars indicate mean ± SD. (c) Insulitis index calculated for each mouse. Symbols represent individual mice; lines and error bars indicate mean ± 95% confidence interval for each group. Statistically significant pairwise comparisons are shown. $p < 0.01$**.

proportion of islets without insulitis was significantly higher in the high-dose group compared to the control group ($p < 0.01$). To better illustrate the insulitis status of each mouse, an insulitis index was calculated (Fig. 3c). Although no statistically significant differences were observed between the experimental groups (one-way ANOVA, $p = 0.294$, $R^2 = 0.160$), the high-dose group displayed a lower mean insulitis index (1.35) compared to the control (1.93) and low-dose (1.90) groups.

These findings suggest that propolis may reduce immune infiltration in pancreatic islets during the prediabetic stage.

3.3. *Propolis affects the distribution of T cell subsets in the pancreatic lymph nodes and spleen*

To investigate whether propolis supplementation influences immune cell composition, T cell subsets were quantified in the pancreatic lymph nodes and spleens of female NOD mice at 14 weeks of age. Single-cell suspensions were analyzed by flow cytometry, and the total number of T cells, $CD4^+$ T helper cells, and $CD4^+Foxp3^+$ regulatory T cells were measured.

In the pancreatic lymph nodes (Fig. 4a–c), a trend toward decreasing cell counts across all T cell subsets was observed with increasing propolis dose, although this did not reach statistical significance for total T cells (one-way ANOVA, $p = 0.062$, $R^2 = 0.348$) or regulatory T cells ($p = 0.067$, $R^2 = 0.341$). In contrast, an opposite pattern emerged in the spleen (Fig. 4d–f), where higher propolis doses appeared to elevate T cell subset counts, again without reaching significance for total T cells ($p = 0.065$, $R^2 = 0.324$) or regulatory T cells ($p = 0.058$, $R^2 = 0.334$). Substantial intragroup variability was noted for all T cell subsets examined, and the consistently moderate effect sizes (R^2 approximately 0.32-0.35) observed across these non-significant comparisons suggest the study may have been underpowered to detect true differences in these subsets.

Among T cell subsets analyzed in both the pancreatic lymph nodes and spleen, only the lymphatic $CD4^+$ T helper cells demonstrated statistically significant differences between experimental groups (Kruskal-Wallis, $p = 0.020$). Post hoc pairwise analysis (Dunn's test) revealed that this difference was attributable to a significant reduction in $CD4^+$ T helper cell counts between the control and high-dose groups ($p = 0.029$, $r = 0.82$), representing a large effect size; the control-versus-low-dose and low-dose-versus-high-dose comparisons did not reach significance ($p > 0.999$, $r = 0.24$; and $p = 0.169$, $r = 0.58$, respectively). In the spleen, $CD4^+$ T helper cell counts showed a similar upward trend with increasing propolis dose (one-way ANOVA, $p = 0.067$, $R^2 = 0.320$). Notably, although absolute numbers of T cell subsets showed a trend toward change with propolis dose, no significant differences were found in their relative proportions within each tissue.

These findings suggest that propolis may modulate local T cell populations in the pancreatic draining lymph nodes during the prediabetic stage.

3.4. *Propolis influences serum cytokines*

To assess systemic immune signaling, cytokine concentrations were measured in serum from female NOD mice at 14 weeks of age. Serum levels of IL-1β, IL-4, IL-6, IL-10, IFN-γ, and TNF-α are shown in Fig. 5a-f.

Significant group-level differences were detected for IL-4 (one-way ANOVA, $p = 0.010$, $R^2 = 0.534$), IL-10 (one-way ANOVA, $p = 0.013$, $R^2 = 0.466$), and IFN-γ (Kruskal-Wallis, $p = 0.004$). For IL-4, pairwise comparisons showed significantly lower concentrations in both the low-dose ($p = 0.026$) and high-dose ($p = 0.018$) groups compared with

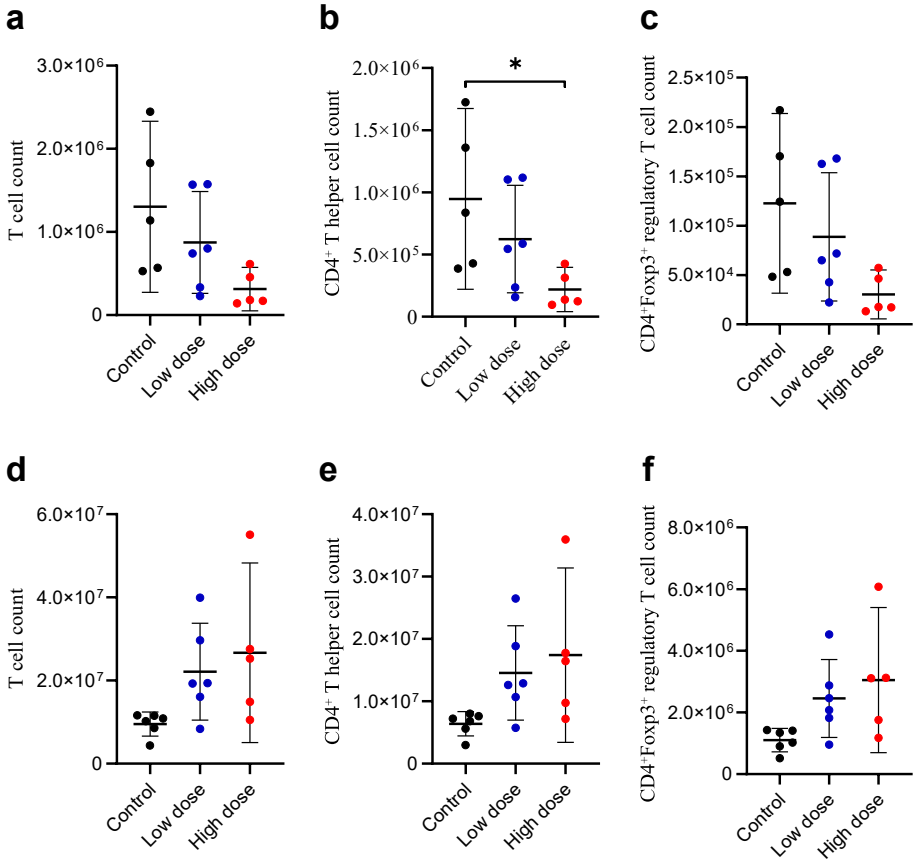

Fig. 4. T cell subset counts in pancreatic lymph nodes and spleen from female NOD mice in the control, low-dose, and high-dose propolis groups at 14 weeks of age (spleen: n = 6 control, 6 low-dose, 5 high-dose; lymph node: n = 5 control, 6 low-dose, 5 high-dose, following loss of one control sample during processing). Total T cell counts (a), $CD4^+$ T helper cells (b), and $CD4^+Foxp3^+$ regulatory T cells (c) in the pancreatic lymph nodes. (d-f) The same T cell subsets measured in the spleen. Symbols represent individual mice; lines and error bars indicate mean ± 95% confidence interval for each group. Statistically significant pairwise comparisons are shown. $p < 0.05$*.

control, with no significant difference between low- and high-dose groups ($p = 0.925$). For IL-10, concentrations were significantly higher in the high-dose group compared with both control ($p = 0.025$) and low-dose ($p = 0.018$) groups, with no significant difference between control and low-dose ($p = 0.982$). For IFN-γ, concentrations were significantly higher in the high-dose group compared with control ($p = 0.007$, $r = 0.92$), with no significant differences between control and low-dose ($p = 0.259$, $r = 0.50$) or between low-dose and high-dose ($p = 0.478$, $r = 0.42$).

Serum IL-1β, IL-6, and TNF-α did not differ significantly between groups. IL-6 and TNF-α showed moderate effect sizes despite non-significance ($R^2 = 0.180$ and 0.340, respectively), while IL-1β showed no significant pairwise differences (Brown-Forsythe/Welch ANOVA, all $p > 0.19$).

These findings indicate that high-dose propolis was associated with a systemic cytokine shift characterized by reduced IL-4, increased IL-10, and increased IFN-γ, while IL-1β, IL-6, and TNF-α were largely unaffected. The interpretation of this cytokine shift is discussed further below.

3.5. *Propolis changes the microbiomics of NOD mice*

Beta diversity analysis, based on weighted (Bray-Curtis) and unweighted (Jaccard) distance matrices, revealed significant differences among all tested groups (Fig. 6a and b), suggesting both qualitative and quantitative alterations in gut microbial composition between the high-dose, low-dose, and control groups.

Further examination using ANCOM identified *Akkermansia muciniphila* and *Heminiphilus faecis* as significantly enriched in the high-dose propolis group compared to the control group (Fig. 6c). Moreover, although not reaching statistical significance, the family Muribaculaceae exhibited a trend toward increased relative abundance in both treatment groups, with some individual mice showing levels approaching 50%.

Collectively, these data indicate that oral administration of propolis modulates gut microbiota composition in NOD mice.

4. Discussion

The purpose of this study was to investigate the effect of dietary propolis on the development of autoimmune diabetes in NOD mice. Propolis administration was associated with a delay in diabetes onset in both the low- and high-dose groups, most pronounced in the high-dose group; however, cumulative diabetes incidence at 30 weeks did not differ significantly between groups. This indicates that under the conditions tested, propolis delayed but did not prevent disease onset, and the protective effect should be interpreted as a transient modulation of disease kinetics rather than a disease-modifying or preventive intervention. This delay coincided with several immunological changes during the prediabetic phase, including reduced immune infiltration in the islets of Langerhans, altered distribution of T cells between immune compartments, changes in systemic cytokine levels, and shifts in gut microbiota composition.

The cytokine profile in the high-dose group, characterized by significantly increased IFN-γ and IL-10 alongside a significant reduction in IL-4, is compatible with a Th1-associated shift accompanied by a

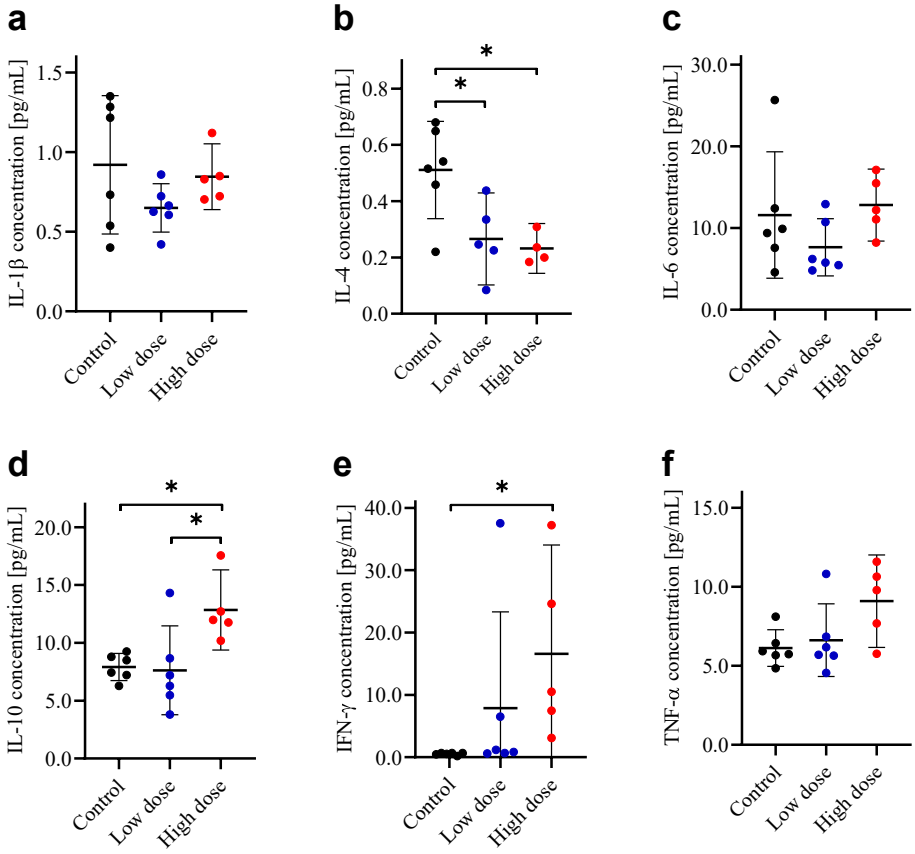

Fig. 5. Serum concentrations of IL-1β (a), IL-4 (b), IL-6 (c), IL-10 (d), IFN-γ (e), and TNF-α (f) in NOD mice in the control, low-dose, and high-dose propolis groups at 14 weeks of age (n = 6 control, 6 low-dose, 5 high-dose, except IL-4: n = 6 control, 5 low-dose, 4 high-dose, due to sample availability). Symbols represent individual mice; lines and error bars indicate mean ± 95% confidence interval for each group. Statistically significant pairwise comparisons are shown. $p < 0.05$*.

regulatory (IL-10) component. At least two interpretations of this shift are worth considering. One possibility is that it reflects a 'regulated Th1-associated profile,' in which the Th1 activity itself is non-pathological and effectively contained by IL-10, consistent with the concurrent reduction in islet infiltration rather than accelerated disease onset. Alternatively, since IFN-γ is a canonical driver of β-cell-directed autoimmunity, the same shift could instead reflect ongoing or enhanced autoimmune activation that the IL-10 response only partially restrains. The data cannot distinguish between these readings, and neither is favored here. Substantial intragroup variability, particularly in IFN-γ levels within the low- and high-dose groups, may reflect individual differences in responsiveness to propolis. Both IL-10 and IL-4 showed significant differences across multiple pairwise comparisons, indicating a consistent and reproducible cytokine shift rather than an isolated or chance finding; the precise magnitude of this shift, however, should be interpreted with appropriate caution given the small group sizes underlying these comparisons.

The cytokine pattern observed is consistent with, but does not establish, engagement of TLR4-NF-κB signaling and IL-12-mediated Th1 differentiation, as direct measurement of TLR4 expression, NF-κB activation, IL-12 signaling, STAT4 activation, or NK-cell activity was not performed in this study. This proposed pathway should therefore be regarded as a mechanistic hypothesis generated from the cytokine data and prior literature. As outlined in the introduction, IL-12-mediated activation of Th1 and NK cell responses could account for the elevated IFN-γ levels observed in the high-dose group. IL-12p70 was included in the multiplex cytokine panel; however, concentrations fell below the assay's lower limit of quantification, consistent with the known low physiological expression of this heterodimer under non-stimulated conditions, precluding reliable quantification and further limiting the ability to test this pathway directly in the present study. TNF-α levels showed a modest, non-significant increase in the high-dose group, which may indicate partial engagement of TLR4-NF-κB signaling without triggering broad inflammatory cascades. The absence of increases in IL-1β and IL-6, together with the known NF-κB-inhibitory activity of several propolis constituents, is consistent with a selective rather than generalized activation pattern. Again, in the absence of direct pathway measurements, this remains an interpretive framework rather than a demonstrated mechanism. Future studies incorporating TLR4/NF-κB reporter assays, IL-12/STAT4 signaling readouts, or NK-cell functional assays would be needed to test this hypothesis directly.

This proposed TLR4-NF-κB/IL-12-mediated Th1 signaling pattern is consistent with previous studies. Zhu et al. demonstrated that supercritical CO_2-extracted propolis activates the TLR4-NF-κB signaling cascade and enhances pro-inflammatory cytokine production in immunosuppressed mice (H. Zhu et al., 2024). Orsatti and Sforcin also found that propolis upregulates TLR4 expression in spleen cells and can protect against stress-induced immunosuppression (Orsatti and Sforcin, 2012). Sy et al. likewise demonstrated that oral propolis administration increased IFN-γ secretion and promoted a Th1-skewed response in an OVA-sensitized asthma model, thereby ameliorating asthma and supporting its role in enhancing cell-mediated immunity (Sy et al., 2006). These studies support the plausibility of the TLR4/IL-12/Th1 hypothesis outlined above, but direct pathway data in the present model are still needed for confirmation.

Flow cytometric analysis provided further insight into the immunemodulatory effects of propolis. In the high-dose group, T cells were redistributed between compartments, with reduced numbers in the pancreatic lymph nodes and increased numbers in the spleen. Given that pancreatic lymph nodes drain the pancreas, this pattern is consistent

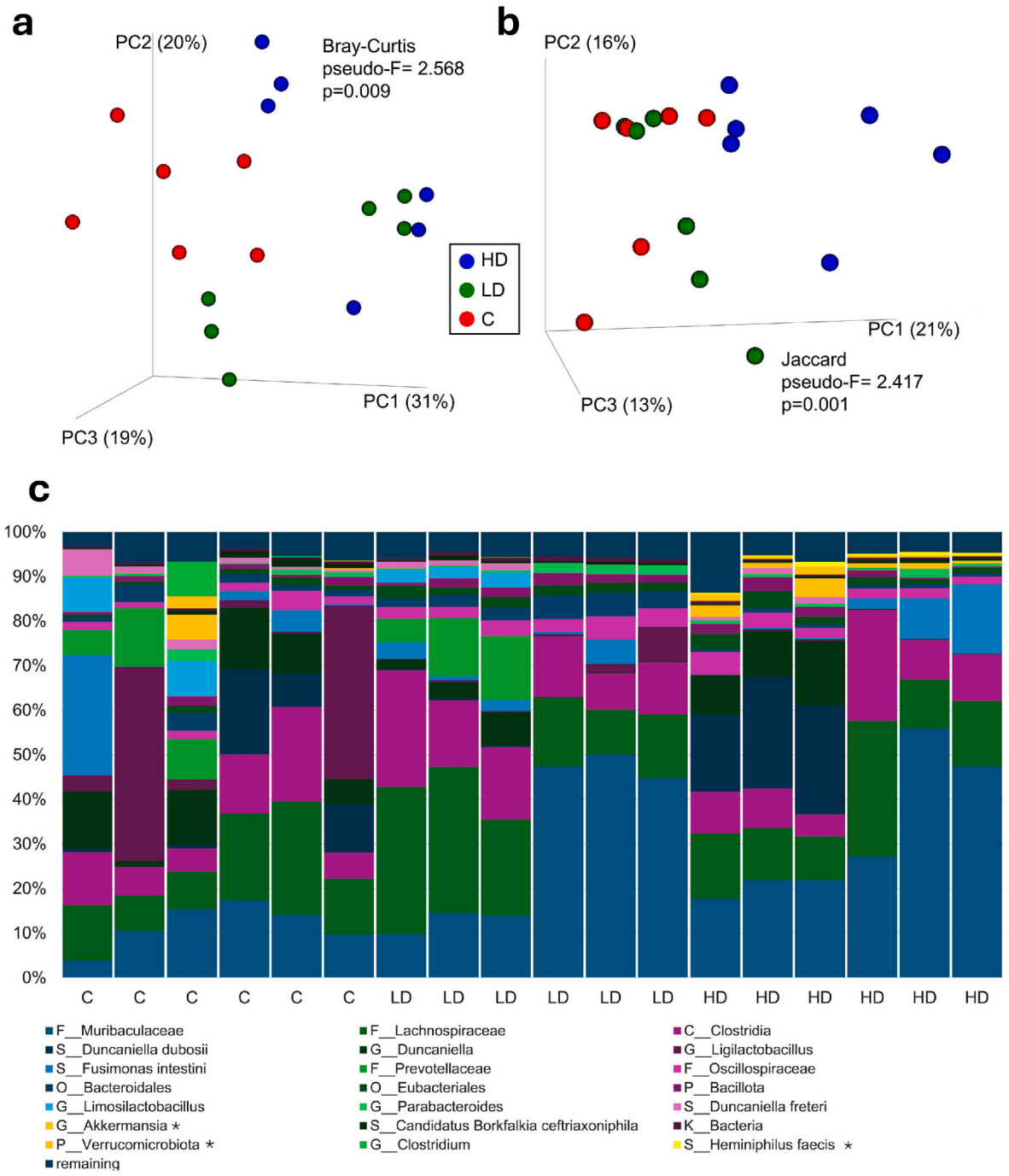

Fig. 6. Gut microbiota composition in female non-obese diabetic (NOD) mice following propolis administration (n = 6 per group). (a) Principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity, with each point representing an individual mouse colored by treatment group. (b) PCoA based on the Jaccard index. (c) Stacked bar plot showing the relative abundances of bacterial taxa in fecal samples from control (C), low-dose propolis (LD), and high-dose propolis (HD) groups at 14 weeks of age. Each bar represents an individual mouse, and taxa are colored by family, genus, or species as indicated in the legend.

with reduced local immune activity and the attenuated insulitis observed in the high-dose group. Notable intragroup variability was observed in T cell counts across all subsets and tissues; nonetheless, the significant, large-effect-size reduction in $CD4^+$ T helper cells in the pancreatic lymph nodes represents a specific and reproducible finding. Although absolute numbers of T cell subsets showed a trend toward change with propolis dose, the relative proportions of these subsets within each tissue did not differ significantly between groups.

Analysis of fecal samples indicated that propolis administration was associated with alterations in gut microbiota composition, particularly *Akkermansia muciniphila*. Because microbiota was assessed at a single time point, without metabolomic or metagenomic functional analyses and without interventional experiments such as fecal microbiota transplantation or antibiotic depletion, these findings should be interpreted as associative rather than mechanistic. Previous studies in NOD mice have demonstrated that manipulating gut microbiota can influence autoimmune diabetes development: early-life vancomycin treatment markedly altered the microbiota, leading to an expansion of *A. muciniphila* and a reduced incidence of diabetes (Hansen et al., 2012). Likewise, direct administration of *A. muciniphila* altered microbial composition, enhanced mucus production, modulated innate immune signaling, and delayed diabetes onset (Hänninen et al., 2018). This

establishes biological plausibility for a microbiota-immune link but does not demonstrate that the compositional shifts observed here are causally involved in the delayed onset observed in the present study. Notably, *Heminiphilus faecis*, a recently described member of the *Muribaculaceae* family, was also significantly enriched in the high-dose propolis group, whereas the *Muribaculaceae* family as a whole showed a non-significant trend toward increased abundance in propolis-treated groups. *H. faecis* has not previously been linked to T1D, and although members of the Muribaculaceae have been associated with gut-restorative effects in metabolic disease models (Y. Zhu et al., 2024), this enrichment is reported here as a novel, hypothesis-generating observation rather than an established contributor to the delayed diabetes phenotype. The gut microbiota data thus represent a genuine, statistically supported compositional shift associated with propolis administration, providing a concrete basis for future mechanistic investigation rather than mechanistic evidence for the immunomodulatory or disease-delaying effects of propolis itself.

Some limitations should be considered when interpreting these findings. First, statistical power was limited as immune profiling was performed in small cohorts (n = 5-6/group) determined by resource and animal availability constraints rather than a formal power calculation, and substantial intragroup variability was observed in several cytokine and flow cytometric measures. Non-significant findings, particularly for TNF-α and T cell subset counts in the pancreatic lymph nodes and spleen, may therefore reflect insufficient power rather than a true absence of effect, and larger cohorts in future studies would help distinguish these possibilities. Second, the immunological and metabolic characterization performed was partial. Diabetes status was determined by blood glucose threshold alone, without glucose or insulin tolerance testing or serum insulin/C-peptide measurement, so a delay in hyperglycemia detection cannot be distinguished from a genuine improvement in glycemic regulation. Similarly, insulitis scoring reflects immune cell infiltration rather than β-cell mass or function directly; islet immunostaining and apoptosis markers, which were not assessed in this study, would be needed to clarify the functional reserve of β-cells, and the conclusion that propolis preserves islet function should therefore be regarded as suggestive rather than directly demonstrated. Flow cytometry was restricted to $CD3^+$, $CD4^+$, $FoxP3^+$, and $CD45^+$ populations and did not include $CD8^+$ T cells, Th17 cells, or antigen-presenting cells, and serum cytokine measurements do not capture local expression within the pancreas or its draining lymph nodes. Finally, diabetes was defined using a blood glucose threshold of >12.0 mmol/L, lower than the >13.9 mmol/L used in some NOD studies (Chen et al., 2020; Boldison et al., 2021), which should be considered when comparing incidence and onset timing across studies.

To our knowledge, this is the first study to evaluate the immunological and disease-associated effects of propolis in the NOD mouse model. The present findings indicate that dietary propolis is reproducibly associated with a delay, though not prevention, of autoimmune diabetes, accompanied by statistically supported changes in islet infiltration, T cell distribution, systemic cytokines, and gut microbiota composition. These associations provide a well-defined starting point for future mechanistic work; however, the specific biological pathways underlying these associations remain to be established. Future work should delineate the underlying cellular and molecular mechanisms, with particular attention to antigen-presenting cells, TLR signaling, and cell trafficking. It will also be important to identify which propolis constituents drive these effects and whether propolis can be combined with other immunomodulatory interventions to enhance prophylactic strategies for T1D.

5. Conclusion

In conclusion, dietary supplementation with propolis was associated with a delay in the onset of autoimmune diabetes in female NOD mice without altering final cumulative incidence. This delay was accompanied by reduced immune infiltration of pancreatic islets, a redistribution of T cells between pancreatic lymph nodes and spleen, and a systemic cytokine shift characterised by increased IFN-γ and IL-10 and reduced IL-4 in the high-dose group. Dietary propolis intake was further associated with reproducible changes in gut microbiota composition, including enrichment of *Akkermansia muciniphila*, previously linked to immune modulation in related contexts, and *Heminiphilus faecis*, a novel finding not previously linked to T1D; whether this microbial shift directly contributes to the observed immunological changes remains to be established.

Taken together, these findings indicate that propolis, a resin long used in traditional medicine for inflammatory and immune-related conditions, is reproducibly associated with dose-dependent changes in immune parameters during the prediabetic stage, along with changes in gut microbiota composition. These results provide a statistically supported preclinical foundation linking propolis's traditional immunomodulatory reputation to measurable biological activity in an autoimmune disease model; the specific mechanisms underlying these associations, however, remain to be established through targeted functional studies. This work therefore provides a solid basis for further investigation of propolis' molecular mechanisms and translational relevance in autoimmune disease.

CRediT authorship contribution statement

Jonas Vind: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft. **Camilla Hartmann Friis Hansen:** Conceptualization, Formal analysis, Methodology, Supervision, Writing – review & editing. **Magni Steintún:** Investigation, Writing – review & editing. **Lukasz Krych:** Formal analysis, Investigation, Writing – review & editing. **Henrik Munch Jørgensen:** Funding acquisition, Supervision, Writing – review & editing. **Julie Christine Antvorskov:** Funding acquisition, Supervision, Writing – review & editing. **Violetta Aru:** Supervision, Writing – review & editing. **Søren Balling Engelsen:** Supervision, Writing – review & editing. **Knud Josefsen:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – review & editing.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2026.122300.

Data availability

Data will be made available on request.

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