

Article

Covalent Arabinoxylans Nanoparticles Enable Oral Insulin Delivery and Gut Microbiota Modulation in Diabetes

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Abstract

Arabinoxylans (AX) are polysaccharides capable of forming covalent gels stable under variations in pH and temperature. They are fermentable by the colonic microbiota, making them appropriate carriers for colon-targeted oral drug delivery, including insulin. This study aimed to fabricate covalent AX nanoparticles loaded with insulin (NPAXI) using a 0.25 (AX/insulin) mass ratio and to evaluate their colon-targeted capacity to improve glycemic control in diabetic rats. In parallel, we assessed gut microbiota modulation as a secondary outcome, derived from the prebiotic fermentation of AX, considered an additional benefit. NPAXI, produced by coaxial electro spraying, displayed a mean diameter of 661 nm, a zeta potential of −31 mV, and high insulin encapsulation efficiency. Bioassay demonstrated that a single oral NPAXI dose restored normoglycemia for 9 h, starting 15 h post-administration. Gut microbiota analysis revealed that while insulin alone increased Lactobacillaceae, it failed to suppress Enterobacteriaceae. NPAXI treatment, however, promoted beneficial taxa such as Muribaculaceae and Prevotellaceae and reduced proinflammatory families like Desulfovibrionaceae and Helicobacteraceae. These microbial shifts paralleled the improved glycemic profile, suggesting a synergistic interaction between AX and insulin in reestablishing gut microbial homeostasis and metabolic regulation. Overall, NPAXI represents a promising strategy for colon-targeted oral insulin delivery, offering additional microbiota-modulating benefits.

Keywords: arabinoxylans; nanoparticles; oral insulin; diabetes; gut microbiota



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1. Introduction

Diabetes is a chronic disease affecting millions worldwide and constitutes a significant public health concern in Mexico. According to the National Health and Nutrition Survey [1], the prevalence of diabetes rose from 14.4% in 2006 to 18.3% in 2022. This high occurrence presents a growing challenge for the healthcare system in terms of patient care and long-term management. Despite advancements in therapeutic interventions, subcutaneous

injections remain the standard method for insulin administration. However, this approach is invasive and often painful, which can lead to poor treatment adherence [2,3].

Additionally, subcutaneous insulin delivery may present limitations such as poor systemic absorption and risk of hypoglycemia, prompting intensive efforts to develop alternative oral delivery systems [4,5]. Multiple animal studies support the potential of colon-targeted oral insulin delivery systems as adjunctive strategies for diabetes management [6–9]. Among natural polysaccharides used for drug encapsulation, arabinoxylans (AX) have arisen as favorable candidates due to their covalent gel-forming capacity and prebiotic, antioxidant, and biocompatible properties [10,11]. In addition, the oral intake of AX has been associated with gut microbiota protection or even beneficial modifications in the microbiota structure [12,13].

The gut microbiota comprises a diverse set of microorganisms residing in the digestive tract, comprising bacteria, archaea, viruses, and eukaryotic microbes. It is estimated that around 100 trillion of these microorganisms reside primarily in the distal colon [14,15]. This microbial community performs essential functions, including digestion and nutrient metabolism, protection against pathogens, and systemic immunomodulation. In healthy individuals, the predominant phyla are Bacteroidetes and Firmicutes. However, its composition can be altered by various factors, such as disease, genetic predisposition, drug use, and diet [16,17]. These alterations, known as dysbiosis, involve an imbalance between commensal symbionts and pathobionts and have been related to the occurrence of diabetes [18,19]. Several studies have shown that dietary interventions with prebiotic fibers can restore microbial balance, increase the generation of short-chain fatty acids, and improve glycemic control [20–23]. Due to their fermentability and their capability to specifically stimulate the development of favorable bacteria, AX is emerging as a promising dietary component for the modulation of intestinal microbiota in patients with diabetes, with the potential to generate synergistic effects when used as colon-targeted insulin delivery systems [24–26].

AX are non-starch polysaccharides present in cereals, such as maize, rye, rice, sorghum, and wheat, as well as in their by-products. The study of AX and its properties have regained interest worldwide, considering that they have been recognized as safe ingredients (GRAS) [27]. Chemically, AX consist of a β -(1-4)-linked xylose main backbone beside side α -L-arabinoses linked through α -(1-3) or α -(1-2) glycosidic bonds [28,29]. Arabinose residues might be esterified with ferulic acid (FA) via O-5 position, forming ferulated AX [10,30]. These AX resist enzymatic hydrolysis in the superior gastrointestinal tract (GIT), reaching the colon region intact, where they are fermented through the resident microbiota, producing short-chain fatty acids and promoting the growth of beneficial bacteria [31,32].

The esterified FA present in AX enables oxidative cross-linking into stable covalent gels via enzymatic (e.g., laccase or peroxidase) or chemical agents (e.g., ferric chloride or ammonium persulfate), resulting in dimeric and trimeric structures that link AX chains into a three-dimensional network [10]. These gels are pH-stable, porous, hydrophilic, and fermentable in the colon, making them ideal for encapsulating bioactive molecules, such as insulin [33]. Previous reports have demonstrated the potential of AX-based beads and microspheres as insulin carriers [33–35].

Our research group was the first to develop AX-based nanoparticles incorporating insulin [36], with a patent in Mexico and internationally [37]. Nevertheless, in those preceding studies, the AX used presented very high FA acid content (8–12 $\mu\text{g}/\text{mg}$), which impaired fermentability and delayed hypoglycemic effects up to 24 h post-administration in diabetic rats [36]. One promising source of AX is dried distillers' grains with soluble (DDGS), a by-product of the maize bioethanol industry, which yields ferulated AX suitable

for nanoparticle formation [38,39]. Nonetheless, before the present study, no research had explored the use of DDGS-derived AX for colon-targeted insulin delivery. This work aimed to fabricate covalently cross-linked insulin-loaded AX nanoparticles (NPAXI) using AX extracted from DDGS at a 0.25 (AX/insulin) mass ratio and to evaluate their colon-targeted capacity to improve glycemic control in diabetic rats following oral administration. In parallel, we assessed gut microbiota modulation as a secondary outcome, derived from AX fermentation, considered an additional benefit.

2. Materials and Methods

AX were isolated from DDGS and characterized as previously reported [40]. The AX exhibited an arabinose-to-xylose branching ratio of 1.16 and a FA content of 7.30 $\mu\text{g}/\text{mg}$. Laccase from *Trametes versicolor* (E.C. 1.10.3.2) was used as the cross-linking agent. Recombinant human insulin and other analytical-grade reagents were acquired from Sigma Chemical Co. (St. Louis, MO, USA). For subcutaneous administration, a commercial isophane insulin suspension (NPH 100 IU/mL, Humulin[®], Eli Lilly and Company, Indianapolis, IN, USA) was used.

2.1. Cross-Linking Process

Gelling kinetics of AX dispersions with and without insulin were monitored in the presence of laccase (1.675 nkat/mg AX) using a dynamic oscillatory rheometer (Discovery HR-2, TA Instruments, New Castle, DE, USA). The insulin-to-AX ratio was 0.25 (w/w). Storage modulus (G') and loss modulus (G'') values were recorded for 1.5 h at 25 °C, with a frequency of 0.25 Hz and 5% strain. After gelation, a frequency sweep from 0.01 to 10 Hz at 5% strain was conducted [33].

2.2. Phenolic Acid Analysis

The content of FA, FA dimers (di-FA), and FA trimers (tri-FA) in the gels was quantified by high-performance liquid chromatography (HPLC) using an Alltima C18 column (250 $\times$ 4.6 mm, Alltech Associates, Deerfield, IL, USA) with a diode-array detector (Waters 996, Millipore Co., Milford, MA, USA) [41].

2.3. Nanoparticle Fabrication

Covalent AX nanoparticles (NPAX) and insulin-loaded AX nanoparticles (NPAXI) were fabricated using a coaxial electrospray system (Profector Life Sciences, Dublin, Ireland) as described by De Anda-Flores et al. [39]. The core solution (1% w/v AX with 0.25% insulin) was loaded into the inner needle, and the laccase solution (1.675 nkat/mg AX) was placed in the external needle. Both solutions were delivered at a flow rate of 1 mL/h under a voltage of 10 kV. Nanoparticles were collected in mineral oil under continuous stirring (500 rpm) at 25 °C for 24 h.

2.4. Characterization of Nanoparticles

Size distribution, polydispersity index, and zeta potential of NPAX and NPAXI were determined by dynamic light scattering (DLS) using a Möbiuζ instrument (Wyatt Technology Corp., Santa Barbara, CA, USA) at a wavelength of 532 nm and detection angle of 163.5°. Particle morphology was assessed by transmission electron microscopy (TEM) using a JEM 2010F microscope (JEOL, Tokyo, Japan) [39]. Insulin encapsulation efficiency was determined by quantifying the unencapsulated insulin using the Bradford assay [42]. Insulin encapsulation efficiency was determined by quantifying the non-encapsulated (free) and total insulin content using the Bradford assay [42]. The encapsulated insulin was calculated by subtracting the free insulin from the total insulin. A bovine serum albumin (BSA) standard curve was used (0.1 to 1.4 mg/mL). 100 μL of free or total insulin was mixed with

3 mL of Bradford reagent and gently vortexed. Samples were incubated for 15 min at room temperature, transferred to UV-VIS cuvettes, and the absorbance was recorded at 595 nm using a Cary 60 UV-VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). All measurements were performed in triplicate and confirmed across independent batches.

2.5. Bioassay

Male Wistar rats (200–250 g) were used in this study, provided by the Universidad de Sonora. Animals were acclimatized for 15 days with free access to water and a standard diet (Formulab Diet 5008, LabDiet, St. Louis, MO, USA). Environmental conditions were maintained at 23 ± 2 °C, with 12-h light/dark cycles and $60 \pm 5\%$ humidity. All procedures were approved by the Ethics Committee of the Centro de Investigación en Alimentación y Desarrollo (CIAD) for studies involving animals (CE/001/2015, 14 April 2015) and followed NOM-062-ZOO-1999 guidelines for laboratory animal care [43].

Diabetes was induced in rats by intraperitoneal injection of streptozotocin (STZ) (55 mg/kg body weight). On day 3 post-induction, rats with blood glucose values over 250 mg/dL (tail vein employing an Accu-Chek Performa glucometer, Roche, Mannheim, Germany) were considered diabetic [44,45].

After a 12-h fasting period, five experimental groups of three rats each were randomly formed based on glucose levels: (1) healthy controls (no intraperitoneal injection), (2) untreated diabetic rats, (3) diabetic rats with subcutaneous insulin (2 IU/kg), (4) diabetic rats treated with NPAX, and (5) diabetic rats treated with NPAXI (50 IU insulin/kg body weight). Mature NPAX and NPAXI were washed with ethanol:water (30:70, 50:50, 70:30 *v/v*), vortexed for 1 min, and centrifuged ($2000 \times g$, 10 min, 20 °C). The supernatant was removed, and the nanoparticles were resuspended in ultrapure water. Washed nanoparticles were incorporated in 1 mL of Milli-Q water and administered orally via stainless-steel gavage. Blood glucose levels were monitored from 0 to 24 h post-administration using a glucometer.

2.6. Gut Microbiota Analysis

2.6.1. DNA Extraction and Sequencing

Fecal samples obtained at the end of the trial were homogenized using a FastPrep 5G (MP Biomedicals, Solon, OH, USA) for a final sample size of $n = 3$ per treatment. DNA from fecal samples was extracted according to the instructions of the manufacturer using the FastDNA[®] SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). The construction of libraries was performed following the 16S Metagenomic Sequencing Library guide with modifications. PCR amplification of 16S rRNA V3 regions was performed using the 338F/533R bacterial primer pair (338F: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACTCC TACGGGAGGCAGCAG-3' and 533R: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGA CAGTTACCGCGGCTGCTGGCAC-3'), with a 24 μ L reaction volume containing 10 μ L Platinum[®] PCR SuperMix (Invitrogen, Carlsbad, CA, USA), 0.2 M of each forward and reverse primer, 6 μ L nuclease-free water and 1 μ L of DNA template. The following PCR conditions were used: an initial denaturation at 95 °C for 30 s, followed by 30 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. A final extension was performed at 72 °C for 7 min. The first-stage PCR was cleaned using magnetic beads, and samples were visualized on a 1.5% agarose gel and quantified using the Qubit 3.0 fluorometer (Invitrogen, Carlsbad, CA, USA). A second PCR to index the amplicons was performed with Platinum[®] PCR SuperMix to add Nextera DNA indexes and sample multiplexing. The resulting amplicons were cleaned again using magnetic beads and quantified using the Qubit 3.0 fluorometer (Invitrogen). Before pooling the libraries, the PCR products were normalized to 4 nM and then pooled by adding 10 μ L of each library. Libraries were diluted at 1 nM, denatured with 0.1 N NaOH, and combined with 5% (*v/v*) denatured 4 nM PhiX

as a control. The sequencing was performed in an Illumina MiniSeq platform, using the MiniSeq Reagent Kit v2, yielding 2×150 bp paired-end reads. Raw reads were uploaded to NCBI, BioProject SRA accession: SUB15369900.

2.6.2. Sequence Data Preprocessing and Diversity Analysis

An initial set of 750,263 raw reads of the V3 16S rRNA hyper-variable region was imported and processed using Quantitative Insights Into Microbial Ecology 2 (QIIME2), version 2023.5 [46]. Raw sequences were preprocessed using the plugin DADA2 to apply the denoise-16S method to the sequences [47]. Reads were trimmed at the 1-pb position and truncated at the 150-bp position, according to the median quality score of $<Q30$, and the detected chimeric sequences were removed. Then, 506,423 filtered reads from 15 samples were considered for further analysis. After the sequence quality control step, 428 amplicon sequence variants (ASVs) were assigned to taxonomy using a full-length, pre-trained classifier (SILVA_138) with OTUs clustered at 99% identity. A rooted phylogenetic tree was constructed to measure phylogenetic diversity (Faith and UniFrac). ASVs were aligned with MAFFT [48], and the resulting alignment was used to build a phylogenetic tree with FastTree software (version 2.1.11) [49] by using the align-to-tree-might-fast tree pipeline from the q2-phylogeny plugin. Before diversity analysis, library samples were rarefied to 29,113 sequences. The sequencing depth was evaluated through rarefaction curves, which reached a clear asymptote for all samples, indicating sufficient coverage and even sequencing effort across treatments (Figure S1).

The statistical significance of diversity analysis was estimated and performed with the online software based on R MicrobiomeAnalyst (<https://www.microbiomeanalyst.ca>, accessed on 26 May 2025) [50,51]. Alpha diversity profiling was estimated using the diversity measures Chao1, Shannon, Simpson, and Fisher at the feature level. The Kruskal-Wallis statistical test ($p < 0.05$) was used to estimate differences. Beta diversity was calculated to estimate differences between pairs of intestinal microbial communities in rats. Distance matrices were calculated at the feature level using the Bray–Curtis dissimilarity, Jaccard, Weighted, and Unweighted UniFrac distances. Distance matrices were visualized using the 2D ordination plot. A pairwise comparison of distance matrices was performed using the PERMANOVA (Permutational Multivariate Analysis of Variance) statistical analysis.

2.7. Statistical Analysis

Results were reported as mean $\pm$ standard deviation. Student's *t*-test and Mann-Whitney U test were used for group comparisons, with significance set at $p < 0.05$.

3. Results and Discussion

3.1. Cross-Linking Process

To evaluate the gelation capacity of the AX used and confirm that insulin incorporation did not inhibit cross-linking, the gelation kinetics of 1% (*w/v*) AX dispersions with and without insulin were recorded (Figure 1a). Both formulations showed a continuous increase in storage modulus (G'), indicating progressive gel formation, with G'' remaining lower, characteristic of solid-like behavior. The gelation time (*tg*), defined as the time at which $\tan \delta = 1$ (G''/G'), occurred at approximately 2 min for AX and 4 min for AX-insulin dispersions, consistent with previous reports using maize AX [36,52].

At the end of the kinetics, AX-insulin gels exhibited a higher G' (344 Pa) compared to AX-only gels (224 Pa). This increased elasticity has been associated with polymer phase separation and has been reported in similar insulin-containing AX gels [33]. Mechanical spectra (Figure 1b) demonstrated that G' remained constant across the frequency range,

while G'' values were lower and frequency-dependent, typical behavior of covalently cross-linked hydrogels [53].

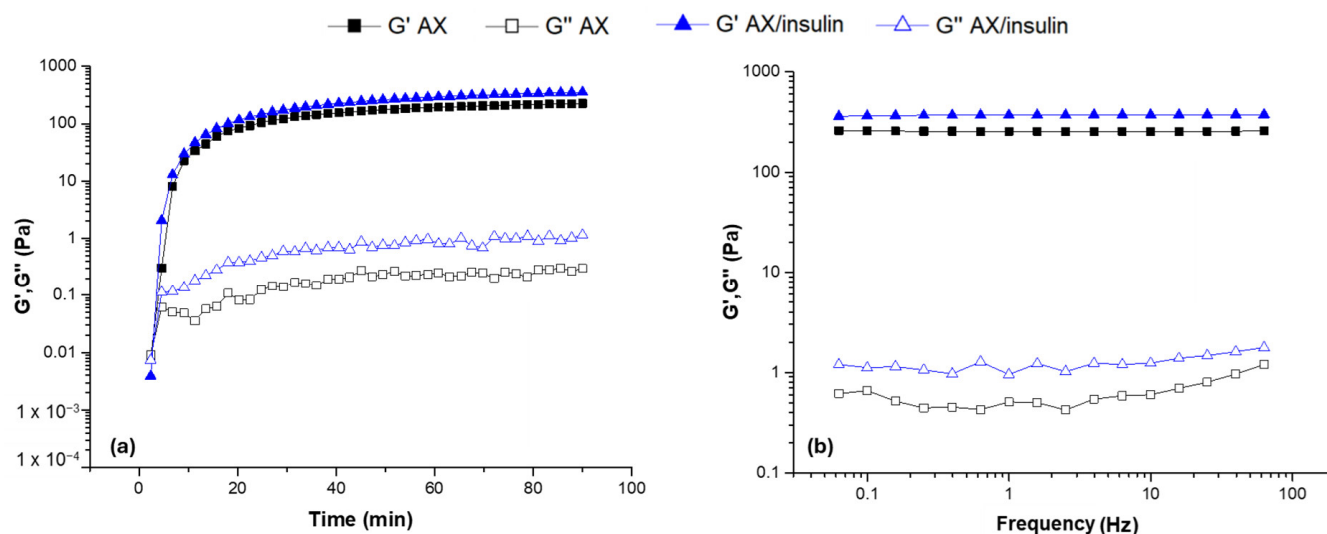


Figure 1. (a) Gelation kinetics of 1% (*w/v*) AX dispersions with and without insulin induced with laccase at 0.25 Hz and 5% strain; (b) Mechanical spectra of AX gels with and without insulin measured at 5% strain. Samples were analyzed at 25 °C.

3.2. Phenolic Acid Analysis

The HPLC analysis of the final gels confirmed the generation of FA dimers (di-FA) and trimers (tri-FA), supporting the creation of a covalently cross-linked network capable of retaining insulin. Isomeric forms such as 8-5', 8-O-4', and 5-5' di-FA were detected, with 8-5' being the most abundant (74%) in both formulations (Table 1). The total content of di-FA and tri-FA was significantly higher in AX-insulin gels, correlating with the increased G' value. It has been suggested that AX-insulin phase separation promotes local areas of denser cross-linking [33].

Table 1. Covalent cross-linking content in AX and AX-insulin gels.

Cross-Linking Structure	Content (μg/mg)	
	NPAX	NPAXI
Dimers of ferulic acid isomers (di-FA)		
8-5'	1.10 ± 0.12 ^a	1.39 ± 0.08 ^a
8-O-4'	0.22 ± 0.04 ^a	0.28 ± 0.03 ^a
5-5'	0.17 ± 0.03 ^a	0.24 ± 0.04 ^a
Total of di-FA	1.49 ± 0.32 ^b	1.91 ± 0.05 ^a
Trimer of ferulic acid (tri-FA)	0.31 ± 0.07 ^b	0.42 ± 0.04 ^a

Values are presented as means ± standard deviations of triplicates (n = 3). Different letters between columns indicate significant differences ($p \leq 0.05$).

3.3. Nanoparticle Fabrication and Characterization

Coaxial electrospraying enabled the successful production of NPAX and NPAXI at low polymer concentrations (1% *w/v*), with rapid gelation (2–4 min). Transmission electron microscopy (TEM) confirmed the spherical morphology and smooth surfaces of the nanoparticles (Figure 2). The resulting particles showed minimal aggregation. Insulin encapsulation efficiency in NPAXI reached 95%, indicating high retention within the nanoparticles.

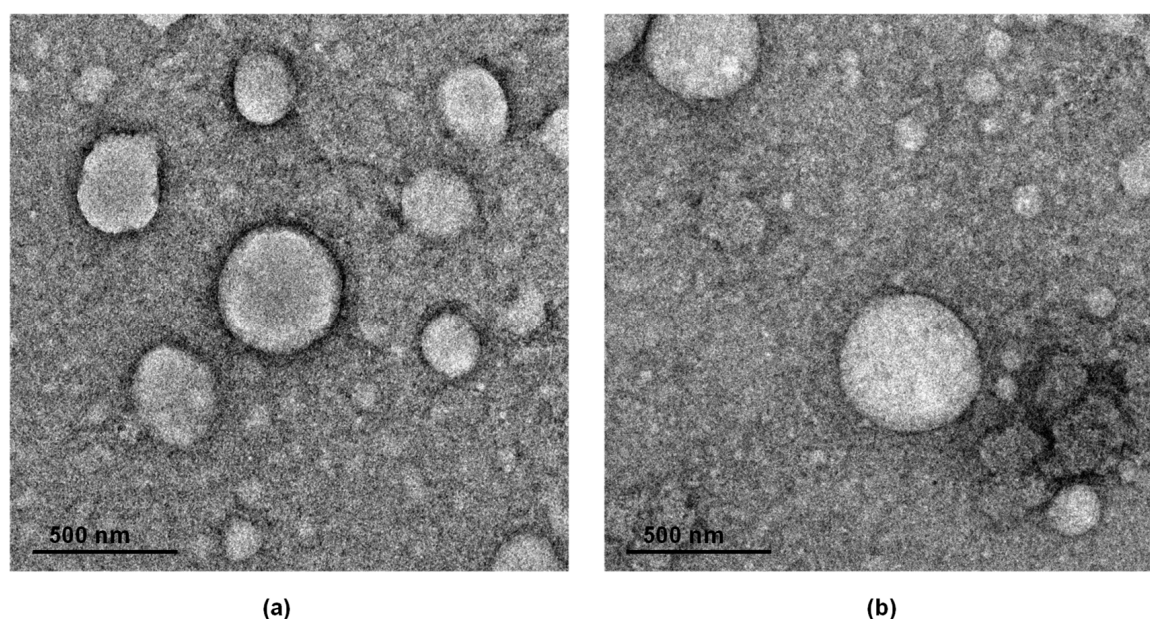


Figure 2. Transmission electron microscopy images of (a) NPAX and (b) NPAXI. Negative staining with 1% (*w/v*) phosphotungstic acid.

Particle size and zeta potential results are presented in Table 2. NPAX and NPAXI exhibited average diameters of 731 nm and 661 nm, respectively, and polydispersity index (PDI) ranges of 12–198% and 12–220%, respectively, for NPAX and NPAXI. These results align with previous work using AX from maize pericarp [36]. Both NPAX and NPAXI showed negative zeta potential (~ -31 mV), with no statistically significant differences between them.

Table 2. Size distribution, polydispersity, and zeta potential of NPAX and NPAXI determined by dynamic light scattering.

Material	Size Range (nm)	Polydispersity Index Range (%)	Zeta Potential (mV)
NPAX	346–1115	12–198	-41 ± 5^a
NPAXI	191–1131	12–220	-31 ± 7^a

Values are means and standard deviation of three replicates. Equal letters within the same column indicate no significant differences ($p \leq 0.05$).

From a pharmaceutical perspective, positively charged nanoparticles can enhance cellular uptake via interactions with negatively charged cell membranes [54]. However, the negative zeta potential observed in NPAXI could reduce cell internalization in the upper GIT, limiting premature absorption and promoting insulin delivery to the colon. Once in the colon, fermentation by local microbiota enables insulin release, which can then be absorbed via transcytosis [55]. Regarding the influence of nanoparticle size on gastrointestinal transit and release kinetics, intestinal mucus pores are typically in the 100–200 nm range while NPAX and NPAXI present average diameters of 731 nm and 661 nm, respectively, favoring to stay within the gastrointestinal lumen and resist absorption or rapid clearance by the mucus layer in the upper GIT, thus facilitating transit to the colon [56]. Concerning the high PDI registered in NPAX and NPAXI, for a colon-delivery mechanism, it is not necessarily a drawback; indeed, it may be advantageous by preventing the entire dose from being rapidly degraded, thereby favoring a sustained release [57].

3.4. Bioassay

The bioassay results are shown in Figure 3. Healthy (normoglycemic) rats maintained fasting blood glucose levels between 70 and 150 mg/dL throughout the experiment.

STZ-treated rats exhibited glucose levels greater than 250 mg/dL, along with polydipsia and polyuria, confirming the induction of diabetes [58,59]. During fasting, a mild drop in glucose was observed in all groups, followed by a rebound at 24 h, likely due to hepatic glycogen mobilization, a known response in rodents after prolonged fasting [60,61].

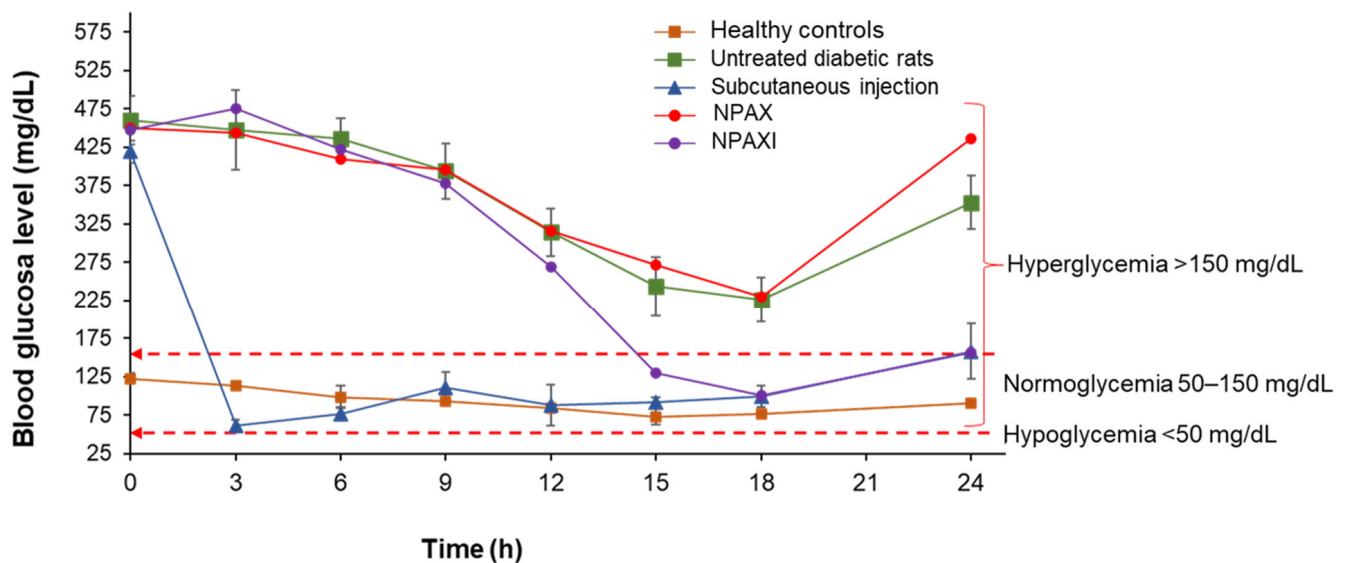


Figure 3. Blood glucose levels of healthy controls, untreated diabetic rats, diabetic rats treated with intermediate-acting subcutaneous insulin (2 IU/kg animal), and diabetic rats treated with NPAX and NPAXI (50 IU/kg animal).

Untreated diabetic rats remained hyperglycemic (with blood glucose levels greater than 150 mg/dL) throughout the study. Rats receiving subcutaneous insulin-maintained glucose levels within the physiological fasting range from 3 to 18 h post-injection, returning to hyperglycemia (158 mg/dL) by 24 h, consistent with the known pharmacokinetics of intermediate-acting insulin [62,63]. NPAXI-treated rats exhibited a delayed glucose-lowering response, reaching a value within the normoglycemic range (50–150 mg/dL) at the 15-h time point after oral administration, consistent with insulin release triggered by gastrointestinal transit and colonic fermentation; this effect was sustained for ~9 h. Notably, the NPAXI effect observed here (15 h) was earlier than in previous AX-based nanoparticles (24 h) [36], likely due to the enhanced fermentability of DDGS-derived AX [38].

NPAXI produced a glucose-lowering effect in diabetic rats for up to ~9 h following a single oral dose, with a delayed onset consistent with colonic fermentation-dependent release. After 15, 18, and 24 h of NPAXI administration, glucose levels in diabetic rats were maintained within the normoglycemic range (50–150 mg/dL). These findings confirm that DDGS-derived AX covalent nanoparticles enable oral insulin delivery targeted to the colon. The proposed mechanism involves oral administration of NPAXI, followed by colonic fermentation, insulin release, and absorption. A hypothetical administration schedule could involve dosing at 5:00 PM to ensure circulating insulin by 8:00 AM the following day, thereby eliminating the need for a pre-breakfast subcutaneous injection. Nevertheless, complementary subcutaneous insulin may be necessary at the beginning of NPAXI treatment, depending on several patient characteristics, such as age, diet, activity levels, and colonic microbiota, among others. Future clinical trials are necessary to explore this strategy.

3.5. Gut Microbiota Analysis

The diversity of gut microbiota compared to that of healthy organisms was influenced by both the diabetic condition and the treatments. Healthy rats exhibited higher diversity,

as measured by the Chao1, Fisher, and Simpson indexes, compared with the treated groups (Insulin, NPAX, and NPAXI). Diabetic rats exhibited lower richness (Chao1 and Fisher) but increased evenness (Shannon and Simpson) (Figure 4, Table 3), indicating a simplified yet more evenly distributed microbial community, likely reflecting the selective pressure imposed by hyperglycemia and inflammation.

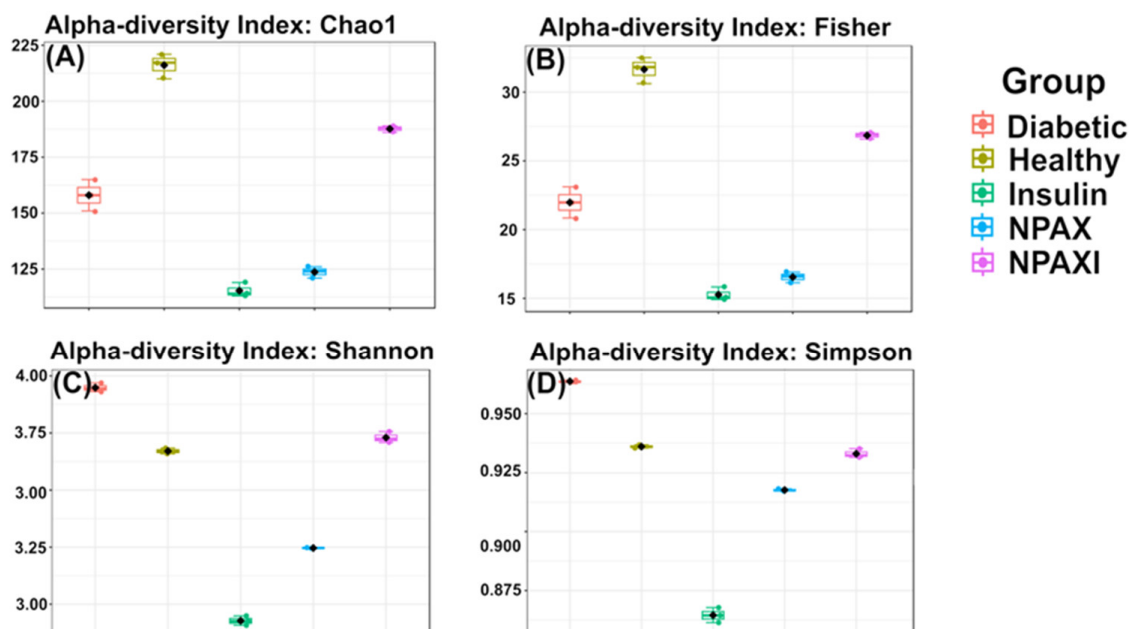


Figure 4. Alpha diversity indices of intestinal microbiota in rats estimated as Chao1 (A), Fisher (B), Shannon (C), and Simpson (D). Alpha diversity analyses were assessed at the feature level.

Table 3. Alpha diversity indexes pairwise comparison. *p*-value significance was estimated by using the Kruskal-Wallis statistical method among treatments.

Pair Comparison	Chao1	Fisher	Shannon	Simpson
Healthy vs. Diabetic	4.628×10^{-4}	4.0396×10^{-4}	1.0427×10^{-4}	4.0617×10^{-6}
Healthy vs. Insulin	7.0322×10^{-5}	1.2223×10^{-4}	6.3367×10^{-6}	3.9715×10^{-4}
Healthy vs. NPAX	7.0322×10^{-5}	3.1141×10^{-4}	2.5006×10^{-4}	9.646×10^{-6}
Healthy vs. NPAXI	7.0322×10^{-5}	0.0091612	0.034011	0.10046
Diabetic vs. Insulin	0.0031449	0.0091612	4.3355×10^{-7}	2.774×10^{-4}
Diabetic vs. NPAX	0.0075845	0.0091612	2.4175×10^{-4}	2.8435×10^{-8}
Diabetic vs. NPAXI	0.014702	0.0091612	3.5553×10^{-4}	8.4495×10^{-4}
Insulin vs. NPAX	0.02644	0.026195	0.0013696	9.6871×10^{-4}
Insulin vs. NPAXI	7.3359×10^{-5}	4.5554×10^{-5}	2.2479×10^{-6}	3.2062×10^{-5}
NPAX vs. NPAXI	1.8496×10^{-5}	1.1479×10^{-5}	8.2555×10^{-4}	0.0035253

At the phylum level, diabetic rats exhibited an altered gut microbiota profile with increased Bacteroidetes (29%) and Proteobacteria (5.5%), and a severe reduction in Actinobacteria (0.08%), compared to healthy controls with higher Actinobacteria (16.3%) and Firmicutes (75.7%) (Figure 4). Insulin treatment partially restored this balance by increasing Actinobacteria (9.71%) and maintaining Firmicutes at 76.93%, suggesting a moderate reversion of dysbiosis. NPAX treatment alone was less effective in restoring Actinobacteria while causing a marked increase in Proteobacteria (11.65%). The NPAXI group exhibited further increases in Bacteroidetes (29.84%) and Proteobacteria (22.17%), accompanied by a decrease in Firmicutes (47.55%), indicating complex microbial shifts likely influenced by both insulin and AX fermentation. This could be associated with the conditions generated

by insulin, probably regulating hyperglycemia, oxidative stress, and intestinal motility and secretions [64,65].

Importantly, all treatments prevented the expansion of Epsilonbacteraeota, a phylum associated with gut barrier dysfunction and inflammation, indicating a protective effect on gut integrity [66,67].

The effect of disease and treatments was reflected in changes to the overall microbiota profile, as shown by all beta diversity analyses (Figure 5), which were also translated into significant changes at the family level (Figures 6 and 7). In this regard, insulin alone increased Lactobacillaceae (38.25%) and Atopobiaceae (0.69%), two families associated with healthy microbiota in rodents [68,69], while failing to reduce Enterobacteriaceae (3.72%), a family associated with inflammation and microbiota imbalance [70]. NPAX treatment increased beneficial Lachnospiraceae (43.44%) but also promoted Enterobacteriaceae (11.16%). The most favorable microbial profile was observed in the NPAXI group, with enrichment of fermentative taxa such as Muribaculaceae (10.97%) and Prevotellaceae (9.75%), and reductions in pro-inflammatory families like Desulfovibrionaceae and Helicobacteraceae [71]. These compositional changes indicate enhanced microbial metabolic function and a reduced inflammatory tone in the gut.

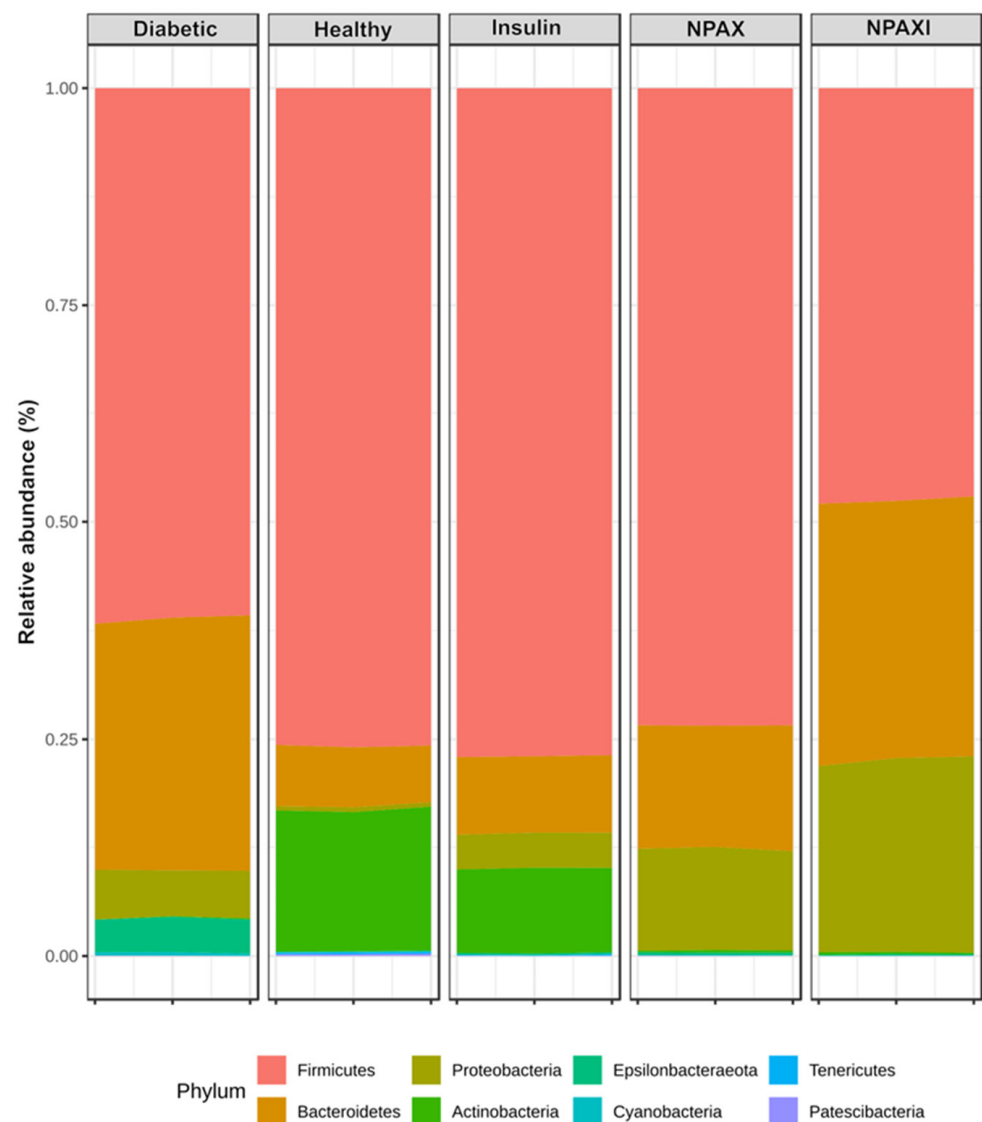


Figure 5. Stacked bar plot representing the bacterial relative abundance at the phylum level of the rat intestinal microbiota.

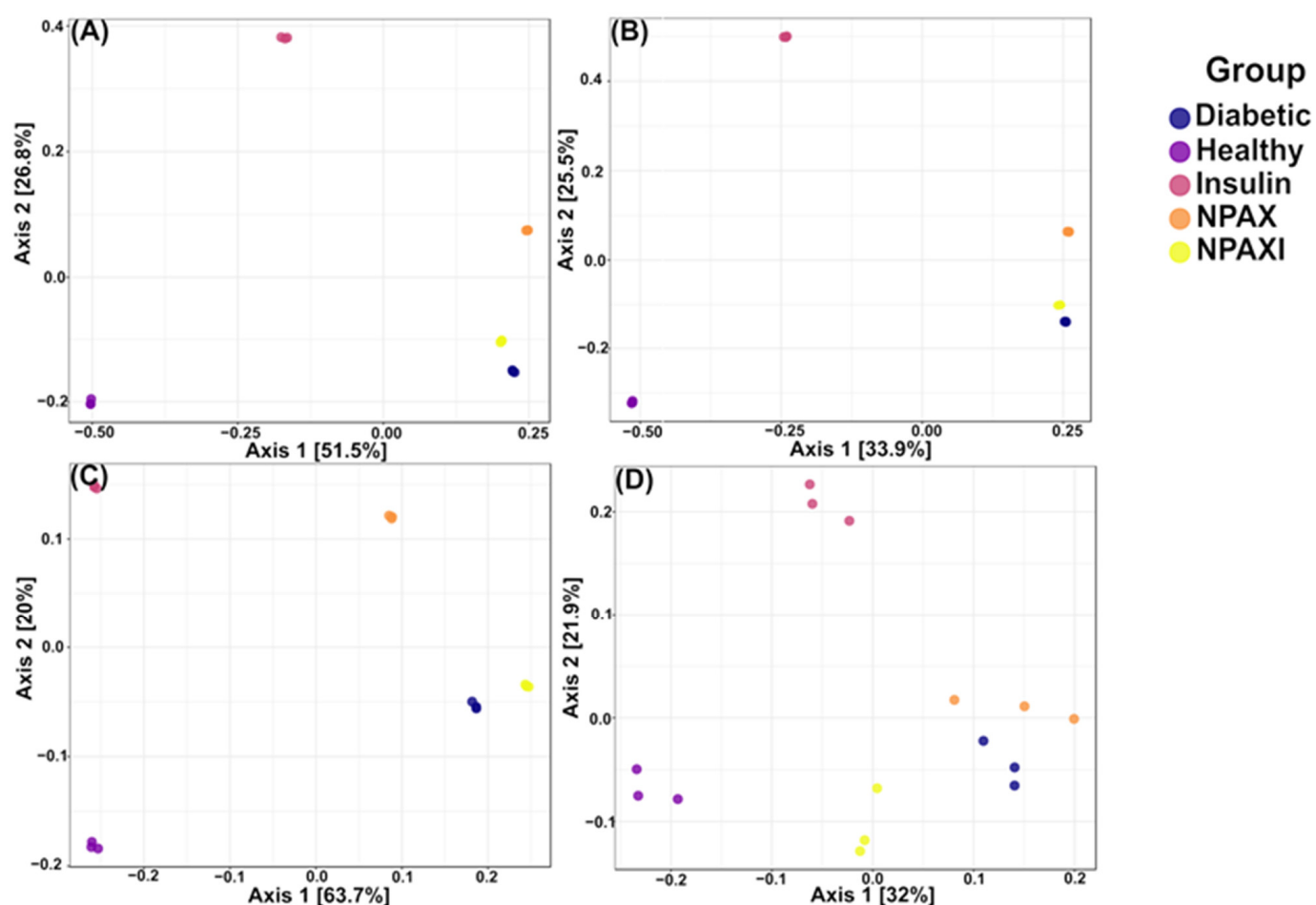


Figure 6. The 2D ordination plots show the β diversity (qualitative and quantitative (dis)similarities) between rat gut microbiota samples. Matrices were estimated by the Bray-Curtis distance (A), Jaccard (B), Weighted Unifrac (C), and Unweighted Unifrac (D) ($p < 0.05$) indices.

Although the observed microbial shifts parallel the metabolic improvements, the present findings could also be interpreted as associative, not purely mechanistic. The microbiota analysis relied on 16S rRNA amplicon sequencing, which provides compositional but not functional resolution. In particular, the production of short-chain fatty acids (SCFAs), a key metabolic output linking microbiota activity to glycemic control, was not directly quantified. Furthermore, given that AX contain esterified FA residues capable of oxidation and radical scavenging, it is plausible that FA monomers or crosslinking dimers exerted direct antioxidant or microbiota-modulating effects independent of insulin. Future work integrating metabolomic assays and targeted SCFA quantification will be required to confirm these functional interactions and clarify the contribution of FA-derived structures to the overall bioactivity of NPAXI.

Interestingly, these microbiota shifts paralleled the glycemic responses observed in vivo. The delayed onset and prolonged duration of normoglycemia in NPAXI-treated rats coincided with an increased abundance of SCFA-producing and fiber-degrading bacteria, which are known to enhance insulin sensitivity and glucose metabolism. The reduction of pro-inflammatory taxa further supports the hypothesis that microbiota modulation contributed to improved systemic glycemic control. Therefore, the hypoglycemic effect of NPAXI appears to result not only from colonic insulin release but also from microbiota-mediated enhancement of intestinal and metabolic homeostasis.

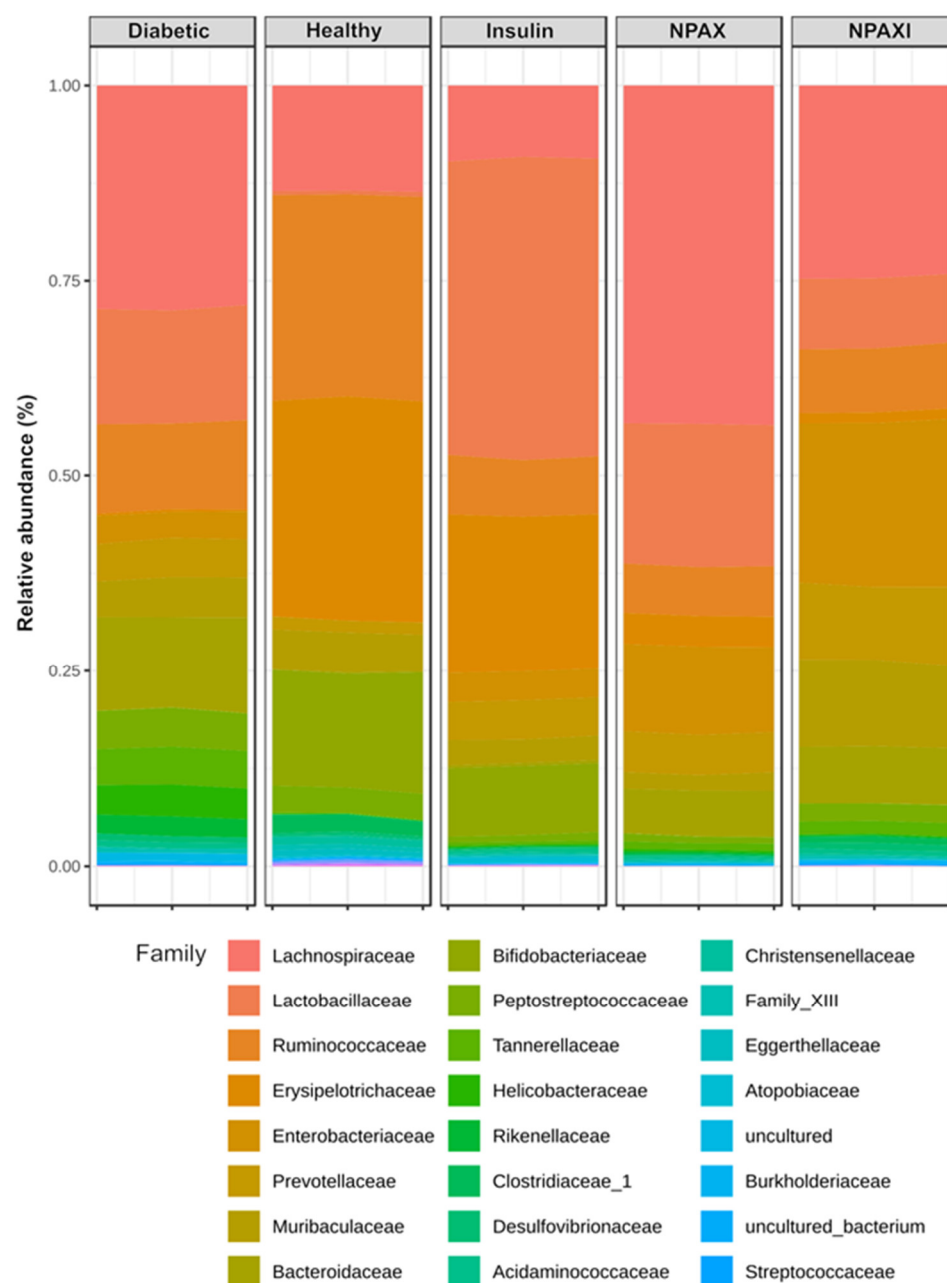


Figure 7. Stacked bar plot representing the bacterial relative abundance at the family level of the rat intestinal microbiota.

4. Conclusions

This study demonstrates that covalent nanoparticles loaded with insulin (NPAXI), fabricated from DDGS-derived AX, represent an encouraging strategy for colon-targeted oral insulin delivery. NPAXI effectively restored normoglycemia in diabetic rats for up to 9 h following a single oral dose, with a delayed onset consistent with colonic fermentation-dependent release. Importantly, NPAXI not only delivered insulin efficiently but also favorably modulated the gut microbiota. The treatment enriched fermentative and health-associated bacterial families, such as Muribaculaceae and Prevotellaceae, while reducing pro-inflammatory taxa, including Desulfovibrionaceae and Helicobacteraceae. These microbiota changes likely contributed to improved metabolic outcomes, highlighting the dual therapeutic action of NPAXI through both pharmacological insulin delivery and microbiota-mediated support of intestinal and systemic homeostasis. Together, these results confirm

the capability of NPAXI as a multifunctional platform for diabetes management and justify further preclinical and clinical evaluation.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/polysaccharides7010003/s1>, Figure S1: Rarefaction curves for all treatments according to their sequence, sample size and species richness.

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