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Enzymatic synthesis of β -galactosylated xylitol derivatives modulates gut microbiota and improves obesity-related metabolic parameters in mice†

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Obesity and its associated metabolic disorders are major global health concerns, highlighting the need for novel dietary interventions. Xylitol, a polyol widely used as a sugar substitute, has shown metabolic benefits beyond its sweetening properties. However, the potential physiological effects of its enzymatically modified derivatives, particularly β -galactosylated xylitol (XylGal), remain largely unexplored. In this study, we evaluated the impact of XylGal and unmodified xylitol (Xyl) on metabolic health and gut microbiota composition in a murine model of diet-induced obesity. Lean and obese C57BL/6J mice received daily doses of Xyl (50 mg kg⁻¹) or XylGal (50 and 100 mg kg⁻¹) for seven weeks. Our findings indicate that both Xyl and XylGal significantly reduced body weight gain, adipose tissue accumulation, and liver weight in obese mice, without affecting food intake. Additionally, Xyl and XylGal modulated glucose homeostasis, with Xyl-treated mice exhibiting improved glucose tolerance. A significant reduction in inflammatory cytokine expression (TNF- α , IL-1 β) in abdominal fat was observed, suggesting decreased macrophage infiltration and attenuation of obesity-induced inflammation. High-throughput sequencing of 16S rRNA revealed that both compounds promoted beneficial bacterial genera, including *Lachnospiraceae* NK4A136 and *Eubacterium xylanophilum*, while reducing potentially obesity-associated taxa such as *Blautia* and *Colidextribacter*. These results suggest that XylGal and Xyl exert prebiotic effects that contribute to their metabolic benefits. Our study provides new insights into the potential of these compounds as functional ingredients for obesity management and metabolic health improvement.

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Introduction

Obesity and its associated metabolic disorders represent a major global health concern, driving the need for innovative

dietary strategies to mitigate their impact.^{1,2} Obesity is a multifactorial disease characterized by excessive fat accumulation and associated with numerous metabolic disorders, including type II diabetes, cardiovascular diseases, and non-alcoholic fatty liver disease. The prevalence of obesity has risen dramatically worldwide, in large part due to sedentary lifestyles and significant changes in dietary patterns over recent decades.³ A key dietary factor implicated in this trend is the excessive consumption of sugars. High sugar intake, commonly from sugar-sweetened beverages and processed foods, has been linked to rapid weight gain, insulin resistance, and metabolic syndrome,^{4,5} contributing to the onset and progression of obesity and its related complications. Recent epidemiological studies have demonstrated a clear association between dietary sugar load and increased risk of metabolic syndrome, highlighting the urgent need to explore alternative dietary interventions that could counteract these adverse effects.^{4,6,7}

In this context, the exploration of non-digestible and modified sugar analogues, such as enzymatically-produced

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oligosaccharides, represents a promising strategy. Unlike common sugars, these modified compounds may provide a lower caloric load while exerting additional metabolic benefits, such as modulation of gut microbiota. This dual function, reducing caloric intake and acting on the gut ecosystem, could be critical for developing new functional foods aimed at the prevention or management of obesity-related disorders.^{8–10} In recent years, polyols such as xylitol have gained significant attention in the food industry as sugar substitutes due to their lower caloric content and beneficial health properties^{11,12} Xylitol, a naturally occurring sugar alcohol, is widely used for its sweetening power and non-cariogenic properties.¹³ In addition, it has been shown to exert favorable metabolic effects, such as attenuating postprandial glycemic responses and improving glucose tolerance.^{14–16} Additionally, previous research has indicated that xylitol can influence lipid metabolism and immune function, suggesting a broader spectrum of metabolic benefits.^{17,18}

Beyond its use as a sugar alternative, xylitol's potential biological effects may be enhanced when enzymatically modified to generate functional derivatives with prebiotic or metabolic modulatory properties^{19–23} Thus, a recent study has explored the *in vitro* prebiotic and metabolic properties of glycosylated polyols, particularly β -galactosylated xylitol derivatives. These compounds have demonstrated the ability to modulate gut microbiota composition, promoting beneficial bacterial populations such as *Bifidobacterium* and *Lactobacillus*, among other genera, and enhancing short-chain fatty acid (SCFA) production.²⁴ Despite these promising findings, studies investigating the *in vivo* physiological effects of β -galactosylated xylitol derivatives and their comparison to xylitol are still scarce. While previous research has explored their impact on cecal fermentation and serum parameters in rats,¹⁹ their role in obesity prevention and metabolic health improvement remains largely unexplored. To address this gap, we hypothesized that β -galactosylated xylitol derivatives exert beneficial metabolic effects by modulating gut microbiota composition and improving metabolic parameters in an *in vivo* model. In this study, we administered these compounds at doses of 50 and 100 mg kg⁻¹ to lean and obese five-week-old male C57BL/6J mice and evaluated their metabolic and gut microbiota modulatory effects. Our results indicate that these derivatives contribute to a beneficial modulation of gut microbiota, which may be a key mechanism underlying their metabolic benefits. Given the recognized role of gut microbiota in energy homeostasis, adipogenesis, and inflammation,^{25–28} our findings highlight the potential of microbiome-targeted interventions in obesity management.

This study provides novel insights into the potential of β -galactosylated xylitol derivatives and xylitol itself as functional ingredients with anti-obesogenic properties. By demonstrating their efficacy *in vivo*, we highlight their relevance not only as sugar substitutes but also as promising dietary interventions for metabolic health improvement.

Experimental

Enzymatic synthesis, purification and characterization of β -galactosylated xylitol derivatives

β -Galactosylated xylitol derivatives were biosynthesized through a transglycosylation reaction catalyzed by the recombinant LacA β -galactosidase from *Lactiplantibacillus plantarum* WCFS1 (1.16 U mL⁻¹). The reaction took place at 37 °C in 50 mM sodium phosphate buffer (pH 6.5) supplemented with 2 mM MgCl₂, using lactose (VWR, Barcelona, Spain) as the galactosyl donor and xylitol (Sigma Aldrich, Stenheim, Germany) as the acceptor. The initial substrate ratio was set at 30:20% (w/w) for xylitol and lactose, following the methodology described by Delgado-Fernandez *et al.*²⁹ Before assessing their *in vivo* effects, the synthesized carbohydrates were purified from the reaction mixtures. The purification process consisted of two key steps as described by Calvete-Torre *et al.*:²⁴ (i) the selective hydrolysis of residual lactose, used as the donor substrate, was carried out using a β -galactosidase from *Kluyveromyces lactis* (Lactozym® Pure 6500 L) (Novozyme, Bagsværd, Denmark), ensuring the preservation of β -galactosylated xylitol structures under optimized conditions described by Julio-Gonzalez *et al.*;³⁰ (ii) free monosaccharides and unreacted xylitol were removed *via* activated charcoal treatment. A solution containing 1 g of oligosaccharides and 6 g of activated charcoal (Darco G60, 100 mesh, Sigma-Aldrich) was prepared in 200 mL of water and stirred for 60 minutes. The mixture was then vacuum-filtered using Whatman no. 1 paper (Whatman International Ltd, Maidstone, UK), and the charcoal was washed three times with 50 mL of water. Oligosaccharides were eluted from the charcoal using 100 mL of 50% (v/v) ethanol, stirred for 30 minutes, and filtered again.³¹ Finally, the purified oligosaccharides were concentrated using a Büchi R-200 rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) at temperatures below 25 °C and subsequently freeze-dried.

The composition of the tested β -galactosylated xylitol derivatives was analyzed in terms of carbohydrate content, expressed as a percentage of total carbohydrates as shown in Table 1. The structural characterization of these compounds was performed using NMR, as described by Delgado-Fernandez *et al.*²⁹

Table 1 Carbohydrate composition of enzymatically produced β -galactosylated xylitol derivatives (%)

Component	Percentage (%)
β -D-Galactopyranosyl-(1 \rightarrow 1)-xylitol	46.3
β -D-Galactopyranosyl-(1 \rightarrow 3)-xylitol	11.4
Xylitol	14.7
Galactooligosaccharides (disaccharides)	5.2
Galactooligosaccharides (trisaccharides)	10.3
Lactose	2.9
Glucose	6.3
Galactose	2.7



Animals

Male C57BL/6J mice were obtained from Janvier (St Berthevin Cedex, France). The animals were housed in T3 type polycarbonate plastic boxes, which were located in the facilities of the Animal Experimentation Center of the University of Granada (Campus of Cartuja). Water was supplied in 500 ml polycarbonate bottles, with a stainless-steel pipette, while pelleted food was placed in the hopper of the box for *ad libitum* availability. All mice were kept at a constant temperature of 24 ± 1 °C, with a 12 hour light-dark cycle. The animals' body weight and food intake were monitored regularly. All experiments were performed following the Guide for the Care and Use of Laboratory Animals (National Institute of Health (NIH) publication no. 85-23, revised 1996) and were approved by our Institutional Commission for the ethical care of animals (Consejería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible. Dirección General de la Producción Agrícola y Ganadera. Protocol no. 18/05/2022/069). At the end of the experiment, the mice were sacrificed to collect tissue samples for further processing.

Experimental groups

Five-week-old C57BL/6J mice were randomly assigned ($n = 6-8$ per group) into seven different groups defined by both diet and compound administration:

Standard diet (lean) groups:

- Lean control (LC)
- Lean treated with xylitol (Xyl) 50 mg kg⁻¹ (LXyl)
- Lean treated with β -galactosylated xylitol derivatives (XylGal) 100 mg kg⁻¹ (LXylGal)

High-fat diet (obese) groups:

- Obese controls (OC)
- Obese treated with Xyl 50 mg kg⁻¹ (OXyl)
- Obese treated with XylGal 50 mg kg⁻¹ (OXylGal50)
- Obese treated with XylGal 100 mg kg⁻¹ (OXylGal100)

The treated mice received the Xyl or XylGal compounds dissolved in water by esophageal gavage daily, and the untreated (control) mice only received the vehicle. Treatment with the compounds continued for 7 weeks. Food intake and body weight was monitored every 3 days, throughout the feeding period. Additionally, stool samples were taken at day 0 and at the end of treatment for analysis. LC, LXyl and LXylGal mice were maintained on a standard maintenance diet (13% calories from fat, 20% calories from protein, 67% calories from carbohydrates; 3.8 kcal g⁻¹) (Global diet 2014, Harlan Laboratories, Barcelona, Spain), whereas OC, OXyl, OXylGal50, and OXylGal100 mice were fed a Western-type High-Fat Diet (HFD) in which 60% of its caloric content came from fat (60.3% of calories from fat (lard), 18.4% calories from protein, 21.3% calories from carbohydrates; 5.4 kcal g⁻¹) (Purified diet 230 HF, Scientific Animal Food & Engineering, Augy, France). This standard-diet control provides a physiologically relevant baseline; future studies including a calorie-matched low-fat diet may further refine our understanding of how fat content *versus* total energy intake shape metabolic and microbiota out-

comes. At the end of treatment, mice were sacrificed under anesthesia (Ketamine 50 mg ml⁻¹ – Atropine 1 mg ml⁻¹ – Diazepam 5 mg ml⁻¹). Liver, abdominal fat, and visceral fat were collected, weighed, and stored at -80 °C until analysis.

The terms 'lean' and 'obese' refer to mice fed a standard diet or a high-fat diet, respectively. Including both dietary states allows us to assess the effects of Xyl and XylGal under normal and obesity-related metabolic conditions. Lean mice served as baseline controls to delineate the metabolic and microbiota changes attributable to each compound in the absence of diet-induced dysregulation, thereby strengthening the evaluation of their efficacy across distinct metabolic states.

To justify the doses used in this study, we first examined the typical xylitol content in common food sources. For example, sugar-free chewing gum generally contains approximately 1 g of xylitol per piece. Considering a typical serving (1 piece) for an average adult weighing 60–70 kg, this corresponds to roughly 14–17 mg kg⁻¹. Using the standard body surface area conversion factor (mouse $K_m = 3$; human (adult) $K_m = 37$) as described in Reagan-Shaw *et al.*,³² the 50 mg kg⁻¹ dose administered to mice translates to a human equivalent dose (HED) of approximately 4 mg kg⁻¹, which equals about 240 mg for a 60 kg human. This indicates that the doses employed in our study are physiologically relevant and can be achieved through the consumption of a realistic number of servings of xylitol-containing foods. Additionally, the safety profile of xylitol is well supported by its regulatory status. Specifically, EFSA has not defined a specific numerical ADI for xylitol, reflecting its low toxicity and safe use at customary dietary levels. Likewise, the FDA's designation of xylitol as GRAS further confirms its safety for use in food products. Thus, the doses applied in our experiments are not only physiologically relevant but also fall within a range that is considered safe for human consumption.

Glucose tolerance test

A glucose tolerance test (GTT) was performed according to the method described by Benedé-Ubieto *et al.*³³ GTT was assessed in mice fasting for 12 hours. Briefly, animals received a 20% glucose solution in water at a dose of 1.5 g kg⁻¹ body weight by intraperitoneal injection, and blood was collected from the tail vein after 0, 15, 45, and 120 minutes. Glucose levels were measured using an Accu-Chek blood glucose meter (Roche Diagnostics, Mainz-Hechtsheim, Germany).

RNA isolation and quantitative RT-PCR

Total RNA was isolated from frozen samples of liver and abdominal and visceral adipose tissue using the RNeasy kit (QIAGEN, Tokyo, Japan) following the manufacturer's protocol. Briefly, tissues were homogenized using a Next Advance Bullet Blender device, then transferred to the supplied columns where the RNA was retained; it was washed repeatedly and then eluted and stored until use at -80 °C. It was quantified using a NanoDrop 1000 Spectrophotometer device (Thermo Scientific). cDNA was synthesized using reverse transcriptase (M-MLV) and oligo (dt)15 Primer from Promega. Real-time



PCR was performed with a Techne thermal cycler (Techne, Cambridge, UK) using the primers described in ESI Table S1.† Quantification was performed using the $\Delta\Delta\text{Ct}$ method. Internal normalization was performed with Glyceraldehyde-3-Phosphate dehydrogenase (GAPDH).

Statistical analysis of *in vivo* study endpoints

Based on our extensive prior experience with similar datasets and through careful visual inspection of the data distribution (*e.g.*, histograms and Q–Q plots), normality and homogeneity of variances were assumed. Accordingly, the mean and standard deviation were calculated for quantitative variables. For the comparison of means among multiple groups, data were analyzed using one-way Analysis of Variance (ANOVA) followed by Tukey's *post hoc* test for multiple comparisons. When comparing two independent groups, the Student's *t*-test was applied with a 95% confidence level. Statistically significant differences were considered to exist when $p < 0.05$.

High-throughput sequencing of 16S rRNA

High-throughput sequencing of 16S rRNA was performed to characterise the taxonomic profiles of a total 35 fecal samples corresponding to seven different groups of mice under study as previously described. Amplicon sequencing of the V3–V4 region was carried out in an Illumina MiSeq instrument at the Sequencing Facilities of “Fundación Parque Científico de Madrid”. For this purpose, the following primers were used: CCTACGGGNGGCWGCAG (forward) and GACTACHVGGGTATCTAATCC (reverse). Then, raw sequencing reads were processed using an in-house script of QIIME2 v.2021.8 software,³⁴ developed in previous works.^{35,36} This script included quality control and filtering of paired-end reads as well as taxonomic identification of Amplicon Sequence Variants (ASVs) at genus level using reference database SILVA 138 release. Raw reads generated have been deposited in the Short Reads Archive (SRA) under BioProject accession number PRJNA1223685.

Bioinformatic analysis

Further bioinformatic analyses were performed on R (v.4.3.2.). Microbial diversity estimators including alpha (Chao1, Shannon, Simpson, Inverse Simpson) and beta (Bray Curtis dissimilarity distance) diversity indices were first calculated using Microbiome (v.1.24.0)³⁷ and Phyloseq (v.1.46.0)³⁸ R packages. To get a graphical overview of microbiota differences among groups of mice, cluster analysis based on Bray Curtis dissimilarity distance, principal coordinate analysis (PCoA) and microbiota composition bar plots were computed using Microbiome (v.1.24.0) R package.³⁷

To better characterise the modulatory effect of Xyl and XylGal compounds on specific microbial genera, statistically significant ($p < 0.05$ and $p_{\text{adj}} < 0.25$) differences in the microbiota composition of groups under study were calculated using advanced statistical methods (ANCOM, ANCOMBC, LefSe and metagenomeSeq) implemented in microbiomeMarker (v.1.8.0) R package.^{39–43}

Finally, statistically significant ($p < 0.05$) correlations between microbial genera and mice metadata (final weight, food consumption, abdominal and visceral fat, liver weight, triglyceride levels and glucose area under the curve, AUC) and cytokine levels (abdominal and visceral increments of IL-1 β and IL-1 β) were computed and expressed as Pearson correlation coefficients using base R (v.4.3.2.) functions.

Results

Impact on obesity-related physiological and anthropometric parameters

In the present study, the dietary intervention in obese mice led to a significant weight increase in untreated animals (OC) compared to those fed a standard diet (Fig. 1A). Treatment with the XylGal compound (50 and 100 mg kg⁻¹) resulted in a significant weight reduction at both doses in obese mice (OXylGal50 and OXylGal100), while in lean mice, the 100 mg kg⁻¹ dose (LXylGal 100) also induced a significant decrease ($p < 0.05$). Similarly, xylitol (50 mg kg⁻¹) also promoted weight loss in both lean (LXyl) and obese (OXyl) mice. A comparison of food intake between treated and untreated animals revealed no significant differences within either the standard diet or HFD groups (Fig. 1B).

The reduced body weight observed in XylGal-treated mice (at both doses) and xylitol-treated mice (Fig. 1C) correlated with lower abdominal and visceral fat accumulation at the end of the study. As shown in Fig. 2A and B, mice treated with xylitol exhibited the lowest fat accumulation.

No significant difference in liver weight was observed between LC and OC ($p > 0.05$; Fig. 2C) however, both Xyl- and XylGal-treated groups (LXyl, LXylGal100, OXyl, OXylGal50, OXylGal100) exhibited significantly lower liver weights compared to their respective diet-matched controls ($p < 0.05$).

Glucose tolerance test (GTT) and glycemic response

In the glucose tolerance test (GTT), both Xyl- and XylGal-treated animals exhibited a reduction in blood glucose levels following glucose overload (Fig. 3A). Given the carbohydrate nature of the tested compounds, their potential impact on glycemia must be considered. Previous studies have shown that xylitol does not alter glycemic profiles post-ingestion and may even improve postprandial glucose levels in rats.¹⁵ Additionally, the digestibility of the XylGal compounds, as analyzed by Rosado *et al.*²³ using an *in vitro* digestion model, suggests that these compounds are poorly absorbed in the small intestine, minimizing their direct impact on blood glucose levels.

A clear difference in glycemic response following glucose overload was observed between lean and obese animals. Xyl-treated animals displayed a significant reduction in blood glucose levels. However, XylGal-treated animals did not show a decrease in the area under the curve (AUC), possibly due to the presence of galactose, despite its predicted limited intestinal absorption (Fig. 3B).



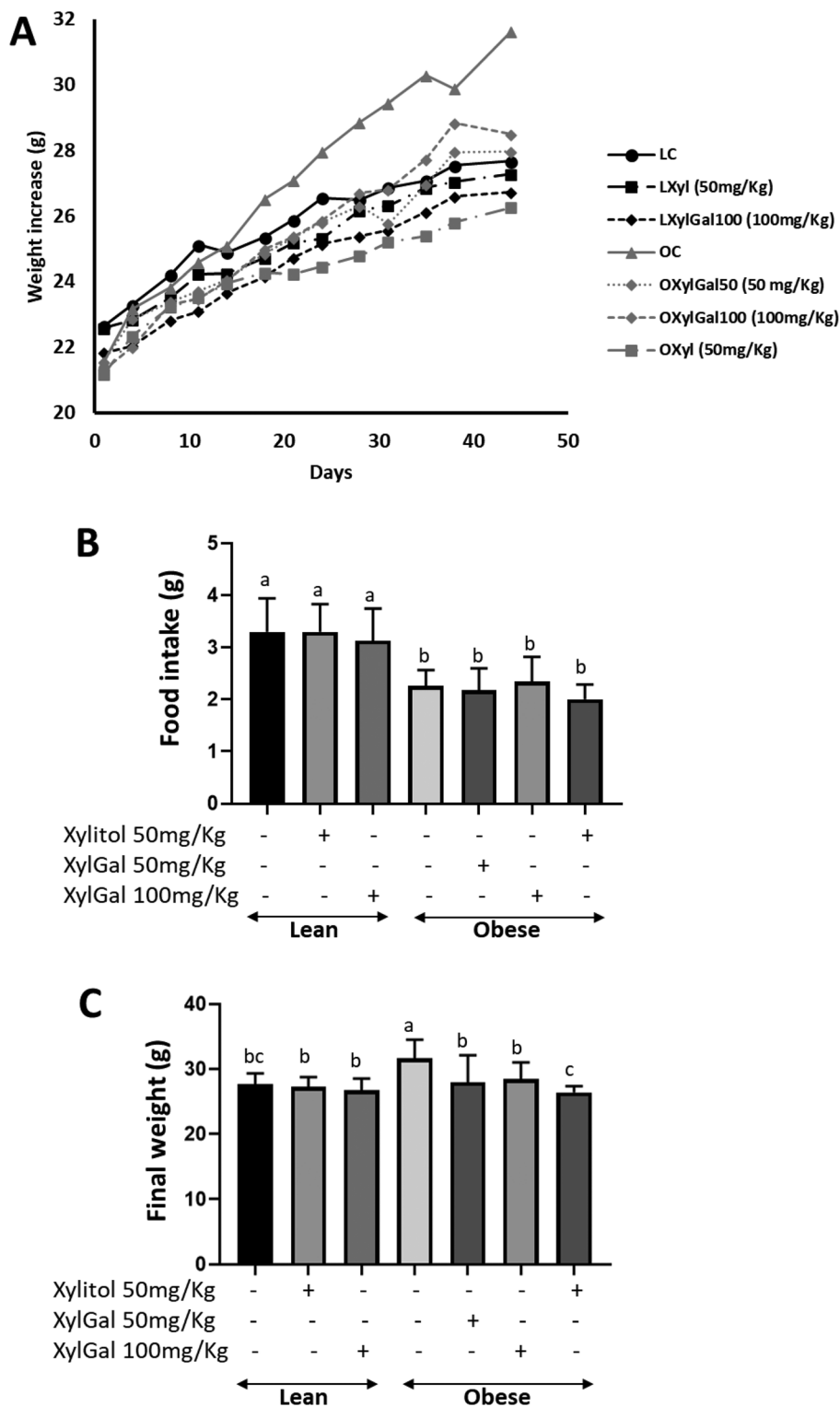


Fig. 1 Effects of the administration of XylGal and Xyl compounds on morphological changes. Weight evolution (A), food consumption per day (B), final weights (C) in lean mice and in mice with an HFD diet. Data are expressed as mean \pm SD ($n = 6-8$ animals per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. Different letters indicate significant differences between groups. $p < 0.05$.



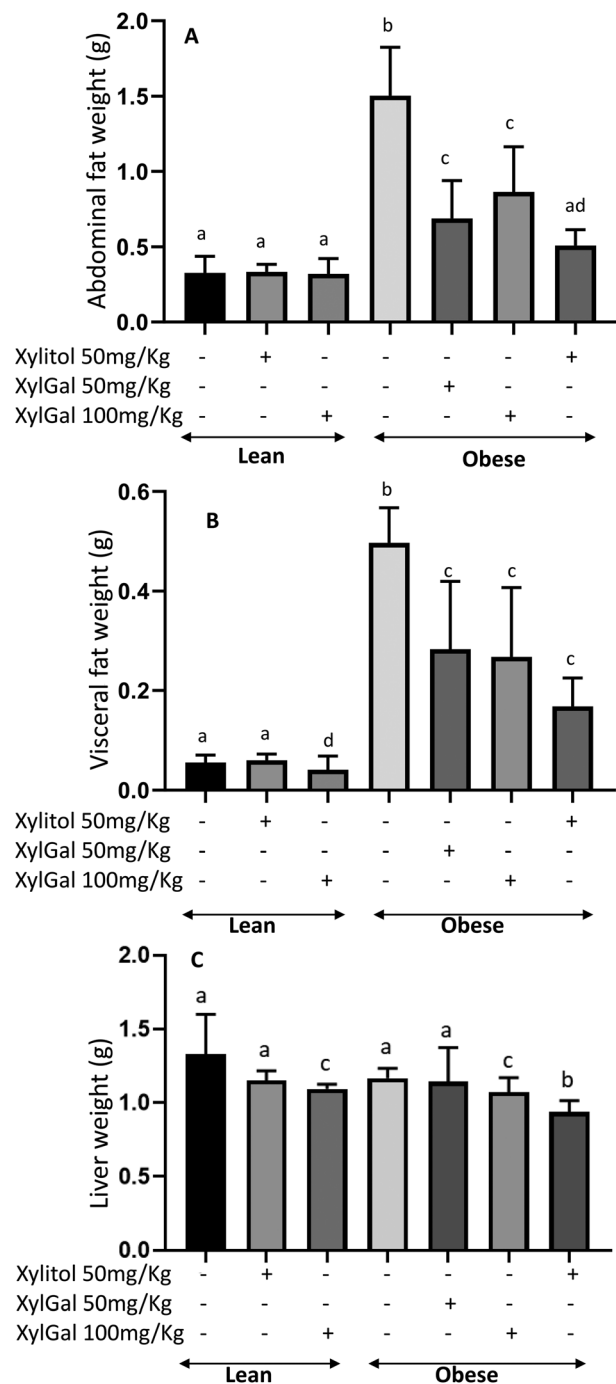


Fig. 2 Effects of the administration of XylGal and Xyl compounds on tissues/organ weight of mice for 7 weeks. Abdominal fat weight (A), visceral fat weight (B), liver weight (C). Data are expressed as mean \pm SD ($n = 6-8$ animals per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. Different letters indicate significant differences between groups. $p < 0.05$.

Inflammatory cytokine expression in adipose tissues

Following RNA extraction from abdominal and visceral adipose tissue, qPCR analysis was performed to assess macrophage and monocyte infiltration in obese animals. As shown in Fig. 4, untreated obese mice exhibited significantly higher

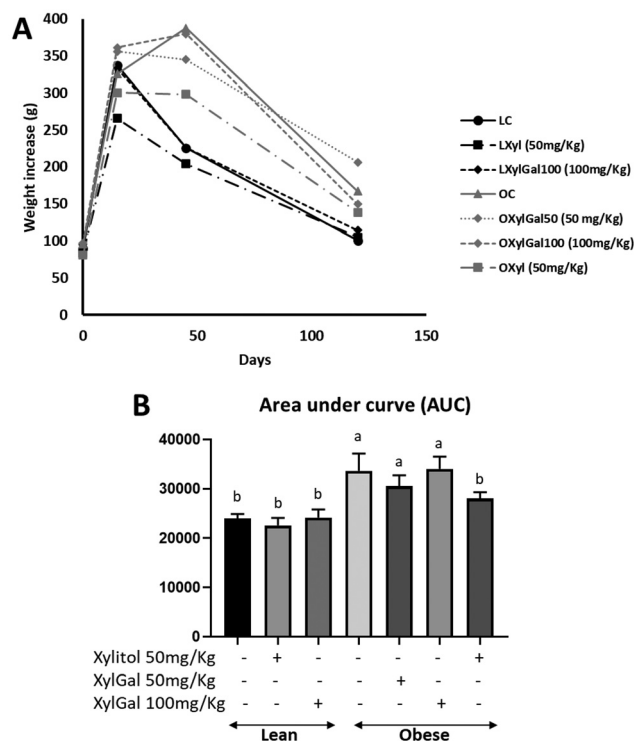


Fig. 3 Effect of intraperitoneal administration of glucose on plasma glucose levels. (A) Plasma glucose levels as a function of time. (B) Area under the curve (AUC). Glucose AUC (unitless), calculated as the integral of blood glucose (mg dL^{-1}) over time (min). Data are expressed as mean \pm SD ($n = 6-8$ animals per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. Different letters indicate significant differences between groups. $p < 0.05$.

expression levels of inflammatory cytokines, such as TNF- α and IL-1 β , in abdominal fat compared to untreated lean mice. Treatment with XylGal (both doses) and Xyl significantly reduced the expression of pro-inflammatory cytokines in adipose tissue, indicating a local reduction in inflammatory signalling that may reflect changes in immune cell activation or presence.

TNF- α production by macrophages infiltrating adipose tissue plays a key role in insulin resistance and the development of type II diabetes in overweight and obese individuals. Consistent with this, the reduced TNF- α expression observed in Xyl-treated obese mice (Fig. 4A) correlates with improved performance in the glucose overload test. Similarly, IL-1 β is known to contribute to obesity-induced metabolic dysfunction, as it plays a key role in promoting systemic inflammation and pancreatic β -cell impairment, ultimately affecting insulin secretion and glucose homeostasis.^{44,45} For XylGal-treated mice, the observed reduction in inflammatory cytokine expression aligns with the decrease in body weight and fat accumulation (Fig. 4B). However, in the glucose overload test, the presence of galactose appears to interfere with the test outcome.

qPCR analysis of visceral fat gene expression yielded results that, while not statistically significant, followed a trend similar



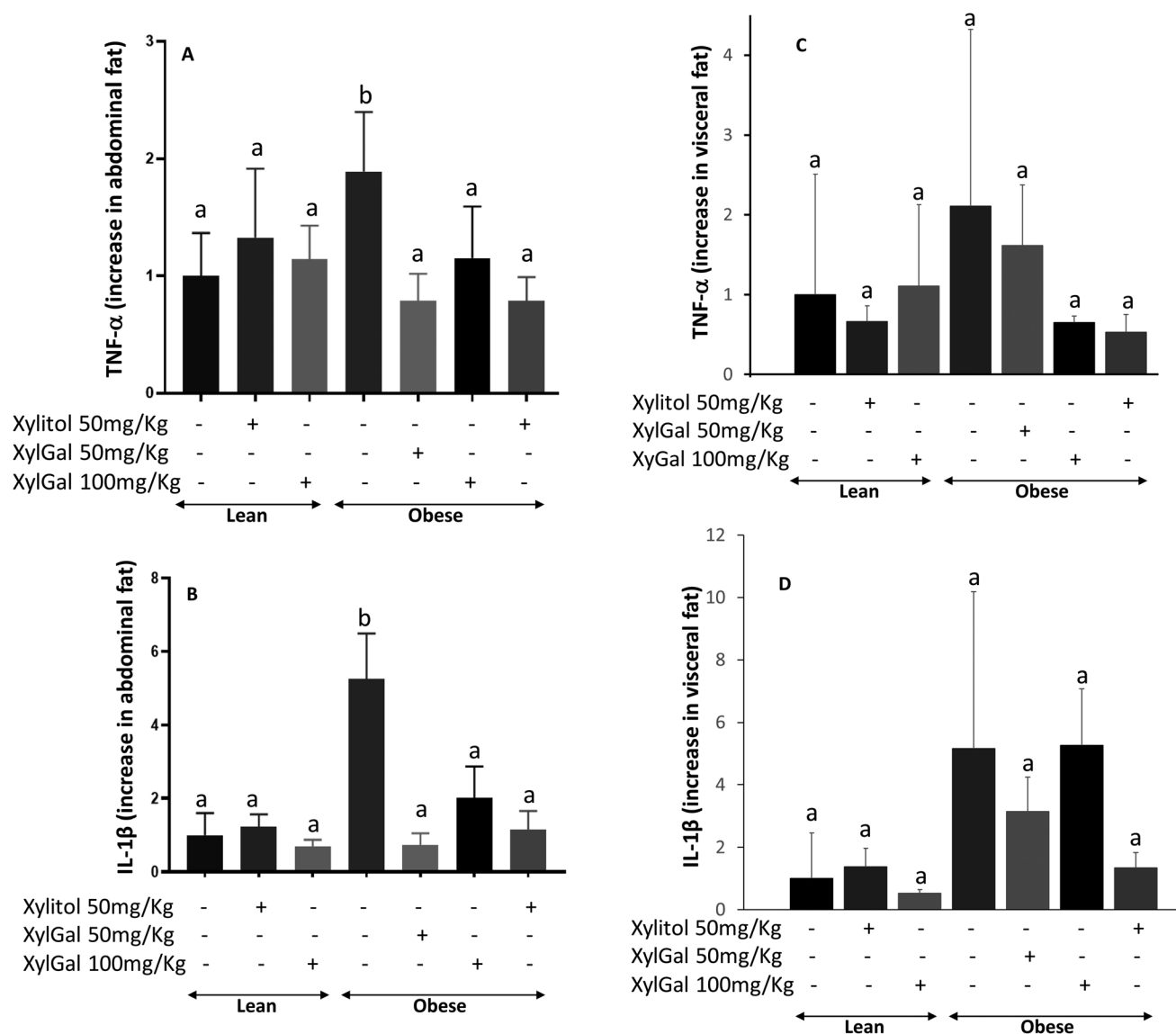


Fig. 4 Effect of the administration of XylGal and Xyl compounds on the expression of inflammatory cytokines: TNF- α (A), IL-1 β (B) in abdominal fat; TNF- α (C), IL-1 β (D) in visceral fat. Data are expressed as mean \pm SD ($n = 6-8$ animals per group). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post hoc* test. Different letters indicate significant differences between groups. $p < 0.05$.

to that observed in abdominal fat. High intra-group variability and the low quantity of visceral fat, particularly in lean and treated obese animals, likely contributed to the lack of statistical significance. Despite this limitation, the observed reduction in proinflammatory cytokines in visceral fat suggests a potential association with lower cardiovascular risk, which could be further explored in longer-term studies (Fig. 4C and D).

Xylitol (Xyl) and β -galactosylated xylitol derivatives (XylGal) promote beneficial genera in lean (normal) and obese mice

The microbiota modulatory effect of Xyl and XylGal on lean and obese mice has been assessed. In addition, potential

dose-dependent (50 and 100 mg kg⁻¹) effects of XylGal in obese mice have been investigated.

Several alpha diversity estimators (Chao1, Shannon, Simpson, Inverse Simpson) measuring microbial diversity within each group of mice were first calculated (Fig. 5). As can be seen, no significant ($p > 0.05$) differences were observed in the Chao1 index measuring the total number of genera determined. In contrast, LXyl showed significantly ($p < 0.05$) lower Simpson and Inverse Simpson indices than obese mice following the same intervention (OXyl). Similarly, LXylGal100 showed significantly ($p < 0.05$) lower Shannon index than obese mice treated with XylGal at two different doses (OXylGal50 and OXylGal100). These results suggest characteristic differences in the microbial diversity of obese and lean



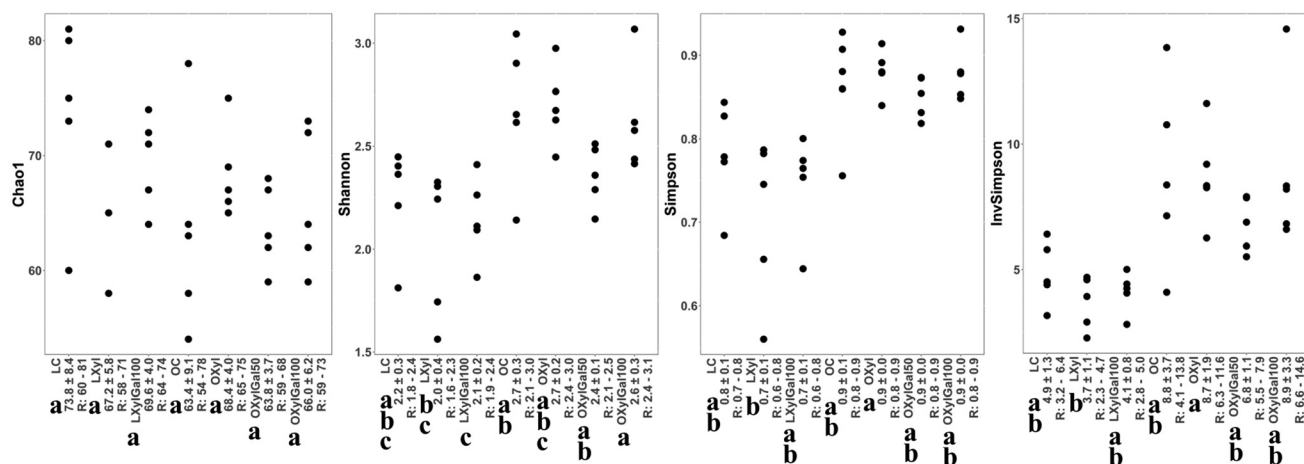


Fig. 5 Comparison of different alpha-diversity indicators (Chao1, Shannon, Simpson and Inverse Simpson, based on taxonomic profiles at genus level) determined in different groups of mice under study: (i) lean control mice (LC), (ii) lean mice supplemented with Xyl at 50 mg kg⁻¹ (LXyl), (iii) lean mice supplemented with XylGal at 100 mg kg⁻¹ (LXylGal100), (iv) obese control mice (OC), (v) obese mice supplemented with Xyl at 50 mg kg⁻¹ (OXyl), (vi) obese mice supplemented with XylGal at 50 mg kg⁻¹ (OXylGal50), (vii) obese mice supplemented with XylGal at 100 mg kg⁻¹ (OXylGal100). These indicators measure the variability of microbial taxa within a sample. a, b, c Statistically significant differences ($p < 0.05$) between groups calculated using non-parametric methods (Kruskal–Wallis test).

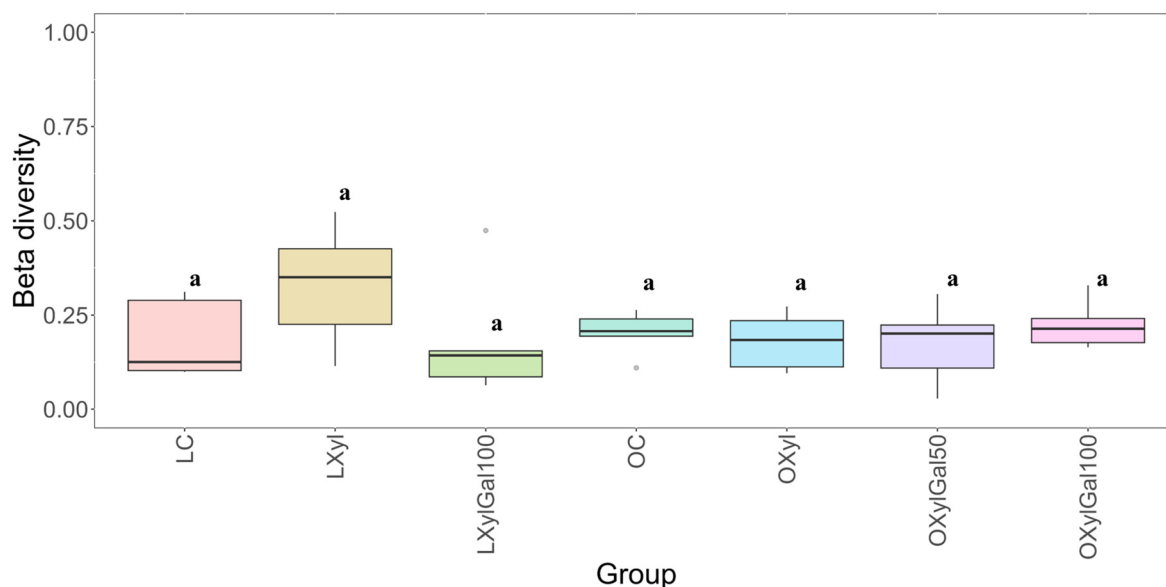


Fig. 6 Beta diversity analysis of the taxonomic profiles of different groups of mice under study: (i) lean control mice (LC), (ii) lean mice supplemented with Xyl at 50 mg kg⁻¹ (LXyl), (iii) lean mice supplemented with XylGal at 100 mg kg⁻¹ (LXylGal100), (iv) obese control mice (OC), (v) obese mice supplemented with Xyl at 50 mg kg⁻¹ (OXyl), (vi) obese mice supplemented with XylGal at 50 mg kg⁻¹ (OXylGal50), (vii) obese mice supplemented with XylGal at 100 mg kg⁻¹ (OXylGal100). Beta diversity analysis was calculated considering microbial taxonomic composition at genus level. Bray–Curtis method was selected for the calculation. a No statistically significant differences ($p > 0.05$) between groups were found.

mice, defined as significant differences in microbial diversity values ($p < 0.05$) between LXyl and OXyl, and LXylGal100, OXylGal50 and OXylGal100. These differences in microbial diversity suggest a different modulatory effect of Xyl and XylGal. While a lower Shannon index typically indicates reduced microbial diversity, this finding may reflect a selective

enrichment of certain taxa in response to the high-dose XylGal treatment, rather than a global loss of microbiota richness.

Then, beta diversity measuring microbial diversity between groups was calculated based on Bray–Curtis dissimilarity distances (Fig. 6). No significant ($p > 0.05$) differences among intervention groups were observed. Bray–Curtis dissimilarity



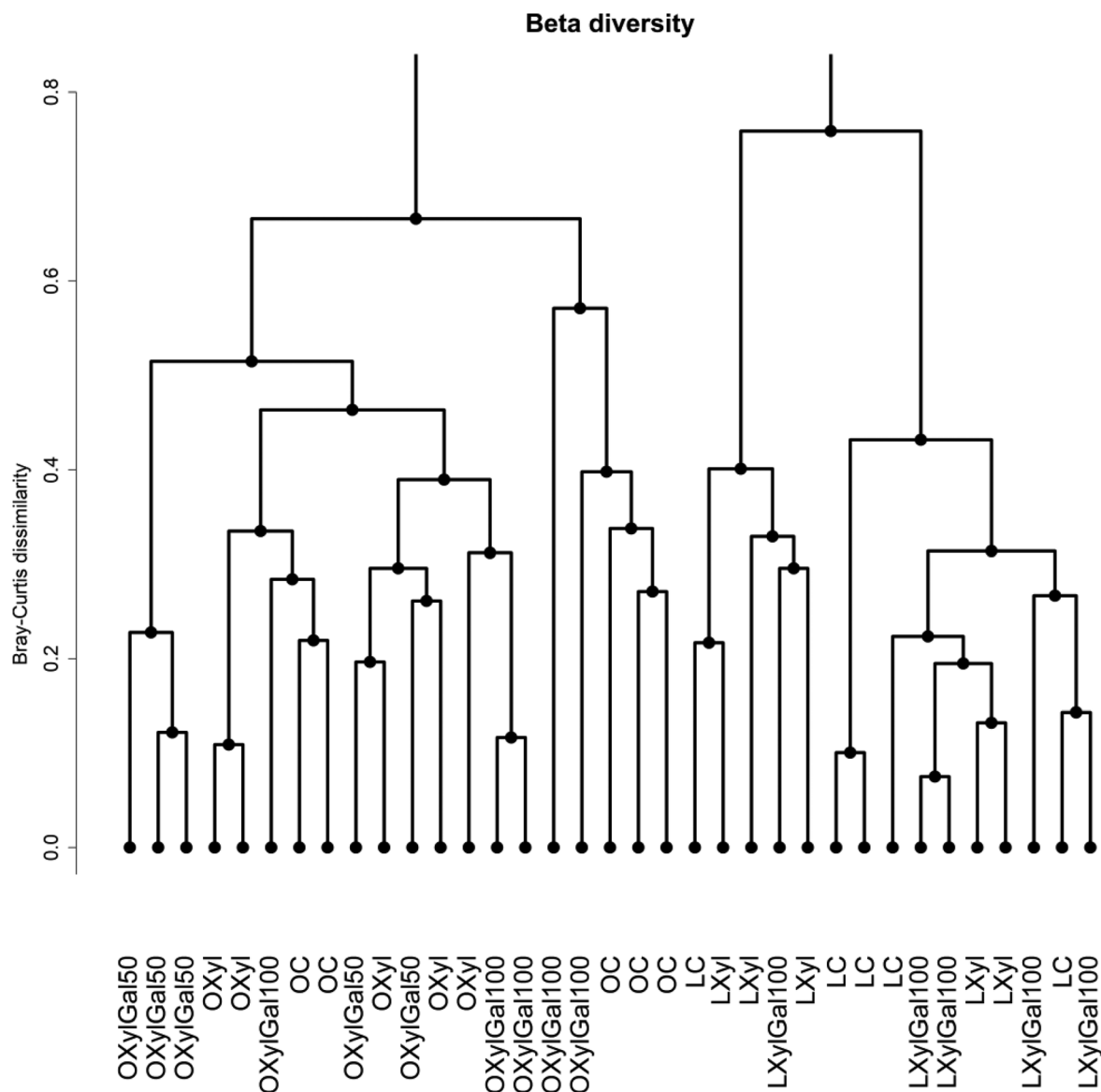


Fig. 7 Cluster analysis of the taxonomic profiles of different groups of mice under study: (i) lean control mice (LC), (ii) lean mice supplemented with Xyl at 50 mg kg⁻¹ (LXyl), (iii) lean mice supplemented with XylGal at 100 mg kg⁻¹ (LXylGal100), (iv) obese control mice (OC), (v) obese mice supplemented with Xyl at 50 mg kg⁻¹ (OXyl), (vi) obese mice supplemented with XylGal at 50 mg kg⁻¹ (OXylGal50), (vii) obese mice supplemented with XylGal at 100 mg kg⁻¹ (OXylGal100). Bray–Curtis dissimilarity method was selected for the calculation.

distances were also used to cluster taxonomic profiles of samples (Fig. 7). As can be seen, cluster analysis completely discriminated lean and obese groups. In addition, samples corresponding to the same substrate (Xyl or XylGal) administered at the same dose (50 or 100 mg kg⁻¹) were clustered together in the same branches. Cluster analysis highlights different microbiota modulatory properties of Xyl and XylGal in obese and lean mice as well as potential dose-dependent effects. Similarly, PCoA discriminated obese and lean groups and samples corresponding to different doses of substrates were differentiated (Fig. 8). However, PCoA classification was less accurate than the one observed for cluster analysis.

Once differences in the total microbiota profiles were determined, differences in the abundance of microbial genera com-

position of intervention groups were investigated (Fig. 9). With regard to lean groups, an unidentified *Muribaculaceae* genus and *Lachnospiraceae* NK4A136 group were the most abundant genera. Major genera found in obese groups include *Blautia*, *Eubacterium fissicatena* group and *Colidextribacter*.

Statistical analysis of the microbiota revealed higher ($p < 0.05$ and $p_{\text{adj}} < 0.25$) abundances of *Bacteroides*, *Clostridia* UCG-014 and *Desulfovibrio* in non-treated mice compared to prebiotic-administered groups (Table 2). These differences were specially accentuated in lean mice. In addition, *Blautia*, *Lachnospiraceae* GCA-900066575, *Lachnoclostridium* and *Eubacterium nodatum* group showed higher ($p < 0.05$ and $p_{\text{adj}} < 0.25$) abundances in non-treated obese mice compared to the rest of intervention groups (Table 2).



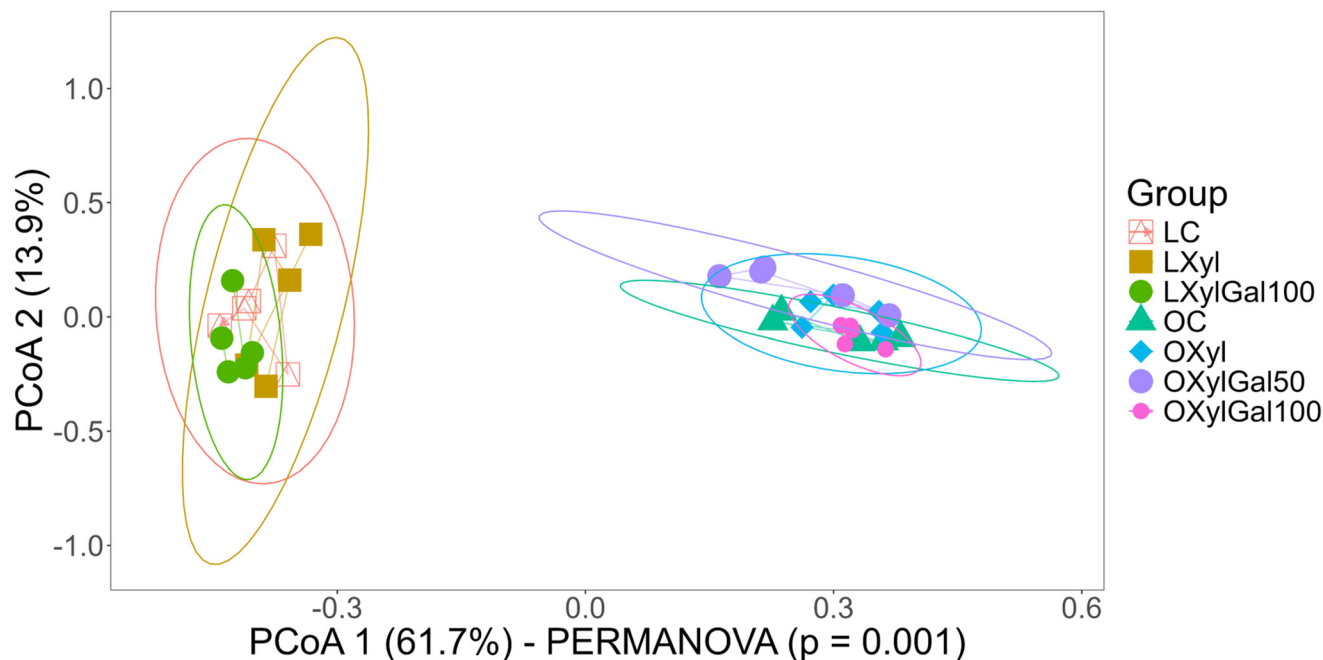


Fig. 8 Principal coordinates analysis (PCoA) of the taxonomic profiles of different groups of mice under study: (i) lean control mice (LC), (ii) lean mice supplemented with Xyl at 50 mg kg^{-1} (LXyl), (iii) lean mice supplemented with XylGal at 100 mg kg^{-1} (LXylGal100), (iv) obese control mice (OC), (v) obese mice supplemented with Xyl at 50 mg kg^{-1} (OXyl), (vi) obese mice supplemented with XylGal at 50 mg kg^{-1} (OXylGal50), (vii) obese mice supplemented with XylGal at 100 mg kg^{-1} (OXylGal100). PCoA: principal coordinate. The percentage of variance explained by each PCoA is indicated in the axis.

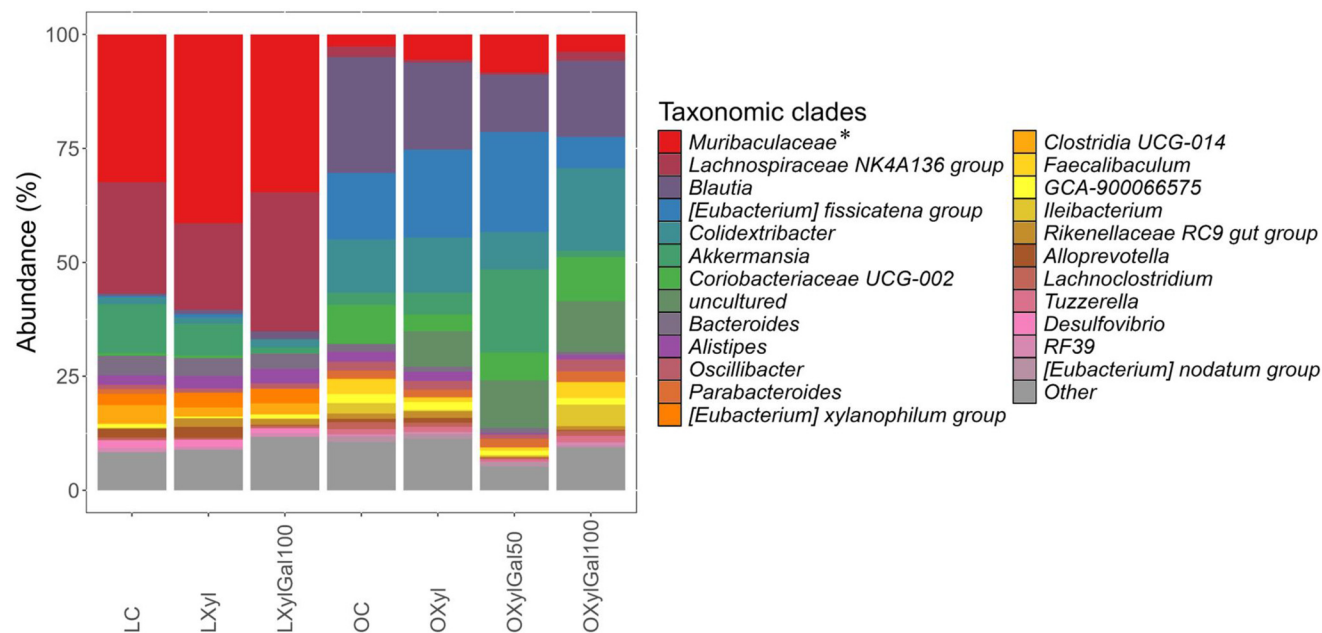


Fig. 9 Most abundant genera found in the taxonomic profiles of different groups of mice under study: (i) lean control mice (LC), (ii) lean mice supplemented with Xyl at 50 mg kg^{-1} (LXyl), (iii) lean mice supplemented with XylGal at 100 mg kg^{-1} (LXylGal100), (iv) obese control mice (OC), (v) obese mice supplemented with Xyl at 50 mg kg^{-1} (OXyl), (vi) obese mice supplemented with XylGal at 50 mg kg^{-1} (OXylGal50), (vii) obese mice supplemented with XylGal at 100 mg kg^{-1} (OXylGal100). Data are expressed as sequence abundance percentages (%). *Unidentified genus belonging to *Muribaculaceae* family.



Table 2 Microbial genera showing statistically significant ($p < 0.05$ and $p_{adj} < 0.25$) differences between groups of mice under study (i) lean control mice (LC), (ii) lean mice supplemented with Xyl at 50 mg kg⁻¹ (LXyl), (iii) lean mice supplemented with XylGal at 100 mg kg⁻¹ (LXylGal100), (iv) obese control mice (OC), (v) obese mice supplemented with Xyl at 50 mg kg⁻¹ (OXyl), (vi) obese mice supplemented with XylGal at 50 mg kg⁻¹ (OXylGal50), (vii) obese mice supplemented with XylGal at 100 mg kg⁻¹ (OXylGal100). Data are expressed as sequence abundance percentages (%). SD: standard deviation

Genera	Higher in:	LC		LXyl		LXylGal100		OC		OXyl		OXylGal50		OXylGal100	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
<i>Bacteroides</i>	LC	4.30	1.98	3.93	3.86	3.34	2.43	1.71	1.05	1.14	0.92	1.12	0.74	0.77	0.42
<i>Clostridia UCG-014</i>	LC	4.08	3.33	2.00	1.79	2.41	1.90	0.05	0.03	0.04	0.04	0.03	0.03	0.01	0.01
<i>Desulfovibrio</i>	LC	1.65	0.67	1.52	0.91	0.97	0.50	0.06	0.12	0.00	0.00	0.02	0.01	0.02	0.01
<i>RF39</i>	LC/LXylGal100	0.87	0.67	0.62	0.64	0.86	0.56	0.42	0.59	0.39	0.17	0.11	0.07	0.73	1.17
<i>Unidentified Muribaculaceae</i>	LXyl	32.44	13.17	41.44	21.44	34.61	13.95	2.64	1.56	5.54	2.93	8.33	2.61	3.78	3.27
<i>Rikenellaceae RC9 gut group</i>	LXyl	0.13	0.09	1.93	1.36	1.33	0.77	1.15	0.47	1.46	0.74	0.21	0.18	0.83	1.07
<i>Alloprevotella</i>	LXyl	1.89	0.83	2.35	2.71	0.19	0.28	0.75	0.32	1.03	0.89	0.10	0.07	0.19	0.09
<i>Lachnospiraceae NK4A136 group</i>	LXylGal100	24.38	14.52	18.96	20.08	30.51	13.93	2.32	2.00	0.65	0.11	0.56	0.38	1.99	0.92
<i>Alistipes</i>	LXylGal100	2.06	0.80	2.69	0.64	3.19	2.41	2.23	1.08	1.97	1.04	0.27	0.12	0.93	1.07
<i>[Eubacterium] xylanophilum group</i>	LXylGal100	2.37	1.30	3.08	4.13	3.12	1.51	0.14	0.18	0.01	0.02	0.04	0.02	0.07	0.14
<i>Blautia</i>	OC	0.52	0.22	0.93	1.06	1.76	1.07	25.43	13.47	19.09	3.67	12.48	4.31	16.64	6.91
<i>GCA-900066575</i>	OC	0.78	0.54	0.33	0.39	0.90	0.45	1.87	0.88	1.76	0.26	0.99	0.25	1.38	0.53
<i>Lachnospiraceae</i>	OC	0.55	0.50	0.39	0.39	0.43	0.29	1.52	0.39	0.93	0.44	0.29	0.18	1.15	0.98
<i>[Eubacterium] nodatum group</i>	OC	0.04	0.02	0.01	0.01	0.05	0.04	1.17	0.58	1.11	0.33	0.92	0.44	0.42	0.42
<i>[Eubacterium] fissicatena group</i>	OXylGal50	0.29	0.32	0.66	1.03	0.00	0.01	14.61	4.22	19.27	9.70	21.99	7.35	6.92	4.28
<i>Akkermansia</i>	OXylGal50	10.80	6.55	6.98	5.34	1.20	2.64	2.66	3.43	4.78	4.99	18.28	12.28	1.37	1.31
<i>Colidextribacter</i>	OXylGal100	1.54	0.94	1.44	0.99	1.84	0.84	11.62	2.07	12.07	2.95	8.14	3.08	18.09	6.05
<i>Coriobacteriaceae UCG-002</i>	OXylGal100	0.54	0.65	0.62	0.39	0.11	0.13	8.61	3.80	3.70	4.09	6.11	6.50	9.75	12.21
<i>Uncultured genus</i>	OXylGal100	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	7.77	3.48	10.42	2.13	11.06	5.09
<i>Oscillibacter</i>	OXylGal100	0.97	0.73	0.87	0.73	1.14	0.80	1.98	0.91	1.94	0.28	0.97	0.41	2.60	0.74
<i>Parabacteroides</i>	OXylGal100	1.06	0.99	0.19	0.15	0.08	0.09	1.64	0.87	1.69	0.80	1.88	0.97	2.33	2.56
<i>Faecalibacterium</i>	OXylGal100	0.11	0.11	0.01	0.01	0.04	0.07	3.32	1.35	0.93	0.70	0.67	0.40	3.49	2.44
<i>Heibacterium</i>	OXylGal100	0.12	0.08	0.02	0.01	0.05	0.08	2.31	0.92	0.28	0.23	0.31	0.19	4.73	4.23
<i>Tuzzerella</i>	OXylGal100	0.14	0.12	0.08	0.10	0.18	0.14	1.15	0.30	1.11	0.73	0.54	0.51	1.37	0.63

Statistical differences: control and obesity groups



Differences in the modulatory effect of substrates under study were observed in agreement with cluster analysis (Fig. 7). In this sense, Xyl administration led to an increment ($p < 0.05$ and $p_{\text{adj}} < 0.25$) in an unidentified *Muribaculaceae* genus, *Rikenellaceae* RC9 gut group and *Alloprevotella* in lean mice (Table 2). Similarly, XylGal administration at 100 mg kg⁻¹ stimulated ($p < 0.05$ and $p_{\text{adj}} < 0.25$) the growth of health-promoting *Lachnospiraceae* NK4A136 group, *Alistipes* and *Eubacterium xylanophilum* group in lean mice (Table 2). It should be noted that the majority of these genera were not promoted by Xyl and XylGal in obese mice. In contrast, XylGal

administration at 50 mg kg⁻¹ resulted in an increment ($p < 0.05$ and $p_{\text{adj}} < 0.25$) in *Eubacterium fissicatena* group and *Akkermansia* in obese mice while high doses (100 mg kg⁻¹) of XylGal promoted a wider variety of genera in these mice including *Parabacteroides* and *Faecalibaculum*.

Finally, statistical correlations ($p < 0.05$) between microbial genera found in the microbiota of different intervention groups and mice metadata and biochemical parameters were determined (Fig. 10). In this regard, several genera including *Blautia*, *Colidextribacter*, *Eubacterium fissicatena* and *Eubacterium nodatum* groups, *Faecalibaculum*, *Ileibacterium*,

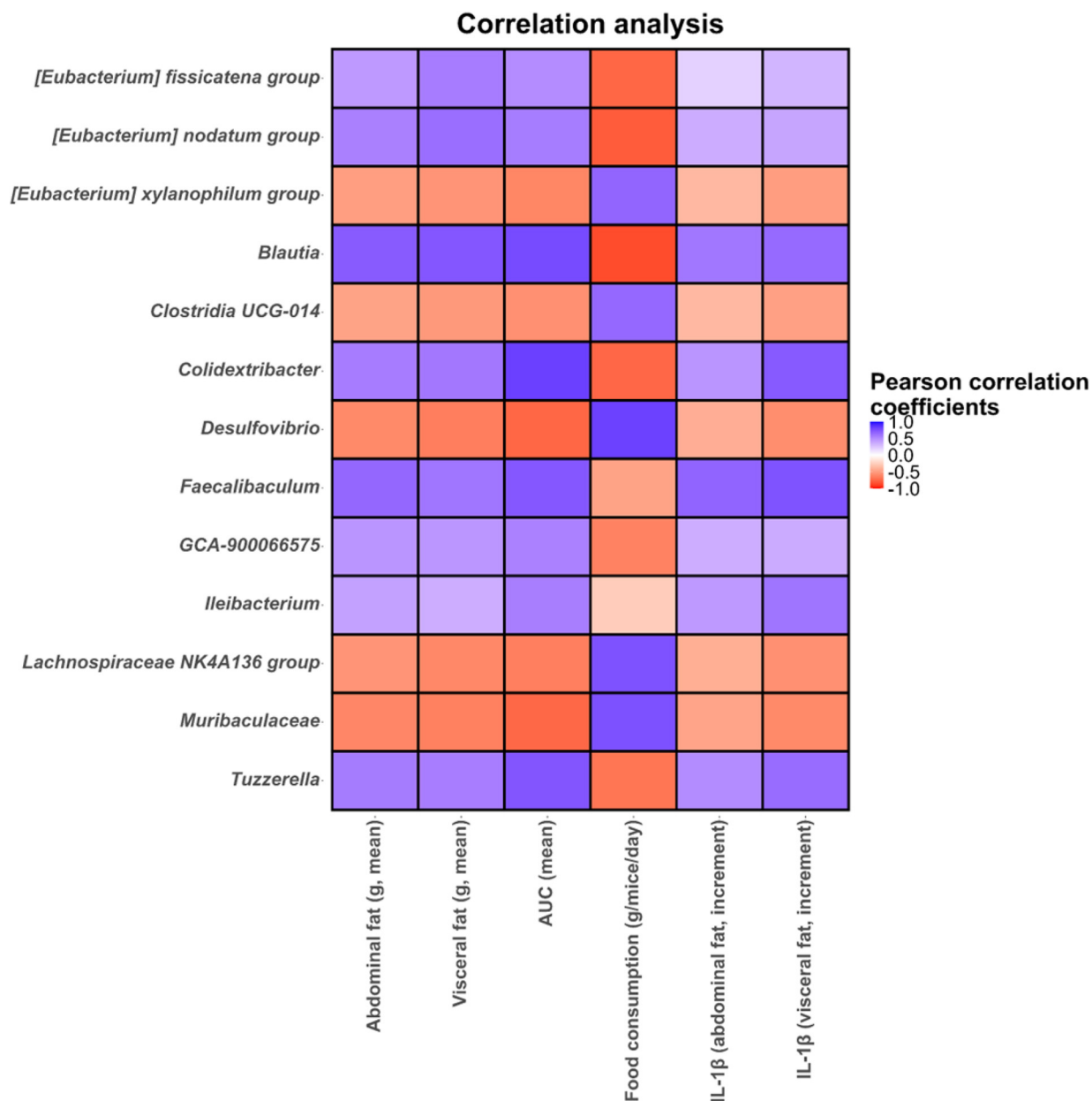


Fig. 10 Correlation heatmaps showing the associations between microbial genera determined in different groups of mice under study and mice metadata (final weight, food consumption, abdominal and visceral fat, liver weight, triglyceride levels and glucose area under the curve, AUC) and cytokine levels (abdominal and visceral increments of IL-1 β and IL-1 β). Blue and red dots indicate positive and negative correlations expressed as Pearson correlation coefficients. Colour intensity is in proportion to magnitude. Data represented correspond to all intervention groups.



Lachnospiraceae GCA-900066575 and *Tuzzerella* showed positive correlations with abdominal and visceral fat and IL-1 β increments and glucose AUC, and negative correlations with food consumption (Fig. 10). These taxa showed high abundances in both treated and untreated obese mice highlighting the higher levels of abdominal and visceral fat, IL-1 β and glucose AUC compared to lean mice. In contrast, *Desulfovibrio*, *Eubacterium xylanophilum* group, *Lachnospiraceae* NK4A136 group and an unidentified *Muribaculaceae* genus showed positive correlations with food consumption and negative correlations with abdominal and visceral fat and glucose AUC (Fig. 10). Most of these taxa showed the highest increments in lean mice treated with Xyl or XylGal highlighting the positive effect of these substrates on biochemical parameters and inflammatory markers associated with obesity.

Discussion

The present study demonstrates that Xyl and XylGal supplementation mitigated obesity-related metabolic disturbances in mice, with significant reductions in body weight and fat accumulation, particularly in abdominal and visceral depots. The inclusion of lean mice provided a necessary control, revealing that while the metabolic impact of Xyl and XylGal is evident in both lean and obese states, the magnitude and mechanisms differ. Future studies may focus on delineating these pathways further. The weight loss observed in treated animals has previously been attributed to the satiating properties of xylitol, as described by Gasmi Benahmed *et al.*¹² However, in our study, no clear evidence of satiety was observed at the doses used, as food intake did not significantly differ between treated and control animals within each dietary group. Interestingly, obese mice exhibited lower overall food consumption than their lean counterparts, yet still gained significantly more weight. This observation is not entirely uncommon, as high-fat diets are more energy-dense and often result in reduced food volume intake despite increased caloric consumption.^{46,47} However, in previous experiments conducted in our laboratory using the same HFD formulation, we observed higher overall food intake in obese mice, suggesting possible variability due to strain, experimental conditions, or gut microbial status.

Despite these nuances, reductions in adipose tissue and liver weight were observed in both Xyl- and XylGal-treated animals. Given that fatty liver is a frequent complication of obesity and liver weight serves as a proxy for hepatic lipid accumulation, these findings suggest a potential role for the compounds in attenuating hepatic steatosis. Similar observations were reported in an 8 week study by Amo *et al.*¹⁵ in rats, where xylitol reduced visceral fat, upregulated genes involved in hepatic fatty acid oxidation, and enhanced lipid degradation in adipose tissue. Conversely, other reports, including those reviewed by Gasmi Benahmed *et al.*,¹² have shown less consistent outcomes, underscoring the complexity

of xylitol's metabolic effects and the need for additional mechanistic studies.

In line with our findings, a recent study in HFD-fed rats treated with β -galactooligosaccharides (GOS) demonstrated reductions in body weight and fat accumulation, which were linked to alterations in gut microbiota composition.⁴⁸ Although our study used β -galactosylated xylitol derivatives rather than GOS, the presence of galactose in XylGal could similarly contribute to prebiotic-like effects. Moreover, glucose homeostasis was significantly improved in Xyl-treated animals, as evidenced by a reduced area under the curve (AUC) in the glucose tolerance test (GTT). However, XylGal did not significantly lower AUC values, possibly due to the presence of galactose, which may have interfered with the test outcome. Additionally, both Xyl and XylGal effectively reduced the expression of inflammatory cytokines in abdominal adipose tissue, demonstrating a significant reduction in the expression of macrophage-associated markers, which may reflect decreased immune cell presence in adipose tissue and suggesting an attenuation of obesity-related low-grade inflammation. While qPCR does not directly quantify immune cell infiltration, the expression levels of macrophage and monocyte markers have been widely used as molecular proxies for tissue inflammation and immune cell recruitment in adipose tissue. Although the visceral fat results did not reach statistical significance, they followed a similar trend, indicating a potential systemic effect of these compounds on inflammation and metabolic health.

These metabolic and inflammatory changes observed *in vivo* correlate with the modulatory effects of Xyl and XylGal on gut microbiota composition, which may underlie some of the observed health benefits. The microbiota modulatory effect of Xyl and XylGal compounds at different doses in obese and lean mice has been investigated. Previous studies suggest that Xyl helps in glycemic and obesity control,¹² in agreement with our results where several genera promoted by Xyl and XylGal were negatively correlated with glucose AUC and obesity-related inflammatory markers (Fig. 10). To our knowledge, this is the first study to examine the effect of Xyl on gut microbiota in an obesity context. Xyl administration led to an increment in *Rikenellaceae* RC9 gut group and *Alloprevotella* in lean mice, while XylGal stimulated the growth of *Lachnospiraceae* NK4A136 group, *Alistipes* and *Eubacterium xylanophilum* group. In obese mice, high doses of XylGal promoted the enrichment of *Parabacteroides* and *Faecalibaculum*. These taxa are considered beneficial gut commensals with established or emerging links to host metabolic health. Among them, members of *Lachnospiraceae*, including the NK4A136 group, have been associated with improved gut barrier integrity and reduced systemic inflammation, particularly in the context of obesity in both humans and mice.^{49,50} They are also considered promising targets for personalized therapeutic interventions to manage cardiometabolic risk.⁵¹ *Eubacterium* species, similarly promoted by XylGal, are characteristic of a healthy gut microbiota compared to taxonomic profiles associated with inflammatory diseases like chronic



spontaneous urticaria.⁵² *Eubacterium* species may improve intestinal dysbiosis in high-fat diet-fed mice,⁵³ and play a major role in immunomodulation and suppression of inflammation in the gut,⁵⁴ in agreement with our results, where *Eubacterium* showed negative correlations with obesity-related inflammatory markers. Other taxa modulated by treatment, including *Rikenellaceae*, *Alistipes* and *Parabacteroides* have been positively associated with circulating lipid metabolites and inversely associated with body mass index. These correlations suggest a potential role for these taxa in lipid metabolism and energy homeostasis. Moreover, these microbial genera have also been involved in the anti-obesity mechanisms following interventions such as sleeve gastrectomy.⁵⁵ This aligns with our observation that Xyl and XylGal supplementation significantly reduced body weight and fat accumulation, potentially mediated by shifts in gut microbiota composition.

In particular, *Parabacteroides* is an emerging probiotic that shows a wide range of metabolic activities, including xylanases and xylosidases.⁵⁶ Previous works involving *in vitro* fermentation experiments of beer bagasse fractions suggest that *Parabacteroides* may ameliorate gut dysbiosis associated with inflammatory diseases.³⁶ Moreover, this genus is inversely associated with obesity among females and participants aged 40–69 years.⁵⁷ Therefore, *Parabacteroides* species have been proposed as next-generation probiotics as potential preventive and therapeutic agents against obesity.⁵⁸ Given the observed metabolic improvements in our study, it is plausible that the increase in *Parabacteroides* following XylGal supplementation contributed to the reduction in obesity-related inflammation and metabolic dysfunction.

Faecalibaculum, likewise enriched in XylGal-treated obese mice, has been negatively associated with body-weight gain in high fat-induced obesity mice supplemented with apple pectin, contributing to obesity control.⁵⁹ The presence of this genus in XylGal-treated obese mice further supports its potential role in modulating body composition and metabolic health.

Interestingly, despite these microbial shifts, the LXylGal100 group showed a significantly lower Shannon diversity index compared to obese controls (Fig. 5). While this reduction may appear unexpected, it likely reflects selective enrichment of health-promoting taxa rather than a loss of microbial richness. Indeed, not all decreases in alpha diversity are indicative of dysbiosis. For example, Zarrinpar *et al.*⁶⁰ demonstrated that time-restricted feeding reduced diversity in mice while improving metabolic outcomes, and Thaïss *et al.*⁶¹ showed that circadian control of the microbiota can lead to transient reductions in diversity with functional gains. Moreover, Thomas *et al.*⁶² reported higher microbial richness in colorectal cancer patients than in healthy individuals, largely due to the presence of ectopic taxa from the oral cavity. Similarly, significantly lower Shannon diversity values have been reported after functional polysaccharide and xylanase supplementation in African catfish⁶³ and mice⁶⁴ with chronic pancreatitis, respectively, suggesting that these interventions selectively promoted certain microbial populations. These

examples reinforce the idea that microbial diversity alone does not fully define gut health, and that composition and function may be more relevant indicators in certain contexts. Thus, the microbial shifts observed in our study may contribute to the metabolic improvements seen in XylGal-treated mice, even in the presence of reduced overall diversity.

Finally, while our study used a standard maintenance diet for the control groups, we acknowledge that a calorie-matched low-fat diet could help more clearly differentiate between effects driven by dietary composition and total energy intake. We have highlighted this point as a limitation and an opportunity for refinement in future study designs.

In conclusion, our study demonstrates that supplementation with Xyl and XylGal improves obesity-related metabolic disturbances, reduces inflammatory markers, and modulates gut microbiota composition in a diet- and dose-dependent manner. Our data suggest that the beneficial effects of XylGal, particularly in the modulation of key microbial genera, could play a pivotal role in reducing metabolic inflammation and adiposity in obese subjects. These findings support further exploration of these compounds as functional ingredients for managing obesity and metabolic dysfunction. Future research should explore the long-term clinical applicability of these compounds and further elucidate the mechanistic links between gut microbiota modulation and improved metabolic outcomes.

Conclusions

Taken together, these findings support a connection between Xyl and XylGal supplementation, metabolic improvements, and gut microbiota modulation. Reductions in body weight, fat accumulation, and inflammatory cytokines observed *in vivo* were accompanied by specific gut microbiota shifts, including an increase in potentially beneficial bacterial genera such as *Lachnospiraceae* NK4A136, *Eubacterium xylanophilum*, and *Parabacteroides*, which have been previously associated with improved gut barrier function, reduced inflammation, and enhanced lipid metabolism. Simultaneously, a decrease in obesity-related taxa such as *Blautia*, *Colidextribacter*, and *Eubacterium fissicatena* was observed, suggesting a potential role in mitigating gut dysbiosis linked to metabolic disorders. While our study was not designed to establish causality, these microbiota modifications may contribute to the observed metabolic benefits, including improved glucose tolerance and reduced obesity-associated inflammation, and suggest a possible prebiotic role for β -galactosylated xylitol derivatives. Future research should investigate the mechanistic pathways linking gut microbial changes to host metabolism outcomes and explore the long-term clinical relevance of these compounds in human studies. These insights may support their development as functional ingredients for metabolic health management.



Author contributions

CS: formal analysis, data curation, writing – original draft, writing – review & editing, methodology; MB: formal analysis, methodology; PDF: formal analysis, methodology; NC: supervision, funding acquisition, conceptualization; BdlR: methodology, validation; RM: methodology, validation; AA: formal analysis, methodology; PU: supervision, writing – original draft, writing – review & editing, resources, methodology, validation; FJM: supervision, funding acquisition, conceptualization, writing – original draft, writing – review & editing.

Data availability

The sequencing data supporting the findings of this study have been deposited in the Short Reads Archive (SRA) under BioProject accession number PRJNA1223685 and can be accessed at <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1223685>. Additional data, including experimental procedures and analysis details, are provided in the ESI† of this article.

Conflicts of interest

There are no conflicts to declare.

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