Original article

Effect of combined metformin and *Lactobacillus plantarum* DAD-13 administration on the Lee index, lipid profile, and visceral adipose tissue histopathology in a rat model of NAFLD

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Abstract

Background: Nonalcoholic fatty liver disease (NAFLD) is associated with obesity, insulin resistance, dyslipidemia, and visceral adipose tissue dysfunction. Metformin and *Lactobacillus plantarum* DAD-13 may act through complementary metabolic and gut-microbiota pathways, but evidence on their combined administration is limited.

Objective: To evaluate the effects of combined metformin and *L. plantarum* DAD-13 administration on the Lee index, lipid profile, and visceral adipocyte number and diameter in a rat model of NAFLD.

Methods: Eighteen male Wistar rats (n = 6 per group) were allocated to a normal-diet group (ND), Western-diet group (WD), or Western-diet plus metformin-probiotic group (MP) for 28 weeks. During the final 10 weeks, the MP group received metformin at 50 mg/kg body weight/day and *L. plantarum* DAD-13 at 1×10^9 colony-forming units/mL, 1 mL/day. Lee index, serum lipid profile, and visceral adipose tissue histopathology were examined. Variable-specific parametric or nonparametric tests were used for analysis.

Results: Adipocyte diameter differed among groups ($P = 0.007$). The MP group had a lower mean adipocyte diameter than the WD group (64.8 *vs.* 72.8 µm; exploratory unadjusted $P = 0.048$). The adipocyte count was similar in both WD and MP groups ($P = 0.655$). Triglycerides differed among groups ($P = 0.013$) but the difference between the WD and MP groups was not significant ($P = 0.130$). No significant differences between groups were observed for the Lee index ($P = 0.226$), high-density lipoprotein cholesterol ($P = 0.159$), low-density lipoprotein cholesterol ($P = 0.389$), or total cholesterol ($P = 0.785$).

Conclusion: Combined metformin and *L. plantarum* DAD-13 administration was associated with a modest reduction in visceral adipocyte diameter compared with WD alone, but did not improve adipocyte count, Lee index, or most lipid outcomes. These exploratory findings do not establish synergism or a broad metabolic benefit.

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Keywords: Adipocyte diameter, *Lactobacillus plantarum* DAD-13, metformin, nonalcoholic fatty liver disease, visceral adipose tissue.

Nonalcoholic fatty liver disease (NAFLD) is a hepatic manifestation of metabolic dysfunction closely associated with visceral obesity, dyslipidemia, type 2 diabetes mellitus, insulin resistance, hypertension, and cardiovascular disease. The pooled prevalence of NAFLD in the Asia-Pacific region is estimated at 29.6%, with particularly high figures reported for Indonesia. [1] Further, NAFLD can progress to steatohepatitis and cirrhosis, potentially leading to hepatocellular carcinoma, liver transplantation, and death. [2 - 4]

Visceral adipose tissue (VAT) contributes to NAFLD through excess fatty acid flux, adipokine dysregulation, insulin resistance, and chronic low-grade inflammation. Diet-induced rodent models are widely used to examine these pathways and tissue-level responses to interventions. [5]

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The gut-liver axis has become a therapeutic target in metabolic liver disease. *L. plantarum* DAD-13 is an Indonesian probiotic strain with reported effects on gut-microbiota composition and intestinal health in overweight adults. [6] Meanwhile, metformin improves insulin sensitivity and activates AMP-activated protein kinase (AMPK), with downstream effects on hepatic lipid metabolism and adipose tissue extracellular matrix remodeling. [7-10]

Considering these potentially complementary pathways, we evaluated combined metformin and *L. plantarum* DAD-13 administration in rats exposed to a Western diet. Since the design excluded metformin-only or probiotic-only groups, we assessed the combined regimen's impact and did not test for pharmacologic synergy.

Materials and methods

Research ethics

This experimental study was approved by the Research Ethics Commission of the Faculty of Medicine, Brawijaya University (no. 190/EC/KEPK-S1-PD/08/2023).

Experimental design and animal allocation

Eighteen male *Rattus norvegicus* Wistar rats aged 8–12 weeks were obtained from PT Indoanilab, Bogor Agricultural University. Healthy rats with shiny white fur and active movement were eligible; rats that became ill or died during the study were excluded. Data from 18 rats were analyzed, with six animals in each group: normal-diet group (ND), Western-diet group (WD), and Western-diet plus metformin-probiotic group (MP). Available study records did not specify a random-sequence generation or allocation-concealment method and did not document investigator blinding.

Dietary induction and feeding procedure

The ND diet consisted of comfeed, wheat flour, and water, providing 104.9 kcal/100 g, comprising 72.0% carbohydrate, 19.3% protein, and 4.0% fat. The Western diet consisted of PARS, wheat flour, egg yolk, goat oil, gelatin, and water, providing 311.0 kcal/00 g, comprising 52.0% carbohydrate, 16.0% protein, and 31.0% fat. Each rat received 40 g of the assigned diet once daily at noon for 28 weeks. The WD and MP groups received the Western diet throughout the 28-week period. This diet protocol was based on a previously reported rat model of diet-induced nonalcoholic steatohepatitis. [11]

Metformin and L. plantarum DAD-13 administration

During the final 10 weeks of the 28-week feeding period, the MP group received metformin and *L. plantarum* DAD-13 once daily. Metformin tablets were ground and suspended in water to achieve a concentration of 50 mg/kg body weight/day. The probiotic was prepared in autoclaved water at 1×10^9 colony-forming units/mL and administered at 1 mL/day. Both interventions were administered orally during the same treatment period. The ND and WD groups did not receive metformin or probiotic supplementation.

Lee index measurement

Body weight and naso-anal length were measured before tissue collection. The Lee index was calculated as follows:

$$Lee\ index = \sqrt[3]{[body\ weight\ (g)]} / naso-anal\ length\ (cm)$$

A Lee index > 0.30 was considered consistent with obesity in the rat model. [12]

Surgical procedure and blood sampling

Rats were fasted for 8 h before sample collection. Ketamine–xylazine anesthesia was administered, followed by cardiac puncture. Up to 500 µL of blood was collected in a serum tube and sent to the Clinical Pathology Laboratory, Faculty of Medicine, Brawijaya University.

Lipid profile measurement

Serum was separated by centrifugation at 5,000 rpm for 10 min. Total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured using ABX Pentra reagents according to the manufacturer-specified enzymatic colorimetric methods.

Visceral adipose tissue histopathology and quantification

Hematoxylin and eosin-stained VAT sections were examined using a CX-23 microscope connected to a camera. Qualitative morphology of adipocyte hypertrophy and hyperplasia was assessed at 400× magnification in ≥ 3 non-overlapping fields per animal. Quantitative cell count and adipocyte diameters were obtained from a standardized field at 100× magnification and summarized for each animal. The available source protocol did not identify the image-analysis software or document assessor blinding; these are acknowledged as methodological limitations.

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Statistical analysis

Analyses were performed using SPSS version 26.0. Continuous data are presented as individual observations with mean ± standard deviation (SD); narrative summaries use means and ranges where source values were available. Normality was assessed with the Shapiro–Wilk test, and homogeneity of variance was evaluated before parametric testing. The Lee index, triglycerides, LDL-C, and total cholesterol were analyzed using one-way analysis of variance; Tukey post-hoc testing was reserved for variables with a significant omnibus result. HDL-C was analyzed using the Kruskal–Wallis test because the distribution was nonnormal and heterogeneous. Adipocyte count and diameter were analyzed using the Kruskal–Wallis test, followed by exploratory pairwise Mann–Whitney U tests. Pairwise nonparametric *P* values are reported as unadjusted because a multiplicity-adjustment method was not documented. All tests were two-sided and $P < 0.05$ considered statistically significant. Descriptive measurements were rounded to one decimal place where scientifically appropriate; for Lee index values, two decimal places were retained because of their scale.

Results

Lee index and lipid profile

Data from all 18 animals were analyzed (n = 6 per group). The MP and ND groups had the highest and lowest mean Lee index values, respectively (0.31 ± 0.02 *vs*. 0.30 ± 0.02). The Lee index did not differ significantly among groups ($P = 0.226$) **(Figure 1)**.

Triglyceride concentrations differed among groups ($P = 0.013$). However, the WD–MP comparison was not significant ($P = 0.130$). No significant group differences were observed for HDL-C ($P = 0.159$), LDL-C ($P = 0.389$), or total cholesterol ($P = 0.785$; **Figure 2).**

Qualitative histopathology of visceral adipose tissue

The ND group showed relatively small adipocytes arranged in lobules, with eccentric round-oval nuclei and clear cytoplasm. The WD group showed enlarged adipocytes, poorly visible lobular architecture, and the lowest apparent cell density within the field of view. The MP group showed an intermediate pattern: smaller adipocytes than those in the WD group, visible lobular organization and connective-tissue septa, and some enlarged cells and dilated capillaries **(Figure 3).**

Quantitative histopathology of visceral adipose tissue

Mean adipocyte counts were 459.0 cells in the ND group (range, 396.0–661.0), 320.0 cells in the WD group (range, 241.0–361.0), and 349.0 cells in the MP group (range, 264.0–448.0). The omnibus Kruskal–Wallis test was significant ($P = 0.010$). In exploratory pairwise testing, the WD and MP groups did not differ ($P = 0.655$; **Figure 4).**

Mean adipocyte diameters were 48.3 ìm in the ND group (range, 41.1–67.1), 72.8 ìm in the WD group (range, 68.8–79.0), and 64.8 ìm in the MP group (range, 55.9–81.3). The omnibus Kruskal–Wallis test was significant ($P = 0.007$). The MP group had a lower mean diameter than the WD group (exploratory unadjusted $P = 0.048$; **Figure 5).**

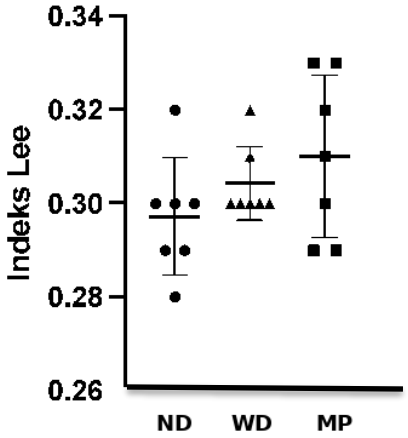


Figure 1. Lee index in the ND, WD, and MP groups. Individual observations and mean ± SD are shown (n = 6 per group). MP, metformin-probiotic; ND, normal-diet; SD, standard deviation; WD, Western-diet.

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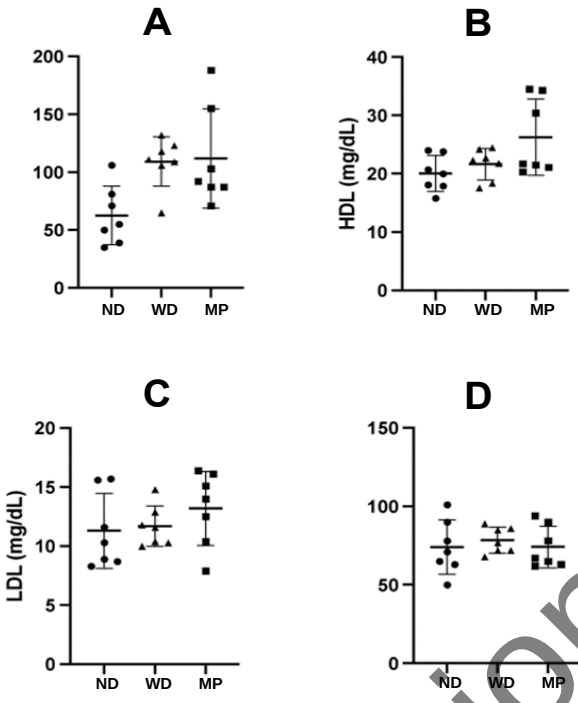


Figure 2. Lipid profile by treatment group: **(A)** triglycerides, **(B)** HDL, **(C)** LDL, and **(D)** total cholesterol and *L. plantarum* DAD-13. Individual observations and mean ± SD are shown (n = 6 per group). HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol; MP, metformin-probiotic; ND, normal-diet; SD, standard deviation; WD, Western-diet.

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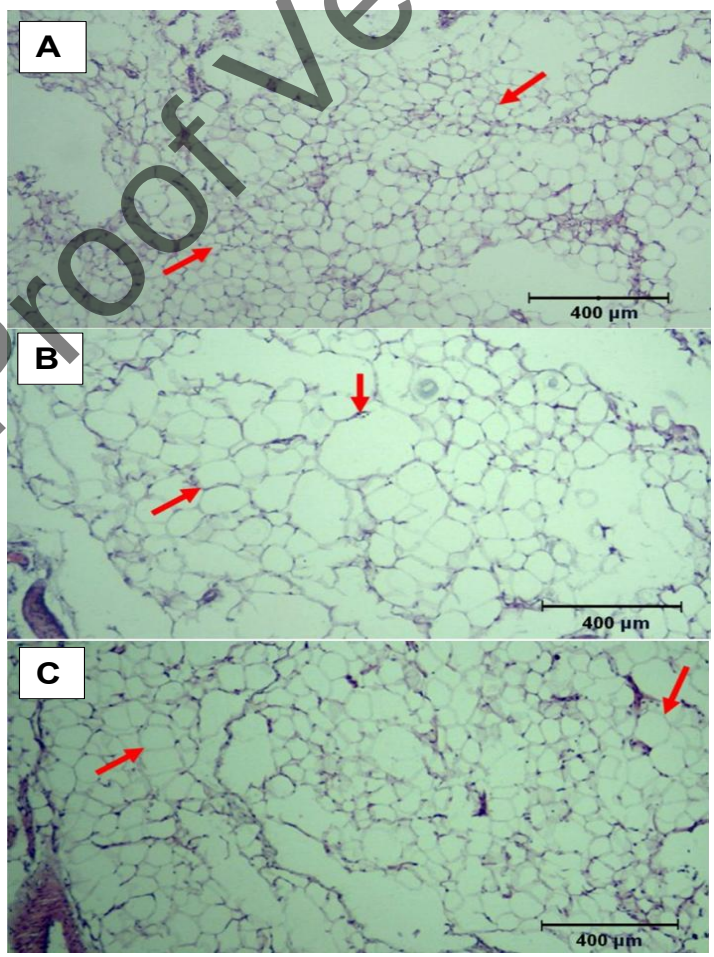


Figure 3. Representative hematoxylin and eosin-stained visceral adipose tissue at 400× magnification. **(A)** ND group, with relatively small adipocytes and preserved lobular architecture; **(B)** WD group, with enlarged adipocytes and reduced apparent cell density; and **(C)** MP group, with an intermediate pattern and smaller adipocytes than in the WD group. Red arrows indicate adipocytes. Scale bar = 400 ìm. MP, metformin-probiotic; ND, normal-diet; WD, Western-diet.

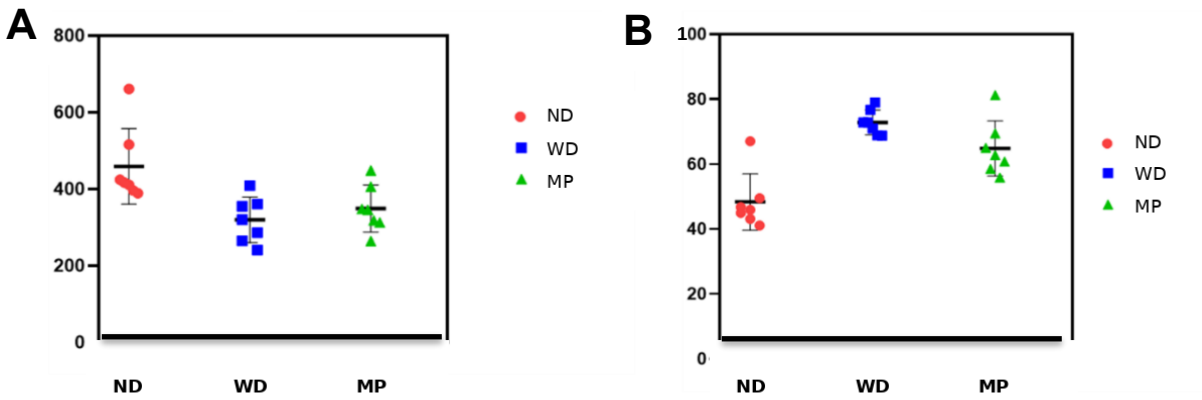


Figure 4. Individual visceral adipose tissue measurements: **(A)** adipocyte count and **(B)** adipocyte diameter. Individual observations and mean ± standard deviation are shown (n = 6 per group). ND, normal diet; WD, Western diet; MP, western diet plus metformin and *L. plantarum* DAD-13.

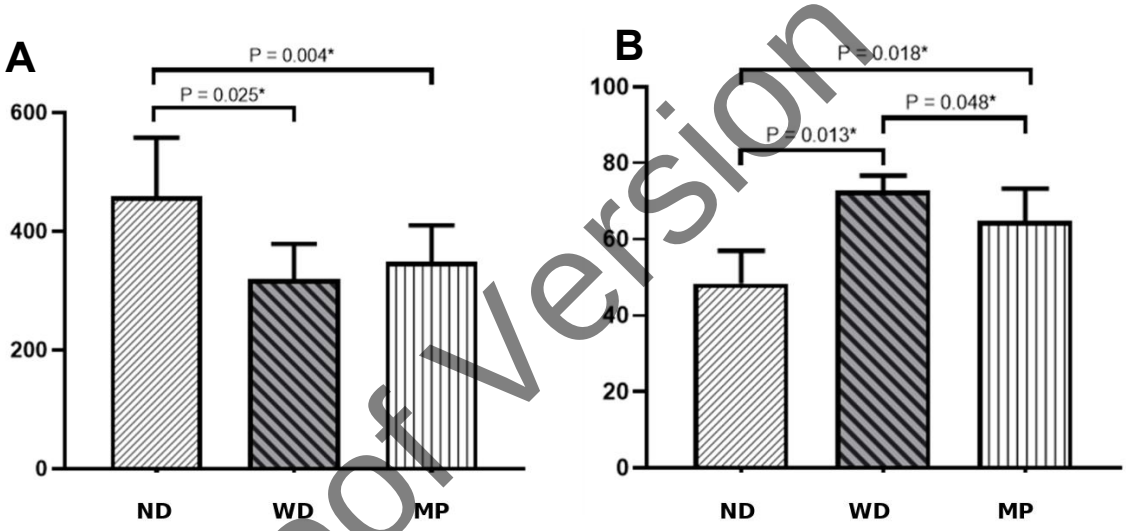


Figure 5. Exploratory pairwise Mann-Whitney U comparisons for **(A)** adipocyte count and **(B)** adipocyte diameter. The displayed *P*-values are unadjusted. ND, normal diet; WD, Western diet; MP, western diet plus metformin and *L. plantarum* DAD-13.

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Discussion

In the MP group, the main finding was a smaller mean visceral adipocyte diameter than in the untreated WD group. This modest tissue-level difference resulted from an exploratory unadjusted pairwise comparison. It was not accompanied by a significant WD–MP difference in adipocyte count, Lee index, triglycerides, HDL-C, LDL-C, or total cholesterol. Accordingly, the results support a preliminary adipose tissue signal rather than a broad metabolic benefit.

The Lee index exceeded the commonly used obesity threshold in all groups but did not differ between the WD and MP groups. Anthropometric indices can be insensitive to changes in fat distribution or lean mass, and probiotic trials have shown variable effects on weight-related outcomes. [13] The higher mean Lee index in the MP group should not be interpreted as evidence of treatment benefit. Future studies should focus on more informative endpoints, such as direct measurements of visceral fat mass or imaging-based body composition.

Several biological pathways could affect adipocyte morphology. Probiotics may alter gut microbial composition, strengthen intestinal barrier function, reduce metabolic endotoxemia, and modify short-chain fatty acid production and inflammatory signaling. [14] In high-fat-diet models, selected *L. plantarum* strains have reduced adipocyte size or attenuated features of metabolic syndrome. [15, 16] Modulation of the intestinal microbiota has been investigated as a strategy

to enhance the response to metformin. [17] The present study did not measure microbial composition, gut permeability, short-chain fatty acids, cytokines, or insulin sensitivity; thus, these mechanisms remain hypotheses.

Evidence regarding concomitant metformin and probiotic therapy is mixed. A clinical comparison in dysglycemia suggested that probiotics may influence gastrointestinal tolerability and metformin adherence, whereas a pediatric NAFLD trial emphasized that probiotic therapy should accompany dietary and lifestyle modification. [18, 19] This current experiment did not include metformin-only or probiotic-only groups so it cannot distinguish the individual contributions or establish any synergy. Furthermore, the treatment duration might have been too short to observe systemic lipid or anthropometric effects.

The overall triglyceride difference likely reflects the dietary model, since the WD and MP groups both received the Western diet and did not differ significantly. Triglyceride accumulation is central to NAFLD biology, but circulating triglyceride concentrations are influenced by diet, hepatic production, peripheral clearance, and fasting conditions. [20] Metformin can improve selected lipoprotein measures, but the magnitude and consistency of these effects vary across studies. [21] The absence of significant WD–MP differences in HDL-C, LDL-C, and total cholesterol therefore argues against lipid profile improvements.

Metformin activates AMPK and affects hepatic gluconeogenesis, fatty acid metabolism, and insulin sensitivity. [22] Probiotic approaches in NAFLD may act through the gut-liver axis and inflammatory pathways. [23] Metformin also influences extracellular matrix remodeling and metabolic signaling in adipose tissue, which offers a plausible explanation for a tissue-specific change that precedes measurable systemic effects. [24] Nevertheless, adipocyte size and number are interrelated, and counts obtained within a fixed field of view can decrease when cells enlarge. Adipose morphology should therefore be interpreted with attention to sampling strategy and tissue architecture. [25]

This study has several limitations. First, the study had a small sample size and undocumented a priori power calculation. Randomization, allocation concealment, and assessor blinding were not documented in the available records. Further, the design lacked metformin-only and probiotic-only groups. The study did not report a liver histology score, hepatic triglyceride content, insulin or glucose indices, inflammatory biomarkers, or microbiome outcomes. Similarly, the image-analysis software and assessor-blinding procedure were not recorded. Finally, pairwise Mann–Whitney U tests were exploratory and unadjusted for multiple comparisons. These limitations increase the risk of imprecision and bias and require cautious interpretation.

Conclusion

Ten weeks of combined metformin and *L. plantarum* DAD-13 administration was associated with a smaller mean visceral adipocyte diameter than with Western diet alone. However, the combined regimen did not significantly improve adipocyte count, Lee index, HDL-C, LDL-C, total cholesterol, or WD–MP triglyceride comparison. These findings are exploratory and warrant confirmation in adequately powered, randomized, blinded studies that include monotherapy groups and mechanistic metabolic and microbiome outcomes.

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Author contributions

SM contributed substantially to study conception and design, data acquisition, literature review, data analysis and interpretation, and drafting of the manuscript. AR contributed to data curation, interpretation, and critical revision of the manuscript. MKH contributed to the methodology, data interpretation, and critical revision of the manuscript. SNN contributed substantially to data acquisition and critical revision of the manuscript. H, NS, and PIDA contributed to the validation and critical revision of the manuscript for important intellectual content. All authors approved the final version submitted for publication and agreed to be accountable for all aspects of the work.

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None.

Conflicts of interest statement

None.

Data sharing statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this manuscript, the authors used Claude (Anthropic) solely to improve the language, grammar, and readability of the text. The tool was not used to generate scientific content, design the study, analyze or interpret the data, or produce figures. After using the tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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