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Wpływ diety wysokotłuszczowej/wysokofruktozowej na rozwój Zespołu policystycznych jajników oraz próba identyfikacji narzędzia dietetycznego sprzyjającego jego terapii

The impact of a high-fat/high-fructose diet on Polycystic Ovary Syndrome and identification of the dietary tool to its treatment

Rozprawa doktorska w dziedzinie nauk rolniczych
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Serdecznie dziękuję wszystkim, którzy przyczynili się do powstania
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ale i źródłem nieustającej motywacji.

Wykaz artykułów naukowych wchodzących w skład zbioru:

1) Pieczyńska-Zając, J. M., Malinowska, A. M., Pruszyńska-Oszmałek, E., Kołodziejski, P. A., Drzymała-Czyż, S., & Bajerska, J. (2024). Effect of a high-fat high-fructose diet on the composition of the intestinal microbiota and its association with metabolic and anthropometric parameters in a letrozole-induced mouse model of polycystic ovary syndrome. *Nutrition* 4 (124), 112450. https://doi.org/10.1016/j.nut.2024.112450 - **140 pkt MNiSW, Impact Factor 4,4**

2) Pieczyńska, J. M., Pruszyńska-Oszmałek, E., Kołodziejski, P. A., Łukomska, A., & Bajerska, J. (2022). The Role of a High-Fat, High-Fructose Diet on Letrozole-Induced Polycystic Ovarian Syndrome in Prepubertal Mice. *Nutrients*, 14(12), 2478. https://doi.org/10.3390/nu14122478 - **140 pkt MNiSW, Impact Factor 5,9**

3) Pieczyńska-Zając, J. M., Malinowska, A., Łagowska, K., Leciejewska, N., & Bajerska, J. (2023). The effects of time-restricted eating and Ramadan fasting on gut microbiota composition: A systematic review of human and animal studies. *Nutrition Reviews* 10, 82(6), 777-793. https://doi.org/10.1093/nutrit/nuad093 - **140 pkt MNiSW, Impact Factor 6,1**

Spis treści

Wykaz skrótów stosowanych w autoreferacie

PCOS – Zespół policystycznych jajników (ang. *Polycystic Ovary Syndrome*)

GnRH - hormon uwalniający gonadotropiny (ang. *gonadotropin-releasing hormone)*

LH – hormon luteinizujący (ang. *luteinizing hormone*)

FSH – hormon folikulotropowy (ang. *follicle-stimulating hormone*)

KKT – krótkołańcuchowe kwasy tłuszczowe (ang. *short chain fatty acids*)

LPS – lipopolisacharyd (ang. *lipopolysaccharide*)

TRE – żywienie ograniczone czasowo (ang. *time-restricted eating*)

RF – post Ramadan (ang. *Ramadan fasting*)

LET – letrozol (ang. *letrozole*)

HF/HFr – dieta wysokotłuszczowa/wysokofruktozowa (ang. *high fat/high fructose diet*)

HOMA-IR – wskaźnik insulinooporności (ang. *Homeostasis Model Assessment of Insulin Resistance*)

StD – dieta standardowa (ang. *standard diet*)

TC – cholesterol całkowity (ang. *total cholesterol*)

HDL-C - cholesterol frakcji lipoprotein o wysokiej gęstości (ang. *high-density lipoprotein cholesterol)*

LDL-C - cholesterol frakcji lipoprotein o niskiej gęstości (ang. *low-density lipoprotein cholesterol*)

CRP – białko C-reaktywne (ang. *C-reactive protein*)

TAC – całkowita pojemność antyoksydacyjna (ang. *total antioxidant capacity*)

NEFA - niezestryfikowane kwasy tłuszczowe (ang. *non-esterified fatty acids*)

QUICKI – ilościowy wskaźnik kontroli wrażliwości na insulinę (ang. *Quantitative Insulin Sensitivity Check Index*)

BMI - wskaźnik masy ciała (ang. *body mass index*)

Streszczenie w języku polskim

Zespół policystycznych jajników (PCOS) to zaburzenie endokrynologiczne i metaboliczne, występujące nawet u 20% kobiet w wieku rozrodczym. Dotychczas nie określono jednoznacznie przyczyny powstawania PCOS, choć uznaje się, że nieprawidłowa dieta, obfitująca w nasycone kwasy tłuszczowe oraz cukry proste ma istotny wpływ na jego rozwój. Od niedawna wskazuje się także na znamienny wpływ mikrobioty jelitowej w patogenezie PCOS. Uznano, że poza kompozycją diety, również czas spożywania posiłków jest czynnikiem wpływającym na skład konsorcjum bakteryjnego. Z tego względu reżim żywienia ograniczonego czasowo (TRE), wydaje się być narzędziem modulującym nie tylko skład i ogólną kondycję mikrobioty jelitowej, ale również zdrowie gospodarza. W związku z powyższym, ***celem głównym badań była ocena wpływu diety wysokotłuszczowej/wysokofruktozowej (HF/HFr) na skład mikrobioty jelitowej oraz zaburzenia metaboliczne i endokrynologiczne w mysim modelu PCOS oraz poszukiwanie terapii dietetycznej, umożliwiającej ich łagodzenie***. Do eksperymentu włączono 32 samice myszy szczepu C57BL/6 w okresie przedpokwitaniowym, które losowo podzielono na 4 grupy, po 8 osobników w każdej. Myszom wszczepiono pellet z letrozolem (LET) (indukcja PCOS) lub placebo i karmiono je dietą HF/HFr lub standardową (StD). Dieta HF/HFr spożywana w okresie przedpokwitaniowym, miała znacznie silniejszy wpływ na skład mikrobioty jelitowej oraz jej zdolność do produkcji metabolitów niż sam LET. Skład mikrobioty korelował również z niektórymi parametrami związanymi z PCOS. Ponadto, dieta HF/HFr sprzyjała rozwojowi zaburzeń metabolicznych oraz hormonalnych, nie tylko u myszy otrzymujących LET, ale także w grupie placebo. Na podstawie przeprowadzonego systematycznego przeglądu literaturowego, zauważono, że TRE częściowo przywraca zaburzone przez dietę wysokotłuszczową, okołodobowe fluktuacje bakterii jelitowych u zwierząt. Reżim ten wpływał korzystnie na poprawę różnorodności oraz stymulację wzrostu prozdrowotnych bakterii (np. *Akkermansia*) u ludzi i zwierząt karmionych StD. Zauważono m.in. dodatnią zależność korelacyjną pomiędzy stężeniem cholesterolu frakcji HDL a bogactwem gatunkowym mikrobioty. Uzyskane rezultaty poszerzają dotychczasową wiedzę na temat znaczenia składu diety, spożywanej w okresie przepokwitaniowym w kontekście rozwoju PCOS, a także możliwości dietoterapeutycznych tej jednostki chorobowej.

Słowa kluczowe: Zespół policystycznych jajników, zaburzenia metaboliczne, zaburzenia endokrynologiczne, mikrobiota jelitowa, żywienie ograniczone czasowo

Streszczenie w języku angielskim (Summary)

Polycystic Ovary Syndrome (PCOS) is an endocrine and metabolic disorder affecting up to 20% of women of reproductive age. The cause of PCOS has not been clearly defined, although an improper diet, high in saturated fatty acids and simple sugars, is considered to have a significant impact on its development. More recently, the significant influence of the gut microbiota in the pathogenesis of PCOS has also been pointed out. It has been recognized that, in addition to the composition of the diet, the timing of meal consumption is also a factor in the composition of the bacterial consortium. Therefore, a time-restricted feeding (TRE) regimen, appears to be a tool to modulate not only the composition and overall condition of the gut microbiota, but also the health of the host. *The main objective of this study was to evaluate the effects of a high-fat/high-fructose (HF/HFr) diet on the composition of the gut microbiota and metabolic and endocrine disorders in a mouse model of PCOS, and to search for a dietary therapy to alleviate them.* Thirty-two female prepubertal C57BL/6 mice were included in the experiment and randomly divided into 4 groups, with 8 individuals in each group. Mice were implanted with a letrozole (LET) pellet (PCOS induction) or a placebo and fed a HF/HFr or standard diet (StD). The HF/HFr diet consumed during the prepubertal period, had a significantly stronger effect on the composition of the gut microbiota and its ability to produce metabolites than LET alone. The composition of the microbiota also correlated with some parameters, associated with PCOS. In addition, the HF/HFr diet caused the development of metabolic and endocrine disorders, not only in mice receiving LET, but also in the placebo group. Based on a systematic review of the literature, it was noted that TRE partially restored the circadian fluctuations of gut bacteria disrupted by the high-fat diet in animals. The regime had beneficial effects on improving the diversity and stimulating the growth of health-promoting bacteria (e.g. *Akkermansia*) in humans and animals fed a StD. Moreover, a positive correlation was observed between HDL fraction cholesterol concentration and species richness of the microbiota. The results add to the existing knowledge of the importance of the composition of the diet consumed during the post-pubertal period in the context of the development of PCOS, as well as the dietary therapeutic options for this disease.

Key words: Polycystic Ovary Syndrome, metabolic disorders, endocrine disorders, intestinal microbiota, time-restricted eating

Wstęp

Zespół policystycznych jajników (PCOS; ang. *Polycystic Ovary Syndrome*) jest stosunkowo powszechnym zaburzeniem endokrynologicznym i metabolicznym, występującym u około 6-20% kobiet w wieku rozrodczym (Siddiqui i in., 2022). Uważa się, że PCOS ma heterogeniczny charakter, ze względu na występowanie różnych objawów m.in. hiperandrogenizmu, insulinooporności, chorób układu krążenia, otyłości brzusznej, zaburzeń psychicznych oraz niepłodności (Siddiqui i in., 2022). Obecnie wyróżnia się cztery fenotypy PCOS:

- I – klasyczny, z występującym hiperandrogenizmem, *oligomenorrhea* (miesiączkami rzadko występującymi) oraz obrazem policystycznych jajników, rozpoznawanym w badaniu ultrasonograficznym,
- II – z występującym hiperandrogenizmem oraz *oligomenorrhea,*
- III – z występującym hiperandrogenizmem oraz obrazem policystycznych jajników, rozpoznawanym w badaniu ultrasonograficznym,
- IV – z występującym obrazem policystycznych jajników, rozpoznawanym w badaniu ultrasonograficznym oraz *oligomenorrhea* (Fauser i in., 2012).

Patomechanizm PCOS nie został dotychczas w pełni wyjaśniony, prawdopodobnie ze względu na jego wieloczynnikowy charakter (Bachelot, 2016), obejmujący hiperandrogenizm, zaburzenia owulacji, nieprawidłowe wydzielanie hormonu uwalniającego gonadotropiny (GnRH; ang. *gonadotropin-releasing hormone*) i wynikające z niej wzmożone wydzielanie hormonu luteinizującego (LH; ang. *luteinizing hormone*), skutkujące zaburzonym stosunkiem LH do FSH (hormonu folikulotropowego; ang. *follicle-stimulating hormone*), co z kolei powoduje nasiloną jajnikową produkcją androgenów a zmniejszoną estrogenów. Czynniki te oddziałują na siebie wzajemnie, nasilając swoje działanie i powodując tzw. „błędne koło" (Harada, 2022).

Obecnie naukowcy wciąż debatują czy podwyższone stężenie androgenów u kobiet z PCOS pochodzi z sekrecji jajnikowej, pod wpływem nadaktywnego wydzielania GnRH i tym samym LH, czy też podwyższone stężenie androgenów wpływa na działanie przysadki mózgowej w okresie dojrzewania i/lub dorosłości, kształtując i utrzymując w ten sposób nadmierne wydzielanie GnRH i LH (Hajam i in., 2024). Zapoczątkowanie tych zaburzeń ma już prawdopodobnie miejsce w nieprawidłowo przebiegającym okresie dojrzewania. U dziewcząt z nieregularnymi, bezowulacyjnymi cyklami zaobserwowano przetrwanie przedpokwitaniowego wydzielania gonadrotropin, a mianowicie znacznie wyższych amplitud

szczytów GnRH i w związku z tym także wyższych stężeń LH oraz niższych stężeń FSH (Gajewska, 2000).

Jednak dotychczas nie określono jednoznacznie bezpośredniej przyczyny zaistnienia tych zaburzeń, choć wskazuje się na znaczenie takich czynników jak predyspozycja genetyczna, przewlekła ekspozycja na substancje zaburzające funkcjonowanie układu hormonalnego (np. bisfenol A) czy też nieprawidłowy skład diety (Merkin i in., 2016). Zauważono, że wraz z rozwojem stopnia urbanizacji, sposób żywienia przeciętnego człowieka zaczął upodabniać się do tzw. diety „zachodniej" (Wu i in., 2023), obfitującej w nasycone kwasy tłuszczowe oraz cukry proste (Edwin Thanarajah i in., 2023). Wyniki systematycznego przeglądu, przeprowadzonego przez De Amicis i in. wskazują, że wśród młodzieży obserwuje się wysokie spożycie żywności ultraprzetworzonej o niskiej wartości odżywczej i wysokiej wartości energetycznej (De Amicis i in., 2022), a także napojów słodzonych i energetyzujących, które stanowią dla młodzieży główne źródło fruktozy w diecie (Giussani i in., 2022). Niepokojący wydaje się fakt, że nawet ok. 75% dzieci w wieku przedszkolnym codziennie spożywa co najmniej jeden rodzaj żywności ultraprzetworzonej (Longo-Silva i in., 2017).

Wspomniane błędy żywieniowe w okresie dojrzewania sprzyjają powstawaniu otyłości (Tsan i in., 2021), a w konsekwencji także zaburzeń hormonalnych, prowadzących do rozwoju hiperandrogenemii (Burt Solorzano i in., 2010). W badaniach na modelu zwierzęcym, zauważono, że szczury żywione dietą wysokotłuszczową w okresie przedpokwitaniowym rozpoczęły proces dojrzewania zdecydowanie wcześniej niż szczury, żywione dietą kontrolną, co świadczy o pojawieniu się zaburzeń reprodukcyjnych (Cannady i in., 2000). Potwierdzają to również badania z udziałem dziewcząt (Lee i in., 2007). Wydaje się, że przyczyną tego stanu jest nadmierna zawartość tkanki tłuszczowej w organizmie, wywołana znaczną podażą energii. Skutkuje to zwiększoną syntezą insuliny oraz leptyny, które stymulują przysadkę mózgową do przedwczesnej sekrecji gonadrotropin i tym samym sekrecji hormonów płciowych w jajnikach (Soliman i in., 2014). Rzeczywiście spożycie diety wysokowęglowodanowej, wysokotłuszczowej, ubogiej w błonnik pokarmowy zwiększa istotnie ryzyko rozwoju PCOS (Alomran & Estrella, 2023). Badania z wykorzystaniem modelu zwierzęcego również sugerują, że podaż wysokotłuszczowej diety w okresie przedpokwitaniowym powoduje szereg zaburzeń metabolicznych i reprodukcyjnych, podobnych do tych obserwowanych u kobiet z PCOS (Patel & Shah, 2018). Wyżej wspomniane błędy żywieniowe powielane są w dalszych latach życia (Mizgier i in., 2021). Również Hajivandi i in. zauważyli, że dziewczęta z nadmierną masą ciała,

chorujące na PCOS, pomijają spożywanie głównych posiłków, głównie śniadań, a także często sięgają po przekąski o wysokiej gęstości energetycznej (Hajivandi i in., 2020).

W ostatnich latach, wskazuje się także na istotną rolę mikrobioty jelitowej w patogenezie PCOS (Sun i in., 2023). W 2012 roku sformułowano teorię DOGMA (ang. *dysbiosis of gut microbiota*), w której wskazano, że nieprawidłowy sposób żywienia w przebiegu PCOS, powoduje wzrost bakterii Gram (-), przy jednoczesnym zmniejszeniu obfitości prozdrowotnych bakterii z grupy *Lactobacillus* i *Bifidobacterium*. Przyczynia się to do rozwoju stanu zapalnego i zwiększeniu przepuszczalności jelitowej, prowadząc do tzw. endotoksemii jelitowej. Aktywacja układu immunologicznego upośledza funkcje receptora insulinowego i kolejno sprzyja rozwojowi insulinooporności i hiperinsulinemii, która następnie zwiększa produkcję androgenów w jajnikach i zatrzymuje rozwój pęcherzyków jajnikowych (Tremellen & Pearce, 2012). W tym względzie zauważono, że kobiety, u których obserwuje się zaburzony stosunek stężenia testosteronu do estrogenu, tak jak w przebiegu PCOS, mają niekorzystnie zmienioną mikrobiotę jelitową, wyrażoną m.in. jej obniżoną alfa-różnorodnością czy też istotnie niższą obfitością prozdrowotnych bakterii, np. z rodzaju *Ruminococcus,* produkujących kwas masłowy (d'Afflitto i in., 2022). W kale kobiet z PCOS w porównaniu do kobiet zdrowych zauważono obniżone stężenie krótkołańcuchowych kwasów tłuszczowych (KKT; ang. *short chain fatty acids*), w tym wspomnianego wcześniej kwasu masłowego, co prowadzi do zaburzenia integralności bariery jelitowej (Zhang i in., 2019). Wskutek tego, lipopolisacharyd (LPS; ang. *lipopolysaccharide*), produkowany przez bakterie Gram (-) przechodzi do krwioobiegu i powoduje rozwój stanu zapalnego, zaburzenia gospodarki węglowodanowej, w tym insulinooporność, co sprzyja powstawaniu otyłości (Liu i in., 2018).

W systematycznym przeglądzie literatury potwierdzono, że nieprawidłowa dieta ma wyraźnie negatywny wpływ na skład mikrobioty jelitowej. Spożycie nasyconych kwasów tłuszczowych zaburza nie tylko obfitość, ale i różnorodność drobnoustrojów jelitowych, natomiast wysokie spożycie fruktozy dodatnio koreluje ze zmniejszoną integralnością bariery jelitowej (Jamar i in., 2021). Agus i in. zauważyli, że dieta obfitująca w tłuszcz oraz cukry proste tworzy w jelitach myszy specyficzne środowisko zapalne, sprzyjające wzrostowi prozapalnych bakterii typu Proteobacteria, m.in. *E.coli*, a zmniejszeniu obfitości prozdrowotnych bakterii, produkujących KKT (Agus i in., 2016). Zaburzenia w składzie mikrobioty jelitowej mogą częściowo wyjaśniać związek pomiędzy spożywaniem „diety zachodniej" a występowaniem niezakaźnych chorób przewlekłych, tj. cukrzycy typu II, depresji czy chorób zapalnych jelit (Severino i in., 2024). Jednak do tej pory nie określono

jednoznacznie w jaki sposób dieta typu zachodniego wpływa na mikrobiotę jelitową, stężenie jej metabolitów i tym samym na manifestację objawów klinicznych PCOS.

W związku z tym, że dieta jest kluczowym czynnikiem, wpływającym na skład mikrobioty jelitowej (Cronin i in., 2021), zaczęto poszukiwać reżimu dietetycznego, który mógłby łagodzić dysbiozę jelitową, będącą konsekwencją jej nieprawidłowego składu. Istotnym elementem tych poszukiwań jest zrozumienie funkcjonowania rytmów okołodobowych, regulujących i synchronizujących procesy fizjologiczne, metaboliczne oraz behawioralne, zachodzące w ustroju z sygnałami środowiska zewnętrznego, tzw. „dawcami czasu" (Zhao i in., 2022). Główny zegar biologiczny, tzw. „master clock" znajduje się w jądrze nadskrzyżowaniowym podwzgórza i jego aktywność regulowana jest poprzez światło. Organizm wyposażony jest również w tzw. zegary obwodowe, zlokalizowane w tkankach i narządach, m.in. w wątrobie, mięśniach, tkance tłuszczowej oraz w jelitach, których funkcjonowanie regulowane jest poprzez rytm aktywność-odpoczynek oraz rytm żywienie-post (Golombek & Rosenstein, 2010; Simon i in., 2019). Doniesienia, płynące z badań z udziałem ludzi oraz z wykorzystaniem modeli zwierzęcych, sugerują, że przebieg PCOS prawdopodobnie związany jest z zaburzoną ekspresją genów, kodujących zegar obwodowy, znajdujący się w jajnikach (Zhou & Huddleston, 2021). Zauważono także, że skład mikrobioty jelitowej ulega cyklicznym, okołodobowym zmianom, związanym z czasem przyjmowania pokarmu (Zarrinpar i in., 2014). Dlatego też, reżim żywienia ograniczonego czasowo (TRE; ang. *time-restricted eating*), polegający na przyjmowaniu posiłków w tzw. „oknie żywieniowym" przez 8 godzin, po których następuje 16 godzin poszczenia, może być niejako czynnikiem modulującym skład i ogólną kondycję mikrobioty jelitowej, jak również ogólnie pojęte zdrowie gospodarza (Ye i in., 2020). Za jedną z form TRE uznaje się także muzułmański post Ramadan (RF; ang. *Ramadan fasting*). Zgodnie z założeniem TRE nie wymaga ograniczeń jakościowych i ilościowych w spożywanej diecie, a „okno żywieniowe" powinno być stosowane w ciągu dnia. Natomiast w przypadku RF posiłki przyjmowane są od zachodu słońca do jego wschodu, a ponadto zmianie ulega także jakość diety, wynikająca ze zwyczajowego zwiększonego spożycia ciast, suszonych owoców oraz zmniejszonego spożycia nabiału, jaj i produktów zbożowych (Shatila i in., 2021). Uzyskane dotychczas wyniki sugerują, że TRE może być korzystnym narzędziem dietoterapeutycznym, stosowanym w przebiegu PCOS. Jednak dostępne rezultaty pochodzą z ograniczonej ilości badań (2 z udziałem kobiet z PCOS oraz 2 z wykorzystaniem mysiego modelu PCOS), w których oceniono wpływ TRE na wybrane parametry metaboliczne i hormonalne. W badaniach Ryu i in., 2023 wykazano normalizację

częstości pulsów LH, zaburzonych podażą letrozolu (LET; ang. *letrozole* - niesteroidowy inhibitor aromatazy – enzym przekształcający androgeny nadnerczowe androstendion i testosteron do estronu i estradiolu), po wprowadzeniu TRE na okres 4 tygodni (Ryu i in., 2023), a także ogólną poprawę profilu metabolicznego (w tym masy ciała) i endokrynologicznego, zarówno u kobiet z PCOS jak i w zwierzęcym modelu tej jednostki chorobowej (Feyzioglu i in., 2023; Han i in., 2022; C. Li i in., 2021). Jednakże, w żadnych z tych badań nie dokonano analizy składu mikrobioty, choć zauważono, że stężenie kalprotektyny w kale kobiet z PCOS uległo istotnemu obniżeniu po 6-tygodniach stosowania TRE, co może świadczyć o redukcji stanu zapalnego w jelitach (Feyzioglu i in., 2023).

Biorąc pod uwagę powyższe, **celem głównym** badań realizowanych w ramach niniejszej rozprawy doktorskiej było:

Ocena wpływu diety wysokotłuszczowej/wysokofruktozowej (HF/HFr) na skład mikrobioty jelitowej oraz zaburzenia metaboliczne i endokrynologiczne w mysim modelu PCOS oraz poszukiwanie terapii dietetycznej umożliwiającej ich łagodzenie.

Wyszczególniono również następujące **cele szczegółowe**:

- Opracowanie protokołu indukcji PCOS z wykorzystaniem długouwalniającego się LET u myszy w wieku przedpokwitaniowym.
- Analiza składu mikrobioty jelitowej oraz jej metabolitów (LPS oraz KKT).
- Ocena związku pomiędzy składem mikrobioty jelitowej a parametrami antropometrycznymi, metabolicznymi oraz hormonalnymi, występującymi w przebiegu PCOS.
- Ocena wpływu TRE i RF na skład mikrobioty jelitowej w badaniach na zwierzętach, jak i z udziałem ludzi oraz ustalenie korelacji pomiędzy obfitością wybranych bakterii a parametrami antropometrycznymi i metabolicznymi.

Sformułowano także następujące **hipotezy badawcze**:

1) Jeżeli LET podawany w wieku przedpokwitaniowym, powoduje niekorzystne zmiany w składzie mikrobioty jelitowej i objawy charakterystyczne dla PCOS, to dieta wysokofruktozowa/wysokotłuszczowa zaburzenia te może dodatkowo zaostrzyć.

2) Żywienie ograniczone czasowo korzystnie moduluje skład i różnorodność mikrobioty jelitowej i tym samym sprzyja poprawie parametrów antropometrycznych oraz metabolicznych zarówno w modelu zwierzęcym, jak i u ludzi.

Uzyskane wyniki pozwolą na lepsze zrozumienie mechanizmów patofizjologicznych PCOS, łączących występowanie zaburzeń metabolicznych i hormonalnych ze składem mikrobioty jelitowej oraz jej metabolitami. Ponadto należy podkreślić nowatorski charakter

badania, wynikający przede wszystkim z doboru metody indukcji PCOS przy użyciu długouwalniającego się letrozolu, który dotychczas nie był powszechnie stosowany w Polsce.

Z kolei, określenie wpływu TRE na skład mikrobioty i ocena powiązań tych zmian ze zdrowiem metabolicznym gospodarza, wydaje się być istotne w aspekcie opracowania personalizowanych i alternatywnych metod dietoterapii, poprawiających jakość życia pacjentów, co może stanowić podstawę do prowadzenia dalszych badań klinicznych oceniających efektywność tego reżimu żywieniowego u kobiet z PCOS.

Materiały i metody badań

W badaniach wykorzystano szczep myszy C57BL/6J, ponieważ wykazuje on znaczną podatność na otyłość spowodowaną dietą, objawiającą się większymi przyrostami masy ciała i tkanki tłuszczowej w przeliczeniu na podaż energii oraz wyraźniejszymi zaburzeniami metabolicznymi w porównaniu z innymi szczepami (Black i in., 1998). Ponadto większość samic myszy tego szczepu ma 4–6-dniowe cykle rujowe z wyraźnymi jego etapami, a także wykazują one zwiększoną ekspresję jajnikowego receptora insuliny, co czyni je najbardziej odpowiednim modelem do badań nad PCOS (Dowling i in., 2013).

Eksperyment na modelu zwierzęcym umożliwia ścisłą kontrolę warunków otoczenia, w tym stosowaną dietę, temperaturę czy cykl oświetlenia, co również wpływa korzystnie na jakość i precyzję uzyskanych wyników (VandeBerg & Williams-Blangero, 1997). Terminacja zwierząt umożliwia pobranie treści jelitowej z jelita ślepego, natomiast w przypadku badań z udziałem kobiet z PCOS, jedyną możliwością jest pobranie od nich próbki kału, co wiąże się z ryzykiem, m.in. związanym z możliwością zanieczyszczenia próbki po pobraniu, niewłaściwym sposobem pobrania, transportu lub jej przechowywania (Bharti & Grimm, 2021).

Dołożono wszelkich starań, aby zminimalizować zarówno liczbę wykorzystywanych zwierząt, jak i ich cierpienie. Wielkość próby obliczona została przy użyciu oprogramowania G*Power (RRID:SCR_013726) na podstawie danych, uzyskanych przez Zheng`a i in. (Zheng i in., 2021). Wielkość efektu obliczono na 2,05 na podstawie różnic w parametrze HOMA-IR (ang. *Homeostasis Model Assessment of Insulin Resistance)* pomiędzy grupą kontrolną, karmioną dietą wysokotłuszczową a grupą PCOS, żywioną tą samą dietą. Przy wartości alfa wynoszącej 0,05 i mocy 0,95, wielkość próby wynosiła osiem osobników na grupę.

Do eksperymentu włączono 32 samice myszy szczepu C57BL/6, w wieku przedpokwitaniowym (3 tygodnie), pochodzące z Instytutu Medycyny Doświadczalnej i Klinicznej im. M. Mossakowskiego (Warszawa, Polska). Badanie uzyskało zgodę Lokalnej Komisji Etycznej na podstawie zezwolenia nr 51/2021. Doświadczenie przeprowadzone zostało w zwierzętarni Katedry Fizjologii, Biochemii i Biostruktury Zwierząt, na Wydziale Medycyny Weterynaryjnej i Nauk o Zwierzętach, Uniwersytetu Przyrodniczego w Poznaniu. Po dziesięciodniowym okresie aklimatyzacji, myszy w wieku 4 tygodni zostały losowo rozdzielone do czterech równolicznych grup, po 8 osobników w każdej – 1) Placebo – grupa, otrzymująca podskórnie pellet z placebo, żywiona dietą standardową (StD), 2) Placebo + HF/HFr - grupa, otrzymująca podskórnie pellet z placebo, żywiona dietą HF/HFr, 3) LET -

grupa, otrzymująca podskórnie pellet z letrozolem, żywiona dietą standardową i 4) LET + HF/HFr - grupa, otrzymująca podskórnie pellet z letrozolem, żywiona dietą HF/HFr. Dieta StD składała się z 18% białka, 66% węglowodanów i 16% tłuszczu, a jej wartość energetyczna wynosiła 3.8 kcal/g. Natomiast wartość energetyczna diety HF/HFr wynosiła 4.7 kcal/g diety i składała się z 17% białka, 37,5% węglowodanów (głównie fruktozy) oraz 45,5% tłuszczu. Pellet podano jednorazowo, co znacząco zredukowało cierpienie i stres zwierząt. Podanie pelletu ze stopniowo uwalniającym się LET (3 mg, 50 ug/day), pozwoliło na wywołanie „szczupłego" fenotypu PCOS, obejmującego jedynie charakterystyczne zmiany w obrazie morfologicznym jajników. Dieta i woda były dostępne *ad libitum* przez cały czas trwania doświadczenia. Raz w tygodniu myszy ważono, a w czwartym tygodniu trwania eksperymentu oceniono także spożycie diety, z wykorzystaniem klatek półmetabolicznych. W trakcie ostatniego tygodnia trwania doświadczenia, od myszy zebrano świeży kał, w którym oznaczono stężeniem KKT (masłowy, propionowy oraz octowy) przy wykorzystaniu chromatografii gazowej z detekcją płomieniowo-jonizacyjną. Badania przeprowadzono w Katedrze i Zakładzie Bromatologii, Uniwersytetu Medycznego w Poznaniu. W tym samym tygodniu, oceniono skład ciała zwierząt przy użyciu analizatora składu ciała Bruker Minispec LF90 (USA).

Po zakończonym eksperymencie, dokonano terminacji zwierząt i pobrano krew oraz organy (wątrobę oraz jajniki). Krew odwirowano przy 3500 x g przez 15 minut w 4*C, w celu uzyskania surowicy, którą następnie zamrożono w -80*C w celu przeprowadzenia dalszych analiz. Od zwierząt pobrano wątrobę, w celu oceny jej stłuszczenia oraz jajniki do potwierdzenia indukcji PCOS w badaniu histopatologicznym. W surowicy krwi oznaczono następujące parametry metaboliczne: stężenie glukozy, triglicerydów, cholesterolu całkowitego (TC; ang. *total cholesterol*), cholesterolu frakcji lipoprotein o wysokiej gęstości (HDL-C; ang. *high-density lipoprotein*), cholesterolu frakcji lipoprotein o niskiej gęstości (LDL-C; ang. *low-density lipoprotein*), białka C-reaktywnego (CRP; ang. *C-reactive protein*) i całkowitą pojemność antyoksydacyjną (TAC; ang. *total antioxidant capacity*) testami enzymatycznymi oraz kolorymetrycznymi. Stężenie niezestryfikowanych kwasów tłuszczowych (NEFA; ang. *non-esterified fatty acids*) oznaczono przy użyciu testu enzymatycznego, natomiast stężenie insuliny i testosteronu oznaczono z wykorzystaniem testów immunoenzymatycznych ELISA, zgodnie z instrukcją producenta. Zawartość cholesterolu oraz triglicerydów w wątrobie oznaczono po ekstrakcji lipidów metodą Folch`a i in. (Folch i in., 1957). Ponadto, z jelita ślepego pobrano treść jelitową, z której wyizolowano DNA bakteryjne z wykorzystaniem zestawu QIAamp fecal DNA minikit. Następnie, zabezpieczony materiał zostały przesłany do

firmy Genomed (Warszawa, Polska) w celu przeprowadzenia sekwencjonowania regionu V3-V4 genu kodującego 16S rRNA przy użyciu sekwenatora MiSeq (Illumina, SanDiego, Kalifornia, USA).

Surowe dane analizowano w programie Statistica 13.3.0 (TIBCO Software, Palo Alto, CA, USA; 2017), a dane dotyczące mikrobioty jelitowej w środowisku RStudio (R version 4.0.3 (2020-10-10)) m.in. z wykorzystaniem pakietów phyloseq, microbiome i vegan.

Systematyczny przegląd literaturowy został przeprowadzony zgodnie z zasadami PRISMA (ang. *Preferred Reporting Items for Systematic reviews and Meta-Analyses*) i zarejestrowany w Międzynarodowym Prospektywnym Rejestrze Przeglądów Systematycznych (https://www.crd.york.ac.uk/prospero/; PROSPERO CRD42021278918). Sformułowano następujące pytania badawcze:

- Czy odmienne rodzaje postu (TRE oraz RF) wpływają na różnice w składzie gatunkowym mikrobioty jelitowej, jej różnorodność i potencjał do produkcji metabolitów u ludzi i w modelu zwierzęcym?
- Czy czas spożywania posiłków niezależnie od rodzaju stosowanej diety poprawia skład gatunkowy mikrobioty jelitowej?
- Czy zmiany składu mikrobioty wywołane stosowaniem wybranego reżimu żywieniowego korelują ze zmianami parametrów metabolicznych i antropometrycznych gospodarza?

Strategia wyszukiwania odpowiednich artykułów bazowała na następujących terminach MeSH (ang. *Medical Subject Headings*) - “intermittent fasting” i “gastrointestinal microbiome” oraz słowach kluczowych “Ramadan fasting” i “microbes.” Wyselekcjonowane artykuły poddane zostały ocenie jakościowej z wykorzystaniem narzędzia SYstematic Review Centre for Laboratory animal Experimentation (SYRCLE) dla badań na modelu zwierzęcym oraz Quality Assessment Tool for Observational Cohort and Cross-Sectional Studies, the Quality Assessment Tool for Before–After (Pre–Post) Studies With No Control Group oraz the Quality Assessment Tool of Controlled Intervention Studies dla badań z udziałem ludzi.

Wyniki badań i ich interpretacja

H1: Jeżeli LET podawany w wieku przedpokwitaniowym, powoduje niekorzystne zmiany w składzie mikrobioty jelitowej i objawy charakterystyczne dla PCOS, to dieta wysokofruktozowa/wysokotłuszczowa zaburzenia te może dodatkowo zaostrzyć.

W celu potwierdzenia skuteczności indukcji PCOS, przeprowadzono badanie histopatologiczne jajników. Charakterystyczne dla PCOS zmiany w obrazie jajników, obejmujące występowanie cyst, w tym cyst krwawych, atrezję pęcherzyków jajników oraz zgrubienie warstwy osłonowej pęcherzyków jajnikowych, zauważono nie tylko w grupach, którym podano letrozol, ale także w grupie Placebo + HF/HFr. Potwierdzają to badania Xu i in., w których zauważono, że spożycie diety o charakterze „zachodnim" wywołało szereg zaburzeń reprodukcyjnych u samic makaków (Xu i in., 2015).

W obu grupach, żywionych dietą HF/HFr (Placebo+HF/HFr oraz LET+HF/HFr) obserwowano istotnie wyższą alfa-różnorodność mikrobioty jelitowej w porównaniu do grup żywionych dietą standardową. Z kolei, diagram PCoA, wykorzystujący miarę Braya-Curtis`a, wykazał odmienną beta-różnorodność mikrobioty jelitowej w grupie Placebo+HF/HFr w porównaniu do pozostałych trzech grup, co zostało potwierdzone testami Kruskalla-Wallis`a oraz Dunn`a. W związku z tym, że w literaturze można znaleźć doniesienia sugerujące, iż wyższa alfa-różnorodność świadczy o prawidłowym zdrowiu metabolicznym gospodarza (Manor i in., 2020), to uzyskane wyniki wydają się być w tym względzie kontrowersyjne. Jednakże, w wielu pracach wskazuje się na szereg zaawansowanych połączeń pomiędzy funkcjonalnością, stabilnością i różnorodnością mikrobioty jelitowej, co sugeruje, że parametr ten powinien być zawsze analizowany wyłącznie w połączeniu z analizą składu konsorcjum bakteryjnego oraz ich zdolnością do produkcji metabolitów (Shade, 2017).

W niniejszych badaniach nie obserwowano żadnych istotnych statystycznie międzygrupowych różnic pomiędzy głównymi typami bakterii tj. Bacteroidota oraz Firmicutes. Natomiast bakterie typu Actinobacteriota były obecne tylko w grupie Placebo+HF/HFr, co można częściowo wytłumaczyć ich wyższą obfitością, wynikającą ze spożycia diety wysokotłuszczowej (Kim i in., 2021), natomiast mniejszą w przebiegu PCOS (Yu i in., 2022).

Ponadto, w celu oceny wpływu diety HF/HFr na skład mikrobioty jelitowej porównano ze sobą grupy Placebo oraz Placebo+HF/HFr. W grupie Placebo + HF/HFr zauważono istotnie wyższą obfitość bakterii z rodzaju *Alloprevotella*, *Muribaculum*, *Rikenellla* oraz *Parasuterella,* które według danych literaturowych dodatnio korelują ze stężeniem triglicerydów we krwi oraz

masą ciała, a także istotnie niższą obfitość prozdrowotnych bakterii z rodzaju *Lactobacillus* (Y. Li i in., 2022; Lv i in., 2019).

Z kolei, w celu określenia wpływu letrozolu na skład mikrobioty, porównano ze sobą grupy LET oraz Placebo. Zauważono istotnie wyższą obfitość bakterii z rodzaju *Prevotellaceae_UCG-001* w grupie LET w porównaniu do grupy Placebo. Według danych literaturowych u kobiet z PCOS obserwuje się zwiększoną obfitość bakterii z rodziny *Prevotellaceae*, co wiązane jest z występowaniem przewlekłego stanu zapalnego w organizmie (Giampaolino i in., 2021).

Zestawiono ze sobą także grupy Placebo+HF/HFr oraz LET, aby porównać siłę działania letrozolu oraz diety HF/HFr. W grupie Placebo+HF/HFr zauważono istotnie wyższą obfitość bakterii z rodzaju *Alloprevotella, Muribaculum, Rikenella oraz Clostridia_vadinBB60_group*, a także istotnie niższą obfitość bakterii z rodzaju *Lactobacillus, Butyricicoccus* i *Prevotellaceae_NK3B31_group* w porównaniu do grupy LET. Można więc zasugerować, że dieta HF/HFr ma silniejszy, negatywny wpływ na konsorcjum bakteryjne niż sam LET. Niewielki efekt działania LET na mikrobiotę prawdopodobnie wynika z procesu indukcji PCOS w okresie przedpokwitaniowym. Uważa się bowiem, że złożoność mikrobioty jelitowej rośnie wraz z wiekiem (Saraswati & Sitaraman, 2014). Istotnie Torres i in. zauważyli, że podaż letrozolu w wieku dorosłym wiązała się z bardziej jej wyraźnymi zmianami (Torres i in., 2019).

Ponadto, obie grupy żywione dietą HF/HFr miały istotnie niższe stężenie poszczególnych krótkołańcuchowych kwasów tłuszczowych tj. kwasu masłowego, octowego oraz propionowego w kale niż grupa Placebo, co jest zgodne z wcześniejszymi badaniami wskazującymi, że dieta ta zaburza zdolność mikrobioty do produkcji metabolitów (Sulistyowati i in., 2022). Z kolei, w grupie LET+HF/HFr obserwowano istotnie wyższe stężenie LPS w surowicy krwi (głównego składnika ścian komórkowych bakterii Gram (-)) w porównaniu do obu grup, żywionych dietą standardową (Placebo oraz LET). Można więc stwierdzić, że dopiero połączenie obu czynników (LET oraz diety HF/HFr) osłabia szczelność bariery jelitowej, prowadząc do rozwoju stanu zapalnego (Tucureanu i in., 2017).

W trakcie trwania doświadczenia oceniono także spożycie diety oraz parametry antropometryczne, a z pobranej w trakcie terminacji krwi oraz wątroby, oceniono parametry metaboliczne zwierząt.

Wyłącznie w grupie LET + HF/HFr obserwowano istotnie większy przyrost masy ciała zwierząt, w porównaniu do pozostałych trzech grup. Jednak co ciekawe, zarówno grupa zwierząt indukowana LET, jak i otrzymująca placebo, żywiona dietą HF/HFr wykazywały

znacząco wyższe spożycie diety (mierzone w gramach), w porównaniu do grup kontrolnych żywionych dietą standardową (Placebo oraz LET). Wydaje się, że zdrowe myszy z grupy Placebo + HF/HFr, dzięki protekcyjnemu wpływowi estrogenów, mogą wykazywać zwiększony wydatek energetyczny, co stanowi mechanizm chroniący je przed przyrostem masy ciała (Huang i in., 2020).

Ponadto, wysokie spożycie diety HF/HFr mogło wynikać z faktu iż spożycie tłuszczu oraz cukrów prostych, m.in. fruktozy, zmienia mózgowe sygnały nerwowe, regulujące równowagę energetyczną, uczucie sytości oraz stymuluje reakcję nagradzania pokarmem, co sprzyja rozwojowi hiperfagii (Sarangi & Dus, 2021). W regulacji zachowań żywieniowych istotną rolę odgrywa układ dopaminergiczny w mózgu, którego sygnalizację aktywuje przyjemny smak żywności oraz jej wartość odżywcza. Zauważono, że słodki smak w jamie ustnej indukuje uwalnianie dopaminy i promuje dalsze spożycie cukrów prostych (Thanarajah & Tittgemeyer, 2020). Podobne rozregulowanie zauważono w przypadku spożycia nadmiernych ilości tłuszczu (Reyes, 2012).

Zauważono także, istotnie wyższą wartość parametru HOMA-IR oraz QUICKI (ang. *Quantitative Insulin Sensitivity Check Index*) w grupie zwierząt LET + HF/HFr w porównaniu do grupy LET, co mogło wynikać z nadmiernej masy ciała myszy. Z kolei, stężenie glukozy było istotnie wyższe w grupie Placebo + HF/HFr w porównaniu do grupy LET. Nie obserwowano jednak żadnych różnic w stężeniu insuliny, parametrze HOMA-β oraz CRP pomiędzy grupami. Z kolei, grupa Placebo, żywiona dietą standardową miała istotnie wyższą całkowitą zdolność antyoksydacyjną TAC w porównaniu do grupy LET + HF/HFr. Istotnie obniżone wartości tego parametru obserwuje się u kobiet z PCOS w porównaniu do kobiet zdrowych (Alipour i in., 2019), a także w przebiegu zaburzeń metabolicznych, wynikających z nadmiernej masy ciała (Mohammadi i in., 2022).

Ponadto, podobnie jak True i in., 2017, w niniejszych badaniach zauważono istotnie wyższe stężenie testosteronu w obu grupach zwierząt spożywających dietę HF/HFr (otrzymujących LET lub placebo) w porównaniu do zwierząt spożywających dietę StD. Niniejsze rezultaty wskazują, że LET podany samodzielnie nie wywołał hiperandrogenizmu, co jest niezgodne z rezultatami otrzymanymi przez Arroyo i in. (Arroyo i in., 2019).

Obie grupy zwierząt żywione dietą HF/HFr w porównaniu do grup żywionych dietą standardową miały także istotnie wyższe stężenie cholesterolu całkowitego, cholesterolu frakcji LDL, cholesterolu frakcji HDL oraz nie-HDL. Z kolei, stężenie NEFA było istotnie podwyższone tylko w grupie Placebo+HF/HFr w porównaniu do pozostałych trzech grup

(Placebo, LET oraz LET+HF/HFr). Prawdopodobnie w wyniku zwiększonej syntezy NEFA, ich upośledzonego katabolizmu wewnątrzkomórkowego, zaburzonego wydzielania triglicerydów lub kombinacji tychże nieprawidłowości w obu grupach żywionych dietą HF/HFr zauważono zapoczątkowanie procesu stłuszczenia wątroby (Basaranoglu, 2013). Pomiędzy grupami nie obserwowano jednak różnic w stężeniu triglicerydów, ponieważ hipertriglicerydemia rozwija się wtórnie w stosunku do stłuszczenia wątroby (Fabbrini & Magkos, 2015), a czas trwania doświadczenia, warunkowany 5-tygodniowym okresem działania letrozolu, mógł być niewystarczająco długi do rozwoju i obserwacji tych zmian (de Moura e Dias i in., 2021).

Sprawdzono także korelacje pomiędzy relatywną obfitością bakterii na poziomie ich typu oraz rodzaju a parametrami związanymi z przebiegiem PCOS, tj. przyrostem masy ciała, zawartością tkanki tłuszczowej, stężeniem testosteronu, parametrem HOMA-IR oraz parametrami gospodarki lipidowej. Na poziomie typu, nie zaobserwowano żadnych istotnych korelacji. Natomiast na poziomie rodzaju, zauważono m.in. dodatnią korelację pomiędzy *Turicibacter* a stężeniem TC, cholesterolu frakcji HDL oraz LDL oraz negatywną korelację pomiędzy tymi parametrami a *Lactobacillus.* Zauważono także, że obfitość bakterii z rodzaju *[Eubacterium]_siraeum_group*, *Muribaculum* i *Turicibacter* dodatnio korelowała ze stężeniem testosteronu, przeciwnie do bakterii z rodzaju *Prevotellaceae_NK3B31_group*, których wyższa obfitość, związana była z niższym stężeniem tego hormonu. Ponadto, bakterie z rodzaju *Bilophila* dodatnio korelowały z przyrostem masy ciała zwierząt.

W związku z tym, że udowodniony został niekorzystny wpływ diety HF/HFr na skład mikrobioty jelitowej i wynikające z tego zaburzenia metaboliczne oraz endokrynologiczne sprzyjające powstawaniu PCOS, rozpoczęto poszukiwania reżimu żywieniowego, który mógłby przywrócić homeostazę organizmu. Biorąc pod uwagę znaczenie rytmu dobowego dla prawidłowego funkcjonowania organizmu sformułowano więc następującą hipotezę:

H2: Żywienie ograniczone czasowo korzystnie wpływa na skład i różnorodność mikrobioty jelitowej i tym samym sprzyja poprawie parametrów antropometrycznych oraz metabolicznych zarówno u ludzi jak i w modelu zwierzęcym.

Do systematycznego przeglądu literatury włączono 7 prac z udziałem ludzi (4 prace w których stosowano TRE oraz 3 prace, w których oceniono wpływ stosowania Ramadanu na skład i różnorodność mikrobioty jelitowej) oraz 9 prac z wykorzystaniem modeli zwierzęcych – mysz i szczur (7 TRE, 3 prace uwzględniające reżim podobny do Ramadanu). Zauważono,

że stosowanie przez ludzi zarówno TRE jak i Ramadanu, prowadziło do wzrostu alfa-różnorodności mikrobioty jelitowej. Zmiana ta wydaje się być korzystna, ponieważ poziom alfa-różnorodności odzwierciedla stan zdrowia metabolicznego człowieka oraz kondycję jelit (Manor i in., 2020). Jednak jak już wspomniano wcześniej, nie należy rozważać tego parametru jako indywidualnego indykatora stanu mikrobioty jelitowej, ponieważ jest to zbyt dużym uproszczeniem, zapewniając tylko ograniczony wgląd w mechanizm funkcjonowania środowiska mikrobioty (Shade, 2017). Ponadto, Turnbaugh i in. oraz Ma i in. również zauważyli, że niższa alfa-różnorodność mikrobioty nie zawsze związana jest z gorszą jej kompozycją czy też z nieprawidłowym stanem zdrowia metabolicznego gospodarza (Ma i in., 2012; Turnbaugh i in., 2008).

W przypadku badań na zwierzętach, zauważono, że dieta wysokotłuszczowa zaburza cykliczne fluktuacje, zachodzące w mikrobiocie jelitowej, jednakże zastosowanie TRE pozwala na częściowe ich przywrócenie (Ye i in., 2020; Zarrinpar i in., 2014). Natomiast RF spowodował całkowite odwrócenie rytmów okołodobowych w mikrobiocie jelitowej, ze względu na wprowadzenie okna żywieniowego podczas fazy odpoczynku (He i in., 2021).

Na poziomie typu bakterii, żaden z badanym reżimów nie wywołał zmian w obrębie obfitości Firmicutes oraz Bacteroidetes. Jedynie w dwóch badaniach z wykorzystaniem zwierząt, żywionych dietą wysokotłuszczową lub litogeniczną w TRE lub RF (He i in., 2021; Ye i in., 2020) oraz w badaniach z udziałem ludzi, stosujących RF (Ali i in., 2021; Su i in., 2021) zauważono zwiększoną obfitość Proteobacteria. Jednak do tej pory, nie oceniono jednoznacznie oddziaływania tego typu bakterii na organizm z człowieka. Z jednej strony obfitość Proteobacteria wydaje się być pozytywnie związana z objawami klinicznymi zespołu metabolicznego (Bradley & Pollard, 2017), z drugiej strony sugeruje się ich udział w promowaniu homeostazy i stabilności jelitowych bakterii beztlenowych (Moon i in., 2018).

Z kolei, na poziomie rodzaju, w badaniach z udziałem ludzi, stosujących post w Ramadanie, zauważono wzrost obfitości *Faecalibacterium*, które poprzez produkcję kwasu masłowego, wykazują silne przeciwzapalne działanie w jelitach (Pittayanon i in., 2019). Z kolei w badaniach na zwierzętach zauważono niejednoznaczne zmiany w obfitości bakterii *Lactobacillus*, a mianowicie spadek w grupach spożywających dietę wysokotłuszczową w TRE oraz wzrost lub brak obserwowanych zmian w grupach, które spożywały dietę standardową w tym reżimie. *Lactobacillus* również korzystnie wpływa na kondycję bariery jelitowej m.in. poprzez zwiększoną produkcję śluzu (Dempsey & Corr, 2022).

Ponadto, poszczenie, niezależnie od stosowanego reżimu, spowodowało wzrost obfitości *Akkermansia* zarówno w badaniach z udziałem ludzi jak i zwierząt, żywionych wyłącznie standardową dietą. Wskazuje to na zależność pomiędzy liczebnością tego rodzaju bakterii a spożywaną dietą, ponieważ odwrotny trend zauważono w przypadku spożywania diety wysokotłuszczowej (Dantas Machado i in., 2022). W obrębie tego rodzaju bakterii, niezwykle korzystne właściwości przypisuje się zwłaszcza bakterii *Akkermansia muciniphila*, której obfitość jest związana m.in. z redukcją stanu zapalnego oraz poprawą wrażliwości na insulinę (Naito i in., 2018).

Można zasugerować więc, że zarówno TRE jak i RF wpływają na skład mikrobioty jelitowej, jednakże wpływ obu reżimów dietetycznych wydaje się mniej znaczący wówczas, gdy stosowana jest dieta wysokotłuszczowa.

Tylko w dwóch badaniach z udziałem ludzi wykazano związek pomiędzy składem mikrobioty a stanem zdrowia gospodarza. Zauważono dodatnią zależność korelacyjną pomiędzy stężeniem HDL-C a bogactwem gatunkowym mikrobioty po zastosowaniu TRE (Zeb i in., 2020). Su et al. zauważył pozytywną korelację pomiędzy wielkością wskaźnika BMI a obfitością bakterii typu Proteobacteria, a także negatywną korelację pomiędzy tym samym parametrem a obfitością bakterii klasy *Negativicutes* oraz *Selenomonadales* (Su i in., 2021). Jednakże niewielka liczba prac, w których oceniano związek pomiędzy parametrami metabolicznymi oraz antropometrycznymi a składem mikrobioty jelitowej, nie pozwala na jednoznaczne stwierdzenie czy obserwowane zmiany są istotnie ze sobą związane.

Podsumowanie

Na podstawie przeprowadzonych badań, sformułowano następujące wnioski:

- Dieta HF/HFr spożywana w okresie przedpokwitaniowym, miała znacznie silniejszy wpływ na skład mikrobioty jelitowej niż LET, powodując m.in. istotnie niższą obfitość prozdrowotnych bakterii z rodzaju *Lactobacillus* oraz wyższą obfitość niekorzystnych bakterii z rodzaju *Muribaculum* czy *Rikenella*, a także na zdolność mikrobioty do produkcji krótkołańcuchowych kwasów tłuszczowych.
- Indukcja LET sprzyjała powstawaniu „szczupłego" fenotypu PCOS, obejmującego jedynie charakterystyczne zmiany w obrazie morfologicznym jajników. Jednoczesne podawanie diety HF/HFr sprzyjało powstawaniu „klasycznego" fenotypu PCOS, objawiającego się licznymi zaburzeniami metabolicznymi i hormonalnymi. Ponadto, zaburzenia metaboliczne i reprodukcyjne, podobne do tych towarzyszących PCOS, rozwinęły się także w grupie myszy, którym podawano placebo i żywiono je dietą HF/HFr.
- Obserwowane zależności korelacyjne m.in. dodatnie pomiędzy rodzajem *Turicibacter* a stężeniem cholesterolu całkowitego, HDL-C oraz LDL-C, ujemne pomiędzy tymi parametrami a rodzajem *Lactobacillus*, a także ujemne pomiędzy rodzajem *Prevotellaceae_NK3B31_group* a stężeniem testosteronu oraz dodatnie pomiędzy rodzajem *Bilophila* a przyrostem masy ciała, wskazują, że skład mikrobioty istotnie wpływa na parametry metaboliczne, antropometryczne oraz hormonalne, związane z PCOS.
- Reżim żywienia ograniczonego czasowo zarówno u ludzi jak i w modelu zwierzęcym wpływa korzystnie na skład mikrobioty jelitowej poprzez poprawę jej różnorodności oraz stymulację wzrostu prozdrowotnych bakterii, m.in. z rodzaju *Akkermansia* u ludzi oraz zwierząt, żywionych dietą standardową, co wpływa korzystnie na poprawę parametrów metabolicznych i masę ciała gospodarza. Jednak reżim ten wykazuje tylko częściową zdolność do przywracania zaburzonych przez spożywanie diety wysokotłuszczowej okołodobowych fluktuacji składu mikrobioty zwierząt.

Niniejsze badania, choć przeprowadzone na modelu zwierzęcym, pozwalają poszerzyć dotychczasową wiedzę na temat wpływu żywienia na rozwój i funkcjonowanie układu rozrodczego także w grupie młodych dziewcząt. W tym względzie należy jednoznacznie podkreślić, że rozwój PCOS i nasilenie manifestacji klinicznych są w znacznej mierze uwarunkowane rodzajem stosowanej diety, w tym zawartością w niej cukrów prostych,

szczególnie fruktozy oraz nasyconych kwasów tłuszczowych. Ponadto przeprowadzone badania pozwoliły zgłębić temat zależności pomiędzy określonym sposobem żywienia, składem i funkcjonowaniem mikrobioty jelitowej i jej metabolitów a zdrowiem gospodarza. Wyodrębniono konkretne rodzaje bakterii korelujące z parametrami antropometrycznymi (*Bilophila* z przyrostem masy ciała zwierząt), gospodarki lipidowej (m.in. *Turicibacter* oraz *Lactobacillus* ze stężeniem cholesterolu całkowitego, HDL-C oraz LDL-C) oraz hormonalnej ([*Eubacterium]_siraeum_group, Muribaculum, Turicibacter* oraz *Prevotellaceae_NK3B31_group* ze stężeniem testosteronu). Ponadto, biorąc pod uwagę wpływ czasu podawania pokarmu na rytm okołodobowy mikrobioty jelitowej, jak i wyraźne zależności korelacyjne pomiędzy bogactwem gatunkowym mikrobioty oraz jej składem na poziomie typu oraz klasy a wybranymi parametrami antropometrycznymi (BMI) oraz gospodarki lipidowej (HDL-C), uznano, że reżim żywienia ograniczonego czasowo może stać się potencjalnym narzędziem terapeutycznym PCOS. Jednak pamiętać należy, że w oknie żywieniowym należy spożywać zbilansowane pod względem składu i wartości odżywczej posiłki.

Obok aspektów poznawczych niniejsze badania niosą również możliwość ich praktycznego zastosowania. Zbilansowana dieta, ograniczająca spożycie cukrów prostych (szczególnie fruktozy) oraz nasyconych kwasów tłuszczowych, już od najmłodszych lat, może ograniczyć ryzyko rozwoju PCOS ze wszystkimi jego powikłaniami. Z kolei, reżim żywienia ograniczonego czasowo może być obiecującym narzędziem dietetycznym łagodzącym te nieprawidłowości, choć na ten moment nie powinien stanowić dietoterapii pierwszego rzutu, ze względu na niewystarczającą ilość potwierdzonych danych.

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Basic nutritional investigation

Effect of a high-fat high-fructose diet on the composition of the intestinal microbiota and its association with metabolic and anthropometric parameters in a letrozole-induced mouse model of polycystic ovary syndrome

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ABSTRACT

Objective: It has been suggested that dysbiosis of the gut microbiota is associated with the pathogenesis of Polycystic Ovary Syndrome (PCOS), and that improper diet can aggravate these changes. This study thus aimed to investigate the effects of a high-fat/high-fructose (HF/HFr) diet on the gut microbial community and their metabolites in prepubertal female mice with letrozole (LET)-induced PCOS. We also tested the correlations between the relative abundance of microbial taxa and selected PCOS parameters.
Research methods & procedures: Thirty-two C57BL/6 mice were randomly divided into four groups (n = 8) and implanted with LET or a placebo, with simultaneous administration of a HF/HFr diet or standard diet (StD) for 5 wk. The blood and intestinal contents were collected after the sacrifice.
Results: Placebo + HF/HFr and LET + HF/HFr had significantly higher microbial alpha diversity than either group fed StD. The LET-implanted mice fed StD had a significantly higher abundance of *Prevotellaceae_UCG-001* than the placebo mice fed StD. Both groups fed the HF/HFr diet had significantly lower fecal levels of short-chain fatty acids than the placebo mice fed StD, while the LET + HF/HFr animals had significantly higher concentrations of lipopolysaccharides in blood serum than either the placebo or LET mice fed StD. Opposite correlations were observed between *Turicibacter* and *Lactobacillus* and the lipid profile,
Conclusion: HF/HFr diet had a much stronger effect on the composition of the intestinal microbiota of prepubertal mice than LET itself.

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disease that affects 6% to 20% of women of reproductive age and is associated with a high risk of infertility, obesity, and insulin resistance [1]. Although genetic, neuroendocrine, metabolic, environmental, and lifestyle-related factors are known to cause PCOS, its etiology remains unclear.

There is growing evidence that dysbiosis of the gut microbiota is associated with the pathogenesis of PCOS. A recent review confirmed that PCOS women with altered testosterone/estrogen profiles had different gut microbiota compositions, including in beta diversity and a lower alpha diversity than healthy women [2]. Moreover, changes in the relative abundances of specific taxa of gut bacteria have been correlated with clinical manifestations of PCOS, such as obesity and insulin resistance [3]. Kelley et al. [4] were the first to confirm changes in gut microbiota after induction of PCOS in mice with letrozole (LET), including a significant decrease in the total gut microbial species count and phylogenetic richness. Moreover, Torres et al. observed that LET-induced PCOS in adult mice was associated with a distinct shift in gut microbial

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diversity, unlike in LET-induced PCOS in pubertal mice [5]. This finding shows that the timing of androgen exposure in animal models may significantly affect metabolism dysregulation and the gut microbiome in PCOS.

Furthermore, improper diet, such as a high-fat diet (HFD), can aggravate the intestinal dysbiosis in LET-induced PCOS in mice [6]. The study of Zheng et al. [6] also observed that the influence of LET on gut bacteria was not as significant as that of HFD; they showed that the abundance of the *Vibrio* genus significantly increased in the LET treatment group, that the *Bacteroides* and *Phascolarctobacterium* genera were enriched in the HFD group, and that the *Bacteroides, Phascolarctobacterium, Blautia, Parabacteroides, Akkermansia, [Ruminococcus]_torques_group*, and *Anaerotruncus* genera were enriched in the LET group fed with HFD [6]. High levels of consumption of highly processed food that is rich in simple sugars—particularly fructose and saturated fat—have been associated with obesity and metabolic disorders [7,8]. The group that is most vulnerable to these effects is young people around adolescence, who are overexposed to diets high in fats and sugar, and especially in fructose [9], from soft drinks, energy drinks, and fruit juices [10]. It is also known that this type of diet can affect the composition of the gut microbiota [11]. However, the exact direction of these microbial changes and their effects on the severity of PCOS symptoms have not been unequivocally assessed so far.

It is worth noting that the use of an animal model of PCOS allows the observation and validation of new biomarkers related to this disease [12]. It also enables identification of the molecular mechanisms that underlie the metabolic features of PCOS, which may result in the development of innovative treatment methods [13]. The application of letrozole in the induction of PCOS allows one-time subcutaneous implantation, while the use of androgens often involves daily injections, which may translate into higher levels of stress in animals [14]. Moreover, the use of letrozole enables induction in both the "lean" PCOS phenotype when used individually, and in the "classic" phenotype, when its effect is enhanced by a factor causing metabolic disorders, such as improper diet [14,15].

This study thus aims to investigate the effects of high-fat/high-fructose (HF/HFr) diet on gut microbial community and their metabolites in prepubertal female mice with letrozole-induced PCOS. We moreover intended to determine whether there is a correlation between the relative abundance of microbial taxa and selected parameters associated with PCOS, such as body weight gain, adipose tissue, blood testosterone concentration, Homeostasis Model Assessment of Insulin Resistance (HOMA-IR), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), and Castelli's Risk Index. The findings of this study may help achieve a better understanding of the effects of the HF/HFr diet and LET-induced PCOS on the composition of the gut microbiota, and this would be valuable for further study of new PCOS therapies.

Materials and methods

Experimental animals and treatment

In PCOS animal models, it is advised to induce the disease during the prepubertal period, as this leads to more stable PCOS outcomes than when it is induced after puberty [16]. Thirty-two prepubertal female C57BL/6 mice (average body weight 13.5 g) with an age of 3 wk were therefore exposed to LET for a period of 5 wk [16]. The animals were purchased from Mossakowski Institute of Experimental and Clinical Medicine, Polish Academy of Sciences, Warsaw, Poland and housed in the vivarium at the Department of Physiology, Biochemistry and Animal Biostructure, part of the Faculty of Veterinary Medicine and Animal Sciences at Poznań University of Life Sciences. The animals were allowed to adapt to the laboratory environment for 10 d. All animals were housed in standard polycarbonate cages and maintained in a controlled environment with a temperature of 21 ± 1°C, humidity of 55%–65%, and a 12-h light–dark cycle. After acclimatization, at 4 wk of age, the mice were randomly assigned to four groups: (1) Placebo, consisting of mice injected with a placebo pellet and fed a standard diet (StD) (n = 8); (2) Placebo + HF/HFr, consisting of mice injected with a placebo pellet and fed the HF/HFr diet (n = 8); (3) LET, consisting of mice injected with a LET pellet and fed a standard diet (n = 8); and (4) LET + HF/HFr, consisting of mice injected with a LET pellet and fed the HF/HFr diet (n = 8). Subcutaneous implantation of continuous release letrozole (3 mg, 50 μg/d) or a placebo pellet was performed to induce PCOS or to create a control group. Both the active product and the placebo control pellets contained a matrix of carrier–binder consisting of cholesterol, lactose, celluloses, phosphates, and stearates, with the only difference being that the pellets inducing PCOS had an additional active substance, letrozole. The letrozole was purchased from Innovative Research of America. Induction of PCOS was confirmed through histopathological examination of the ovaries. The methodology and results of this are presented in our previous article [15].

Two groups of mice were fed a standard laboratory diet (3.8 kcal/g, energy supply ratio: protein 18%, carbohydrate 66%, fat 16%). The other two groups were fed the HF/HFr diet (4.7 kcal/g, energy supply ratio: protein 17%, carbohydrate 37.5% (mainly fructose), fat 45.5%). The experimental diets were bought from Morawski Animal Feed (Kcynia, Poland). The animals had unlimited access to water and food throughout the experimental period. Once a week, the animals were weighed using a Sartorius MSE2202S-100-D0 precision balance (Germany). During the last week of the experiment, the body composition of the mice was analyzed by in vivo time-domain nuclear magnetic resonance using a Bruker Minispec LF90 body analyzer (USA). The study was approved by the Local Ethical Commission under permission No. 51/2021 and was carried out in line with the ARRIVE 2.0 guidelines for animal research [17].

Sample collection

Fresh fecal samples were collected from all groups of mice during the last week of the experiment and were immediately frozen at −80°C until the analysis was performed. After the end of the experiment (5 wk), two individuals from each group were randomly selected until the number of individuals was depleted. These individuals were sacrificed by decapitation between the two time points ZT 3 (9 am) and ZT5 (11 am). Blood was collected in nonheparinized tubes. The blood was centrifuged (3500 × g, 15 min, 4°C) to obtain serum samples, which were then frozen at −80°C for future biochemical analysis. The intestinal contents of the cecum were collected and the bacterial DNA was immediately isolated using the commercially available QIAamp fecal DNA minikit (QIAGEN, Hilden, Germany), following the manufacturer's protocol.

Biochemical analysis

The concentration of lipopolysaccharides in blood serum was measured using an immunoassay (ELISA) kit obtained from Sunlong Biotech (Hangzhou, Zhejiang, China). Serum glucose (GLU), triglycerides (TG), total cholesterol (TC), HDL-C, and LDL-C were measured using commercially available colorimetric and enzymatic assays from Pointe Scientific (Lincoln Park, MI, USA). The concentrations of insulin and lipopolysaccharide (LPS) in blood serum were measured using an immunoassay (ELISA) kit from Sunlong Biotech (Hangzhou, Zhejiang, China). Testosterone level was determined using an immunoassay (ELISA) kit from LDN (Nordhorn, Germany). The optical density of these samples was measured using a Synergy 2 microplate reader (Biotek, Winooski, VT, USA).

Calculation of the HOMA-IR and Castelli indices

Insulin resistance and β-cell function were evaluated using the Homeostasis Model Assessment Method with the following formula:

HOMA − IR = fasting glucose [mmol/L] × fasting insulin [μIU/mL]/22.5 [18]

Castelli's Risk Index I (CRI-I) was calculated as follows:

CRI-I = total cholesterol [mg/dL] / high-density lipoprotein [mg/dL] [19]

Fecal SCFA analysis

Determination and quantification of short-chain fatty acids (SCFAs) in the mice feces were performed by gas chromatography coupled with flame ionization detection (GC-FID) [20]. Thawed stool samples weighing 100 mg were homogenized with a spatula and then acidified with 50% sulfuric acid. After centrifugation, 50 μL of internal standard solution (IC6, concentration 330 μM) was added. This mixture was extracted using 1 mL of ethyl ether and centrifuged (5 min, 2800 × g). The extraction was repeated three times and 3 mL of the organic phase was collected each time. Finally, 0.5 μL of the harvested organic phase was injected into the gas chromatograph (GC) for analysis. The individual acids were quantified using gas chromatography equipped with a flame ionization detector (Agilent 7890 series II Agilent Technologies, Santa Clara, CA, USA) and a BPX 70 column (BPX70, 25 m × 0.22 mm ID × 0.25 μm, SGE Analytical Science, Ringwood, Australia). The acids were identified by mass spectrometry (Agilent 5975C, Agilent Technologies, Santa Clara, CA, USA). Peak integration was performed using MSD ChemStation (Agilent Technologies, Santa Clara, CA, USA). Acid concentrations are expressed in [μmol/g] [20].

Gut microbiota analysis

Bacterial DNA isolated from the cecal contents of the mice was sent to Genomed (Warsaw, Poland) for 16S rRNA gene, V3–V4 region sequencing using a MiSeq platform with paired-end (PE) technology, 2 × 300 nt (Illumina, San Diego, CA, USA). Specific sequences of the 341F and 785R primers (metagenomic 16S rRNA analysis) were used to amplify the selected region and to prepare the library. PCR was performed using a Q5 Hot Start High-Fidelity 2X Master Mix under the reaction conditions recommended by the manufacturer. Bioinformatic analysis of the raw sequences was performed using QIIME 2 software. OTUs were classified to taxonomic levels based on the Silva 138 reference sequence database.

Statistical analysis

The sample size was calculated using G*Power software (RRID:SCR_013726), following the previous study of Zheng et al. [6]. The sample size was calculated to be eight mice per group on the basis of the differences in HOMA-IR between the HFD (high-fat diet) group (0.8 ± 0.26) and the PCOS+HFD group (1.21 ± 0.11), with an alpha value of 0.05 and a power of 0.95. The normality of the data distribution was tested using the Shapiro–Wilk test. The Kruskal–Wallis test was then used for nonnormally distributed data, such as SCFA concentrations, and the Tukey HSD test was used for normally distributed data [21], such as LPS concentration. Both tests were carried out using Statistica 13.3.0 (TIBCO Software, Palo Alto, CA, USA; 2017). A *P* value of less than 0.05 was considered statistically significant.

The microbiota composition was analyzed using RStudio (R version 4.0.3 (2020-10-10)) using a set of packages that included *phyloseq, microbiome*, and *vegan*. The taxa were filtered by removing all those not assigned to any phylum. Only taxa with abundances over 0.25% in at least one sample were left in the dataset [22]. In total, 302 OTUs were identified. All analyses of gut microbiota composition were performed on the basis of the relative abundances (RA) of the OTUs. As the data was not normally distributed, the Kruskal–Wallis test and the Dunn test (with *P* adjusted using the method of Benjamini and Hochberg) were used to assess differences in the RA of the individual taxa, grouped at different taxonomic levels between the study groups. To assess the association between study groups and microbial β-diversity, a bacterial distance matrix was constructed using the Bray–Curtis distance, and PCoA and PERMANOVA analysis was performed. The α-diversity was compared between groups using the Shannon and Simpson indices. These indices was calculated for the samples using QIIME (v1.7.0) based on the rarefied OTU counts. Correlations between the relative abundance of microbiome genera and the metabolic and anthropometric markers were calculated using Spearman's correlation test. Only strong correlations ($q < 0.01$, $R > 0.6$) are presented in the body of this report, while other correlations ($q < 0.05$, $R < 0.06$) are shown in Supplementary Figure S3. The microbiota features that differentiate intestinal microbiota were characterized using the LEfSe method (with the strategy of multi-class analysis all-against-all) for biomarker discovery [23], which uses the Kruskal–Wallis rank–sum test to detect features with significantly different abundance levels between assigned taxa, and which performs an LDA to determine the effect size of each feature. A q value of less than 0.05 was considered statistically significant. All the results are presented in the tables and figures as arithmetic means ± standard deviations (SD).

Results

Anthropometric, hormonal, and metabolic parameters

The anthropometric, hormonal and metabolic results have been thoroughly described previously [15]. In brief, the LET+HF/HFr group saw significantly greater weight gain than did the LET group, by approximately 11.9%. Additionally, the LET+HF/HFr group exhibited significantly increased testosterone levels and deteriorated lipid profile and HOMA-IR. Only in the Placebo+HF/HFr group were similar changes observed, other than for changes in insulin sensitivity. Both the LET+HF/HFr and Placebo+HF/HFr groups developed polycystic ovaries. Although the LET-treated group did not display endocrine or metabolic abnormalities, polycystic ovaries were nonetheless observed.

Intestinal microbiota diversity

The alpha diversity metric determined at the OTU level with the Shannon index showed that the Placebo group had significantly lower diversity and richness than the Placebo + HF/HFr group (3.48 ± 0.25 vs. 4.06 ± 0.14, $q < 0.001$) or the LET + HF/HFr group (3.48 ± 0.25 vs. 3.82 ± 0.21, $q < 0.05$). Significantly lower alpha-diversity was also observed in the LET group than in the Placebo + HF/HFr group (3.62 ± 0.27 vs. 4.06 ± 0.14, $q < 0.001$) (Fig. 1). Similar differences were observed using Simpson's index (Placebo vs. Placebo + HF/HFr 0.92 ± 0.03 vs. 0.96 ± 0.01, $q < 0.001$; Placebo vs. LET + HF/HFr 0.92 ± 0.03 vs. 0.95 ± 0.01, $q < 0.01$; Placebo + HF/

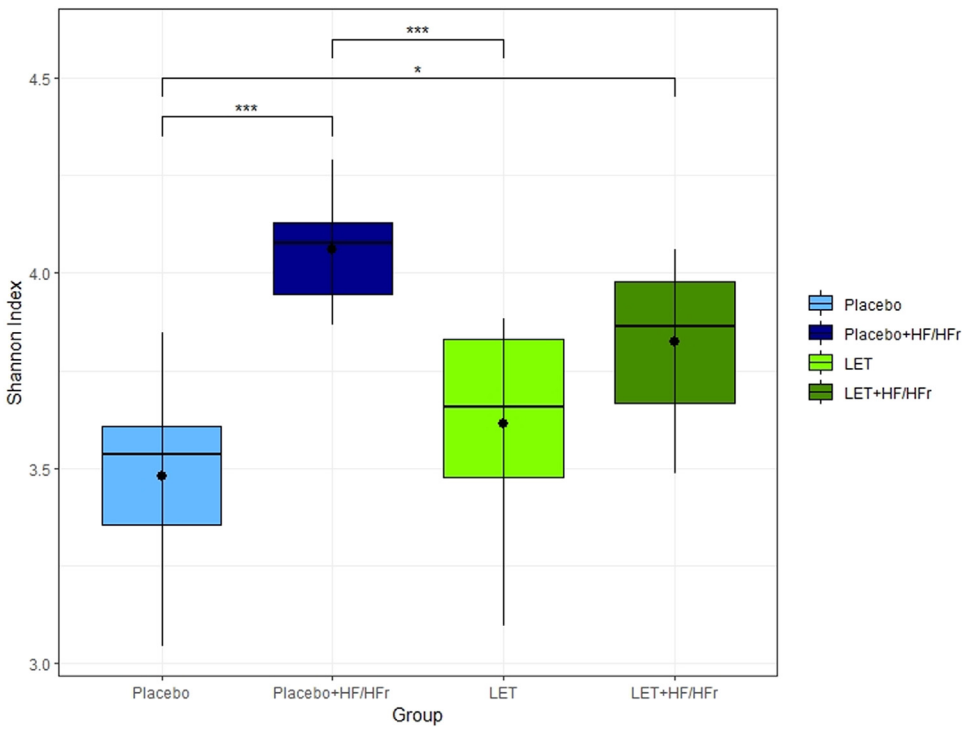

Fig. 1. Boxplots comparing cecal microbial α-diversity (measured by the Shannon Index) of experimental groups. *: $q < 0.05$; **: $q < 0.01$; ***: $q < 0.001$.

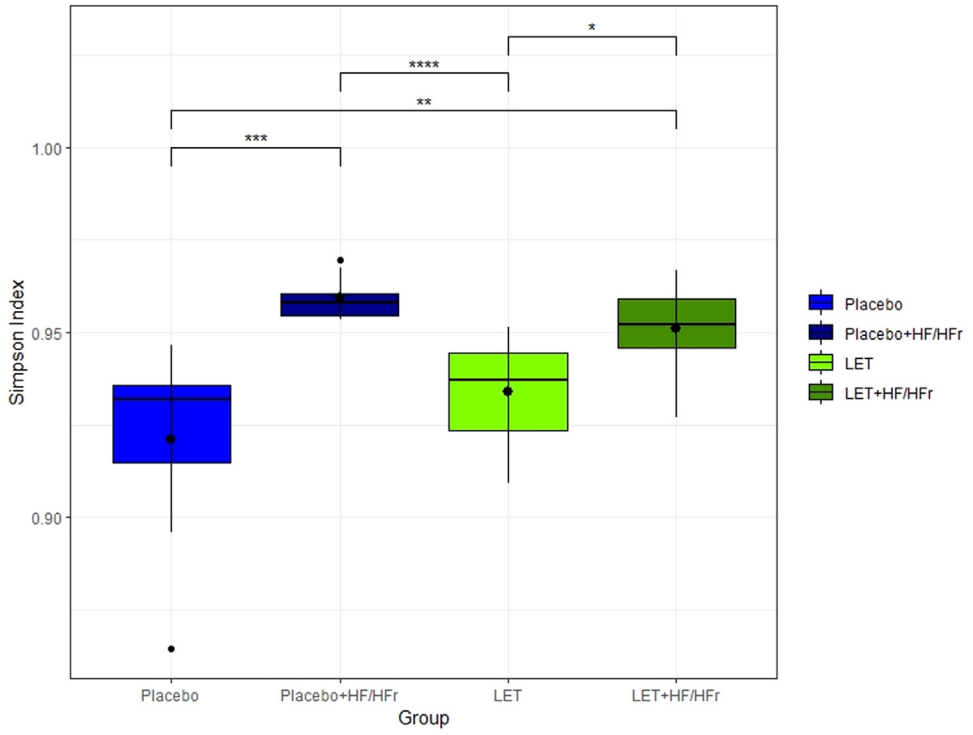

Fig. 2. Boxplots of the cecal microbial α-diversity (measured by Simpson's index) of the experimental groups. *: $q < 0.05$; **: $q < 0.01$; ***: $q < 0.001$.

HFr vs. LET 0.96 ± 0.01 vs. 0.93 ± 0.01, $q < 0.001$). However, the use of Simpson's index allowed us to note significant differences between the LET and LET + HF/HFr groups (0.93 ± 0.01 vs. 0.95 ± 0.01, $q < 0.05$) (Fig. 2).

A PCoA plot using the Bray–Curtis dissimilarity metric demonstrated a distinct clustering of the Placebo + HF/HFr group than the other three groups (Fig. 3). The significance of these differences was confirmed by the Kruskal–Wallis and Dunn tests, which compared coordinate values for points located within each group on the *x* axis (Placebo HF/HFr and LET: $q < 0.001$; Placebo HF/HFr and LET + HF/HFr: $q < 0.01$; Placebo HF/HFr and Placebo: $q < 0.001$).

Composition of the intestinal microbiota

Bacterial abundances and prevalence at the phylum and genus levels are compared and presented in Table 1, while comparisons of other taxonomic levels (class, order, and family) are presented in Supplementary Table S1.

The five major phyla in our study groups are shown in Figure 4. *Firmicutes* and *Bacteroidota* were the most abundant phyla in all groups. Moreover, the *Firmicutes:Bacteroidota* ratio did not significantly differ across groups (data not shown).

On the phylum level, *Actinobacteriota* was present only in the Placebo + HF/HFr group (0.13% ± 0.13%). Differences between groups in the majority of remaining phyla were not statistically significant.

To determine the effects of the HF/HFr diet, mice in the Placebo +HF/HFr group were compared with those in the Placebo group. On the genus level, the Placebo + HF/HFr group had a significantly higher abundance than the Placebo group for *Alloprevotella* (5.54% ± 1.69% vs. 1.06% ± 0.90%, $q < 0.01$), *Muribaculum* (2.16% ± 0.32% vs. 0.81% ± 0.31%, $q < 0.01$), *Rikenella* (1.17% ± 0.51% vs. 0.33% ± 0.18%, $q < 0.01$), and *Parasuterella* (0.22% ± 0.15% vs. 0.001% ± 0.004%, $q < 0.01$). *Lactobacillus* abundance and prevalence were also significantly lower in the Placebo + HF/HFr group than in the Placebo group (0.001% ± 0.003% vs. 0.11% ± 0.12%, $q < 0.001$; prevalence 12,5% vs. 100%).

Furthermore, to determine the effects of LET, animals in the LET group were compared with those in the Placebo group. The LET group had a significantly higher abundance of *Prevotellaceae_UCG-001* than did the Placebo group (0.66% ± 0.29% vs. 0.29% ± 0.55%, $q < 0.01$).

The Placebo + HF/HFr and LET groups were then analyzed in order to compare the effects of both LET and HF/HFr. The Placebo + HF/HFr group showed significantly higher abundances than the LET group for *Alloprevotella* (5.54% ± 1.69% vs. 1.50% ± 1.10%, $q < 0.01$), *Muribaculum* (2.16% ± 0.32% vs. 0.74% ± 0.33%, $q < 0.001$), *Rikenella* (1.17% ± 0.51% vs. 0.42% ± 0.24%, $q < 0.01$), and *Clostridia_vadinBB60_group* (11.14% ± 2.59% vs. 4.40% ± 1.99%, $q < 0.01$). However, the Placebo + HF/HFr group had significantly lower abundances of *Lactobacillus* (0.001% ± 0.003% vs. 0.06% ± 0.09%, $q < 0.05$), *Butyricicoccus* (0.04% ± 0.04% vs. 0.38% ± 0.46%, $q < 0.05$), and *Prevotellaceae_NK3B31_group* (0.00% ± 0.00% vs. 0.21% ± 0.25%, $q < 0.001$). The prevalence of *Prevotellaceae_NK3B31_group* was 0% in the Placebo + HF/HFr group.

Finally, the LET+ HF/HFr group was compared with the Placebo+ HF/HFr group to determine whether LET and HF/HFr had an additive harmful effect on the composition of the intestinal microbiota. The Placebo + HF/HFr group also had significantly higher abundances of *Muribaculum* (2.16% ± 0.32% vs. 1.02% ± 0.42%, $q < 0.01$), *Romboutsia* (0.69% ± 0.54% vs. 0.01% ± 0.03%, $q < 0.01$), *Turicibacter* (0.63% ± 0.32% vs. 0.07% ± 0.16% $q < 0.01$), *Clostridium_sensu_stricto _1* (0.23% ± 0.24% vs. 0.01% ± 0.04% $q < 0.01$), and *Clostridia_vadinBB60_group* (11.14% ± 2.59% vs. 3.47% ± 1.79%, $q < 0.001$) than did the LET + HF/HFr group.

Furthermore, linear discriminant analysis (LDA) effect size (LEfSe) was determined in order to identify significant differentially abundant microbiota. The results of this analysis are presented in Supplementary Figures S1 and S2. The results for genus taxonomic levels are comparable with those from the previous

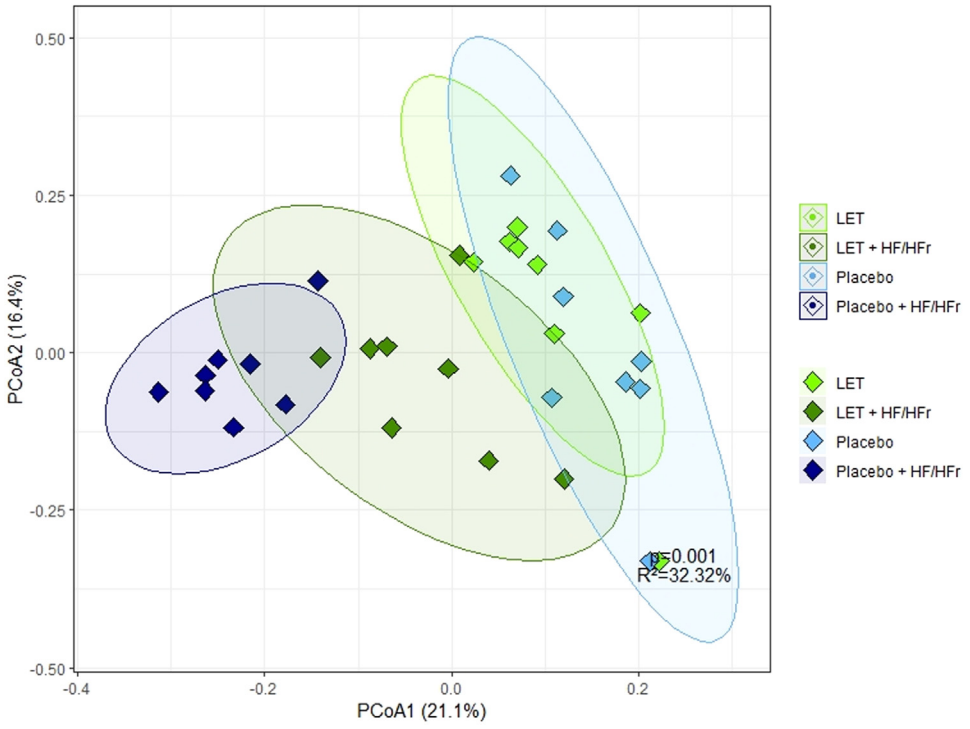

Fig. 3. PCoA plot of microbial β-diversity (based on the Bray–Curtis distance) of cecal samples showing PERMANOVA results on associations with study groups.

Table 1
Relative abundances and prevalence of selected taxa at the phylum and genus levels (values are presented as percentages)

Taxonomic level	Placebo			Placebo + HF/HFr			LET			LET + HF/HFr		
	Mean	SD	Prevalence	Mean	SD	Prevalence	Mean	SD	Prevalence	Mean	SD	Prevalence
PHYLUM												
Bacteroidota	37.67	6.60	100	48.22	7.21	100	46.08	7.62	100	50.54	14.05	100
Firmicutes	38.97	10.23	100	33.94	6.55	100	34.40	5.43	100	30.52	13.06	100
Campylobacterota	18.04	5.30	100	12.75	3.45	100	14.34	5.28	100	13.73	3.96	100
Deferribacterota	3.96	1.50	100	1.99	0.78	100	3.73	1.98	100	2.92	1.40	100
Cyanobacteria	0.52	0.32	100	1.15	1.60	100	0.41	0.34	100	0.59	0.89	87.5
Proteobacteria	0.32	0.27	100	0.97	0.64	100	0.33	0.51	100	0.49	0.57	100
Desulfobacterota	0.24	0.22	100	0.44	0.37	100	0.28	0.21	100	0.73	0.63	100
Patescibacteria	0.18	0.25	50	0.22	0.14	100	0.17	0.19	66,7	0.15	0.16	87.5
Verrucomicrobiota	0.09	0.14	100	0.20	0.15	100	0.26	0.40	100	0.34	0.34	100
Actinobacteriota	0.00 ^{a}	0.00	0	0.13 ^{b}	0.13	100	0.00 ^{a}	0.00	0	0.00 ^{a}	0.00	0
GENUS												
Clostridia_vadinBB60_group	8.69 ^{bc}	4.19	100	11.14 ^{c}	2.59	100	4.40 ^{ab}	1.99	100	3.47 ^{a}	1.79	100
Alloprevotella	1.06 ^{a}	0.90	87.5	5.54 ^{b}	1.69	100	1.50 ^{a}	1.10	77.8	3.04 ^{ab}	2.51	75
Muribaculum	0.81 ^{a}	0.31	100	2.16 ^{b}	0.32	100	0.74 ^{a}	0.33	100	1.02 ^{a}	0.42	100
Rikenella	0.33 ^{a}	0.18	100	1.17 ^{b}	0.51	100	0.42 ^{a}	0.24	100	1.05 ^{b}	0.76	100
Prevotellaceae_UCG-001	0.29 ^{a}	0.55	75	0.36 ^{ab}	0.17	100	0.66 ^{b}	0.29	100	0.32 ^{ab}	0.15	100
Butyricicoccus	0.19 ^{ab}	0.18	87.5	0.04 ^{a}	0.04	100	0.38 ^{b}	0.46	88.9	0.05 ^{a}	0.05	87.5
Prevotellaceae_NK3B31_group	0.18 ^{ab}	0.32	50	0.00 ^{a}	0.00	0	0.21 ^{b}	0.25	100	0.01 ^{a}	0.02	12.5
ASF356	0.15 ^{b}	0.25	75	0.35 ^{b}	0.25	87.5	0.04 ^{a}	0.11	11.1	0.00 ^{a}	0.00	0
Lactobacillus	0.11 ^{b}	0.12	100	0.001 ^{a}	0.003	12.5	0.06 ^{b}	0.09	77.8	0.00 ^{a}	0.00	0
Parasutterella	0.001 ^{a}	0.004	12.5	0.22 ^{b}	0.15	75	0.09 ^{ab}	0.11	77.8	0.14 ^{b}	0.15	87.5
Romboutsia	0.00 ^{a}	0.00	0	0.69 ^{b}	0.54	87.5	0.00 ^{a}	0.00	0	0.01 ^{a}	0.03	25
Turicibacter	0.00 ^{a}	0.00	0	0.63 ^{b}	0.32	100	0.00 ^{a}	0.00	0	0.07 ^{a}	0.16	50
Clostridium_sensu_stricto_1	0.00 ^{a}	0.00	0	0.23 ^{b}	0.24	87.5	0.00 ^{a}	0.00	0	0.01 ^{a}	0.04	12.5
GCA-900066575	0.00 ^{a}	0.00	0	0.16 ^{b}	0.13	87.5	0.11 ^{ab}	0.14	55.6	0.13 ^{ab}	0.21	62.5
Bifidobacterium	0.00 ^{a}	0.00	0	0.13 ^{b}	0.13	100	0.00 ^{a}	0.00	0	0.00 ^{a}	0.00	0
[Eubacterium]_siraeum_group	0.00 ^{a}	0.00	0	0.13 ^{b}	0.16	87.5	0.00 ^{a}	0.00	0	0.00 ^{a}	0.00	0
[Eubacterium]_coprostanoligenes_group	0.00 ^{a}	0.00	0	0.06 ^{b}	0.11	75	0.00 ^{a}	0.00	0	0.07 ^{b}	0.13	75

Results are expressed as means ± SDs (n = 8 per group). Values with different letters (a, b, c) show statistically significant differences ($q < 0.05$).

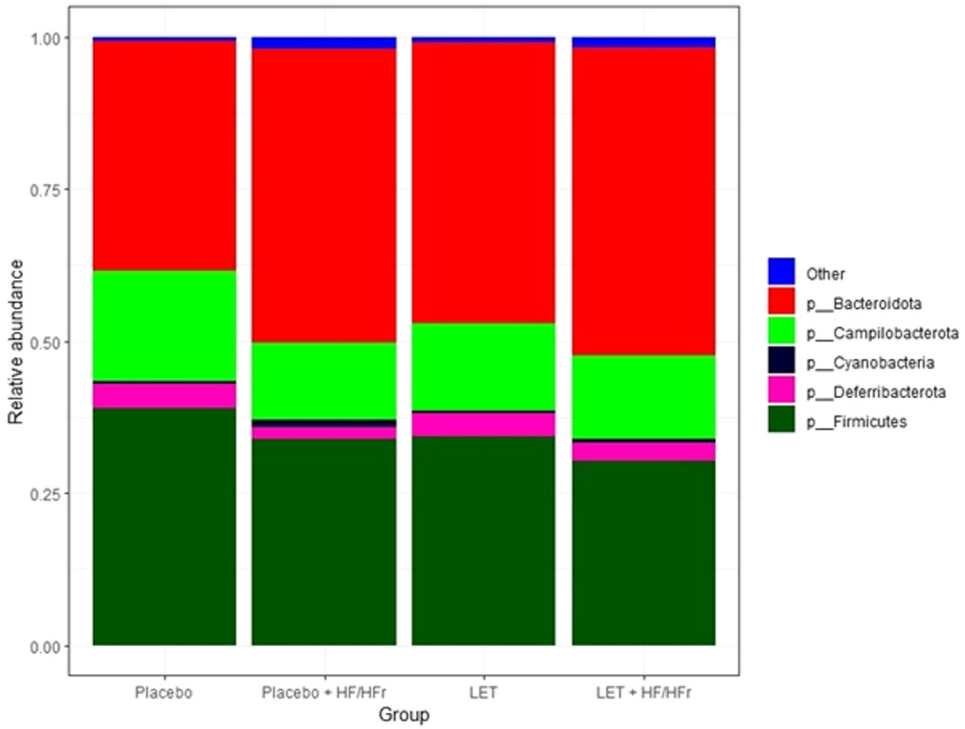

Fig. 4. Relative abundances of gut microbiota on the phylum level.

analysis, but there are notable discrepancies on the phylum level. The LET + HF/HFr group was characterized by the presence of *Bacteroidota*, Placebo + HF/HFr by the presence of *Proteobacteria*, and the Placebo group had an increased abundance of bacteria from *Deferribacterota*.

Bacterial metabolites

Both groups fed the HF/HFr diet (the Placebo + HF/HFr and the LET + HF/HFr groups) showed significantly lower fecal levels of three short-chain fatty acids—acetic, propionic, and butyric acid—than did the Placebo group ($P < 0.05$). The LET + HF/HFr group had a significantly higher plasma lipopolysaccharide (LPS) concentration than the Placebo and LET groups ($P < 0.05$). Detailed values are presented in Table 2.

Correlation of microbial relative abundance with selected parameters associated with PCOS

Potential correlations of relative abundance in bacterial phyla and genera were investigated for all mice using measurements of seven metabolic parameters associated with PCOS. The parameters tested were body weight gain, adipose tissue, concentration of testosterone, HOMA-IR, TG, LDL-C, and Castelli's Risk index.

No significant correlations were observed on the phylum level. On the genus level, however, significant correlations were noted between the abundance of selected genera and lipid profile parameters. Figure 5 shows only the strongest Spearman correlations ($q < 0.01$, $R > 0.6$).

We note that there are opposing correlations of *Turicibacter* and *Lactobacillus* abundances with total cholesterol concentrations (R = 0.65, R = -0.63, respectively, $q < 0.01$). A positive correlation was observed between the concentration of HDL-C and the abundance of the *[Eubacterium]_coprostanoligenes_group* (R = 0.64, $q < 0.01$) and *Turicibacter* (R = 0.66, $q < 0.01$). In turn, abundances of *Lactobacillus* and *Butyricicoccus* were inversely correlated with the concentration of the HDL-L cholesterol fraction (R = -0.7, R = -0.64, respectively, $q < 0.01$). Furthermore, higher abundances of *[Eubacterium]_coprostanoligenes_group, Romboutsia, Turicibacter*, and *Rikenella* were also associated with higher concentrations of LDL-C (R = 0.61, R = 0.61, R = 0.60, R = 0.61, respectively, $q < 0.01$), unlike in the case of *Lactobacillus* and *Prevotellaceae_NK3B31_group* (R = -0.72, R = -0.65, respectively, $q < 0.01$).

There was also a positive correlation between the abundance of *[Eubacterium]_siraeum_group* (R = 0.52, $q < 0.05$), *Muribaculum*

Table 2
Levels of selected short-chain fatty acids in the feces of mice and the concentrations of LPS in blood

Parameters/study groups	Placebo	Placebo + HF/HFr	LET	LET + HF/HFr
Acetic acid (μmol/L)	30.41 ± 7.04 ^{b}	4.16 ± 0.47 ^{a}	10.09 ± 3.45 ^{ab}	3.85 ± 0.74 ^{a}
Propionic acid (μmol/L)	1.49 ± 0.45 ^{b}	0.30 ± 0.09 ^{a}	0.52 ± 0.14 ^{ab}	0.30 ± 0.10 ^{a}
Butyric acid (μmol/L)	2.63 ± 0.96 ^{b}	0.29 ± 0.09 ^{a}	0.61 ± 0.12 ^{ab}	0.32 ± 0.08 ^{a}
LPS (μg/mL)	23.44 ± 4.43 ^{a}	26.47 ± 5.00 ^{ab}	23.52 ± 2.21 ^{a}	28.78 ± 7.86 ^{b}

Data are presented as means ± standard deviations and should be read horizontally. LPS concentration was analyzed by one-way analysis of variance followed by Tukey's HSD test, while SCFA content was analyzed using the nonparametric Kruskal–Wallis test.
Values with different letters show statistically significant differences ($P < 0.05$).
LPS, lipopolysaccharide; HF/HFr, high-fat/high-fructose diet; LET, letrozole.

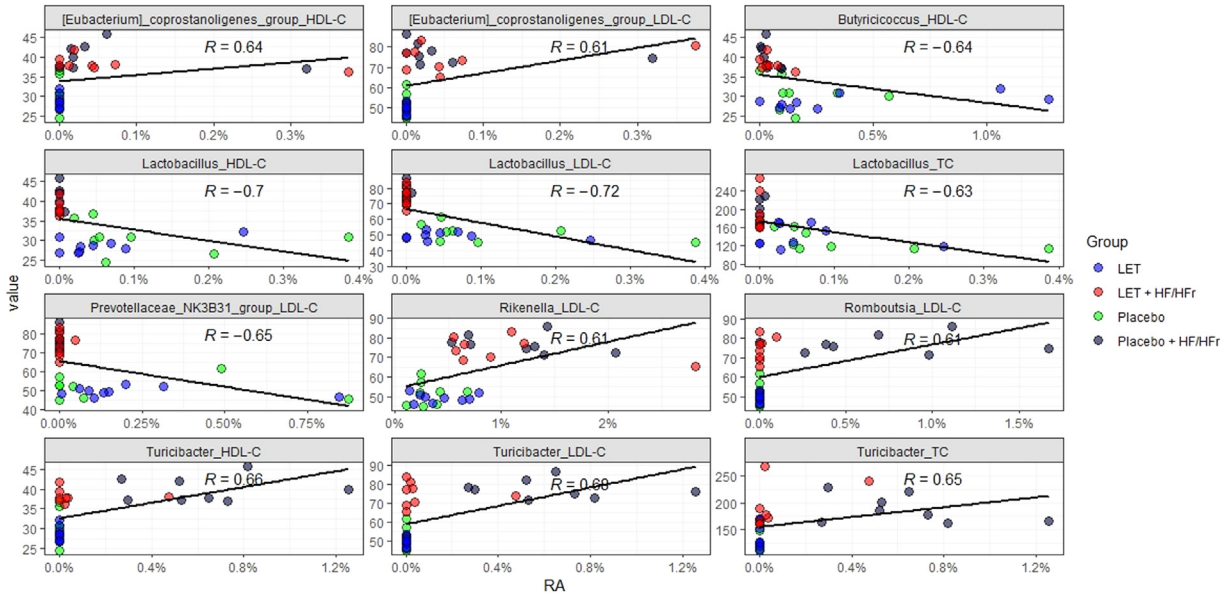

Fig. 5. Spearman correlations between abundances of genera and lipid metabolism parameters associated with PCOS. The significance of all the correlations is < 0.01. TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

(R = 0.52, q < 0.05), *Turicibacter* (R = 0.52, q < 0.05), and testosterone concentration. On the other hand, the *Prevotellaceae_NK3B31_group* was negatively associated with testosterone concentration (R = -0.58, q < 0.05). In addition, a positive association between *Bilophila* abundance and body weight was also noted (R = 0.52, q < 0.05) (all data shown in Supplementary Figure S3).

Discussion

Contrary to expectations, alpha diversity proved to be significantly higher in the Placebo + HF/HFr and LET + HF/HFr groups than in the Placebo and LET groups fed the standard diet. Several studies [24,25] have shown that the alpha diversity of intestinal microbiota is associated with the health of the host, with lower values being associated with metabolic or endocrine disorders like PCOS [5]. On the other hand, some authors have also emphasized that the expectation that high alpha diversity of intestinal microbiota is always beneficial is an overly simplistic one [26]. Very complex correlations exist between the diversity of gut microbiota and its stability and functionality [27]. Moreover, some studies [28,29] have indicated that lower alpha diversity does not always mean a poorer community or poorer health [27]. Our results may in fact suggest that alpha diversity is not a universal parameter that can be analyzed alone, without taking into account the composition and functioning of the intestinal microbiota [27].

Similarly to us, Aho et al. [30] found higher alpha diversity and lower levels of SCFAs in people suffering from Parkinson's disease compared to healthy subjects; this is probably due to a rearrangement in the composition of the intestinal microbiota. Indeed, in both our groups fed the HF/HFr diet (Placebo + HF/HFr and LET + HF/HFr), significantly lower concentrations of individual SCFAs in the feces were observed than in the Placebo group fed the standard diet; this is consistent with the results of Sulistyowati et al. [31], who confirmed that there was a negative impact of HFD on the ability of microbiota to produce metabolites. Moreover, the LET group that was fed the standard diet did not differ significantly from the other groups in terms of SCFA concentrations, while Zhang et al. showed that women with PCOS have reduced SCFAs level compared to healthy women [32]. A reduction in the abundance of bacteria producing SCFAs, especially butyric acid, may negatively affect the integrity of the intestinal barrier and mucosal immunity [33]. Indeed, our LET + HF/HFr group also had significantly higher LPS concentrations than either group fed a standard diet (placebo and LET), which suggests that only the combination of improper diet and letrozole caused damage to the intestinal barrier. Moreover, it is known that the LPS produced by Gram(-) bacteria enters the bloodstream and may cause inflammation, insulin resistance, and obesity [3]. We noted in our previous article that only the LET + HF/HFr group showed significantly higher body weight gain and developed carbohydrate metabolism disorders, while the remaining three groups—LET, Placebo and Placebo + HF/HFr—did not [15].

We noted no significant differences on the phylum level, except in the case of *Actinobacteria*, which was present only in the Placebo + HF/HFr group. Zheng et al. [6], however, noted a greater abundance of this phylum in a group combining letrozole with HFD. Although it has been suggested that the abundance of *Actinobacteria* is related to the amount of fat in the diet [34] and to excess body weight [28], the study of Lindheim et al. [35], showed the reduced relative abundance of this phylum in PCOS patients. This result may partly explain why these bacteria were not observed in the LET + HF/HFr group.

More pronounced differences were observed at the genus level, with *Lactobacillus* abundance being significantly lower in the groups fed HF/HFr (Placebo + HF/HFr and LET + HF/HFr groups) than in the groups fed a standard diet (Placebo and LET). It can be unequivocally concluded that the abundance of this genus is related to the diet, and other studies have also shown that the HFD diet significantly reduces the abundance of bacterial taxa from this genus [36]. We also observed a negative correlation between *Lactobacillus* and lipid profile, particularly relating to TC and LDL-C. Furthermore, in the mice fed HF/HFr (the Placebo + HF/HFr and LET + HF/HFr groups), significantly higher abundances of *Rikenella* and *Parasuterella* were observed. It has been suggested that *Rikenella* is strongly associated with serum triglyceride concentrations [37]. We also noted that the abundance of this genus correlates with elevated LDL-C levels. Moreover, *Parasuterella* has been found in the microbiota of people with excess body weight [38] and

associated metabolic disorders [39]. Indeed, significant metabolic abnormalities have been observed in both groups fed HF/HFr (the Placebo + HF/HFr and LET + HF/HFr groups), in which *Parasuterella* was enriched—further exacerbated by letrozole [15].

Equally interesting is the significantly higher abundance of certain genera, such as *Muribaculum, Romboutsia, Turicibacter,* and *Clostridium_sensu_stricto _1*, in the Placebo + HF/HFr group, as compared to the other three groups, including even the LET + HF/HFr group. Also, when considering the beta-diversity parameter, we can observe that only the Placebo + HF/HFr group differs from the other groups. Unlike Zheng et al. [6], we did not observe any synergistic effect of the HF/HFr diet with letrozole here. The age of PCOS induction with letrozole should be taken into account: Torres et al. noted that administration of letrozole at a prepubertal age was associated with the development of marked metabolic abnormalities, but with only minor changes in the composition of the gut microbiota, as compared to mice in which PCOS was induced in adulthood [40]. Both human and animal studies have showed the dependence of the intestinal microbiota on the organism's maturity, and this applies particularly to the complexity of microbiota, which increases with age [41,42]. This is probably due to the developing hormonal system, although the mechanism is not yet known [43].

Indeed, we noted only a few changes in the LET group, such as the significantly higher abundance of *Prevotellaceae_UCG-001* than in the Placebo group and the significantly higher abundance of *Prevotellaceae_NK3B31_group* than in the LET + HF/HFr group. An increase in the number of different genera from the Prevotellaceae family has been observed among women with PCOS, and this is often associated with increased inflammation in the organism [44,45]. However, these reports are inconclusive, as Zeng et al. noted a dramatic decrease in the abundance of *Prevotellaceae* in women with PCOS [39]. Interestingly, we observed a negative correlation between *Prevotellaceae_NK3B31_group* and testosterone and LDL-C, which is consistent with the results of Zeng et al. [39].

It can thus be suggested that our induction of PCOS at prepubertal age (in 4-wk old animals) led to us no noting many significant differences between the microbiota composition of the LET group and that of the other groups; neither did we observe any intensification in these differences as a result of the interaction of letrozole and the HF/HFr diet. Furthermore, Paris et al. [46] noted that diet has a stronger effect on the composition of the intestinal microbiota than PCOS pathology *per se*, which is consistent with our results.

However, diet may also affect gut microbiota favorably, though this depends on the relative identity and abundance of the constituent bacterial populations [47]. It has been suggested that positive regulation of gut microbiota composition is mediated mainly by phenolic acids released from plant polyphenols and SCFAs derived from the fermentation of dietary fiber by commensal bacteria in the gut [48]. Both phenolic acids and SCFAs have anti-inflammatory properties [49], which also act beneficially in PCOS [50]. Moreover, it has been suggested that probiotic supplementation may also alleviate PCOS-associated hormonal and metabolic disorders [51].

Our study has some limitations. First of all, we assessed the composition of the microbiota at only one point in time, so we did not observe any potential fluctuating disorders that might have resulted from an improper diet or the presence of PCOS. In addition, the composition of the microbiota was assessed from intestinal contents, while the level of SCFAs was determined from the feces, on account of the limited amount of intestinal content that could be obtained from the mice.

Furthermore, we are also aware of the occurrence of coprophagous behavior in experimental mice. First of all, it should be noted that this phenomenon affects the composition of the microbiota in the small intestine, but has a much less pronounced effect on the microbiota in the large intestine [52]. There are several ways to prevent coprophagia, for example using cups placed under the tail of the mice. However this is not useful for female mice, due to their anatomical structure and the accumulation of urine in the cups [53]. In addition, young animals, due to their high activity, would need to be kept in isolation to prevent the cup from being moved or bitten. However, mice are social animals and the need to maintain their well-being precludes their isolation [54]. There are also numerous reports that inhibiting coprophagous behavior in animals adversely affects their body weight, height, and biochemical parameters, and promotes the development of inflammation, which may affect experimental results [55–57].

This study also possesses a number of strengths. To the best of our knowledge, ours is the first study to assess differences in the composition of gut microbiota resulting from a HF/HFr diet and LET-induced PCOS in prepubertal mice. Moreover, our assessment of the composition of the microbiota was performed on the basis of the intestinal content, which is considered a more precise method than assessment based on feces. It has also been suggested that cecal microbiota might be more revealing of HF/HFr diet-induced proinflammatory stimuli (such as LPS and bacterial DNA), because some differences have been detected on the taxonomic level in the fecal microbiota and the caecum microbiota [58].

Conclusions

The HF/HFr diet provided to the experimental animals had a much stronger effect on the composition of the intestinal microbiota of prepubertal mice than did the letrozole itself. However, the age of PCOS induction may be a key factor in our results. Moreover, the correlations we observed indicate that the composition of the gut microbiota has a significant effects on parameters associated with PCOS, and particularly on the correlation between the *Turicibacter* and *Lactobacillus* genera and the lipid profile. However, these require observations further exploration in human studies, which will allow the development of macrobiotic profiles along with individualized diets and probiotic therapy adapted to them.

Declaration of competing interest

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

CRediT authorship contribution statement

Joanna Maria Pieczyńska-Zając: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Anna Maria Malinowska:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Ewa Pruszyńska-Oszmałek:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Paweł Antoni Kołodziejski:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Sławomira Drzymała-Czyż:** Writing – review & editing, Methodology, Data curation. **Joanna Bajerska:** Writing – review & editing, Formal analysis, Conceptualization.

Institutional review board statement

The study was approved by the Local Ethical Commission under permission no. 51/2021 and was performed in line with the ARRIVE 2.0 guidelines for animal research [17].

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.nut.2024.112450.

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The Role of a High-Fat, High-Fructose Diet on Letrozole-Induced Polycystic Ovarian Syndrome in Prepubertal Mice

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Abstract: This study aims to investigate the effects of a high-fat, high-fructose (HF/HFr) diet on metabolic/endocrine dysregulations associated with letrozole (LET)-induced Polycystic Ovarian Syndrome (PCOS) in prepubertal female mice. Thirty-two prepubertal C57BL/6 mice were randomly divided into four groups of eight and implanted with LET or a placebo, with simultaneous administration of an HF/HFr/standard diet for five weeks. After sacrifice, the liver and blood were collected for selected biochemical analyses. The ovaries were taken for histopathological examination. The LET+HF/HFr group gained significantly more weight than the LET-treated mice. Both the LET+HF/HFr and the placebo-treated mice on the HF/HFr diet developed polycystic ovaries. Moreover the LET+HF/HFr group had significantly elevated testosterone levels, worsened lipid profile and indices of insulin sensitivity. In turn, the HF/HFr diet alone led to similar changes in the LET-treated group, except for the indices of insulin sensitivity. Hepatic steatosis also occurred in both HF/HFr groups. The LET-treated group did not develop endocrine or metabolic abnormalities, but polycystic ovaries were seen. Since the HF/HFr diet can cause substantial metabolic and reproductive dysregulation in both LET-treated and placebo mice, food items rich in simple sugar—particularly fructose—and saturated fat, which have the potential to lead to PCOS progression, should be eliminated from the diet of young females.

Keywords: polycystic ovary syndrome; pre-pubertal mice; high-fat and high-fructose diet; metabolic disorders; endocrine disorders

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1. Introduction

Polycystic ovary syndrome (PCOS) is one of the most common hormonal disorders among women of reproductive age. It significantly impairs their fertility and increases the risk of obesity, type 2 diabetes, hyperlipidemia, and cardiovascular disease [1]. Although the exact cause of PCOS is unknown, recent reviews of the PCOS research have found that genetic susceptibility is associated with PCOS and that environmental factors—such as endocrine disruptors and poor diet—are likely to play an important role in the expression of those genetic traits [2]. PCOS often manifests in the early reproductive years; puberty has been suggested as a critical developmental time period for the development and pathology of PCOS [3]. One factor commonly believed to be a risk factor for the development of adolescent PCOS is excess body weight [4]. With normal body weight, the level of total testosterone physiologically increases and the concentration of sex hormone binding globulin (SHBG) decreases, leading to an increase in the concentration of free testosterone;

in obese girls, these changes are much more pronounced, leading to hyperandrogenemia [5]. This occurs because excessively developed adipose tissue is unable to properly secrete leptin and adiponectin, which are responsible for regulating androgen concentrations [6]. Moreover, excessive body weight is often associated with the development of insulin resistance (IR), where higher insulin concentrations stimulate the ovaries to secrete more androgens [7].

At present, young people are increasingly subjected to significant exposure to diets rich in saturated fat, sugar, and fructose in particular [8]. Soft drinks, energy drinks, fruit juices and nectars, and more generally, free sugars in liquid form represent the main source of fructose consumed by today's young generation, especially obese individuals. Of course, fructose is also contained in fruit, but in minimal quantities compared to the weight of the fruit itself [9]. This type of dietary environment has undoubtedly contributed to the alarming increased prevalence of childhood obesity [10]. The results of the survey by Pathak and Nichter imply that the childhood exposure of girls to a diet high in saturated fat and simple sugars may also cause hormone disturbances during the prepubescent reproductive maturation period, leading to lifelong ovarian dysfunction and the progression of PCOS [11].

Many animal models have been developed with the aim of coming to a fuller understanding of the potential mechanisms underlying PCOS. Prepubertal exposure to the aromatase inhibitor letrozole (LET) has been used for this purpose [12]. Although there are inconclusive data on metabolic disorders resulting from exposure to LET [13–15], polycystic changes in ovaries have occurred and disturbed endocrine parameters have been observed; these include elevated levels of testosterone and luteinizing hormone and reduced levels of estradiol, progesterone, and follicle-stimulating hormone [16,17]. The results of earlier studies indicate that the addition of high-fat diet to the LET model affords good metabolic aberrations, along with ovarian cysts [1,18,19]. However, all these studies were conducted among adult female rodents. Pilot studies employing chronic, mild elevation of androgen in prepubertal rhesus macaques maintained through young adulthood demonstrated that these female monkeys developed metabolic and ovarian dysfunction when they were exposed to a high-fat, calorie-dense diet [20,21]. These findings revealed for the first time that diet can directly modulate the reproductive and metabolic symptoms associated with hyperandrogenemia. A similar effect is caused by high-fat and high-sugar (HF/HS) diets administered in the prepubertal period. However, it has been suggested that polycystic ovaries and hyperandrogenism develop secondarily to the IR induced by the tested diet [22].

Although the increased consumption of highly processed food, rich in simple sugar particularly fructose and saturated fat, has been associated with obesity and metabolic disorders in young people, the effects of this diet on the symptoms of PCOS around the time of puberty are not clear. This study aims to investigate the effects of a HF/HFr diet on metabolic/endocrine dysregulations associated with letrozole-induced PCOS in prepubertal female mice.

2. Materials and Methods

2.1. Experimental Animals and Treatment

Every effort was made to minimize both the number of animals used and their suffering. We calculated the sample size using G*Power software (RRID:SCR_013726); the sample size of the mice was also determined in accordance with Zheng et al. [23]. The effect size was calculated to be 2.05 on the basis of the differences in HOMA-IR between the control HFD (high fat diet) group and the PCOS+HFD group. With an alpha value of 0.05, a sample size of eight mice per group would yield a power of 0.95.

Thirty-two (32) prepubertal C57BL/6 mice (average body weight 13.5 g) with an age of three weeks were involved in the experiment. The animals were purchased from the Mossakowski Institute of Experimental and Clinical Medicine, Polish Academy of Sciences, Warsaw, Poland, and were housed in the vivarium at the Department of Physiology,

Biochemistry and Animal Biostructure, part of the Faculty of Veterinary Medicine and Animal Sciences at Poznań University of Life Sciences. They were allowed to adapt to the laboratory environment for ten days. All animals were housed in standard polycarbonate cages and maintained in a controlled environment, with a temperature of 21 ± 1 °C, humidity of 55–65%, and a twelve-hour light–dark cycle. After acclimatization, at four weeks of age, the mice were randomly assigned to four groups: (1) mice receiving placebo pellet fed a standard diet (n = 8); (2) mice receiving placebo pellet fed the HF/HFr diet (n = 8); (3) mice receiving LET pellet fed a standard diet (n = 8); and (4) mice receiving LET pellet fed the HF/HFr diet (n = 8). Subcutaneous implantation of continuous release letrozole (3 mg, 50 μg/day) or the placebo pellet was performed to induce PCOS or form a control group, respectively. Letrozole was purchased from Innovative Research of America.

Such young animals were used because PCOS should be induced in the prepubertal period [13]. Two groups of mice were fed with a standard laboratory diet (3.8 kcal/g, energy supply ratio: protein 18%, carbohydrate 66%, fat 16%). In turn, the other two groups were fed the HF/HFr diet (4.7 kcal/g, energy supply ratio: protein 17%, carbohydrate 37.5% (mainly fructose), fat 45.5%). The experimental diets were bought from Morawski Animal Feed (Kcynia, Poland).

The animals had unlimited access to water and food throughout the experimental period. Once a week, the animals were weighed with a Sartorius MSE2202S-100-D0 (Germany) precision balance. During the fourth week of the experiment, dietary consumption was assessed by randomly selecting four mice from each group and placing them in a semi-metabolic cage for 3 days. For this purpose, the diet provided and the diet that remained uneaten were weighted, and the difference was calculated to give the weight consumed. The study was approved by the Local Ethical Commission under permission No. 51/2021 and was performed in line with the ARRIVE 2.0 guidelines for animal research [24].

2.2. Sample Collection

The animals were sacrificed after five weeks of the experiment by decapitation. Blood was collected into nonheparinized sample bottles. The blood was centrifuged (3500× g, 15 min, 4 °C) to obtain serum samples, which were subsequently kept frozen at −80 °C until needed for biochemical assays. The liver was collected and frozen in liquid nitrogen.

Immediately after the last blood draw, ovary samples were rapidly removed from the animals. Ovaries from each mouse were fixed in 10% formalin (formaldehyde in saline). The ovaries were stored at 4 °C in 50 mL of 20% sucrose in PBS for 24 h before sectioning. Ovary sections of 4–5 μm thickness were obtained using a cryostat (CM1860 Ag Protect; Leica Biosystems, Warsaw, Poland) and collected on microscopic slides (Menzel-Glaser, SuperFrost Ultra Plus, Thermo Scientific, Budapest, Hungary). Subsequently, the sections were stained with hematoxylin-eosin (Sigma-Aldrich, Madrid, Spain) in line with the standard histological procedures; they were cover-slipped with DPX Mountant for Histology (Sigma-Aldrich, Madrid, Spain). Slides were examined under a light microscope (Leica, DM500, Leica Biosystems) and analyzed with LAS 4.9 software (Leica Biosystems). The ovarian preparations were analyzed in terms of the number of follicles and their diameters, the corpus luteum, and the thickness of the theca layer and the follicular wall.

2.3. Serum Biochemical Analysis

Serum glucose (GLU), triglycerides (TG), total cholesterol (TC), HDL-C, LDL-C, C-reactive protein (CRP), and total antioxidant capacity (TAC) were measured using commercially available colorimetric and enzymatic assays from Pointe Scientific (Lincoln Park, MI, USA). The concentration of non-esterified fatty acids (NEFA) was determined using an enzymatic test from Wako (Oxoid, Dardilly, France). Concentrations of insulin in blood serum were measured using an immunoassay (ELISA) kit obtained from Sunlong Biotech (Hangzhou, Zhejiang, China). The level of testosterone was analyzed using an immunoassay (ELISA) kit from LDN (Nordhorn, Germany). Liver cholesterol and triglycerides were analyzed after lipid extraction using a cholesterol and triglycerides kit (Pointe Scientific,

Lincoln Park, MI, USA), as described by Folch et al. [25]. The total antioxidant capacity was measured using the TCA method with a TBARS Assay Kit. (Cayman Chemical, Ann Arbor, MI, USA). The optical density of the samples was measured using a Synergy 2 microplate reader (Biotek, Winooski, VT, USA).

2.4. Calculation of the HOMA-IR, HOMA-β, and QUICKI Indices

Insulin resistance and β-cell function were evaluated using the Homeostasis Model Assessment Method. The HOMA-IR was calculated using the following formula:

$$\text{HOMA} - \text{IR} = \text{fasting glucose [mmol/L]} \times \text{fasting insulin [}\mu\text{IU/mL]}/22.5$$

HOMA-β was calculated using the following equation: $\text{HOMA} - \beta = \text{FI} \times 20/(\text{FG} - 3.5)$, where FI is fasting insulin (in μU/mL) and FG is fasting glucose (in mmol/L).

The quantitative insulin sensitivity check index (QUICKI) was calculated using the following formula:

$$\text{QUICKI} = 1 \log (\text{fasting glucose [mg/dL]}) + \log (\text{fasting insulin [}\mu\text{IU/mL]})$$

Non-HDL was calculated using the following formula: Non-HDL = total cholesterol (mg/dL) − HDL (mg/dL).

2.5. Statistical Analysis

The results were statistically evaluated using Statistica 13.3.0 (TIBCO Software, Palo Alto, CA, USA; 2017). The results are presented in the tables and figures as arithmetic means ± standard deviations (SD), and the data in some figures are presented as medians with boxes and whiskers representing the interquartile range and the 5th–95th percentiles (GraphPad Prism 9.3.1. (471), GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) was used to compare the mean values of variables among the groups. Tukey's post hoc test was used to identify the significance of pairwise comparison of mean values among the groups. Values with different letters (a, b) show statistically significant differences ($p < 0.05$, a < b).

3. Results

There were no significant differences in body weight at the beginning of the experiment (placebo-treated mice: 14.6 ± 1.2 g; placebo-treated mice on HF/HFr diet: 15.0 ± 1.5 g; LET-treated mice: 14.7 ± 1.5 g; and LET-treated mice on HF/HFr diet 14.5 ± 0.6 g; data not shown).

Both the placebo and the LET-treated group of mice on the HF/HFr diet showed significantly higher diet consumption than the corresponding controls ($p < 0.05$). Moreover, the LET+HF/HFr group ate significantly more than mice receiving the placebo ($p < 0.05$). On the other hand, placebo-treated mice on the HF/HFr diet ate significantly more ($p < 0.05$) than LET-treated mice (Figure 1A). After 35 days of the experiment, mice from the LET+HF/HFr group had gained significantly more weight (by a factor of 1.3; $p < 0.05$) than the related control. Moreover, this group of mice showed a significantly higher weight gain than placebo-treated mice either on the control diet ($p < 0.05$) or on the HF/HFr diet ($p < 0.05$; Figure 1B).

Both placebo- and LET-treated mice on the HF/HFr diet showed significantly ($p < 0.05$) higher cholesterol concentrations than the corresponding controls. Moreover, the latter group had a significantly ($p < 0.05$) higher cholesterol concentration than the placebo-treated mice. In turn, the placebo-treated mice on the HF/HFr diet had substantially higher cholesterol levels than the LET-treated mice ($p < 0.05$; Table 1). Exactly the same dependencies were observed for the concentrations of HDL cholesterol, non-HDL cholesterol, and LDL cholesterol. TG concentration and TC/HDL ratio fluctuated at the same level in all four groups, and no significant differences were observed here. Substantially higher levels of plasma NEFA were seen in the HF/HFr placebo-treated mice than in the related

controls ($p < 0.05$). This parameter in the placebo-treated mice on the HF/HFr diet was also considerably higher ($p < 0.05$) than in the LET-treated group and even than in the LET+HF/HFr group. HF/HFr feeding also disturbed the hepatic lipid metabolism of the experimental mice. The hepatic concentrations of both TC and TG increased remarkably compared to the control ($p < 0.05$) after the placebo mice had fed on the HF/HFr diet. The hepatic concentrations of TG in this group were also significantly higher than in the LET-treated group ($p < 0.05$). Additionally, the hepatic concentrations of TG were also significantly higher ($p < 0.05$) in the LET+HF/HFr group than in the related control. In this group of mice, the hepatic concentrations of both TC and TG were also significantly higher than in placebo mice fed a standard diet (Figure 2A,B). Plasma glucose levels were significantly higher ($p < 0.05$) by a factor of 1.2 in the placebo-treated mice on the HF/HFr diet than in the LET-treated mice. The LET+HF/HFr group of mice had significantly higher values ($p < 0.05$) of the HOMA-IR and QUICKI indices than the related controls. No significant differences in HOMA-β and CRP levels were observed between groups, while the placebo-treated mice fed the standard diet had a significantly higher TAC, by a factor or 4.9, than the LET+HF/HFr group.

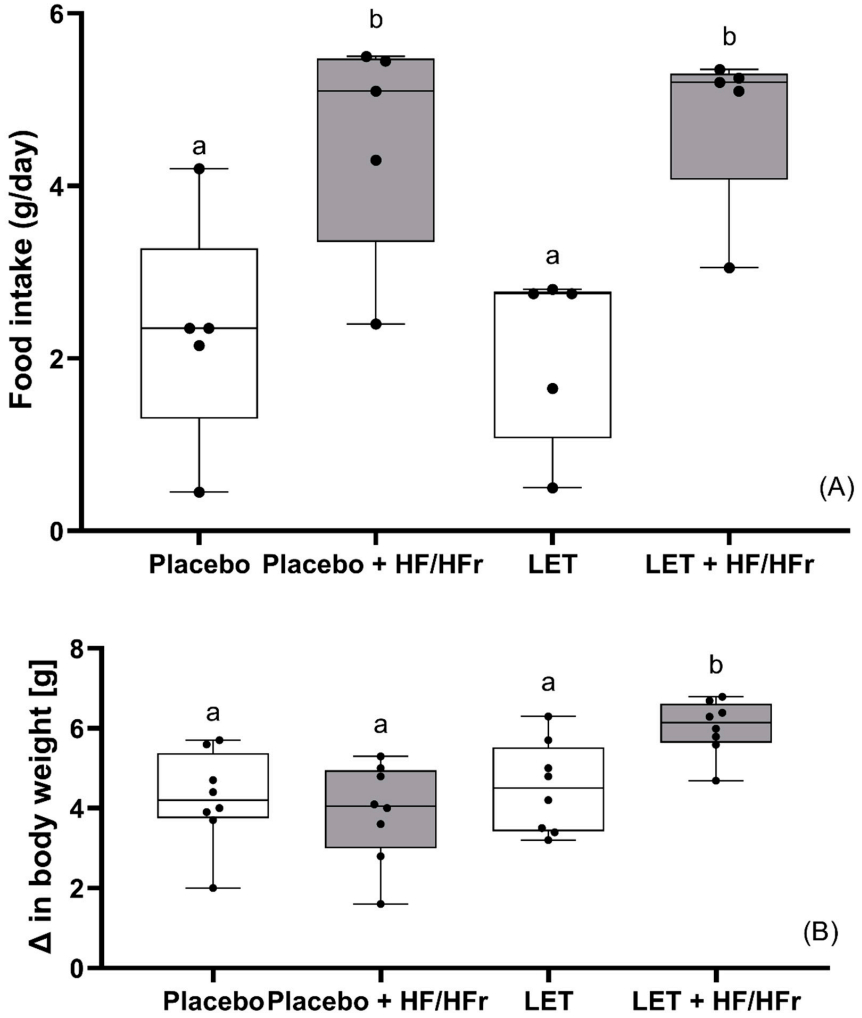

Figure 1. Effects of HF/HFr (high fat/high fructose) diet on food intake (**A**) and changes in body weight (**B**) in placebo or LET-treated (letrozole-treated) mice. The data are presented as medians, with boxes and whiskers representing the interquartile range and the 5th–95th percentiles ($n = 8$ per group), analyzed using one-way ANOVA followed by Tukey's post hoc test. Values with different letters show statistically significant differences ($p < 0.05$).

Table 1. Effects of the HF/HFr diet on metabolic parameters in placebo and LET-treated mice.

Variables	Placebo		Letrozole	
	Control	HF/HFr	Control	HF/HFr
Total cholesterol (mg/dL)	131.9 ± 21.8 [a]	188.4 ± 26.0 [b]	137.6 ± 23.0 [a]	191.7 ± 39.8 [b]
LDL cholesterol (mg/dL)	51.6 ± 5.9 [a]	77.1 ± 4.9 [b]	49.5 ± 2.5 [a]	74.5 ± 6.2 [b]
HDL cholesterol (mg/dL)	30.8 ± 4.1 [a]	40.0 ± 3.2 [b]	28.9 ± 1.8 [a]	38.3 ± 1.6 [b]
Non-HDL cholesterol (mg/dL)	101.2 ± 22.0 [a]	148.4 ± 28.3 [b]	108.7 ± 23.7 [a]	153.4 ± 40.6 [b]
Triglycerides (mg/dL)	141.2 ± 16.4	135.9 ± 21.3	136.2 ± 16.6	128.7 ± 12.9
TC/HDL ratio	4.4 ± 0.9	4.8 ± 1.0	4.8 ± 0.9	5.0 ± 1.2
NEFA (mmol/L)	1.09 ± 0.05 [a]	1.24 ± 0.06 [b]	1.14 ± 0.11 [a]	1.12 ± 0.06 [a]
Glucose (mg/dL)	119.9 ± 14.0 [ab]	140.1 ± 23.0 [b]	113.9 ± 12.0 [a]	136.3 ± 18.4 [ab]
Insulin (mU/L)	2.9 ± 0.8	3.4 ± 1.5	2.6 ± 0.7	3.4 ± 0.7
HOMA-IR	0.9 ± 0.3 [ab]	1.0 ± 0.2 [ab]	0.7 ± 0.2 [a]	1.2 ± 0.3 [b]
HOMA-β	18.5 ± 2.9	17.7 ± 10.9	20.3 ± 9.3	20.0 ± 8.0
QUICKI	0.8 ± 0.1 [ab]	0.7 ± 0.1 [ab]	0.8 ± 0.1 [a]	0.7 ± 0.1 [b]
CRP (mg/L)	25.4 ± 4.2	27.2 ± 1.5	27.5 ± 2.9	29.4 ± 3.0
TAC (μmol/L)	6.5 ± 5.6 [a]	2.8 ± 1.1 [ab]	4.9 ± 3.5 [ab]	1.6 ± 0.5 [b]

Results are expressed as mean ± SD (n = 8 per group). Values with different letters (a,b) show statistically significant differences ($p < 0.05$, Tukey's post hoc test). NEFA: non-esterified fatty acids; TC: total cholesterol; HDL: high-density lipoprotein; LDL: low-density lipoprotein; HOMA-β: homeostasis model assessment of β-cell function; HOMA-IR: homeostasis model assessment of insulin resistance; QUICKI: quantitative insulin sensitivity check index; CRP: C-reactive protein; TAC: total antioxidant capacity; HF/HFr: high fat, high fructose.

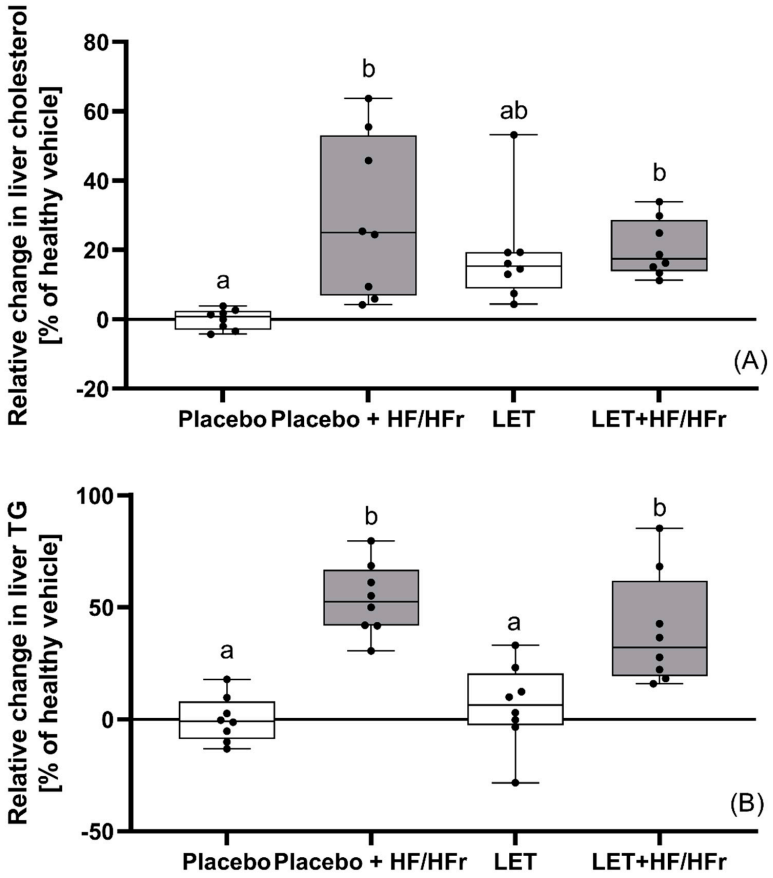

Figure 2. Terminal changes in the liver cholesterol (**A**) and triglycerides (**B**). The data are presented as medians, with boxes and whiskers representing the interquartile range and the 5th−95th percentiles (n = 8 per group), analyzed using one−way ANOVA followed by Tukey's post hoc test. Values with different letters show statistically significant differences ($p < 0.05$).

Serum testosterone concentration was robustly higher by a factor of 1.6 in the LET+HF/HFr group of mice ($p < 0.05$, Figure 3) compared to the control. Moreover, testosterone levels in the placebo mice on the HF/HFr diet were significantly higher ($p < 0.05$) than in the LET-treated group. In the LET-treated group, the LET+ HF/HFr group, and the placebo mice on the HF/HFr diet, an increased number of corpus luteum were observed compared to the placebo-treated mice on the standard diet. Moreover, the placebo mice on the HF/HFr diet had the largest number of ovarian follicles of the four groups. This group also had the thickest granulosa layer in the follicle, while in both the LET-treated and the LED+HF/HFr groups, this layer's thickness was visibly reduced. Furthermore, the LET+HF/HFr group had the greatest follicle diameter and the thickest theca folliculi. Follicular atresia and the presence of cysts were observed not only in both the LET-treated groups but also in the group of placebo-treated mice on the HF/HFr diet. Interestingly, the changes in ovarian morphology in the LET-treated mice were milder than in the LET+HF/HFr group, at least partly due to the smaller number of cysts and lesser degradation of the granular cell layer (Figures 4–9).

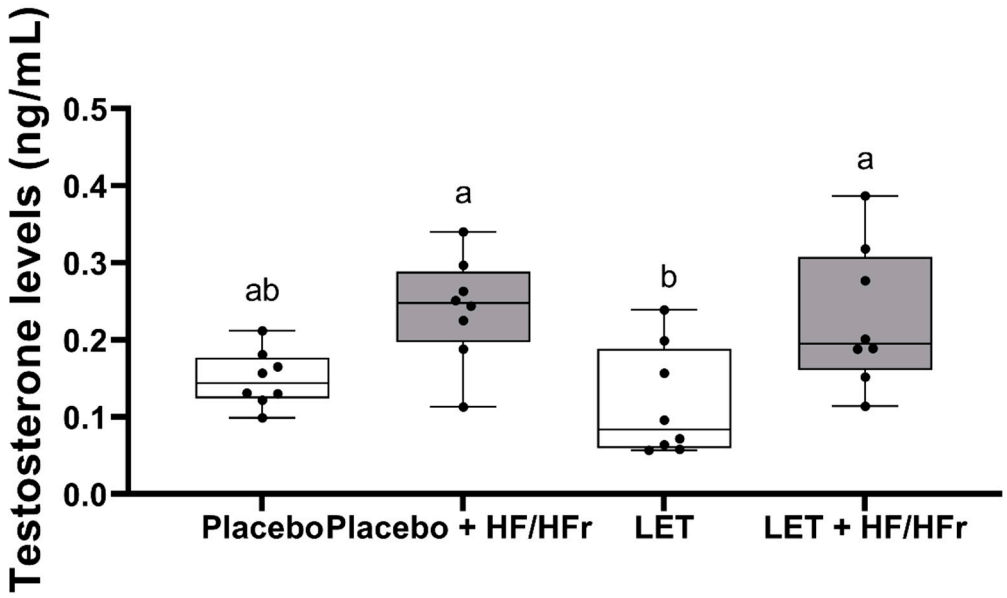

Figure 3. Effects of HF/HFr diet on testosterone levels in placebo or letrozole-treated mice. The data are presented as medians, with boxes and whiskers representing the interquartile range and the 5th−95th percentiles ($n = 8$ per group), analyzed using one-way ANOVA followed by Tukey's post hoc test. Values with different letters show statistically significant differences ($p < 0.05$).

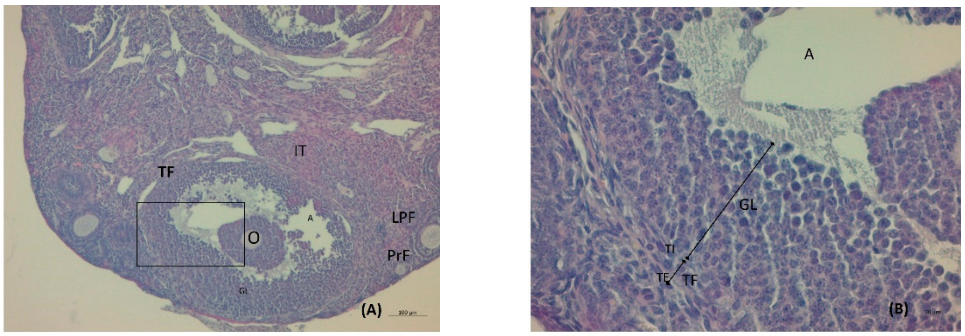

Figure 4. Histological sections of the ovaries of intact mice: tertiary follicle. O: oocyte; GL: granulosa layer; A: antrium; LPF: late primary follicle; PrF: primodial follicle; IT: interstitial tissue; TF: theca folliculi; TE: theca externa; TI: theca interna (H&E; power 100×(**A**)/400×(**B**)).

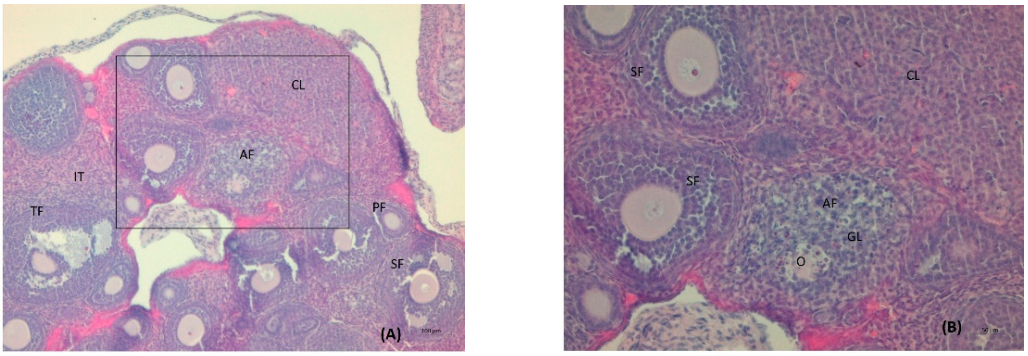

Figure 5. Histological sections of the ovaries with atretic follicles (AF) in intact mice on the HF/HFr diet. TF: tertiary follicle; PF: primary follicle; SF: secondary follicle; CL: corpus luteum; IT: interstitial tissue; O: oocyte; GL: granulosa cells in the process of degradation (H&E; power 100×(**A**)/200×(**B**)).

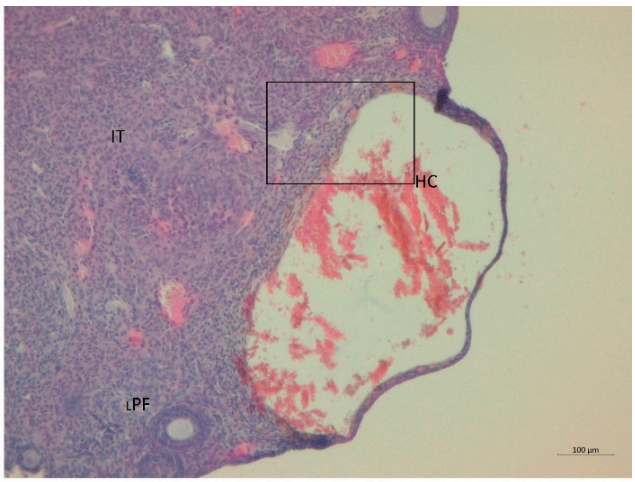

Figure 6. Histological sections of ovaries with hemorrhagic cysts (HC) in LET-treated mice. LPF: late primary follicle; IT: interstitial tissue (H&E; power 100×).

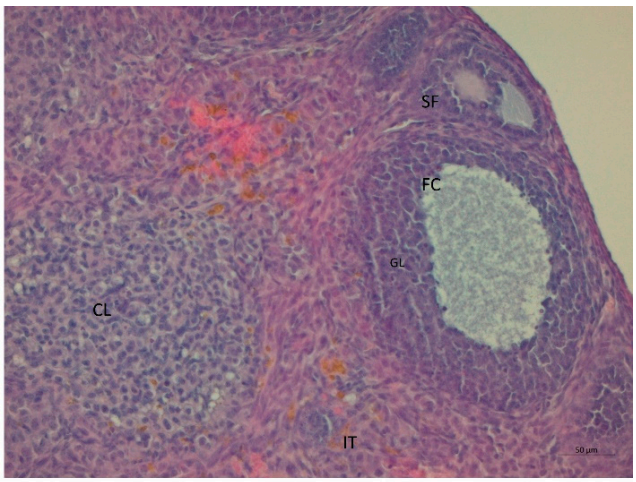

Figure 7. Histological sections of ovaries with follicular cysts (FC) in LET-treated mice. GL: granulosa cells; CL: corpus luteum; IT: interstitial tissue (H&E; power 200×).

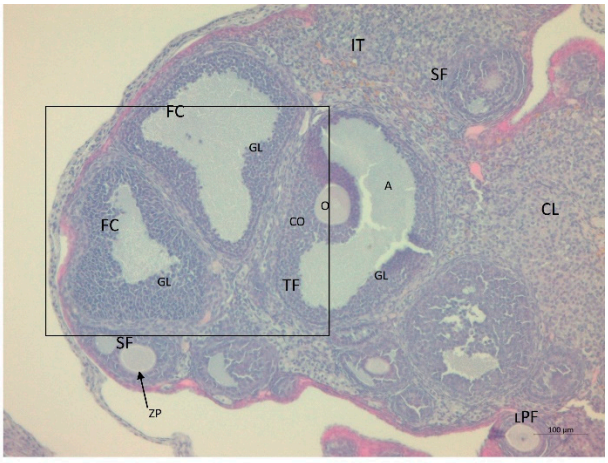

Figure 8. Histological sections of ovaries with follicular cysts (FC) in LET-treated mice on the HF/HFr diet. TF: tertiary follicle; O: oocyte; GL: irregular granulosa layer; CO: cumulus oophorus; A: antrium; LPF: late primary follicle; SF: secondary follicle; ZP: zona pellucida; IT: interstitial tissue (H&E; power 100×).

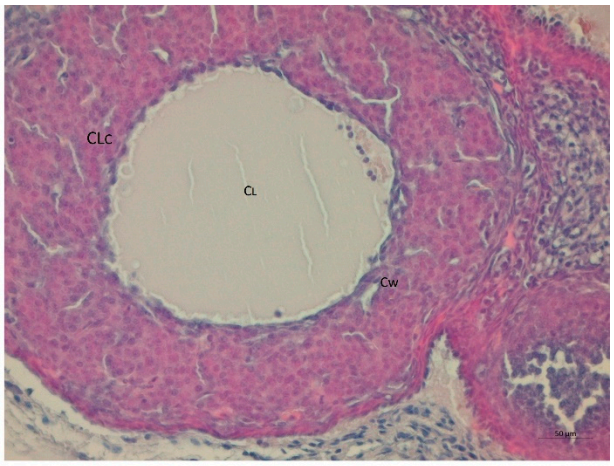

Figure 9. Histological sections of ovaries with corpus luteum cysts (CLc) in LET-treated mice on the HF/HFr diet. Cw: cyst wall; CL: cyst lumen (H&E; power 400×).

4. Discussion

Since PCOS often manifests in the early reproductive years, puberty is considered to be a critical time period for the development of PCOS. Indeed, our study demonstrated that five weeks of HF/HFr feeding initiated at the prepubertal age provoked some reproductive and metabolic features of PCOS in LET-treated female mice. More specifically, in the LET+HF/HFr group of mice, elevated testosterone levels and morphological changes in the ovaries were seen, suggesting PCOS. Indeed as was shown previously, elevated serum testosterone levels are related to endocrine imbalances and contribute to PCOS symptoms such as ovarian dysfunction and irregular ovarian or estrous cycles [26]. The LET+HF/HFr group of mice also had a worsened lipid profile (TC, LDL-C, HDL-C and non-HDL, except of TG) and insulin sensitivity indices (HOMA-IR and QUICKI). Hepatic steatosis also occurred in this group of mice. Our findings are in line with those of previous studies showing that the use of letrozole with a high-fat diet may induce or worsen the symptoms of PCOS [1,25]. More specifically, Xu et al. noted that twelve weeks of administration of LET with a high-fat diet in female Sprague–Dawley rats induced anovulatory cycles and polycystic ovary morphology, body weight gain, elevated testosterone levels, abnormal glucose and lipid metabolism, as well as insulin resistance [27]. Begum et al. indicated that twelve weeks of administration of the LET+HF diet in Wistar female rats induced additional glucose intolerance [1]. It should, however, be highlighted that all PCOS symptoms seen

in our study developed as early as week five of the experiment when LET+HF/HFr was administrated; moreover, hepatic steatosis also occurred. Worsened insulin sensitivity indices, as observed in the LET-treated mice on the HF/HFr diet, seem to be associated with the excess body weight of mice. Indeed, obesity is associated with inflammation and the generation of reactive oxygen species that have a potent role in inducing insulin resistance [15]. In line with this, in our LET-treated mice on the HF/HFr diet, we observed substantial decreases in total antioxidant capacity (TAC) compared to placebo-treated mice on a standard diet. This indicates that the occurrence of PCOS is associated with oxidative stress in PCOS women, which may even contribute to the pathogenesis of this disorder [28]. Agreeing with this, a case-control study showed statistically significant decreases in the TAC levels of women with PCOS as compared to the control group [29].

Interestingly, it was seen in our study that LET itself did not lead to the development of any endocrine or metabolic abnormalities in experimental mice, but polycystic ovaries were observed. In contrast, Arroyo et al. and Skarra et al. demonstrated that five weeks of LET treatment resulted in the hallmarks of PCOS, including elevated testosterone and luteinizing hormone (LH) levels, acyclicity, and the appearance of cystic ovarian follicles [13,30]. However, despite the hormonal variations shown in different animal studies, letrozole in general manifests good reproducibility for PCOS-like features in rodents [15] and is believed to cause the lean reproductive phenotype of PCOS [31].

The HF/HFr diet itself may also lead to some features of PCOS. As was observed in our study, five weeks of exposure to the HF/HFr diet significantly elevated serum testosterone of the female mice and also disturbed some lipid parameters (TC, LDL-C, HDL-C and non-HDL, except of TG). Elevated levels of glucose and polycystic changes in ovaries were also observed. However, worsened insulin sensitivity was not observed in this model. Roberts et al. indicated that a high-fat, high-sugar diet given for eleven weeks led to hyperinsulinemia but not to hyperandrogenemia in experimental rats [22]. However, elevated testosterone levels in that study were predictive of a high number of ovarian cysts [22]. In our study, the elevated testosterone levels seen in a group of mice on an HF/HFr diet seem to be the effect of higher levels (though not statistically significant levels) of insulin. Indeed, insulin acts directly through its own receptor in PCO theca cells to increase androgen production [32]. Interestingly, metabolic disturbances in the placebo-treated mice fed the HF/HFr diet were seen even when the body weight of those mice did not increase significantly. It was also surprising that, despite the equally high consumption of the HF/HFr diet, only the LET-treated mice gained significantly more weight, while the body weight of the placebo mice did not differ from that of the other groups. Similar results were obtained by Patel and Shah, but this was associated with a reduction in food intake, which was not observed in our experiment [33]. Huang et al. explained that female rodents are relatively resistant to hyperphagia and weight gain in response to a high-fat diet, in part due to the effects of estrogen, which suppress food intake and increase energy expenditure [34].

Since increased prevalence of NAFLD has been reported in women with PCOS [35], we also assessed the hepatic accumulation of TC and TG in our experimental mice. More specifically, the hepatic accumulation of both TC and TG increased remarkably after the placebo-treated mice were fed the HF/HFr diet, as compared with control. In LET-treated mice on the HF/HFr diet, only the TG level was significantly higher than that of the related control. The accumulation of excess triglycerides in hepatocytes is generally the result of the increased delivery of non-esterified fatty acids (NEFAs), increased synthesis of NEFAs, impaired intracellular catabolism of NEFAs, impaired secretion as triglyceride, or a combination of these abnormalities [36]. In our study, only higher levels of the plasma NEFA in the mice fed a HF/HFr diet compared to placebo-treated mice was seen. Interestingly, despite the visible NAFLD, we did not observe significant differences in serum triglyceride concentration, as hypertriglyceridemia develops secondarily to hepatic steatosis [37] and the period of 5 weeks was likely insufficient for its full appearance [38].

5. Conclusions

Our findings reveal for the first time that HF/HFr feeding given around puberty may directly stimulate reproductive and metabolic symptoms, not only in LET-treated mice but also in placebo-treated mice. These findings indicate that a diet that is highly processed, high in simple sugars (particularly fructose), and high in saturated fats may, if eaten every day, have a great impact on PCOS progression in young females. Food products that are rich in these ingredients should therefore be eliminated from the diets of women with PCOS, especially younger women. Furthermore, the combination of the HF/HFr diet with LET causes visible metabolic disorders, comparable to those found in women with PCOS. Moreover, hepatic steatosis also occurred. Thus, this animal model can be used to the test various options for PCOS treatment. In turn, the model based on letrozole alone was not sufficient to induce the above-mentioned disorders, although it caused visible changes in the morphological structure of the ovaries.

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Systematic Review

The effects of time-restricted eating and Ramadan fasting on gut microbiota composition: a systematic review of human and animal studies

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Context: *It is well known that the microbiome undergoes cyclical diurnal rhythms. It has thus been hypothesized that meal timing may affect gut microbial composition, function, and host health.* ***Objective:*** *This review aims to examine the effects of time-restricted eating (TRE) and Ramadan fasting (RF) on the composition of the gut microbiota in animal and human studies. The associations between composition of microbiota and host metabolic parameters are also examined.* ***Data Sources:*** *A search was performed on the PubMed, Cochrane, Scopus, and Web of Science databases up to December 31, 2022. The search strategy was performed using the Medical Subject Heading (MeSH) terms "intermittent fasting" and "gastrointestinal microbiome" and the key words "Ramadan fasting" and "microbes."* ***Data Extraction:*** *Seven human studies (4 TRE and 3 RF) and 9 animal studies (7 TRE, 2 RF-like) were retrieved.* ***Data Analysis:*** *TRE and RF in human studies lead to an increase in gut microbial community alpha-diversity. In animal studies (both TRE and RF-like), fasting is not associated with improved alpha-diversity, but enhancement of microbial fluctuation is observed, compared with high-fat diet ad libitum groups. Within Firmicutes and Bacteroidetes phyla, no specific direction of changes resulting from fasting are observed in both animals and human. After TRE or RF, a greater abundance of the* Faecalibacterium *genus is observed in human studies; changes in* Lactobacillus *abundance are found in animal studies; and increases in* Akkermansia *are seen both in humans and in animals fed a feed-pellet diet. Only 2 human studies show a beneficial correlation between microbiota changes and host metabolic (HDL cholesterol) or anthropometric parameters (body mass index).* ***Conclusions:*** *These findings support the importance of both regimens in improving the gut microbiota composition. However, based on results of animal studies, it can be suggested that diet remains the essential factor in forming the microbiota's environment.*
Systematic Review Registration: *PROSPERO registration no. CRD42021278918.*

Key words: *chrononutrition, fasting, gut microbiota, host health, microbial diversity.*

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INTRODUCTION

Circadian rhythms represent an endogenous timekeeping system that regulates and synchronizes behavior, physiology, and metabolism with external cues known as zeitgebers, thus establishing homeostasis.[1] The light–dark cycle is the most important zeitgeber, but other stimuli such as temperature and the presence of food can also act as zeitgebers.[2] Circadian rhythms are regulated by a master clock located in the suprachiasmatic nucleus of the hypothalamus,[3] as well as by peripheral clocks in other tissues, including the liver, muscle, adipose tissue, and even the gut. At the molecular level, the circadian clock consists of multiple sets of transcription factors that regulate gene expression, operating in a series of feedback loops.[4]

The gut microbiota provides many benefits to the host, biosynthesizing vitamins and essential amino acids and generating important metabolic byproducts, including short-chain fatty acids, such as butyrate, propionate, and acetate, that act as major energy sources for intestinal epithelial cells, and which may therefore strengthen the mucosal barrier.[5] Diet is a key factor for gut microbiota composition and metabolism, and several studies have investigated the effects of different dietary components, including dietary fiber, on the gut microbiota.[6,7] On the other hand, a high-fat diet (HFD) has been shown to adversely alter the composition of the gut microbiota, reducing microbial diversity and depleting the abundance of beneficial bacteria, including *Bifidobacterium* and *Akkermansia*,[8] which are believed to have beneficial effects on body weight and on carbohydrate metabolism parameters.[9]

It is known that the microbiome undergoes cyclical diurnal rhythms.[10] The greatest peak in bacteria of the Bacteroidetes and Veruccomicrobia phyla can be observed in rodents during feed-deprivation periods. The number of these bacteria gradually decreases with the approach of the feeding period, and bacteria of the Firmicutes phylum instead dominate.[10] This is a particularly important aspect that should be taken into account in the methodology of research on the composition of the microbiota, because these cyclical changes can be observed only in the intestinal contents collected during the circadian termination of rodents.[10,11] Therefore, the assessment of animal microbiota directly from the intestinal contents seems to be more accurate than from the feces, both human and animal, which allows the observation of changes in only 1, often impossible to determine, time point.

It is also hypothesized that meal timing may also affect the gut microbiome, with implications for host health.[5] One dietary regimen that may affect peripheral oscillations is time-restricted eating (TRE), a pattern where food intake is restricted to certain hours of the day (most often an 8-h period), with no limitation on nutrient quality or quantity.[11] One form of TRE is Ramadan fasting (RF), a regimen that is common among Muslims.[12] Those practicing RF fast from sunrise to sunset, eating 2 or 3 meals after sunset. However, during Ramadan, there is also a change in the quality of the diet, with increased consumption of cakes, sweetened drinks, vegetables, and dried fruits, and decreased consumption of fats, dairy products, eggs, and cereal products.[13] Meals are mainly consumed during the day in TRE, but in RF they are mainly consumed at night, which may have an effect on gut microbiota composition and metabolic health of the host.

Considering that both dietary regimens may be significant modulators of health and microbiota diversity, the aim of this systematic review is to summarize the effects of the TRE and RF regimens on the composition of the gut microbiota in both animal and human studies. Extensive research using both animal models[10,11,14,15,16] and humans[17,18] demonstrates that both TRE and RF yield beneficial changes in the metabolic parameters associated with obesity; for this reason, the aim was also to investigate whether the changes in these host metabolic parameters are associated with changes in the composition of the gut microbiota.

METHODS

Study eligibility

This systematic review was registered in the International Prospective Register of Systematic Reviews (CRD4202 1278918) and was conducted in line with the principles of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement (Table S4 *in the Supporting Information online*).

Search strategy and inclusion/exclusion criteria

A search was performed by J.M.P.-Z. and J.B. on the PubMed, Cochrane, Scopus, and Web of Science databases from January 1, 2005, up to December 31, 2022. The search strategy was performed using both Medical Subject Heading (MeSH) terms and key words. The search for TRE used the terms "intermittent fasting" (a MeSH term) OR "ramadan fasting" (a key word). For gut microbiota, the search was carried out using the terms "gastrointestinal microbiome" (a MeSH term) OR "microbes" (a keyword).

The Population, Intervention, Comparison, Outcomes, and Study (PICOS) design criteria were used to identify all of the quantitative research studies for the present literature review (Table 1). Any

Table 1 **PICOS (Population, Intervention, Comparison, Outcomes, and Study) criteria for inclusion of studies**

Parameter	Description
Population	Humans aged 18–65 years Rodents older than 6 weeks
Intervention	Time-restricted eating or Ramadan fasting regimen for at least 3 weeks
Comparison	Nonfasting/ad libitum groups Baseline parameters
Outcomes	Changes in the composition of gut microbiota at different taxonomic levels (assessed by 16S rRNA) Alpha- and beta-diversity Associations between the composition of the gut microbiota and host metabolic parameters or body weight

interventional and observational studies that met the following eligibility criteria were included: (1) study participants were humans aged 18–65 years or rodents older than 6 weeks who underwent TRE or RF for at least 3 weeks; outcomes included changes in the composition of the gut microbiota at different taxonomic levels (assessed by 16S rRNA) and its alpha- and beta-diversity. Selected associations between the composition of the gut microbiota and host metabolic parameters or body weight (secondary outcomes) were also evaluated. Systematic reviews, case reports, articles written in a language other than English, and papers in which a treatment arm (other than TRE/RF) included exercise, calorie restriction, or weight-loss supplementation were excluded.

The search results from all of the databases were collected in the Mendeley tool (Mendeley Desktop Version 1.19.8), where duplicates were removed. A 2-phase search strategy was subsequently used by 2 independent reviewers (J.M.P.-Z. and J.B.) up to December 31, 2022. In phase 1, the eligibility of each study was assessed on the basis of its title and abstract. Studies that had questionable suitability were provisionally included, with a final decision made in phase 2. In phase 2, full articles were retrieved and assessed against the eligibility criteria. Reference lists of original and review articles were screened to ensure that all relevant studies had been included. Any disagreement over the eligibility of an article for this study was resolved through discussion with K.Ł., A.M., and N.L. The search strategy is summarized in Fig. 1.

Data extraction

The following data were extracted from the animal studies: author, type of animal model, number of animals and their age, type of intervention, control conditions, intervention diet, and duration of the study. The following outcomes were extracted from the animal studies: type of material (colonic or fecal) taken to test the composition of the microbiota, the variable gene region selected for gene sequencing, abundance of microbial taxa at the phylum and genus level, the alpha-diversity and beta-diversity parameters, as well as other study findings, such as associations between changes in the microbiota and host metabolic markers.

The following data were extracted from the human studies: author, study design, number of participants, age (years), type of intervention, control conditions, and duration of the study. The following outcomes were extracted from the human studies: type of material (feces), the variable gene region selected for gene sequencing, abundance of microbial taxa at the phylum and genus level, alpha-diversity and beta-diversity parameters, and other study findings, such as associations between changes in microbiota and host metabolic markers.

Any disputes regarding the appropriateness of including or excluding a given study were resolved by discussion between the authors.

Quality assessment

For the rodent model studies, the Systematic Review Centre for Laboratory Animal Experimentation (SYRCLE) risk-of-bias assessment tool was used.[19] For the human studies, the Quality Assessment Tool for Observational Cohort and Cross-Sectional Studies, the Quality Assessment Tool for Before–After (Pre–Post) Studies With No Control Group, and the Quality Assessment of Controlled Intervention Studies from the National Institutes of Health (NIH) National Heart, Lung, and Blood Institute[20] were used.

RESULTS

Due to the nature of the data, the limited number of studies, the large heterogeneity they displayed, the range of designs used, the various methodologies for determining microbiota, and various ways of presenting the results, it was decided to systematically summarize the current evidence, rather than performing a quantitative meta-analysis. Differences between groups and changes within the study group before and after the intervention are reported. In order to unify the text, the abbreviation TRE is used in reference to both the human and animal studies.

Reviewed studies

Overall, 331 articles were identified and the final analysis included 7 human studies and 9 animal studies from

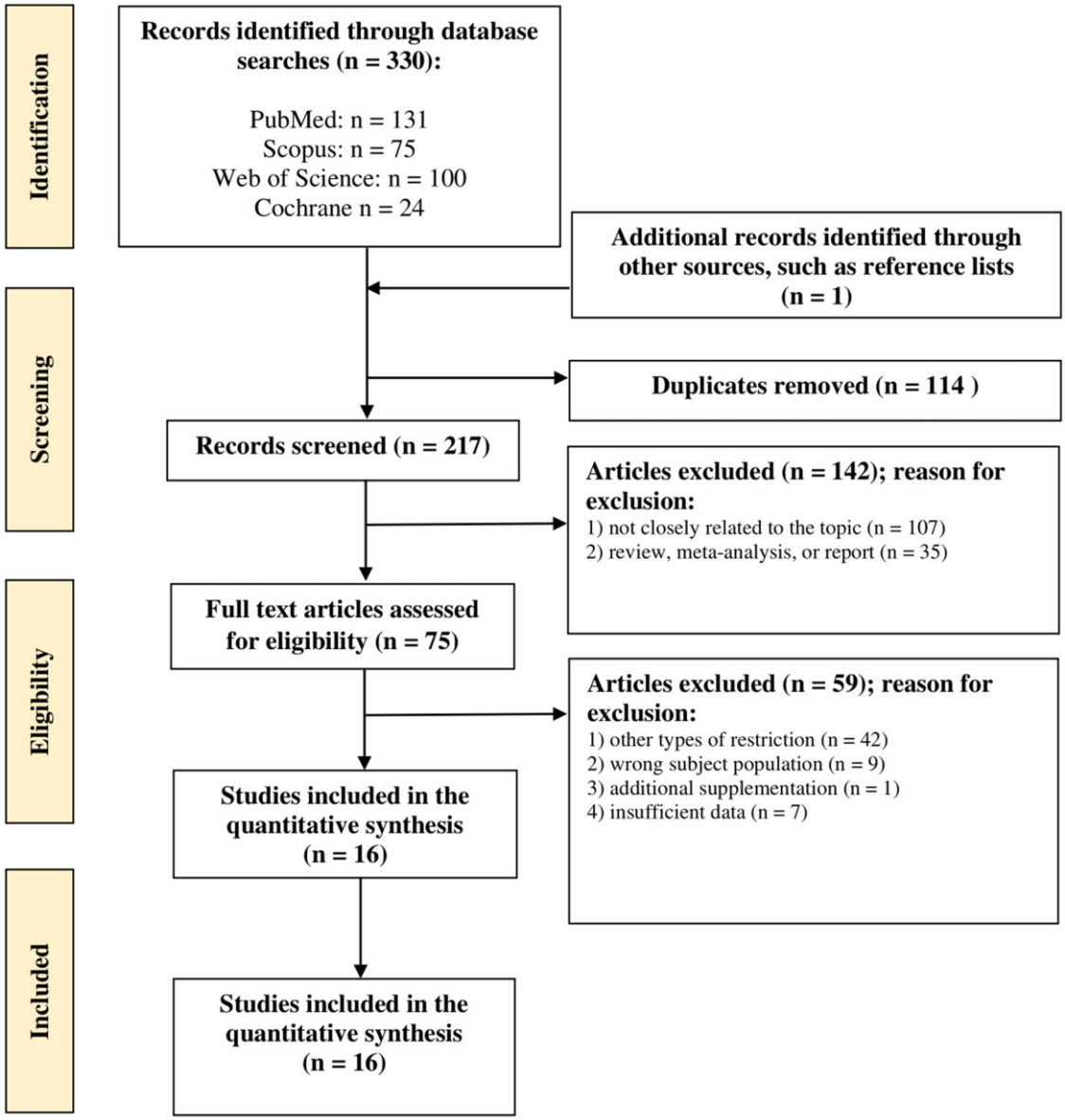

Figure 1 **Flow diagram showing the study selection process.**

16 papers (Fig. 1). The quality of all included studies are rated "good" or "fair." None of the studies were of "poor" quality. All animal studies were assessed as "good." In turn, 2 of the human studies[21,22] were rated as "fair." One of them, which was cross-sectional, gave no specific inclusion or exclusion criteria and no exact recruitment period.[22] The other study, which was a randomized controlled trial, gave no specific randomization method and did not calculate a minimum sample size, despite this being necessary in this type of study.[21]

It is worth mentioning that, due to the nature of TRE and RF, it is impossible to blind the research participants and the animal caregivers. For this reason, blinding is not taken into account when assessing the quality of studies. Tables S1 and S2 (*see the Supporting Information online*) present the full details.

The publication dates were between January 6, 2020,[21] and February 22, 2022,[23] for the human studies and between December 2, 2014,[10] and July 5, 2022,[24] for the animal studies. The duration of TRE or RF differed between the studies, from 25 days[21,22] to 12 weeks[25] in the human studies and from 4 weeks[15] to 48 weeks[26] in the animal studies.

Of the 9 rodent studies, 7 were conducted in mice—specifically Kunming mice (2 studies),[11,14] C57BL/6J mice (5 studies),[10,15,16,24,27] and BALB/c mice (1 study).[28] One study was conducted in DPP-IV–Fischer rats.[26] Their details, housing, and diet treatments are presented in Table 2.[10,11,14,15,16,24,26,27,28] All

Table 2 **Details of the animal studies**

Source	Animal models	Model	n	Specimens	Variable gene region for gene sequencing	Age	Dietary regimen	Intervention diet	Control	Duration	Comparison	Outcomes		
												Alpha-diversity	Beta-diversity	Taxonomic composition
Zarrinpar et al, 2014[10]	C57BL/6J mice	Male	72	Cecal contents	V1-V3	10-wk-old	TRE 8/16	HFD	HFD AL; CD AL	8 wk	TRE (HFD) vs AL (HFD)	X	X	X
											TRE (HFD) vs AL (CD)			
Ye et al, 2020[11]	Kunming mice	Male	60	Rectal contents	V3-V4	8-wk-old	TRE 8/16	HFD	HFD AL; CD AL	8 wk	TRE (HFD) vs AL (HFD)	X	X	X
											TRE (HFD) vs AL (CD)			
He et al, 2021[15]	C57BL/6J mice	Male	60	Cecal contents	NR	6-wk-old	RF-like 12/12	LD	LD AL	4 wk	RF-like (LD) vs AL (LD)		X	X
Machado et al, 2022[24]	C57BL/6J mice	Male	54	Ileal/cecal contents	V1-V3 (cecal)	8-wk-old	TRE 8/16	HFD	HFD AL; CD AL	8 wk	TRE (HFD) vs AL (HFD)	X	X	X
					V3-V4 (ileal)						TRE (HFD) vs AL (CD)			
Hu et al, 2018[14]	Kunming mice	Male	40	Cecal contents	V1-V3	9-wk-old	TRE 8/16	CD	CD AL	2 mo	TRE (CD) vs AL (CD)	X	X	X
Li et al, 2020[27]	C57BL/6Jlvri mice	Male	15	Feces	V3-V4	7-wk-old	TRE 8/16	CD	CD AL	1 mo	TRE (CD) vs AL (CD)	X	X	X
	C57BL/6Jlvri mice	Male	15	Feces	V3-V4	7-wk-old	TRE 12/12	CD	CD AL	1 mo	TRE (CD) vs AL (CD)	X	X	X
	C57BL/6Jlvri mice	Male	15	Feces	V3-V4	7-wk-old	TRE 4/20	CD	CD AL	1 mo	TRE (CD) vs AL (CD)	X	X	X
van der Merwe et al, 2020[16]	C57BL/6J mice	Male	43	Feces/cecal contents	NR	12-wk-old	TRE 8/16	HFD	HFD AL	6 wk	TRE (HFD) vs AL (HFD)	X	X	X
Palomba, 2021[26]	DPP-IV–Fischer rats	Male	16	Feces	V4	8-wk-old	TRE 8/16	CD	CD AL	48 wk	TRE (CD) vs AL (CD)			X
Su et al, 2022[28]	BALB/c mice	Male	14	Feces	V3-V4	6-wk-old	RF-like 8/16	CD	CD AL	1 mo	RF-like (CD) vs AL (CD)	X	X	X

Abbreviations: AL, ad libitum; CD, chow (feed-pellet) diet; HFD, high-fat diet; LD, lithogenic diet containing 1.25% cholesterol and 0.5% cholic acid; NR, not reported; RF-like, Ramadan-like fasting; TRE, time-restricted eating; X, data presented; 8/16, 8-hour eating window, 16-hour fasting; 12/12, 12-hour eating window, 12-hour fasting; 4/20, 4-hour eating window, 20-hour fasting.

rodents were 6 weeks of age or older to ensure maturity. Each of the articles used an 8-hour eating window and a 16-hour fasting/feed-deprivation period, with the single exception of Li et al[27] who used not only the traditional 8/16 regimen but also a 12/12 regimen (12 h of access to feed and 12 h of feed deprivation) and a 4/20 regimen (4 h of access to feed and 20 h of feed deprivation). Moreover, in 2 animal studies,[15,28] the eating window was during the resting phase of the rodents, which more closely mimics the Ramadan pattern.[15] The most common diets were feed-pellet diets and HFDs, although in 1 study a lithogenic diet was used.[15] The control for the TRE or Ramadan-like fasting was the same, or feed-pellet diet given ad libitum (AL).

Of the 7 human studies, 2 were randomized controlled trials,[21,23] 1 study had a quasi-experimental design,[25] and 1 study was cross-sectional.[22] The 3 RF human studies all had an observational design.[17,18,29] Six studies enrolled apparently healthy individuals,[17,18,21,22,23,29] while 1 study enlisted patients with obesity.[25] All participants were aged from 18 to 56 years (Table 3[17,18,21,22,23,25,29]). Four studies[21,22,23,25] explored the effects of TRE (with an 8-h eating window and a 16-h fast vs a normal diet), while in 1 of these studies TRE was divided into early and mid-day.[23] The other 3 studies were undertaken during the month of Ramadan with a 7-hour or 8-hour eating window during the night[17,18,29]; in the study by Su et al,[18] 2 study groups were included—one younger and one middle-aged—and were analyzed separately. In addition, the results of the young cohort were analyzed in relation to the baseline parameters, while those of the middle-aged cohort were compared with the results of a nonfasting control group.[18]

Method of microbiome assessment

Fecal specimens from all of the human studies (n = 7) were analyzed for gut microbiota composition. Of the 9 rodent studies, 5 used cecal, ileal, or rectal contents[10,11,14,15,24]; 3 studies used fecal material[26–28]; and 1 study used both types.[16] The 16S rRNA amplicon sequencing method was used in both human and animal studies. In the animal studies, 3 of them use the V3-V4 hypervariable region,[11,27,28] 2 studies used the V1-V3 region,[10,14] 1 study used the V4 region,[26] and 1 study used the V1-V3 region for cecal content and the V3-V4 region for ileal contents.[24] The rest do not specify a region.[15,16] In the case of human studies, 4 studies used the V3-V4 region,[22,23,28,29] 2 studies used the V4 region,[17,25] and 1 study used the V1-V3 region.[21]

Table 3 **Details of the human studies**

Source	Study design	Participants	n	Age, y	Specimens	Intervention	Control	Duration	Comparison	Variable gene region for gene sequencing	Outcomes		
											Alpha-diversity	Beta-diversity	Taxonomic composition
Time-restricted eating													
Gabel et al, 2020[25]	Quasi-experiment	Obese adults	14	25–65	Feces	TRE 8/16	—	12 wk	Post vs pre	V4	X		X
Zeb et al, 2020[22]	Cross-sectional	Healthy males	30	18–38	Feces	TRE 8/16	AL	25 d	TRE vs AL	V3-V4	X	X	X
Zeb et al, 2020[21]	RCT	Healthy males	80	> 18	Feces	TRE 8/16	AL	25 d	TRE vs AL	V1-V3	X	X	X
Xie et al, 2022[23]	RCT	Healthy adults	82	eTRE: 28.68 ± 9.707 mTRE: 31.08 ± 8.438 AL: 33.57 ± 11.6	Feces	eTRE 8/16 mTRE 8/16	AL	5 wk	TRE vs AL	V3-V4	X		X
Ramadan fasting													
Ozkul et al, 2020[17]	Observational	Healthy adults	9	31–56 (45.0 ± 9.7)	Feces	RF 7/17	—	29 d	Post vs pre	V4	X	X	X
Su et al, 2021[18]	Observational	Healthy males (young cohort)	30	18.63 ± 1.75	Feces	RF 8/16	—	30 d	Post vs pre	V3-V4	X	X	X
	Observational	Healthy adults (middle-aged cohort)	37	NF: 42.6 ± 7.9; F: 39.9 ± 6.4	Feces	RF 8/16	AL	30 d	RF vs AL	V3-V4	X	X	X
Ali et al, 2021[29]	Observational	Healthy adults	34	18–40	Feces	RF	—	1 mo	Post vs pre	V3-V4	X	X	X

Abbreviations: AL, ad libitum; eTRE, early TRE; F, fasting; mTRE, mid-day TRE; NF, nonfasting; NR, not reported; RCT, randomized controlled trial; RF, Ramadan fasting; TRE, time-restricted eating; X, data presented; 8/16, 8-hour eating window, 16-hour fasting; 7/17, 7-hour eating window, 17-hour fasting.

Outcomes of microbiome assessment

The synthesized results from the animal and human studies report on 2 major taxonomic levels, P (phylum) and G (genus), of bacterial taxa, although not all of these are analyzed in each group and in each study.[11,15,16,18,18,23–27] Table 4[10,11,14,15,16,24,26,27,28] presents the effects of TRE on the composition, alpha-diversity, and beta-diversity of microbiota in the animal studies. Table 5[17,18,21,22,23,25,29] presents the effects of TRE and RF on the composition, alpha-diversity, and beta-diversity of microbiota in the human studies. Due to the large number of results from the animal studies, Table 4 presents only those changes that concerned phyla or genera repeated in most studies. The full results are presented in Table S3 (*see the Supporting Information online*).

Effects of TRE and Ramadan-like fasting on composition, alpha-diversity, and beta-diversity of microbiota in the animal studies

Seven out of the 9 studies analyzed alpha-diversity using the Shannon,[10,11,14,16,24,28] Chao1, ACE (abundance-based coverage estimator),[11,14] Simpson,[14,27,28] and Faith's phylogenetic diversity (PD)[24] indicators. Three studies that administered a feed-pellet diet under the TRE regimen found no changes in alpha-diversity.[14,27] In the remaining 4 studies,[10,11,16,24] where an HFD is administered following TRE, the alpha-diversity results were inconclusive. More specifically, in 2 studies,[10,11] the TRE regimen was found to not alter alpha-diversity in animals consuming an HFD. The study by van der Merwe et al,[16] on the other hand, was the only one in which the TRE regimen, despite an HFD, preserves microbiota diversity. However, in relation to the control groups fed the feed-pellet diet, Zarrinpar et al[10] noted a decrease in alpha-diversity, while Ye et al[11] found an increase in this parameter. Interestingly, the study by Machado et al[24] found the result to be dependent on the alpha-diversity indicator used. No changes in the ileal or cecal contents were observed with the Shannon index, while Faith's PD pointed to a decrease in alpha-diversity in the animals fed an HFD under the TRE regimen, as compared with the control animals fed a feed-pellet diet AL. Only 1 of the Ramadan-like fasting studies assessed the alpha-diversity, and found no changes.[28]

Cyclicality and fluctuations in the microbiota were also analyzed, but only in studies in which an HFD or lithogenic diet was administered (TRE[10,11,26] or Ramadan-like fasting,[15] respectively). For each operational taxonomic unit (OTU), the percentage of total reads was calculated for each mouse and then averaged

Table 4 **Effects of TRE regimen at the phylum and genus level, and in alpha- and beta-diversity in animal studies**

Source	Source of microbiota	Comparison	Phyla: differences between groups	Phyla: differences between phases	Genera: differences between groups	Genera: differences between phases	Alpha-diversity	Beta-diversity
Circadian termination								
Zarrinpar et al, 2014[10]	Cecum sample; every 4 h over 24 h (ZT1, 5, 9, 13, 17, 21)	HFD TRE vs HFD AL	↔Firmicutes ↔Bacteroidetes ↔Veruccomicrobia	NR	↗ *Oscillibacter* (0.40 ± 0.08% vs 0.13 ± 0.04%) ↘ *Lactobacillus* (0.97 ± 0.49% vs 3.70 ± 1.01%)	↓ *Lactococcus* (light phase) (2.66 ± 0.84% vs 0.45 ± 0.16%) ↓ *Lactobacillus* (dark phase) (3.62 ± 1.49% vs 0.06 ± 0.04%)	↔	√
Zarrinpar et al, 2014[10]	Cecum sample; every 4 h over 24 h (ZT1, 5, 9, 13, 17, 21)	HFD TRE vs CD AL	↔Firmicutes ↔Bacteroidetes ↔Veruccomicrobia	NR	↘ *Lactobacillus* (0.97 ± 0.49% vs 3.70 ± 1.01%)	NR	↓	√
Ye et al, 2020[11]	Rectal samples; ZT0, ZT8, ZT12, and ZT20	HFD TRE vs HFD AL	↘ Firmicutes (58.04 ± 9.33% vs 34.10 ± 13.49%) ↗ Bacteroidetes (39.28 ± 17.08% vs 27.02 ± 13.06%) ↔ Proteobacteria ↔ Actinobacteria	Light phase ZT0 ↔ Firmicutes, ↔ Bacteroidetes ZT8 ↔ Firmicutes, ↔ Bacteroidetes Dark phase ZT12 ↔ Firmicutes, ↔ Bacteroidetes ZT20 ↓ Firmicutes (35.04 ± 9.38% vs 52.77 ± 7.73%), ↑Bacteroidetes (57.58 ± 10.77% vs 29.27 ± 11.56%)	NR	NR		
Ye et al, 2020[11]	Rectal samples; ZT0, ZT8, ZT12, and ZT20	HFD TRE vs CD AL	↗ Firmicutes (47.89 ± 12.86% vs 34.1 ± 13.49%) ↘ Bacteroidetes (39.28 ± 17.08% vs 61.34 ± 12.99%) ↗ Proteobacteria (9.471 ± 5.918% vs 2.34 ± 1.38%) ↔ Actinobacteria	Light phase ZT0 ↑ Firmicutes, ↓ Bacteroidetes ZT8 ↔ Firmicutes, ↔ Bacteroidetes Dark phase ZT12 ↑ Firmicutes, ↓ Bacteroidetes ZT20 ↔ Bacteroidetes, ↔ Firmicutes	NR	NR	↔	√

(continued)

Table 4 **Continued**

Source	Source of microbiota	Comparison	Phyla: differences between groups		Phyla: differences between phases	Genera: differences between groups		Genera: differences between phases	Alpha-diversity	Beta-diversity
He et al, 2021[14]	Cecal samples after 4 wk of LD	LD RF-like vs LD AL	NR	Light phase ZT0	↑ Firmicutes ↓ Verucomicrobia ↔ Actinobacteria ↔ Proteobacteria ↔ Bacteroidetes	NR	NR		NR	√
				ZT4	↑ Proteobacteria ↔ Firmicutes ↔ Veruccomicrobia ↔ Actinobacteria ↔ Bacteroidetes					
				ZT8	↑ Firmicutes ↓Bacteroidetes ↑ Proteobacteria ↑ Actinobacteria ↔ Veruccomicrobia					
				Dark phase ZT12	↑ Proteobacteria ↓ Actinobacteria ↔ Veruccomicrobia ↔ Firmicutes ↔ Bacteroidetes					
				ZT16	↑ Bacteroidetes ↑ Actinobacteria ↔ Veruccomicrobia ↔ Firmicutes ↔ Proteobacteria					
				ZT20	↔ Veruccomicrobia ↔ Firmicutes ↔ Actinobacteria ↔ Proteobacteria ↔ Bacteroidetes					
Machado et al, 2022[24]	Ileal samples; ZT1, ZT4, ZT9, ZT13, ZT17, ZT21; after HFD	HFD TRE vs HFD AL	↔ Bacteroidetes			↑ *Enterococcus* ↑ *Staphylococcus* ↑ *Lactococcus* ↑ *Colidextribacter* ↑ *Blautia* ↑ *Tuzzerella* ↑ *Angelakisella* ↑ *Helicobacter* ↑ *Parasutterella* ↓ *Ruminococcus* ↓ *Lachnoclostridium* ↓ *Turicibacter* ↓ *Alistipes* ↓ *Akkermansia*	Light phase	↑ *Ruminococcaceae/Lactococcus* ↓ *Turicibacter/Enterococcus* ↑*Enterococcus/Lactococcus*	↔ (Shannon index) ↔ (Faith's PD)	√
							Dark phase	↔ *Ruminococcaceae/Lactococcus* ↓ *Turicibacter/Enterococcus* ↔ *Enterococcus/Lactococcus* ZT13 ↑ *Staphylococcus*		

(continued)

Table 4 **Continued**

Source	Source of microbiota	Comparison	Phyla: differences between groups	Phyla: differences between phases	Genera: differences between groups		Genera: differences between phases	Alpha-diversity	Beta-diversity
Machado et al, 2022[24]	Ileal samples; ZT1, ZT4, ZT9, ZT13, ZT17, ZT21; after HFD	HFD TRE vs CD AL	↘ Bacteroidetes		↑ *Staphylococcus* ↑ *Leuconostoc* ↑ *Colidextribacter* ↑ *Blautia* ↑ *Enterococcus* ↑ *Tuzzerella* ↑ *Lactococcus* ↓ *Turicibacter* ↓ *Lachnoclostridium* ↓ *Ruminococcus* ↓ *Monoglobus* ↓ *Alistipes*	Light phase Dark phase 	↓ *Ruminococcaceae/Lactococcus* ↓ *Turicibacter/Enterococcus* ↔ *Enterococcus/Lactococcus* ↓ *Ruminococcaceae/Lactococcus* ↓ *Turicibacter/Enterococcus* ↓ *Enterococcus/Lactococcus* ZT13 ↔ *Staphylococcus*	↔ (Shannon index) ↓ (Faith's PD)	√
Machado et al, 2022[24]	Cecal samples; ZT1, ZT4, ZT9, ZT13, ZT17, ZT21; after HFD	HFD TRE vs HFD AL	NR	NR	NR	NR		↔ (Shannon index) ↔ (Faith's PD)	√
Machado et al, 2022[24]	Cecal samples; ZT1, ZT4, ZT9, ZT13, ZT17, ZT21; after HFD	HFD TRE vs CD AL	NR	NR	NR	NR		↔ (Shannon index) ↓ (Faith's PD)	√
Termination at 1 ZT point									
Hu et al, 2018[14]	Cecal samples; ZT21; after CD	CD TRE vs CD AL	↗ Firmicutes ↘ Bacteroidetes		↗ *Lactobacillus* ↗ *Roseburia* ↘ *Staphylococcus*			↔	√
Li et al, 2020[27] (16 h of fasting)	Fecal samples; day 30 and day 60; after CD	CD TRE vs CD AL	NR		↗ *Akkermansia* ↘ *Alistipes*			↔	√
Li et al, 2020[27] (12 h of fasting)	Fecal samples; day 30 and day 60; after CD	CD TRE vs CD AL	No taxonomic differences		No taxonomic differences			↔	√
Li et al, 2020[27] (20 h of fasting)	Fecal samples; day 30 and day 60; after CD	CD TRE vs CD AL	No taxonomic differences		No taxonomic differences			↔	√
van der Merwe et al, 2020[16]	Fecal samples; after 6 wk of HFD (T0) and again at 3 wk (T1) & 7 wk (T2)	HFD TRE vs HFD AL	NR		↗ *Ruminococcus* ↗ *Lactococcus* ↗ *Desulfovibrio* ↗ *Enterococcus*			↑	x
	Cecal samples; after HFD	HFD TRE vs HFD AL	↗ Verrucomicrobia (6%) (unknown significance)		↗ *Lactococcus* ↗ *Akkermansia* ↘ *Bilophila*			NR	x

(continued)

Table 4 **Continued**

Source	Source of microbiota	Comparison	Phyla: differences between groups	Phyla: differences between phases	Genera: differences between groups	Genera: differences between phases	Alpha-diversity	Beta-diversity
Palomba,2021[26]	Fecal samples; after 48 wk of CD	CD TRE vs CD AL	NR		↗ *Akkermansia* (number of readings - 4327 vs 15) ↘ *Bilophila* (52 vs 21) ↘ *Lactococcus* (386 vs 46) ↘ *Ruminococcus* (11 948 vs 2979) ↘ *Turicibacter* (576 vs 167) ↔ *Lactobacillus* ↔ *Oscillibacter* ↔ *Roseburia* ↔ *Alistipes* ↔ *Desulfovibrio* ↔ *Colidextribacter* ↔ *Blautia* ↔ *Tuzzerella* ↔ *Angelakisella* ↔ *Helicobacter* ↔ *Parasutterella* ↔ *Lachnoclostridium* ↔ *Monoglobus*		NR	NR
Su et al, 2022[28]	Fecal samples; day 0 and day 30; after CD	CD RF-like vs CD AL	↗ *Firmicutes* (52.79 ± 7.48% vs 67.53 ± 4.84%) ↘ Bacteroidetes (38.79 ± 4.93% vs 24.39 ± 6.12%)		NR		↔	√

Abbreviations: AL, ad libitum; CD, chow (feed-pellet) diet; HFD, high-fat diet; LD, lithogenic diet containing 1.25% cholesterol and 0.5% cholic acid; NR, not reported; PD, phylogenetic diversity; RF-like, Ramadan-like fasting; TRE, time-restricted eating; ZT, zeitgeber time; ↓, significant decrease; ↔, nonsignificant effect; ↑, significant increase; ↗, enrichment in comparison to control group; ↘, depletion in comparison to control group; √, changes in beta-diversity, X, no changes in beta-diversity.

Table 5 **Effects of the TRE and RF regimens at the phylum and genus levels, and on the alpha- and beta-diversity, in human studies**

Source	Phylum	Genus	Alpha-diversity	Beta-diversity
Time-restricted eating				
Gabel et al, 2020[25]	↔ Firmicutes ↔ Bacteroidetes	NR	↔	NR
Zeb et al, 2020[22]	Most abundant in the TRE group: Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria	Most abundant in TRE group: *Faecalibacterium*, *Dialister*, *Prevotella_9* Less abundant in TRE group: *Alloprevotella*, *Prevotella_7*, *Prevotella_2*	↑	NR
Zeb et al, 2020[21]	Most abundant in TRE group: Bacteroidetes	Most abundant in TRE group: *Prevotella_9*, *Prevotella_2*	↑	√
Xie et al, 2022[23] (eTRE group)	No taxonomic differences	No taxonomic differences	↑	NR
Xie et al, 2022[23] (mTRE group)	No taxonomic differences	No taxonomic differences	↔	NR
Ramadan fasting				
Ozkul et al, 2020[17]	↑ Bacteroidetes	↑ *Roseburia* ↑ *Akkermansia* ↑ *Bacteroides* ↑ *Butyricicoccus* ↑ *Faecalibacterium*, ↑ *Allobaculum*, ↑ *Eubacterium*, ↑ *Dialister*, ↑ *Erysipelotrichi*	↑	√
Su et al, 2021[18] (young cohort)	↑ Firmicutes (40.56 ± 13.90% → 56.41 ± 14.49%) ↑ Proteobacteria (5.55 ± 4.56% → 7.85 ± 4.53%) ↓ Bacteroidetes (53.14 ± 15.81% → 34.37 ± 12.51%)	↑ *Faecalibacterium* (5.62 ± 2.96% → 9.47 ± 5.93%) ↓ *Prevotella_9* (21.79 ± 17.69% → 7.63 ± 10.20%)	↑	√
Su et al, 2021[18] (middle-aged cohort)	NR	↗ *Agathobacter* (5.42 ± 4.56% → 12.57 ± 13.75%) ↗ *Blautia* (6.19 ± 6.36% → 9.71 ± 5.66%) ↘ *Megamonas* (1.53 ± 3.45% →0.04 ± 0.86%)	↔	√
Ali et al, 2021[29]	↑ Proteobacteria	↑ *Klebsiella* ↓*Coprococcus* ↓*Clostridium_XIVa*	↔	X

Abbreviations: eTRE, early TRE; mTRE, mid-day TRE; NR, not reported; RF, Ramadan fasting; TRE, time-restricted eating; ↓, significant decrease; ↔, nonsignificant effect; ↑, significant increase; ↗, enrichment in comparison to control group; ↘, depletion in comparison to control group; √, changes in beta-diversity; X, no changes in beta-diversity.

per time point per condition. These data were analyzed to detect cyclical variation.[30] Zarrinpar et al[10] and Machado et al[24] showed that the microbiome of mice fed an HFD under the TRE regimen exhibited greater fluctuations than the microbiome of the mice consuming an HFD given AL, despite using different approaches based on OTUs and amplicon sequence variants (ASVs), respectively. However, Zarrinpar et al[10] noted that the number of OTUs that change cyclically was the same in both groups fed the HFD, whether by TRE or AL, and was lower than in the group fed the feed-pellet diet AL, while Machado et al observed that the number of cyclic ASVs in the HFD TRE group was similar to that in the feed-pellet diet AL group, and higher than in the HFD AL group. He et al[15] noted a significant decrease in the variability of OTUs under the Ramadan-like fasting regimen, unlike Zarrinpar et al[10] who use standard TRE. OTUs are defined as a cluster of sequences that have a sequence identity above a certain threshold, typically above 97%. On the other hand, ASV is an exact sequence variant or amplicon sequence variant, which is created as a result of a methodological change involving the increased use of denoising methods. Therefore, ASV-based approaches have a higher sensitivity in detecting bacterial strains present compared with OTUs, but sometimes at the expense of specificity.[31]

Beta-diversity was analyzed in 8 out of 9 studies using principal coordinate analysis based on Bray–Curtis distances,[16,24,27,28] weighted UniFrac,[14,15,24] or the Jaccard dissimilarity index.[10] One study uses principal component analysis.[11] Seven studies in which animals were fed an HFD/feed-pellet diet[10,11,14,24,27] with TRE or a lithogenic/feed-pellet diet under a Ramadan-like fasting regimen[15,28] showed differences in beta-diversity between the intervention (feed deprivation) and control (AL) groups. Interestingly, after feeding an HFD with TRE, Ye et al[11] observed a difference only in relation to the feed-pellet diet given AL. In turn, van der Merwe et al[16] noted no such changes between groups fed an HFD with TRE or AL.

All of the animal studies showed the relative abundances of the 2 taxonomic levels—phylum and genus, and all of the changes discussed were statistically significant unless otherwise noted. The data are presented in Table 4. The TRE studies in which animals were fed a feed-pellet diet showed a growth trend in the Firmicutes phylum. In fuller detail, 2 studies showed a greater abundance of the Firmicutes phylum in the TRE group fed a feed-pellet diet or HFD than in the case of the feed-pellet diet given AL.[11,14] Interestingly, an increase in the Firmicutes phylum was noted in the Ramadan-like fasting studies of He et al[15] and Su et al,[28] in which the feeding window with the lithogenic or feed-pellet diet was during the light (resting) phase. The results for the Bacteroidetes phylum were the opposite in the TRE and RF-like studies.[11,14,15,24,28] In 1 study in which animals were fed an HFD under a TRE regimen, the abundance of Firmicutes was notably lower—by approximately 23.9% in total and by approximately 17.7% at the ZT20 point (dark phase)—than in the animals fed the HFD AL.[11]

Interestingly, in the microbiota of the mice fed an HFD according to the TRE regimen, Ye et al[11] and Zarrinpar et al[10] observed an increase in the fluctuations of Firmicutes and Bacteroidetes, compared with both control groups (HFD and feed-pellet diet given AL, respectively).[10,11] On the other hand, He et al[15] noted a decrease in microbiota fluctuations, especially with regard to Bacteroidetes and Firmicutes, after a lithogenic diet during the resting phase (Ramadan-like fasting).

At the genus level, 1 study found a greater abundance of *Lactobacillus* (by 854 reads) in animals consuming a feed-pellet diet under the TRE regimen than in animals fed this diet al.[14] Zarrinpar et al[10] provided time-restricted access to the HFD diet and observed a decrease in the relative abundance of *Lactobacillus* (by 2.7%), in comparison to the control groups fed both a feed-pellet diet and an HFD given AL. Interestingly, Zarrinpar et al also found that TRE has a beneficial effect on maintaining the cyclic variability of this genus, as no changes were observed in the group consuming the feed-pellet diet, while an increase in cyclicity was observed in the group consuming the HFD. In turn, Machado et al[24] observed that the cyclicity of *Lactobacillus* was preserved only in the groups fed the HFD diet (whether AL or TRE), but that in the TRE group there was a sharp decrease in fluctuations in *Lactobacillus* in the dark phase compared with the HFD AL group.

The results regarding the effects of TRE on *Lactococcus* were inconclusive. In the microbiota of mice fed an HFD in the TRE regime, Zarrinpar et al[10] noted a decrease (by 2.2%) in the relative abundance of *Lactococcus* in comparison to the group fed an HFD AL. This contrasts with the findings of van der Merwe et al[16] and Machado et al[24] who observed an overall greater relative abundance of this genus in mice also fed an HFD under TRE. Interestingly, in 1 study by Zarrinpar et al,[10] TRE with an HFD halted oscillations in the relative abundance of *Lactococcus*, while the study by Machado et al[24] noted both *Lactococcus* and *Staphylococcus* oscillations only in the TRE HFD group. Furthermore, *Oscillibacter* oscillations were disturbed by an HFD, regardless of the dietary regimen,[10] while *Streptococcus* oscillations in HFD TRE were the same as in the feed-pellet diet AL group and greater than for the HFD AL.[24] Moreover, van der Merwe et al[16] also showed a greater abundance of *Ruminococcus* in mice fed an HFD in TRE than in the control HFD AL group. In turn, in a study by Palomba,[26]

mice fed a feed-pellet diet under TRE were observed to have significantly lower abundances of the *Ruminococcus* genus than a group fed a feed-pellet diet given AL. Interestingly, although it can be assumed that the discrepancy in *Ruminococcus* might be due to differences in the type of diet used (HFD and feed-pellet diet), Machado et al[24] also noted a decrease in the abundance of *Ruminococcus* in the TRE HFD group compared with both AL-fed groups (feed-pellet diet and HFD).

Effects of TRE on the composition, alpha-diversity, and beta-diversity of microbiota in human studies

Two human studies observed significantly higher alpha-diversities[21,22] in the TRE group, while, in 1 study, the alpha-diversity remained unchanged after introducing the dietary regimen.[25] In the study by Xie et al,[23] significantly higher alpha-diversity was observed only in the early TRE group and not in the nonfasting control; the mid-day TRE group showed no changes. To assess alpha-diversity, these studies used the Shannon index,[21,25] the Richness index,[21,22,25] and the Chao1 indicator.[23]

Beta-diversity was examined using principal component analysis and principal coordinate analysis with Bray–Curtis distance measurement in 1 study by Zeb et al,[21] while the other study by the same authors used the UniFrac distance.[22] Only the first of these studies[21] showed differences between the TRE and control groups.

All of the human TRE studies presented the relative abundance of microbiota (Table 5) and all changes discussed here were statistically significant unless otherwise noted. In TRE studies at the phylum level, Bacteroidetes was the more abundant phylum, unlike in the control group.[21,22] However, in the study conducted by Gabel et al,[25] no changes were observed in this phylum after dietary intervention.

Analysis at the genus level was performed in both studies conducted by Zeb et al; in one of these, *Faecalibacterium* and *Dialister* were more abundant in the TRE group than in the nonfasting control group. Changes in the *Prevotella* genus were also observed. *Prevotella_9* was most abundant in both studies by Zeb et al,[21,22] while the results for *Prevotella_2* were inconclusive: 1 study showed the most abundance,[21] and another study showed less abundance, in the TRE group, compared with the nonfasting control group.[22] Moreover, Xie et al[23] did not observe any changes at either taxonomic level.

Effects of RF on the composition, alpha-diversity, and beta-diversity of microbiota in human studies

Two human studies[17,18] observed an increase in the alpha-diversity over the baseline parameters, while another study noted no changes with respect to either the baseline parameters[29] or to the nonfasting control group (a middle-aged cohort).[18] To assess the alpha-diversity, these studies used the Shannon index,[17,18,29] Simpson's index,[18,29] OTUs Richness,[17] the Chao1 index, and the ACE index.[29]

Beta-diversity was analyzed in all of the studies using principal coordinate analysis, based either on Bray–Curtis distances[18,29] or on the unweighted and weighted UniFrac algorithm[17]; nonetheless, significant differences were only seen in 2 studies.[17,18]

All of the human RF studies presented relative abundances on the 2 taxonomic levels—phylum and genus—and all changes discussed here were statistically significant unless otherwise noted. The data are presented in Table 5. In 1 of the 3 studies, an increase of 18.8% in Firmicutes at the phylum level was observed in the younger cohort over the pre-RF value.[18] Furthermore, both of the studies by Ali et al[29] and Su et al,[18] which were conducted among young cohorts, observed increases in Proteobacteria, by 4.7% and 2.3%, respectively. One study by Ozkul et al[17] reported an increase in Bacteroidetes, while another by Su et al[18] described a decrease in this phylum by 18.8% compared with baseline values. Moreover, the study by Ozkul et al[17] was the only one to assesses the Firmicutes: Bacteroidetes (F/B) ratio; this proved to be elevated both before and after the application of the Ramadan dietary habits, so no changes were observed.

One the genus level, an increase in *Faecalibacterium* (by 3.9%) was observed in 2 of 3 studies.[17,18] There was also an increase in the relative abundance of *Roseburia*, *Akkermansia*, *Bacteroides*, *Butyricicoccus*, *Allobaculum*, *Eubacterium*, *Dialister*, *Erysipelotrichi*,[17] and *Agathobacter* by 7.2%; of *Blautia* by 3.5%[18]; and of *Klebsiella*[29] compared with baseline. In turn, a decrease in *Prevotella_9* by 14.2% and in *Megamonas* by 1.5% was also observed by Su et al.[18]

Associations between composition of the microbiota and host metabolic markers caused by TRE or RF in human studies

Only 2 studies[18,21] showed any correlation between host metabolic markers or body weight and gut microbiota composition. In one of the studies conducted by Zeb et al,[21] a positive relationship was shown between HDL concentration and the richness of the intestinal microbiome after TRE ($r = 0.42$, $P = .0289$). Su et al[18] further observed a positive correlation between body mass index values and the abundance of OTUs belonging to the phylum Proteobacteria alongside a negative correlation between body mass index and abundance of the class Negativicutes and the order Selenomonadales after RF ($P < .05$).

DISCUSSION

It is accepted that the feeding–fasting cycle affects host metabolism[10]; however, little is known regarding the essential characteristics of the changes that occur in the gut microbiota, and even less about the correlation between microbiota changes and host metabolic parameters. This study is the first systematic review to summarize the effects of TRE and RF on specific taxonomic groups of gut microbiota and to examine the correlations between the composition of microbiota and host metabolic parameters in both humans and animals.

This systematic review reveals that TRE may restore the cyclical fluctuation of major phyla within the gut microbiome of mice fed an HFD.[10,11] However, TRE in the presence of an HFD does not lead to the microbial dynamic becoming as dynamic as is observed in mice fed a feed-pellet diet,[10,11] indicating that diet is an important factor in forming the gut microbial environment. Ye et al[11] indicated that the circadian microbial rhythm of mice fed an HFD given under the TRE regimen is opposite to mice fed an HFD AL. At the same time, the major microbial phyla in the mice fed the feed-pellet diet AL oscillate with a diurnal pattern: During the night, when rodents are active, the Firmicutes count is at its highest, while Bacteroidetes are lower in number; during the day, when the rodents are resting, the latter have higher numbers.[11] These rhythmic changes in the abundance of major phyla may occur on account of the fact that Firmicutes are more effective than Bacteroidetes at obtaining energy from food (hence, they increase when food is consumed). This would explain why both the abundance of bacteria of the Firmicutes phylum and greater F/B ratio are associated with obesity.[32] It should be noted that the introduction of an eating window during the resting phase (Ramadan-like fasting) in mice also results in an inversion of the circadian rhythm of these major phyla.[15]

Cyclical fluctuations in specific members of the gut microbiota contribute to microbial diversity, and likely represent a mechanism by which the microbes affect the host's metabolism.[10] Indeed, alpha-diversity is the most common means of assessing not only intestinal microbiota health but also human nutritional status,[33] with lower levels of diversity being associated with obesity and metabolic syndrome.[34] Moreover, it is well known that dietary fiber intake has direct effects on the amount of microbial diversity in the gut.[35] There is no difference in animal studies in alpha-diversity between animals fed AL or those on TRE, regardless of the diet administered. This may be due to the composition of both the intervention and control diets, as Wang et al[8] noted that the increase in alpha-diversity in HFD-fed mice was due to a greater amount of fiber in this diet than in the control diet. Interestingly, in the human studies, the use of both TRE and RF was related to an increase in gut microbial community diversity. Meals eaten during Ramadan contain more foods rich in carbohydrate and fiber,[36] such as soups, porridges, legumes, and whole grains,[37] which could partially explain the difference in gut microbial diversity before and after RF. Unfortunately, dietary fiber consumption was not assessed in any of the RF studies examined here, while in TRE studies, Zeb et al[21,22] did not show any difference in fiber intake. The lack of change is probably due to the fact that TRE does not impose any restrictions on diet quality[11]; however, animal studies where the quality of the diet is under control allow us to examine the relationship between TRE and the quality of the diet.

In turn, changes in beta-diversity were observed mainly in animal studies using TRE, as well as in the 2 of 3 human studies that tested RF.[17,18] While alpha-diversity as a measure of microbiome diversity is applicable to a single sample, beta-diversity is a measure of the similarity or dissimilarity of 2 communities.[38] It can thus be pointed out that fluctuations in the gut microbiome are important for host metabolism, and not necessarily for species richness, which is determined mainly by diet.[10]

This systematic review shows that TRE alters the average abundance of the main microbial phyla—Firmicutes and Bacteroidetes. In animal studies, the direction of such changes may be associated with the kind of diet that is given under the TRE regimen (HFD[10,11,24] or feed-pellet diet[14,28]). In 2 of the 4 human TRE studies, Bacteroidetes were more abundant than in the nonfasting control groups,[21,22] while the human RF studies were inconsistent.[17,18] Bacteria of the Bacteroidetes phylum are responsible for the production of acetic acid, which can be successfully converted to butyric acid—although only if the microbiome has the appropriate F/B ratio.[39] Furthermore, acetic acid accumulates in the hypothalamus and, through a series of reactions, suppresses appetite, which may be useful in the treatment of obesity.[39] Moreover, obesity is associated with an elevated F/B ratio.[40] There is also a correlation between this indicator and eating behaviors[41]; it can thus be suggested that, in the case of people with obesity who often snack under the influence of emotions,[42] regardless of the time of day or feelings of hunger,[43] the optimal F/B ratio may be disturbed by the virtually uninterrupted availability of foods.[44] The human study by Ozkul et al[17] was the only one to evaluate the F/B ratio and note that it remained high after RF, so no significant changes were observed (data not shown).

In 2 studies in which animals were fed the HFD or lithogenic diet under the TRE[11] and RF-like regimens,[11,15] an enrichment in the Proteobacteria phylum was seen. Similar changes were observed in the human RF studies.[15,25] The effect of Proteobacteria on the body's functioning seems to be controversial: on one hand, it has been suggested that Proteobacteria contribute to homeostasis of the anaerobic environment in the gut tract, and thus to the stability of the strictly anaerobic microbiota[45]; on the other hand, an increase in abundance of Proteobacteria may be associated with metabolic syndrome.[46]

At the genus level, *Akkermansia* abundance seems to be dependent on the type of diet administered in TRE (enrichment after a feed-pellet diet[16,26,27] and decrease after an HFD[24]). On the other hand, only the RF study by Ozkul et al[17] among the human studies showed an increase in the *Akkermansia* genus. An increase in the abundance of these bacteria seems to be extremely favorable, as it has been noted that *Akkermansia muciniphila* causes an increase in the expression of genes associated with immune responses and in the strengthening of the gut barrier function.[44] It is also indicated that *A. muciniphila* affects glucose and lipid metabolism through the production of mucin, which improves the strength of the intestinal barrier and stimulates the immune system to secrete anti-inflammatory cytokines.[47] Moreover, although *A. muciniphila* is a G(-) bacterium, it is not associated with endotoxemia and, more importantly, it reduces the concentration of endotoxins resulting from consuming an HFD.[46] *Akkermansia muciniphila* is also inversely correlated with the occurrence of insulin resistance and obesity.[47]

The increase in the abundance of Faecalibacterium, which was observed only in human RF studies, and their significantly greater abundance than in the control group, is also interesting.[17,18,22] By producing butyric acid and other short-chain fatty acids, this genus is strongly associated with intestinal health and also has a strong anti-inflammatory effect. Less abundant Faecalibacterium is observed in individuals with irritable bowel syndrome[48] and in those with depression[49] or Parkinson's disease.[50] It can therefore be suggested that this change caused by RF seems to be beneficial.

The results for the *Lactobacillus* genus are inconclusive. In the animal studies alone, consumption of an HFD under TRE was associated with a decrease in the abundance of this genus,[10] while the intake of a control diet led to either a greater abundance of this genus[14] or no change.[26] In general, *Lactobacillus* is associated with good intestinal health,[51] because it strengthens the intestinal barrier function by increasing mucus production or stimulating release of antimicrobial peptides and providing a competitive resistance against pathogens.[52] However, further studies are needed to determine whether these changes are directly related to the dietary regimen or just to the type of diet.

There are some reports that suggest that beneficial changes in host metabolic parameters may be the direct result of changes in the microbiota induced by TRE. For instance, Wang et al[53] showed that the TRE-dependent increase in *Prevotellaceae* abundance in the microbiome of swine was negatively correlated with blood levels of 2-amino-butyrate, suggesting a reduced risk of cardiovascular disease. Zeb et al[21] suggested that TRE reduces the risk of developing metabolic disease precisely by regulating the level of serum HDL caused by the microbiome in humans, while Su et al[18] indicated that RF can be associated with beneficial changes in body mass index. Unfortunately, the small number of studies associating microbiota changes with improvements in metabolic or anthropometric parameters induced by TRE makes it impossible to unequivocally state whether the observed microbial and metabolic changes are actually related.

The articles included in this systematic review have some limitations. In the human studies that examined fecal microbiota composition, it can be difficult to determine the exact time and method of collection (eg, sample storage and process sterility); in many cases, this leads to an inability to compare results between studies.[54] These differences undoubtedly have a large effect on the measured microbiome composition. The same consideration applies to the examination of animal feces, as it is also not possible to collect them immediately after expulsion. Furthermore, assessing human or animal gut microbiota composition at 1 point in time (whether fecal or intestinal) makes it impossible to observe cyclical circadian fluctuations in the microbiota. The results of the included studies lead us to conclude that the microbiota can be most accurately assessed from the intestinal contents collected at circadian termination.

Some studies show that different primer pairs may affect the microbial profile. Primers spanning more than 1 V region generally enhance precision in identifying bacteria, as compared with a single region. The studies reviewed in this article mostly used the V3-V4 or V1-V3 regions. It has been shown that the V3-V4 region slightly outperforms the other region combinations, and thus might be recommended for the analysis of human gut samples.[55] Another limitation of this review is that the studies it considers are based not only on different study populations (humans and animals) but also on different intervention protocols with, for example, eating windows being during the day or

during night; this may cause some ambiguity and make interpretation difficult.

Moreover, although only 2 human studies were assessed negatively as "fair"[21,22] and the rest of the studies were assessed as "good," there are some aspects that, although not covered by the tools used, could improve the quality of the research. First, no animal study evaluated fiber intake. Although 5[17,18,21,22,29] of the 7 human studies assessed the composition of the diet, 3 of them also did not assess fiber consumption.[17,18,29] The precise estimation of fiber consumption in both human and animal studies is indeed a valuable result, but the opportunity to detect a correlation between the consumption of individual nutrients and the composition of microbiota should not be overlooked, as it would increase the quality of these studies.

This study also has a number of strengths. To the best of knowledge, ours is the first study to discuss the changes in microbiota composition caused by TRE and RF in both animal and human studies. This systematic review includes both preclinical studies in animals and preliminary studies in humans, in order to discuss the effects and potential differences resulting not only from genetic variation but also from the material collected for the microbiome study.

CONCLUSION

These findings support the importance of TRE and RF in improving gut microbiota composition. However, based on the results of animal studies, it can be suggested that diet remains the essential factor in forming its environment. Since only a small number of studies link changes in the microbiota with improvements in metabolic or anthropometric parameters induced by the regimens studied, it is impossible to unequivocally state whether all the observed microbial and metabolic changes are actually related. Further research should thus include metagenomics and microbial and host metabolomics in their methodology to better understand the potential correlations between microbes and host health. It should be pointed out that data in this field remain limited, especially among human studies, and so it is difficult to draw meaningful conclusions about the effects of the TRE and RF on specific taxonomic groups of gut microbiota. Moreover, more precise inspection of the human diet and of the time of specimen collection is necessary to better interpret studies of the gut microbiome, and to better understand the host–microbiome relationship.

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Supporting Information

The following Supporting Information is available through the online version of this article at the publisher's website.

Table S1 **Evaluation of the quality of the animal studies**

Table S2 **Evaluation of the quality of the human studies**

Table S3 **Effects of time-restricted eating (TRE) regimen at the phylum and genus level, and in alpha and beta diversity, in animal studies**

Table S4 **PRISMA checklist**

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Załącznik 2

Dr hab. Paweł Kołodziejski

Katedra Fizjologii, Biochemii i Biostruktury Zwierząt

Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Pieczyńska-Zając, J. M., Malinowska, A. M., Pruszyńska-Oszmałek, E., Kołodziejski, P. A., Drzymała-Czyż, S., & Bajerska, J. (2024). Effect of a high-fat high-fructose diet on the composition of the intestinal microbiota and its association with metabolic and anthropometric parameters in a letrozole-induced mouse model of polycystic ovary syndrome. *Nutrition* 4 (124), 112450 mój indywidualny udział w jej powstaniu polegał na opracowaniu metodologii, przygotowaniu i analizie danych oraz recenzji i edytowaniu manuskryptu.

Data 10.06.2024.....

Podpis

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Załącznik 2

Dr hab. n. med. Sławomira Drzymała-Czyż

Katedra i Zakład Bromatologii

Uniwersytet Medyczny im. Karola Marcinkowskiego w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Pieczyńska-Zając, J. M., Malinowska, A. M., Pruszyńska-Oszmałek, E., Kołodziejski, P. A., Drzymała-Czyż, S., & Bajerska, J. (2024). Effect of a high-fat high-fructose diet on the composition of the intestinal microbiota and its association with metabolic and anthropometric parameters in a letrozole-induced mouse model of polycystic ovary syndrome. *Nutrition* 4 (124), 112450 mój indywidualny udział w jej powstaniu polegał na opracowaniu metodologii, przygotowaniu danych oraz recenzji i edytowaniu manuskryptu.

Data 5/06/2024.

Podpis

Sławomira Drzymała-Czyż

Załącznik 2

Prof. UPP Joanna Bajerska

Katedra Żywienia Człowieka i Dietetyki

Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Pieczyńska-Zając, J. M., Malinowska, A. M., Pruszyńska-Oszmałek, E., Kołodziejski, P. A., Drzymała-Czyż, S., & Bajerska, J. (2024). Effect of a high-fat high-fructose diet on the composition of the intestinal microbiota and its association with metabolic and anthropometric parameters in a letrozole-induced mouse model of polycystic ovary syndrome. *Nutrition* 4 (124), 112450 mój indywidualny udział w jej powstaniu polegał na konceptualizacji, analizie danych oraz recenzji i edytowaniu manuskryptu.

Data 6.06.2024 Podpis

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Załącznik 2

mgr Joanna Pieczyńska-Zając

Katedra Żywienia Człowieka i Dietetyki

Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Pieczyńska, J. M., Pruszyńska-Oszmałek, E., Kołodziejski, P. A., Łukomska, A., & Bajerska, J. (2022). The Role of a High-Fat, High-Fructose Diet on Letrozole-Induced Polycystic Ovarian Syndrome in Prepubertal Mice. Nutrients, 14(12), 2478, mój indywidualny udział w jej powstaniu polegał na konceptualizacji, opracowaniu metodologii, przygotowaniu i analizie danych oraz przygotowaniu manuskryptu.

Data 10.06.2024r.

Podpis

Joanna Pieczyńska-Zając

Załącznik 2

Dr hab. Ewa Pruszyńska-Oszmałek

Katedra Fizjologii, Biochemii i Biostruktury Zwierząt

Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Pieczyńska, J. M., Pruszyńska-Oszmałek, E., Kołodziejski, P. A., Łukomska, A., & Bajerska, J. (2022). The Role of a High-Fat, High-Fructose Diet on Letrozole-Induced Polycystic Ovarian Syndrome in Prepubertal Mice. Nutrients, 14(12), 2478, mój indywidualny udział w jej powstaniu polegał na opracowaniu metodologii, przygotowaniu i analizie danych oraz recenzji i edytowaniu finalnej wersji manuskryptu.

Data 10.06.2024.....

Podpis

.....

Załącznik 2

Dr hab. Paweł Kołodziejski

Katedra Fizjologii, Biochemii i Biostruktury Zwierząt

Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Pieczyńska, J. M., Pruszyńska-Oszmałek, E., Kołodziejski, P. A., Łukomska, A., & Bajerska, J. (2022). The Role of a High-Fat, High-Fructose Diet on Letrozole-Induced Polycystic Ovarian Syndrome in Prepubertal Mice. Nutrients, 14(12), 2478, mój indywidualny udział w jej powstaniu polegał na opracowaniu metodologii, przygotowaniu i analizie danych oraz recenzji i edytowaniu finalnej wersji manuskryptu.

Data 10.06.2024

Podpis

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Załącznik 2

Lek. wet. Anna Łukomska

Katedra Nauk Przedklinicznych i Chorób Zakaźnych

Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Pieczyńska, J. M., Pruszyńska-Oszmałek, E., Kołodziejski, P. A., Łukomska, A., & Bajerska, J. (2022). The Role of a High-Fat, High-Fructose Diet on Letrozole-Induced Polycystic Ovarian Syndrome in Prepubertal Mice. Nutrients, 14(12), 2478, mój indywidualny udział w jej powstaniu polegał na analizie danych oraz recenzji i edytowaniu finalnej wersji manuskryptu.

Data 10.06.2022.

Podpis

Anna Łukomska

Załącznik 2

Prof. UPP Joanna Bajerska

Katedra Żywienia Człowieka i Dietetyki

Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Pieczyńska, J. M., Pruszyńska-Oszmałek, E., Kołodziejski, P. A., Łukomska, A., & Bajerska, J. (2022). The Role of a High-Fat, High-Fructose Diet on Letrozole-Induced Polycystic Ovarian Syndrome in Prepubertal Mice. Nutrients, 14(12), 2478, mój indywidualny udział w jej powstaniu polegał na konceptualizacji, analizie danych oraz recenzji i edytowaniu finalnej wersji manuskryptu.

Data 6.06.2022 Podpis

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Załącznik 2

mgr Joanna Pieczyńska-Zając

Katedra Żywienia Człowieka i Dietetyki

Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Pieczyńska-Zając, J. M., Malinowska, A., Łagowska, K., Leciejewska, N., & Bajerska, J. (2023). The effects of time-restricted eating and Ramadan fasting on gut microbiota composition: A systematic review of human and animal studies. *Nutrition Reviews* 10, 82(6), 777-793 mój indywidualny udział w jej powstaniu polegał na konceptualizacji i opracowaniu protokołu systematycznego przeglądu literaturowego, przeprowadzeniu wyszukiwania literatury, selekcji, ekstrakcji, analizie i interpretacji danych oraz przygotowaniu manuskryptu.

Data 10.06.2024 Podpis

Joanna Pieczyńska-Zając

Dr Anna Malinowska

Laboratorium Mikrobiologii

Wageningen University & Research

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Pieczyńska-Zając, J. M., Malinowska, A. M., Łagowska, K., Leciejewska, N., & Bajerska, J. (2023). The effects of time-restricted eating and Ramadan fasting on gut microbiota composition: A systematic review of human and animal studies. *Nutrition Reviews* 10, 82(6), 777-793 mój indywidualny udział w jej powstaniu polegał na opracowaniu protokołu systematycznego przeglądu literaturowego oraz analizie i interpretacji danych.

Data 24.05.2024

Podpis

A. Malinowska

Załącznik 2

Dr hab. Karolina Łagowska

Katedra Żywienia Człowieka i Dietetyki

Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Pieczyńska-Zając, J. M., Malinowska, A., Łagowska, K., Leciejewska, N., & Bajerska, J. (2023). The effects of time-restricted eating and Ramadan fasting on gut microbiota composition: A systematic review of human and animal studies. *Nutrition Reviews* 10, 82(6), 777-793 mój indywidualny udział w jej powstaniu polegał na opracowaniu protokołu systematycznego przeglądu literaturowego oraz analizie i interpretacji danych.

Data 10.06.2024

Podpis

Karolina Łagowska

Załącznik 2

Dr Natalia Leciejewska

Katedra Fizjologii, Biochemii i Biostruktury Zwierząt

Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Pieczyńska-Zając, J. M., Malinowska, A., Łagowska, K., Leciejewska, N., & Bajerska, J. (2023). The effects of time-restricted eating and Ramadan fasting on gut microbiota composition: A systematic review of human and animal studies. *Nutrition Reviews* 10, 82(6), 777-793 mój indywidualny udział w jej powstaniu polegał na asystowaniu w procesie projektowania przeglądu oraz recenzji powstałego mansukryptu.

Data 06.06.2024

Podpis

Leciejewska Natalia

Załącznik 2

Prof. UPP Joanna Bajerska

Katedra Żywienia Człowieka i Dietetyki

Uniwersytet Przyrodniczy w Poznaniu

Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy Pieczyńska-Zając, J. M., Malinowska, A., Łagowska, K., Leciejewska, N., & Bajerska, J. (2023). The effects of time-restricted eating and Ramadan fasting on gut microbiota composition: A systematic review of human and animal studies. *Nutrition Reviews* 10, 82(6), 777-793 mój indywidualny udział w jej powstaniu polegał na konceptualizacji i opracowaniu protokołu systematycznego przeglądu literaturowego, przeprowadzeniu wyszukiwania literatury, selekcji, ekstrakcji, analizie i interpretacji danych oraz przygotowaniu manuskryptu.

Data 6.06.2024

Podpis

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Załącznik 3

UCHWAŁA NR 51/2021

z dnia 03.09.2021 r.

Lokalnej komisji etycznej do spraw doświadczeń na zwierzętach w Poznaniu

§ 1

Na podstawie art. 48 pkt. 1 ustawy z dnia 15 stycznia 2015r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (Dz. U. poz. 266) po rozpatrzeniu wniosku pt.: ***„Określenie mechanizmu łączącego mikrobiom jelitowy i zaburzenia metaboliczne oraz hormonalne w Zespole Policystycznych Jajników – badania na modelu mysim"*** z dnia **23.08.2021 r.**, złożonego przez **Uniwersytet Przyrodniczy w Poznaniu, Wydział Medycyny Weterynaryjnej i Nauk o Zwierzętach,** adres **ul. Wojska Polskiego 28, 60-637 Poznań** zaplanowanego przez **prof. UPP. dr hab. Joannę Bajerską**
Lokalna Komisja Etyczna:

WYRAŻA ZGODĘ

na przeprowadzenie doświadczeń na zwierzętach w zakresie wniosku.

§ 2

W wyniku rozpatrzenia wniosku, o którym mowa w § 1, Lokalna Komisja Etyczna ustaliła, że:

1. Wniosek należy przypisać do kategorii: **[PB11] Badanie podstawowe kategoria obejmująca wiele układów**
2. Najwyższy stopień dotkliwości proponowanych procedur to**: umiarkowany**
3. Doświadczenia będą przeprowadzane na gatunkach lub grupach gatunków**: mysz domowa *(Mus musculus)* szczep C57BL/6, 3-tygodniowe samice, 32 osobniki**
4. Doświadczenia będą przeprowadzane przez: **prof. UPP dr hab. Joannę Bajerską, dr Ewę Pruszyńską – Oszmałek, dra Pawła Kołodziejskiego**
5. Doświadczenie będzie przeprowadzane w terminie **od 01.10.2021 r. do 30.06.2023**
6. Doświadczenie będzie przeprowadzone w ośrodku: **nie dotyczy**
7. Doświadczenie będzie przeprowadzone poza ośrodkiem w: **nie dotyczy**
8. Użyte do procedur zwierzęta dzikie zostaną odłowione przez ……., w sposób: **nie dotyczy**
9. Doświadczenie zostanie/nie zostanie poddane ocenie retrospektywnej w terminie do … miesięcy od dnia przekazania przez użytkownika dokumentacji, mającej stanowić podstawę dokonania oceny retrospektywnej. Użytkownik jest zobowiązany do przekazania ww. dokumentacji niezwłocznie, tj. w terminie, o którym mowa w art. 52 ust. 2 ustawy **- nie dotyczy**

§ 3

Uzasadnienie:

Celem naukowym projektu jest porównanie składu mikrobioty jelitowej i wybranych parametrów metabolicznych oraz hormonalnych, w grupie myszy zdrowych i z indukowanym Zespołem Policystycznych Jajników (PCOS), karmionych dietą standardową lub dietą wysokotłuszczową/wysokofruktozową (HF/HF). PCOS jest jedną z najczęstszych endokrynopatii w wieku rozrodczym, przyczyniającą się do niepłodności u kobiet. W grupie kobiet z PCOS obserwuje się również występowanie otyłości, insulinooporności, oraz zaburzeń lipidowych. Ponieważ mikrobiota jelitowa i jej metabolity posiadają zdolność do regulowania aktywacji ścieżki szlaku zapalnego, w ostatnim czasie pojawiły się doniesienia wskazujące, że niekorzystna zmiana konsorcjum bakteryjnego może być kluczowym czynnikiem rozwoju PCOS. Wniosek o udzielenie zgody na przeprowadzenie doświadczenia spełnia kryteria, o których mowa art. 47 ust. 1 pkt 2–5 oraz 45 ust. 1 ustawy z dnia 15 stycznia 2015 r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych.

§ 4

Integralną część niniejszej uchwały stanowi kopia wniosku, o którym mowa w § 1

LOKALNA KOMISJA ETYCZNA
do Spraw Doświadczeń na Zwierzętach
Uniwersytet Przyrodniczy w Poznaniu
60-637 Poznań, ul. Wołyńska 35
tel. 61 8487150, fax 61 8466085

(Pieczęć lokalnej komisji etycznej)

PRZEWODNICZĄCA
Lokalnej Komisji Etycznej
do Spraw Doświadczeń na Zwierzętach
dr Paweł Kołodziejski

Podpis Przewodniczącego komisji

Pouczenie:

Zgodnie z art. 33 ust. 3 i art. 40 ustawy w zw. z art. 127 § 1 i 2 oraz 129 § 2 ustawy z dnia z dnia 14 czerwca 1960 r. Kodeks postępowania administracyjnego (Dz. U. 2017, poz. 1257 – t.j.; dalej KPA) od uchwały Lokalnej Komisji Etycznej strona może wnieść, za jej pośrednictwem, odwołanie do Krajowej Komisji Etycznej do Spraw Doświadczeń na Zwierzętach w terminie 14 od dnia doręczenia uchwały.

Na podstawie art. 127a KPA w trakcie biegu terminu do wniesienia odwołania strona może zrzec się prawa do jego wniesienia, co należy uczynić wobec Lokalnej Komisji Etycznej, która wydała uchwałę. Z dniem doręczenia Lokalnej Komisji Etycznej oświadczenia o zrzeczeniu się prawa do wniesienia odwołania przez ostatnią ze stron postępowania, decyzja staje się ostateczna i prawomocna.

Otrzymuje:

1) Użytkownik,
2) Organizacja społeczna dopuszczona do udziału w postępowaniu (jeśli dotyczy)
3) a/a

Użytkownik kopie przekazuje:

- Osoba planująca doświadczenie
- Zespół ds. dobrostanu

UCHWAŁA NR **23/2022**

z dnia 25.02.2022 r.

Lokalnej Komisji Etycznej do spraw doświadczeń na zwierzętach w Poznaniu

§ 1

Lokalna komisja etyczna po rozpatrzeniu wniosku pt.: ***„Określenie mechanizmu łączącego mikrobiom jelitowy i zaburzenia metaboliczne oraz hormonalne w Zespole Policystycznych Jajników – badania na modelu mysim","*** z dnia **14.02.2022 r.**, złożonego przez **Uniwersytet Przyrodniczy w Poznaniu, Wydział Medycyny Weterynaryjnej i Nauk o Zwierzętach adres ul. Wojska Polskiego 28, 60-637 Poznań,** zaplanowanego przez **prof. dr hab. Joannę Bajerską** a dotyczącego**:**

dodatkowych osób przeprowadzających doświadczenia

w ramach wydanej przez komisję zgody uchwałą **nr 51/2021 w dn. 03.09.2021 r.**

WYRAŻA ZGODĘ

na dokonanie zmian w zakresie określonym poniżej.

§ 2

1. Najwyższy stopień dotkliwości proponowanych procedur po zatwierdzonych zmianach to: **nie dotyczy**
2. Zespół prowadzący doświadczenia rozszerza się o następujące osoby (nazwisko i imię, nazwa użytkownika): **mgr Pieczyńską Joannę**
3. Doświadczenie będzie przeprowadzane w terminie od 01.10 2021 r. do 30.06. 2023 r.

§ 3

Uzasadnienie:

Mgr Joanna Pieczyńska odbyła wymagane *Ustawą z dnia 15 stycznia 2015 r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych i Rozporządzeniem MNiSW DU 8.05.2015 poz. 628* szkolenia na podstawie, których uzyskała odpowiednie wyznaczenie.

§ 4

Integralną część niniejszej uchwały stanowi kopia wniosku, o którym mowa w § 1

LOKALNA KOMISJA ETYCZNA
do Spraw Doświadczeń na Zwierzętach
Uniwersytet Przyrodniczy w Poznaniu
60-637 Poznań, ul. Wołyńska 35
tel. 61 8487198, tel. 61 8466085

(Pieczęć lokalnej komisji etycznej)

Podpis wiceprzewodniczącej komisji

Otrzymuje Użytkownik

Pouczenie:

Od decyzji komisji można wnieść odwołanie do Krajowej Komisji Etycznej w terminie 14 od dnia otrzymania uchwały.

Użytkownik kopie przekazuje:

- Osoba planująca doświadczenie
- Zespół ds. dobrostanu