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ALTERAÇÕES NA MICROBIOTA INTESTINAL EM MODELO EXPERIMENTAL DE MENOPAUSA E SUA RELAÇÃO COM A DIETA RICA EM FRUTOSE

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Dissertação apresentada à Coordenação do Curso de Pós-graduação em Ciências da Saúde da Universidade Federal de Alagoas para obtenção do grau de mestre em Ciências da Saúde.

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“Entenda os seus medos, mas jamais deixe que eles sufoquem os seus sonhos.”

(Alice no País das Maravilhas)

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LISTA DE ABREVIATURAS

Ágar infusão cérebro-coração	AICC
Bacilos Gram positivos	BGP
Bacilos Gram negativos	BGN
Body weight	BW
Brain-heart infusion agar	BHIA
Catalase test	Cat
Coagulase-negative *Staphylococcus* …….....	CoNS
Cocos Gram positivos ……...	CGP
Cocos Gram negativos ……	CGN
Colony forming units …….....	CFUs
Comissão de Ética no Uso de Animais …….	CEUA
Conselho Nacional de Controle de Experimentação Animal ……	CONCEA
Control diet (water) ……...	C
Control surgery ……..	sham
Cirurgia controle ……...	sham
Dieta controle (água) …….....	C
Dieta rica em fructose ……...	F
DNAse test	DNAse
Federal University of Alagoas …….....	UFAL
High-fructose diet …….....	F
Gram-negative bacilli	GNB
Gram-negative cocci	GNC
Gram-positive bacilli	GPB
Gram-positive cocci	GPC
Gut microbiota	GM
Laboratory of Cardiovascular and Neuroendocrine Physiology……	LACAN
Levedura ……	L
Mannitol salt ágar ……..	MSA
Medidas repetidas	MR
Microbiota intestinal …….	MI
Ovariectomia …….	ovx
Ovariectomy …….	ovx
Potential of hydrogen	pH
Repeated measures ……	RM
Unidades formadoras de colônias …….....	UFCs
Yeast	Y

RESUMO

Alterações na microbiota intestinal (MI) foram avaliadas em função da menopausa e sua associação com dieta rica em frutose. Ratas Wistar adultas foram submetidas à ovariectomia (ovx; um modelo experimental de menopausa) ou cirurgia sham (controle) e divididas em 4 grupos: ratas alimentadas com dieta rica em frutose (solução a 10% na água de beber; sham-F e ovx-F) e ratas alimentadas com dieta controle (água potável; sham-C e ovx-C). Coletou-se fezes (1g) imediatamente antes da cirurgia (T0), na 4ª (T4), 8ª (T8) e 12ª (T12) semanas subsequentes para processamento, cultivo em ágar sangue 5%, em duplicata, e incubação (24h; 36°C). A contagem manual das unidades formadoras de colônias (UFCs) foi realizada e 10 colônias aleatórias de cada cultura foram subcultivadas em ágar de infusão de cérebro-coração (AICC) para análise quantitativa e qualitativa. A análise bioquímica foi realizada em cocos Gram-positivos (CGP) e bacilos Gram-negativos (BGN). Two-way ANOVA para medidas repetidas (MR) seguido por Student Newman-Keuls (SNK); One-way ANOVA MM ou Friedman MR ANOVA on Ranks seguido por SNK ou Dunnett's; Two-way ANOVA seguido por SNK ou Kruskal-Wallis One-way ANOVA on Ranks seguido por Dunn's; e o teste exato de Fisher foram utilizados, sendo considerado o nível de significância $p<0,05$. Em T0, a quantidade de UFCs observada entre os grupos foi semelhante. Diferentemente, de T4 a T12, os grupos alimentados com frutose apresentaram maior número de UFCs em relação aos respectivos controles ($p <0,05$). O grupo sham-F apresentou várias placas incontáveis (quantidade extremamente alta de UFCs), em que a razão de probabilidade de ocorrência foi de 16,1 vezes maior em sham-F comparado a sham-C. Em contraste, ovx-F apresentou apenas 1 placa incontável em T4, exibindo novamente apenas em T12 ($n = 6$), o que reflete que ovx-F não apresentou diferença na probabilidade de gerar placas incontáveis até T12 comparado a ovx-C. Além disso, a razão da probabilidade de ovx-F apresentar placas incontáveis foi 0,09 menor comparado a sham-F. Do total de 1.320 isolados (T0 a T12) obtidos, 971 isolados eram cocos Gram-positivos (CGP), 222 bacilos Gram-positivos (BGP), 98 bacilos Gram-negativos (BGN), 22 cocos Gram-negativos (CGN) e 7 leveduras (L), sendo estas últimas observadas apenas nos grupos ovx. Exceto para os grupos alimentados com frutose em T4, a maioria dos isolados foram CGP (pelo menos 55%) em todos os grupos. Além disso, todos os grupos em comparação com T0, exceto ovx-C, tiveram aumento considerável em BGP, CGN e/ou BGN em T4 ($p <0,05$). A análise bioquímica de CGP revelou que microorganismos comensais constituíram pelo menos 80% em todos os grupos em qualquer momento, exceto para ovx-F em T8, no qual representou 52,1%. Entre 67 isolados recuperados de BGN, 49 isolados eram fermentadores, 3 isolados eram não-fermentadores e 15 isolados não cresceram em MacConkey. A maioria dos BGN foi obtida em T4 ($n = 51$, onde 48 eram fermentadores, dos quais 32 foram detectados nos grupos ovx). Todos os isolados não-fermentadores ($n=3$) foram negativos em ágar cetrimide, portanto, não identificados como *Pseudomonas aeruginosa*. Apesar dessas variações percentuais, não foram observadas diferenças estatísticas entre os grupos para as análises bioquímicas de CGP ou BGN. Ratas alimentadas com frutose ingeriram mais solução e menos ração que seus respectivos controles ($p <0,05$). Como esperado, os grupos ovx ganharam maior peso corporal (PC) em comparação com grupos sham ($p <0,05$). No entanto, nenhuma diferença foi observada no ganho de PC devido à ingestão de frutose. Observamos que a ingestão crônica de frutose aumentou as UFCs em ratas sham e ovx, porém a ovariectomia evitou o aumento de UFCs. O percentual de BGP aumentou em ratas alimentadas com frutose a partir do T4; enquanto o aumento do BGN e/ou CGN ocorreu apenas na T4 em todos os grupos. Além disso, tais alterações na MI parecem ser independentes do ganho de PC. Concluímos que a ovariectomia atenuou o crescimento excessivo de UFCs, mesmo quando associada à alimentação com frutose, e que as demais alterações observadas na MI em função da dieta rica em frutose são em maioria similares entre ratas intactas e ovariectomizadas.

Palavras-chave: menopausa; doenças metabólicas; frutose; microbiota intestinal; disbiose; dieta.

ABSTRACT

Changes in gut microbiota (GM) were evaluated due to menopause and its association with high-fructose diet. Adult female Wistar rats were subjected to ovariectomy (ovx; an experimental model of menopause) or sham surgery (control) and divided into 4 groups: rats fed high-fructose diet (10% solution in drinking water; sham-F and ovx-F) and rats fed control diet (drinking water; sham-C and ovx-C). Faeces (1g) was collected immediately before surgery (T0), and at the 4^{th} (T4), 8^{th} (T8) and 12^{th} (T12) subsequent weeks for processing, culture in 5% blood agar, in duplicates, and incubation (24h; 36°C). Manual counting of colony forming units (CFUs) was performed and 10 random colonies of each culture were subcultured in brain-heart-infusion agar (BHIA) for quantitative and qualitative analysis. Biochemical analysis was performed in Gram-positive cocci (GPC) and Gram-negative bacilli (GNB). Two-way ANOVA repeated measures (RM) followed by Student Newman-Keuls (SNK); One-way ANOVA RM or Friedman RM ANOVA on Ranks followed by SNK or Dunnett´s; Two-way ANOVA followed by SNK or Kruskal-Wallis One-way ANOVA on Ranks followed by Dunn's; and the Fisher's exact test were used, considering the level of significance as $p< 0.05$. At T0, similar quantity of CFUs was observed between groups. Differently, from T4 to T12, fructose-fed groups presented increased CFUs compared to controls ($p<0.05$). Interestingly, sham-F had several uncountable plates (extremely high quantity of CFUs), in which odds ratio of occurring it was 16.1 times more in sham-F compared sham-C. In contrast, ovx-F had only 1 uncountable plate at T4, displaying it again only at T12 (n=6), reflecting that ovx-F had no difference in odds ratio for uncountable plates until T12 compared to ovx-C. Moreover, ovx-F odds ratio for uncountable plates was 0.09 in relation to sham-F. Of the total of 1.320 isolates (T0 to T12) that were obtained, 971 isolates were Gram-positive cocci (GPC), 222 Gram-positive bacilli (GPB), 98 Gram-negative bacilli (GNB), 22 Gram-negative cocci (GNC) and 7 yeasts (Y), in which the latter was observed only in ovx groups. Except for fructose-fed groups at T4, the majority of isolates were GPC (at least 55%) in all groups at any time. Also, all the groups, but ovx-C, had considerable increase in GPB, GNC and/or GNB at T4 ($p<0.05$) compared to T0. Biochemical analysis of GPC revealed that commensal microorganisms constituted at least 80% in all groups at any time, except for ovx-F at T8, in which it represented 52.1%. Sixty-seven (n=67) GNB recovered isolates, 49 isolates were fermenters, 3 isolates were non-fermenters and 15 isolates did not grow on MacConkey. The majority of GNB were obtained at T4 (n=51, wherein 48 were fermenters, of which 32 were detected in ovx group). All non-fermenters isolates (n=3) were negative on cetrimide agar, thus, non- identified *Pseudomonas aeruginosa*. Despite such percentual variations, no statistical differences were observed between groups in any time for biochemical analysis of GPC or GNB. Fructose-fed rats drank more solution and ate less food than their respective controls ($p<0.05$). In addition, as expected, ovx-F had increased body weight (BW) gain compared to sham groups ($p<0.05$). However, no difference was observed on BW gain due to fructose intake. In conclusion, chronic fructose intake increased CFUs in both sham and ovx females, but ovariectomy prevented the occurrence of overgrowth of CFUs. The percentual of GPB increased in fructose-fed rats from T4 onwards; while the increase of GNB and/or GNC occurred only at T4 and in all groups. Changes in GM seemed to be independently of BW gain. The data herein suggest that ovariectomy attenuated the overgrowth of CFUs, even when it is associated with fructose feeding. Despite that, general changes in GM are likely to be similar to fructose-fed sham rats.

Keywords: menopause; metabolic disease; fructose; gut microbiota; dysbiosis; diet.

INTRODUÇÃO

A pós-menopausa é considerada um período da vida de maior risco para o desenvolvimento de doenças cardiovasculares e alterações metabólicas devido à redução das ações cardiometabólicas do estrógeno e aumento dos andrógenos circulantes [1-4]. Evidências crescentes têm destacado que o estrógeno pode ser importante para a manutenção da saúde da microbiota intestinal (MI) [5], bem como que a MI está relacionado à manutenção do ciclo estral, especialmente na condição da síndrome do ovário policístico [6-7], e que a redução do estrógeno na pós-menopausa pode ser um fator fisiológico capaz de alterar as comunidades microbianas intestinais [8-9]. A MI humana é definida como um ecossistema endógeno que consiste de 10-100 trilhões de microrganismos [10], principalmente espécies bacterianas [11], nos quais os filos Bacterioidetes e Firmicutes são prevalentes em uma microbiota saudável [5].

Além disso, fatores ambientais podem afetar a saúde da MI, por exemplo, excesso de ingestão energética [12], sedentarismo [13-14], alteração do pH [15], uso de antibióticos [16-17] e composição da dieta, como o alto teor de gordura [18], alto teor de frutose [19] e consumo de açúcar no início da vida [20], que já foram associados à disbiose intestinal em modelos humanos e animais de obesidade. Entretanto, ainda permanece desconhecida a influência da condição da menopausa associada à dieta rica em frutose sobre a MI.

Portanto, o presente estudo buscou observar alterações na MI em ratas ovariectomizadas, modelo experimental de menopausa, submetidas à alimentação rica em frutose de forma crônica. Tais resultados poderão contribuir para estudos futuros que visem compreender as ações fisiológicas do estrógeno e/ou do ciclo reprodutivo funcional sobre a MI e sua relação com a dieta alimentar.

SUMÁRIO

CHANGES IN GUT MICROBIOTA IN AN EXPERIMENTAL MODEL OF MENOPAUSE AND ITS RELATIONSHIP WITH HIGH-FRUCTOSE DIET

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ABSTRACT

Objective: We sought to investigate changes in gut microbiota (GM) due the induction of menopause and its association with high-fructose diet.

Methods: Adult female Wistar rats were subjected to ovariectomy (ovx) or sham surgery (control) and divided into 4 groups: rats fed high-fructose diet (10% solution in drinking water; sham-F and ovx-F) and rats fed control diet (water; sham-C and ovx-C). One gram of faeces was collected, processed and cultured in 5% blood agar immediately before surgery (T0), and at the 4th (T4), 8th (T8) and 12th (T12) subsequent weeks. Manual counting of colony forming units (CFUs) was performed and 10 colonies of each culture were randomly subcultured in brain-heart-infusion agar for quantitative and qualitative analysis, and morphological and biochemical tests, $p<0.05$ was considered for statistical analysis.

Results: Fructose-fed groups presented increased CFUs from T4 to T12 compared to their respective controls ($p<0.05$), while ovariectomy prevented the chance of occurring extremely high quantity of CFUs in ovx-F compared to sham-F (odds ratio=0.09; $p<0.05$). At T0, similar pattern of GM distribution was observed among the groups, in which Gram-positive cocci (GPC) were the most abundant (n > 75%). However, compared to T0, all groups, but ovx-C, had considerable increase of GNC and/or GNB at T4 ($p<0.05$), which became similar to T0 at T8 and T12. Differently, a GPB increase in fructose-fed groups from T4 onwards was observed. Commensal microorganisms constituted at least 80% in all groups at any time, except for ovx-F at T8, in which it represented 52.1%. Despite the percentual variations of GPC throughout time, no significant difference was observed. Changes in the number of CFUs or in the pattern of GM seemed to be not related to body weight gain. Thus, we suggested that ovariectomy considerably attenuated the overgrowth of CFUs, even when it is associated with fructose feeding, despite general changes in GM may occur as in fructose-fed intact females.

Keywords: menopause; metabolic disease; fructose; gut microbiota; dysbiosis; diet.

INTRODUTION

Postmenopause is considered a life period of greater risk to develop cardiovascular diseases and metabolic alterations due to reduced estrogen cardiometabolic actions and increased circulating androgens [1-4]. Increasing evidence has highlightening that estrogen may be important to the maintenance of gut microbiota (GM) health [5], as well as that GM is related with maintenance of estrous cycle, especially in the polycystic ovarian syndrome condition [6-7], and that the reduction of estrogen in the postmenopause may be a physiological factor that changes gut microbial communities [8-9]. The human GM is defined as an endogenous ecosystem that consists of 10-100 trillion microorganisms [10], mostly bacterial species [11], in which Bacterioidetes and Firmicutes phylum are prevalent in healthy GM [5].

In addition, environmental factors may affect GM health, for instance, excess energy intake [12], sedentary lifestyle [13-14], pH alteration [15], antibiotics intake [16-17] and diet composition, such as high-fat [18], high-fructose [19,] and early-life sugar consumption [20], which have been associated to gut disbiosis in human or animal models of obesity. In this regard, it remains unknown the influence of the association of menopause condition and high-fructose diet on GM.

Therefore, the present study sought to observe changes in GM in ovariectomized rats, an experimental model of menopause, subjected to high-fructose feeding chronically. These results may contribute to future studies pursuing to comprehend the physiological actions of estrogen or functional reproductive cycle on gut microbiota and its relationship with diet.

METHODS

Animals. Adult female Wistar rats (8-10 wks-old) were obtained from the animal facilities of the Federal University of Alagoas (UFAL) and placed at 3 animals per cage, under 12h light-dark cycle (06:00h and 18:00h), fed commercial food and drinking water *ad libitum*, and kept in the animal facilities of the Laboratory of Cardiovascular and Neuroendocrine Physiology (LACAN) of UFAL for the entire study. Animals were placed individually for 12h (overnight) prior surgery (T0), and at 4th (T4), 8th (T8) and 12th (T12) subsequent weeks for collection of one gram of faeces for the purpose of this study. All animal procedures were approved by the local committee that regulates the use of animals in experimental studies (*Comissão de Ética no Uso de Animais*, CEUA-UFAL #71/2016), which follows the guidelines of the Brazilian Council for Controlling Animal Experiment (*Conselho Nacional de Controle de Experimentação Animal* - CONCEA).

Ovariectomy and experimental groups. For bilateral ovariectomy (ovx) surgery, females (12-14 wks-old) were subjected to tramadol (1-hour prior to surgery, 10 mg/kg; i.p), and the association of ketamine (8.5 mg/kg; i.p) and xilazine (80 mg/kg; i.p). Under aseptic conditions, ovaries were assessed by a 1.0 cm incision in each lateral flank, isolated and removed after clamping and suturing the uterine tube. Part of animals was subjected to control surgery (sham). Muscles and skin were then appropriated sutured, and animals were subjected to a single dose of prophylactic antibiotic (Pentabiótico veterinário Zoetis®; 0,1 ml, i.m), and anti inflamatory fluxinin meglumine (1.0 mg/kg; s.c). After that, animals were separated into 4 groups, as follows: sham-C (n=6) and ovx-C (n=9), which kept drinking water; or sham-F (n=9) and ovx-F (n=9), which switched to 10% fructose solution to drink after surgery for 12 weeks. Animals were weekly weighted and the average of ingestion per cage was obtained, so that the consumption of each cage, as milliliter (for solution) or gram (for food) per day per rat, was estimated.

Microbiological analysis. The one gram of faeces collected were macerated in aseptic conditions for dilution into 20 ml of 0.9% sterile saline and mixed using vortex for 3 min. After 30 min, the supernatant was serially diluted (1:100) and 100 µl were cultured in 5% blood agar in duplicates for incubation (24 h; ± 36°C). Manual counting of colony forming units (CFUs) was performed in countable plates and 10 colonies of each culture (a total of 20 colonies per animal in each time of collection) were randomly

subcultured in brain-heart infusion agar (BHIA; Acumedia®) for posterior Gram staining and stock in BHI-glycerol 20% (-4 °C).

In case less than 10 colonies had grown in a plate, all colonies were collected for analysis. Catalase test and DNAse test agar (Acumedia®) revealed with HCl 1 mol/L were performed for Gram-positive cocci (GPC), and cultivation mannitol salt agar (MSA; Acumedia®) for *Staphylococcus aureus* identification. Gram-negative bacilli (GNB) were cultured in MacConkey agar and cetrimide agar (Acumedia®). Suggestive colonies of fungi were cultured in Chromagar *Candida* (Difco®) for presumptive identification.

Statistical analysis. Differences in body weight and food intake were analyzed by Two-way ANOVA repeated measures (RM), followed by Student Newman-Keuls (SNK) post-hoc test; differences in the percentage distribution of GM, GPC, or GNB inside a group throughout time were analyzed by One-way ANOVA RM or Friedman RM ANOVA on Ranks followed by SNK or Dunnett´s post-hoc test; differences in the percentage distribution of GM, GPC, or GNB between groups in a specific time were analyzed by Two-way ANOVA followed by SNK post-hoc or Kruskal-Wallis One-way ANOVA on Ranks followed by Dunn's post-hoc. Cumulative odds ratio for uncountable plates was analyzed by non-parametric Fisher's exact test. The level of significance for all statistical tests was considered at $p < 0.05$.

RESULTS

Body weight, and ingestion of solution and food. Ovariectomized females presented increased body weight (BW) compared to sham groups (Fig. 1A). No difference in the BW gain was observed due to fructose intake (Fig. 1A); differently, sham-F and ovx-F groups presented increased drinking behavior and reduced ingestion of food compared to their respective control group (Fig. 1B-C). Interestingly, sham-F drank more than ovx-F throughout the experiment (T4, T8, and T12; $p<0.05$), despite similarity in food intake, except for T4, in which ovx-F ate more than sham-F ($p<0.05$).

Quantitative analysis of microbiota fecal. As depicted in Figure 2, sham-F and ovx-F had highgest number of CFUs, in which some plates of these groups became uncountable (extremely high quantity of CFUs growth). No uncountable plate was observed in any group before surgery (T0). Interestingly, sham-F displayed several uncountable plates at T4 (n=5), T8 (n=3) and T12 (n=6), while ovx-F displayed only 1 (one) uncountable plate at T4, none at T8, and 6 (six) at T12, as sham-F did. The odds ratio for uncountable plates in sham-F was 16.1 times more compared to sham-C ($p<0.05$), but no chance was observed between ovx groups. As for the countable plates, there was no statistical difference among the groups at T0 (Fig. 2A). However, at T4 and T8, both sham-F and ovx-F had augmented CFUs compared to respective controls ($p<0.05$; Fig. 2B-C). At T12, no difference was observed in countable plates among the groups. However, it is worth to note that, at this time, most of plates of both fructose groups were uncountable (6 of 9 plates in each group; Fig. 2D).

Qualitative analysis of microbiota fecal

Phenotypic identification. A total quantity of isolates obtained in the present study is summarized in Table 1, considering those which were pure colonies for biochemical analysis; as well as those that had to be excluded of the analysis, for being mixed colonies or having no clear identification on phenotype analysis; or just for not growing, which means that less than 10 colonies were obtained in a certain plate.

A total of 1.320 isolates (T0 to T12; Fig. 3A-P) were obtained, in which 971 isolates were Gram-positive cocci (GPC), followed by 222 Gram-positive bacilli (GPB), 98 Gram-negative bacilli (GNB), 22 Gram-negative cocci (GNC) and 7 yeasts (Y), in which the latter was observed only in ovx groups.

At T0, the pattern of GM distribution was similar between groups, in which GPC was the most abundant isolate, representing more than 75%, (Fig. 3A-D). In contrast, pattern

has changed at T4. As for GPC, all groups except ovx-C presented a significant reduction compared to T0 (Fig. 3A-H). Interestingly, comparing the percentage of GPC at T4 among the groups, this reduction was more prominently in both ovx-F ($p<0.05$ *vs* ovx-C) and sham-F ($p=0.06$ *vs* sham-C). Moreover, the sham-C group presented an increase in GNC isolates at T4 compared to T0 ($p<0.05$), which did not occur in sham-F ($p<0.05$ *vs* sham-C at T4), neither in ovx groups (Fig. 3E-H). The sham-F and ovx-F groups presented otherwise 2.4- and 3.6-fold more GPB, respectively, compared to T0 ($p<0.05$ sham-F *vs* sham-C, and sham-F *vs* ovx-C at T4; no statistical difference was observed between ovx groups though). In addition, comparing T4 to T0, all groups displayed an increase in GNB values, which was statistically different in fructose-fed groups (T4 *vs* T0). No significant difference in GNB or Yeast values was observed among the groups at T4 (Fig. 3A-H).

Differently, the pattern of GM distribution at T8 started to become closer to the one observed in T0. At T8, the percentage of GPC was increased in sham-C, sham-F and ovx-F compared to T4 ($p<0.05$), becoming comparable to T0 (Fig. 3A-L). Also, values for GNC and GNB were greatly reduced, while values for GPB remained unchanged in all groups compared to T4. At this point (T8), there was no statistical difference between groups. On the other way, at T12, GPB reduced in sham-F towards to the pattern at T0, but not in ovx-F, in which values remained similar to T4 and T8. No statistical difference between groups was observed at T12 (Fig. 3O-P).

Biochemical tests

Family Streptococcaceae.

As shown in Table 2, of the 971 GPC isolated, 683 were recovered for biochemical tests, in which 291 were negative for catalase test (Cat-), being it suggestive of Streptococcaceae family (*e.g*: *Enterococcus*, *Streptococcus*, *Lactococcus*). Only 2 of the 20 isolates of an ovx-F rat were positive for DNAse test (DNAse +) at T0. All other GPC isolates Cat- from all groups were negative for DNAse test (DNAse-). There was no statistical difference in the percentage distribution of Streptococcaceae among the groups in any time, neither inside a specific group throughout time (Fig. 4A-P).

Family Staphylococcaceae

As for the others GPC, 392 were positive for catalase test (Cat+), in which 181 isolates were suggestive of Staphylococcaceae family. Of these last, 130 isolates were identified as coagulase-negative *Staphylococcus* (CoNS) and 51 isolates were identified as *S. aureus* (Table 2). No statistical difference was observed between groups in any time for

the percentage distribution of CoNS or *S. aureus*. However, the percentual quantity of CoNS gradually reduced through time in all groups, wherein in ovx-C group, statistical difference was observed between T12 *vs* T4 or T8 ($p<0.05$; Fig. 4, column C-O).

Differently, the percentual quantity of *S. aureus* did not significantly differ throughout time in none of the groups but ovx-F, in which was observed a considerable increase of *S. aureus* at T8 compared to T0, T4 or T12 ($p<0.05$; Fig. 4, column D-P).

As depicted in Table 2, of the remaining 211 Cat+ isolates, a total of 118 were inconclusive, in which 96 were DNAse- and positive for mannitol fermentation (GPC X), while 22 isolates were DNAse+ and negative for fermentation in mannitol (GPC Y). No statistical difference was observed among the groups in any time, neither inside a specific group throughout time (Fig. 4A-P).

It was not possible to perform MSA fermentation test in the remaining 93 Cat+ GPC (not recovered), therefore being inconclusive diagnosis for *Staphylococcus* genus, however 81 isolates of those were DNAse- and 12 isolates were DNAse+ (Table 2). There was no statistical difference among the groups in any time, neither inside a specific group through time (Fig. 4A-P).

Gram-negative bacilli (GNB)

As indicated in Table 2 and Figure 5, a total of 67 GNB isolates were recovered and cultured on MacConkey agar, in which 49 isolates were fermenters, 3 isolates non-fermenters and 15 isolates did not grow on MacConkey. The majority of GNB were obtained at T4 (n=51), mostly in ovx-C and ovx-F (n=16, each group), while no GNB was obtained at T8 (n=0 for all groups). All non-fermenters isolates (n=3) were negative on cetrimide agar, thus, negative to *Pseudomonas aeruginosa*.

Yeast

No yeast (n=7) were recovered after freeze (stock) for culture on Chomagar *Candida* Difco®.

DISCUSSION

The present study aimed to elucidate changes in the pattern of gut microbiota in an experimental model of menopause, induced by ovariectomy, and its association with high-fructose diet. The main finding was that, opposing to what was observed in fructose-fed intact females (sham-F), ovariectomy prevented the occurrence of exacerbated overgrowth of CFUs in fructose-fed females (ovx-F) for at least 8 weeks. Moreover, throughout 12 weeks, ovariectomized controls (ovx-C) did not present significant changes in the percentage distribution of GM phenotypes. Differently, the other groups displayed considerable changes in it especially at T4. Finally, such observations may occur independently of changes in BW, since fructose feeding did not alter the pattern of BW gain in sham or ovx females.

As expected, ovariectomized females presented considerable increase in body weight [21-22,9]. Moreover, ingestion of fructose was increased while ingestion of food was decreased, in both sham-F and ovx-F rats throughout the experiment. In this regard, fructose intake was even higher in sham-F compared to ovx-F, but no change except for T4, was observed in food ingestion between these two groups. The reduction in food intake was an expected result, since sugar intake potentiates the activation of reward systems due to both caloric input and taste [23]. However, such changes in eating behavior (source of energy intake) did not alter the pattern of increasing body weight either in ovariectomized or intact rats. Therefore, the difference in fructose intake between sham-F and ovx-F rats might be mostly due to the reduction in basal metabolic rate in the last ones [24].

Simple sugars, including fructose, are important sources of nutritional substrate for diverse microorganisms [23-24]. In this regard, several studies have shown that GM is influenced by diet and that the increase in sugar intake even for a day can alter GM, and for few days, can significantly decrease its diversity [27-29]. Moreover, changes in GM due to high-sugar diet (glucose and fructose) may occur regardless of obesity, caloric intake, body weight and adiposity index [20]. In the present study, we were not aimed to fully address the diversity of GM; however, despite that, our data corroborates with the literature, since fructose intake greatly increased CFUs in both sham and ovx females compared to controls, which presented significantly different body weight gain, as expected.

Interestingly, the number of uncountable plates, which reflects extreme overgrowth of microorganisms, considerably differed among the groups. While controls displayed no uncountable plates at any time, sham-F had 16.1 more chance of having uncountable plates compared to sham-C. In fact, sham-F presented several uncountable plates from T4 to T12. In contrast, ovariectomy abolished the increased chance of occurring overgrowth of microorganisms due to fructose intake for at least for 8 weeks. In line with this, ovx-F had only one uncountable plate at T4, presenting it more prominently just at T12. Such difference between sham-F and ovx-F may highlight the importance of the synergy of estrogen availability with high-fructose diet. Previous studies have shown that decoupled-estrogen form can increase glycogen bioavailability on intestinal lumen, through intestinal receptors ERα and ERβ, favoring the growth of certain groups of microorganisms (*e.g.*: fermenting bacteria) [5], as it occurs in the vaginal mucous [30-31]. Therefore, the same condition may occur in the GM, since both, estrogen bioavailability and sugar, are benefits to growth gut bacteria [5, 25-26]. Differently, the gradual increase of CFUs in controls throughout time may reflect the effect of individual natural development on GM, since aging may alter CFUs quantitatively and qualitatively [32-33].

In order to better comprehend and discuss the data, it is important to mention some limitations we have encountered performing the present study, which may have accounted for the lack of statistical differences in different parts of the data obtained. First, some isolates either were not pure colony or did not grow or had no clear identification, so those were not included in the analysis (see Table 1); second, we were not able to recover all pure isolates, which reduced the number of samples used in biochemical tests (see Table 2); third, the data mostly had non-parametric distribution, in which as for one or two samples, there was an overlap of minimum quartilies, compromising detecting statistical differences between groups or even in a specific group throughout time.

Analyzing pure isolated colonies, we observed that GPC was the most abundant type at T0 in all groups prevailing it in controls up to T12. Differently, sham-F and ovx-F displayed a considerable reduction in the percentage of GPC at T4, achieving less than 50% of total GM population in these females at that point. Such reduction was accompanied by an increase in GPB, which did not occur in controls. Also, at T4, it was observed an increase in gram-negative bacteria in all groups, which may be, at least in part, related to the administration of the antibiotic compound at ovariectomy (or sham) surgery, as we discuss ahead.

Gram positive Firmicutes are abundant in healthy GM [34], such as Streptococcaceae [35] and Staphylococcaceae [36] families. Non-pathogenic streptococci are essential to maintain the GM healthy, which in association to *Lactobacillus* and *Bifidobacteria* contributes to inhibit pathogens and modulates the expression of inflammatory mediators, such as interleukins, NF-kB and TRL5 receptors [37-38].

The balance between non-pathogenic (commensals) and pathogenic species is crucial to maintain a person's health, since the development and/or progression of different diseases, such as gastroenteritis and inflammatory diseases are related to gut dysbiosis [39-41].

In the present study, biochemical analysis of GPC revealed that our data are in line with the literature, and shows that, despite ovariectomy or 12-wks of fructose feeding, at least 25% of total GPC were Streptococcaceae, and that at least 50% of total GPC in each group were constituted by presumptively commensal microorganisms (DNAse-) [42-44].

There was no significant change throughout time in the percentage of Streptococcaceae in any group. Differently, recent studies have reported that gut Streptococcaceae species were reduced in overweight/obese teenagers after increasing sugar intake [35], while others have reported an increase in Streptococacceae in GM, such as in from pacients with Coronavirus Disease 19 or H1N1 Influenza [45], or fed high-fat diet [46] or Western diet [47].

In the present study, the greatest Staphylococcaceae family isolated were coagulase-negative *Staphylococcus* (CoNS), which was an expected data, since CoNS are abundant saprophytes, inhabiting the skin and mucous (including intestinal mucous) [48], although some species may have clinical relevance (pathogenic potential), such as *S. epidermitis* and *S. haemolyticus* [48]. It is interesting to note that relative percentage of CoNS clearly reduced in all groups along with the experiment. Therefore, in case of part of the detected CoNS were pathogenic in the present study, it would may affect minimally GM.

Intriguingly, ovx-F presented a great increase in *S. aureus* at T8. The clinical relevance of *S. aureus* in GM is due to the fact that this microorganism is closely related to inflammation and infection, for example enterocolitis [49], one of the most common postsurgery infections, which can be accompanied by rapid deterioration [50]. Therefore, one may presume that ovx-F at T8 might have had increased susceptibility to infections and/or inflammatory bowel diseases due to elevated relative percentage of *S. aureus*.

However, none of the females presented any clinical symptom during the 12-week surveyed period. Regarding to this data, it is possible that the gradual decrease of estradiol serum levels associated to high-fructose feeding may have accounted for the increase in *S. aureus*. Although we have not addressed serum levels of estradiol in this study, previous data from the literature has reported that serum level of estradiol of ovariectomized 6 month-old Sprague-Dawley rats are significantly reduced after 2 weeks of ovariectomy; however, such reduction was only about a third of baseline levels, and kept being detected in blood for at least 9 weeks later [51]. We also do not discard the possibility of such increase being, at least in part, a collateral effect of the postsurgical antibiotic administration potencialized by the fructose intake and reduction of circulating estrogen. Anyway, it remains to be answered why and how such increase in *S. aureus* in ovx-F rats did not persist until T12, in which, in contrast, no *S. aureus* was observed (similar profile at T0).

In the present study, were observed that some GPC with Cat+/DNAse- profile were positive for salted mannitol agar (MSA+), so that we called GPC X isolates, probably non-pathogenic species, since some commensal microorganisms can ferment mannitol [52]. Unfortunately, some isolates were lost after stock, making it impossible to differentiate of *S. aureus* from CoNS. However, the majority of inconclusive GPC were DNAse-, thus suggesting presumably non-pathogenic species (*e.g*: CoNS) [42].

We also observed that some GPC with Cat+/ DNAse+ profile and negative for salted mannitol agar (MSA-), named GPC Y isolates, which we assume being potentially pathogenic species, since the production of DNAse enzyme is considered a virulence factor [42-44]. Previous studies have showed that some stains of *Staphylococcus aureus*, including methicillin-resistent *S. aureus* (MRSA – multidrug resistent), may be negative to mannitol fermentation (MSA-) [53-55]. Therefore, complementary tests, such as molecular identification and searching for genes to antibiotic resistant (*e.g.: mecA*), are important to confirm these findings, since MRSA is related to indiscriminate use of antibiotics and a worldwide public health problem [56]. Anyway, even the greatest quantity of GPC Y observed in sham-F at T8 accounted for less than 15% of total GPC in GM.

Interestingly, there was an increase in GPB in fructose groups, especially at T4 and T8. Fermenting microorganisms (*e.g*.: *Lactobacillus* spp. and *Scherichia coli*) use sugars as nutritional substrate [26,57] and previous study has shown that juvenile male rats presented an increase in *Lactobacillus* spp. after increasing fructose consumption [20]. Contrary,

Lactobacillus spp. was reduced in the GM of juvenile male rats fed high-fat diet [18]. A positive relationship between *Lactobacillus* and estrogen levels has also already been well established [5]. However, considering that we also observed 3- to 4-fold increased of GPB in ovx-F from T4 onwards, it is possible that a tough positive relationship between fructose intake and GPB were greater than the estrogen one. Future studies addressing individual eating and drinking behaviour (through metabolic cages, for instance), as well as the decline of blood levels of estrogen in these animals, may be helpful to correlate data individually and clear this question.

As mentioned before, there was an increase in gram-negative bacteria (GNC or GNB) at T4 in all groups, which were clearly reduced at T8 or T12. Since the pattern of GM distribution in all groups were altered at T4, we believe the single dose of the antibiotic compound administered immediately after ovx (or sham) surgery might have accounted for this data, which contains three types of penicillins and two streptomycins [58]. As for the anti-inflammatory used (flunixin meglumine), we are not aware of a study approaching a possible effect of it on GM, as other non-steroidal anti inflammatories do [59].

Here, the administration of postsurgical antibiotic and anti-inflammatory was priorized aiming animal welfare, since it would avoid possible infections and discomfort in animals. The choose of the drugs used considered the fact that penicillins reach concentrations greater than 90% in blood plasma in few hours and damage cell wall biosynthesis, mainly of GP-bacteria, being normally eliminated from 60-90% in urine after 6h of administration [58]. Also, streptomycins reach maximum levels in blood plasma between 30 and 90 minutes and damage protein synthesis deregulation, usually in GN-bacteria, being normally eliminated in the urine at 24h after intramuscular injection [58].

Although the pentabiotic used is considered to have a short-term activity (usually 24h), it is interesting to note that qualitative changes in the GM remained after four weeks of a single administration (T4). We observed a reduction in GPC at T4 in all groups except for ovx-C, which was recovered at T8. The lack of change in GPC in ovx-C at T4 does not exclude the possibility of such reduction had occurred in this group for at least few days after surgery. However, the gradual reduction in estrogen levels along with control diet might have accounted to drawback the GPC changes in this group. Surely, future studies approaching this issue more specifically would be necessary to properly address this question.

Since antibiotics may influence directly on the taxonomic, genetic and even optimization of the GM functionality, depending on the drug class administered and the time of use [16], we do not discard its influence on qualitative changes at T8 and T12, even the pattern at T12 , which mostly resembles T0. More importantly, it remains to be answered whether the administration of the antibiotic altered the influence of ovariectomy and fructose intake.

Still regarding to possible influence of the antibiotic on GM at T8 and T12, it is worth to note that the recovered GNB profiles at T4 considerably differed from the ones at T12. First, at T4, the majority of GNB isolates grew in MacConkey and was fermenter, whereas at T12, most of it did not grow on MacConkey. This alteration seems to be independent of fructose feeding, but yet associated to the pentantibiotic administered, since such variation is apparently homogeneous among the groups at both times.

All GNB that did not grow on MacConkey at T12 were Cat+, suggesting *Pasteurella* genus identification, which is commonly isolated from animals, including rodents, and is considered to have zoonotic potential [60-63]; despite infection and mortality for *Pasteurella* is uncommon in humans, around 20 to 30 deaths occur annually [61].

Here, few yeasts were found, which it was an expected data, with only seven isolates obtained in total. Studies using metagenomics show that more than 90% of the GM are bacteria, in which mostly are from Bacterioidetes and Firmicutes phyla [5,11,65]. Although not well explored in comparation to bacterial communities, gut mycobiota also plays its role in human health, since several diseases such as Crohn's, ulcerative colitis and irritated bowel syndrome are associated with gut fungi [66-67].

Although the present study had been limited to the use of the classical methods, in which is common the loss of isolates due to reduced viability for reactivation, the preliminary results presented here indicated important alterations in GM. These results may contribute to future studies pursuing the interactions between changes in gut microbiota, estrous cycle, diet, possible diseases associated to the menopause condition, and even the use of antibiotics.

CONCLUSION

The present study addresses general changes in gut microbiota (GM) due to fructose consumption in association with an experimental model of menopause. As expected, ovx females had increased BW compared to sham, however no difference in BW due to fructose feeding was observed. Fructose intake markedly increased CFUs. Also, no significant changes were observed in the pattern of GM phenotypes after 12 weeks of ovariectomy.

In our study, we observed an increase in GPB in fructose groups compared to control at T4 onwards. Also, we observed a great change in the pattern of GM distribution in all groups at T4, specially an increase in GNB fermenters in ovx groups and GNC in sham-C, suggesting that the use of a single-dose of a prophylatic antibiotic immediately after surgery may have altered GM for longer than 4 weeks. Therefore, our study may highlight a possible interaction of short-term antibiotic therapy and the effects of ovariectomy and fructose intake on the pattern of changes in GM.

The results obtained here may also contribute to future studies pursuing to elucidate the relationship between inherent comorbidities to menopause associated to nutritional manners, as well as to those seeking food protocols to improve GM behavior, since postmenopausal women have more propensity to metabolic diseases than age-matched men. Furthermore, our data may favor studies that aim to understand pathologies inherent to postmenopause due imunoneuroendocrine axis changes, since these changes are strongly interconnected to GM.

TABLE 1

Table 1 –Isolates obtained from faeces samples of intact (sham) or ovariectomized (ovx) Wistar rats fed fructose solution (-F) or tap water (-C) chronically.

	T0			
Group	**pure colonies**	**mixed colonies**	**no clear ID**	**not grew**
sham-C	74	4	1	41
sham-F	70	31	1	78
ovx-C	95	21	7	57
ovx-F	117	20	0	43
	T4			
Group	**pure colonies**	**mixed colonies**	**no clear ID**	**not grew**
sham-C	58	30	2	30
sham-F	97	14	5	64
ovx-C	119	12	13	36
ovx-F	84	33	5	58
	T8			
Group	**pure colonies**	**mixed colonies**	**no clear ID**	**not grew**
sham-C	75	10	1	34
sham-F	97	25	0	58
ovx-C	99	10	1	70
ovx-F	74	6	0	100
	T12			
Group	**pure colonies**	**mixed colonies**	**no clear ID**	**not grew**
sham-C	46	3	5	66
sham-F	65	13	1	102
ovx-C	88	10	4	78
ovx-F	63	7	0	110

T0 = data obtained immediately before surgery (baseline); T4 = after 4 weeks; T8 = after 8 weeks; and T12 = after 12 weeks of surgery and fructose intake. ID = identification; not grew = number of isolates that did not grow in blood agar.

TABLE 2

Table 2 –Gram-positive cocci and Gram-negative bacilli* isolated from faeces samples of intact (sham) or ovariectomized (ovx) Wistar rats fed fructose solution (-F) or tap water (-C) chronically.

Gram-positive cocci								
T0								
Group	**Reactivated (total)**	**Streptococcaceae**	***Staphylococcus aureus***	**GPC Y (Cat+; D+;M-)**	**GPC X (Cat+; D-; M+)**	**CoNS**	**Inconclusive GPC (Cat +; D+; NR)**	**Inconclusive GPC (Cat +; D-; NR)**
sham-C	56 (66)	31	2	2	5	11	2	3
sham-F	27 (55)	7	0	0	11	6	1	2
ovx-C	38 (81)	12	2	2	3	9	0	10
ovx-F	75 (106)	29	1	3	7	34	0	1
T4								
Group	**Reactivated (total)**	**Streptococcaceae**	***Staphylococcus aureus***	**GPC Y (Cat+; D+;M-)**	**GPC X (Cat+; D-; M+)**	**CoNS**	**Inconclusive GPC (Cat +; D+; NR)**	**Inconclusive GPC (Cat +; D-; NR)**
sham-C	33 (34)	20	0	0	9	3	0	1
sham-F	15 (32)	7	0	0	5	3	0	0
ovx-C	69 (85)	18	4	4	19	22	0	2
ovx-F	21 (37)	10	1	0	4	4	0	2
T8								
Group	**Reactivated (total)**	**Streptococcaceae**	***Staphylococcus aureus***	**GPC Y (Cat+; D+;M-)**	**GPC X (Cat+; D-; M+)**	**CoNS**	**Inconclusive GPC (Cat +; D+; NR)**	**Inconclusive GPC (Cat +; D-; NR)**
sham-C	61 (65)	22	8	1	4	6	4	16
sham-F	37 (58)	23	0	3	2	2	0	7
ovx-C	80 (93)	15	8	0	12	23	1	21
ovx-F	31 (57)	11	12	0	2	1	2	3
T12								
Group	**Reactivated (total)**	**Streptococcaceae**	***Staphylococcus aureus***	**GPC Y (Cat+; D+;M-)**	**GPC X (Cat+; D-; M+)**	**CoNS**	**Inconclusive GPC (Cat +; D+; NR)**	**Inconclusive GPC (Cat +; D-; NR)**
sham-C	42 (45)	29	6	2	1	1	0	3
sham-F	23 (45)	17	3	0	0	0	2	1
ovx-C	57 (68)	32	4	3	9	4	0	5
ovx-F	18 (44)	8	0	2	3	1	0	4

Table 2 (continued)

Gram-negative bacilli	T0				T4			
Group	**Reactivated (total)**	**Fermenter**	**non-Fermenter**	**non MCK**	**Reactivated (total)**	**Fermenter**	**non-Fermenter**	**non MCK**
sham-C	0 (0)	0	0	0	8 (8)	7	0	1
sham-F	1 (1)	1	0	0	11 (15)	9	2	0
ovx-C	0 (0)	0	0	0	16 (25)	16	0	0
ovx-F	1 (1)	0	0	1	16 (18)	16	0	0
	T8				**T12**			
Group	**Reactivated (total)**	**Fermenter**	**non-Fermenter**	**non MCK**	**Reactivated (total)**	**Fermenter**	**non-Fermenter**	**non MCK**
sham-C	0 (5)	0	0	0	0 (0)	0	0	0
sham-F	0 (3)	0	0	0	4 (8)	0	1	3
ovx-C	0 (0)	0	0	0	7 (10)	0	0	7
ovx-F	0 (0)	0	0	0	3 (3)	0	0	3

T0 = data obtained immediately before surgery (baseline); T4 = after 4 weeks; T8 = after 8 weeks; and T12 = after 12 weeks of surgery and fructose intake. non MCK = did not grow on MacConkey; *= presumably biochemically identified.

FIGURE 1

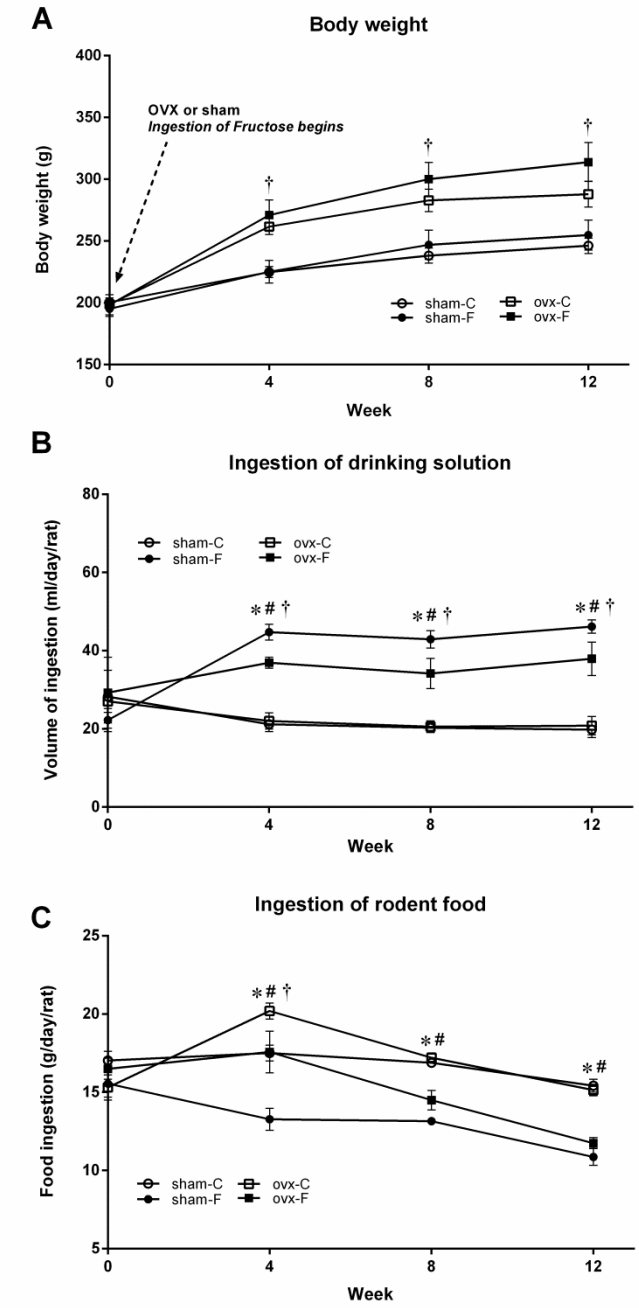

Figure 1 – Body weight gain (A), ingestion of drinking solution (B) and ingestion of rodent food (C) of intact (sham) or ovariectomized (ovx) Wistar rats fed fructose solution (-F) or tap water (-C) chronically. T0 = data obtained immediately before surgery (baseline); T4 = after 4 weeks; T8 = after 8 weeks; and T12 = after 12 weeks of surgery and fructose intake. Data are mean ± SEM. Two-way ANOVA repeated measures followed by Student Newman-Keuls post-hoc test. *p<0.05 ovx-F *vs* ovx-C; # p<0.05 sham-F *vs* sham-C; † p<0.05 ovx-F *vs* sham-F.

FIGURE 2

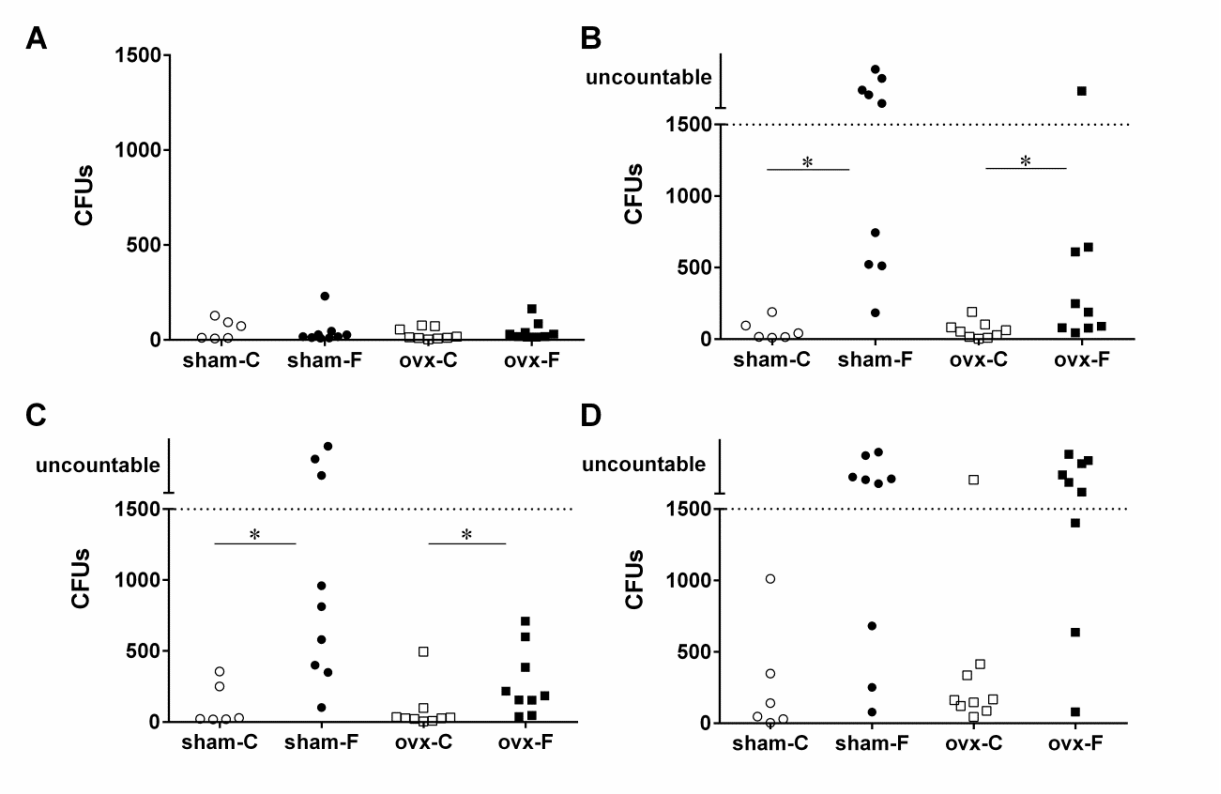

Figure 2 – Arithmetic mean of colonies formation units (CFUs) of intact (sham) or ovariectomized (ovx) *Wistar* rats fed fructose solution (-F) or tap water (-C) chronically. (A) T0 = data obtained immediately before surgery (baseline); (B) T4 = after 4 weeks; (C) T8 = after 8 weeks; and (D) T12 = after 12 weeks of surgery and fructose intake. Two-way ANOVA followed by Student Newman-Keuls (countable plates); *$p<0.05$ (ovx-F *vs* ovx-C or sham-F *vs* sham-C). Cumulative odds ratio for uncountable plates (sum of the data from T0 to T8) was obtained using Fisher's exact test. Odds ratio (OR) between sham-F *vs* sham-C = 16.1 ($p<0.05$); OR ovx-F *vs* sham-F = 0.09 ($p<0.05$); OR ovx-F *vs* ovx-C = not significant ($p=1.0$).

FIGURE 3

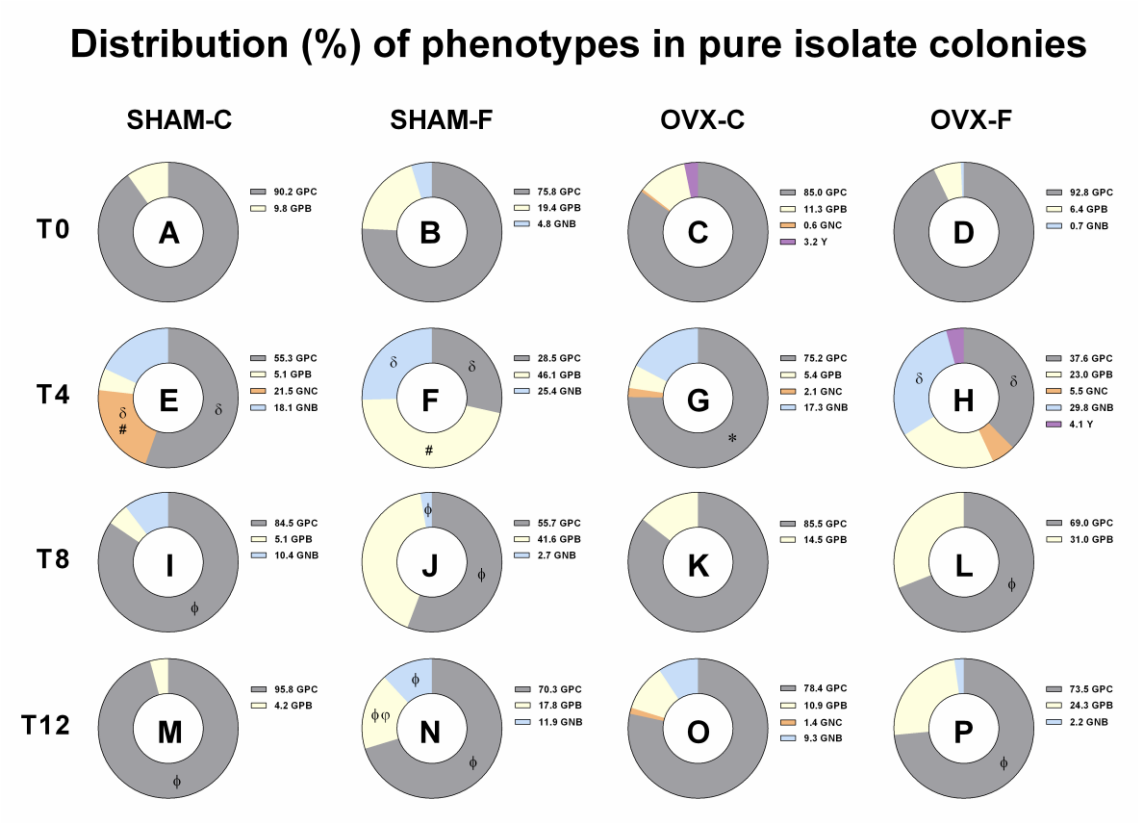

Figure 3 – Average of percentage distribution of the phenotypes identified by Gram stain of pure isolates from feaces of intact (sham) or ovariectomized (ovx) *Wistar* rats fed fructose solution (-F) or tap water (-C) chronically. Line A-D (T0) = data obtained immediately before surgery (baseline); line E-H (T4) = after 4 weeks; line I-L (T8) = after 8 weeks; and line M-P (T12) = after 12 weeks of surgery and fructose intake. GPC= Gram-positive cocci; GNC= Gram-negative cocci; GPB= Gram-positive bacilli; GNB= Gram-negative bacilli; Y= yeasts. The numbers in legends show the average of the percentage for each phenotype. Differences between groups were analyzed by Two-way ANOVA followed by Student Newman-Keuls (SNK) or Kruskal-Wallis One-way ANOVA on Ranks followed by Dunn's post hoc; differences inside a group through time were analyzed by One-way ANOVA repeated measures (RM) or Friedman RM ANOVA on Ranks followed by SNK or Dunnett´s post-hoc test. δ $p<0.05$ *vs* T0; ϕ $p<0.05$ *vs* T4; φ $p<0.05$ *vs* T8 of the same group; * $p<0.05$ ovx-C *vs* ovx-F; # $p<0.05$ sham-C vs sham-F.

FIGURE 4

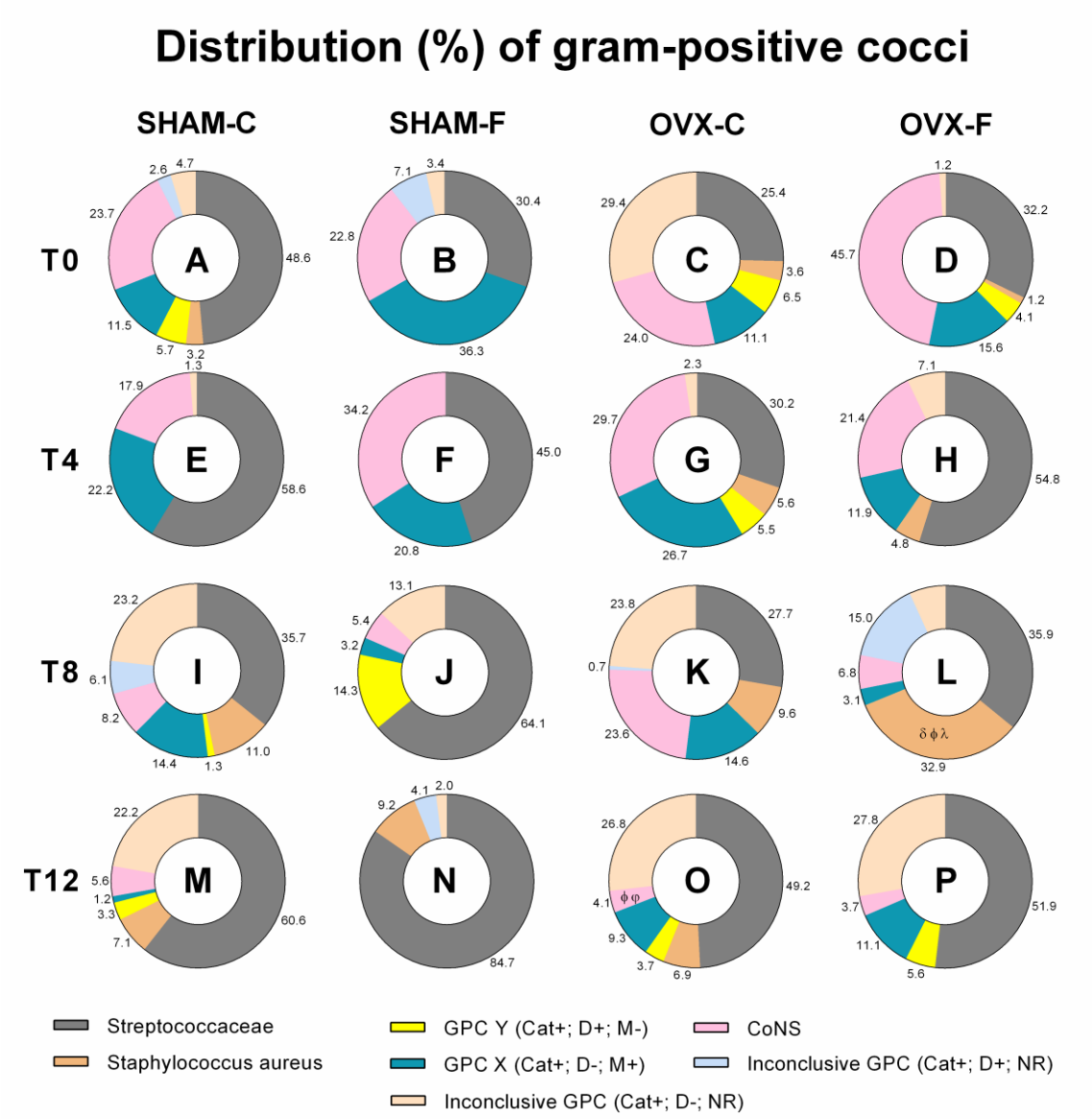

Figure 4 – Percentage distribution of biochemically identified Gram-positive cocci (GPC) recovered from faeces samples of intact (sham) or ovariectomized (ovx) *Wistar* rats fed fructose solution (-F) or tap water (-C) chronically. Line A-D (T0) = data obtained immediately before surgery (baseline); line E-H (T4) = after 4 weeks; line I-L (T8) = after 8 weeks; and line M-P (T12) = after 12 weeks of surgery and fructose intake. The numbers beside each color in plots indicate the average of the percentage for the respective phenotype, as shown in the legend. Differences between groups were analyzed by Two-way ANOVA followed by Student Newman-Keuls (SNK) or Kruskal-Wallis One-way ANOVA on Ranks followed by Dunn's post hoc; differences inside a group through time were analyzed by One-way ANOVA repeated measures (RM) or Friedman RM ANOVA on Ranks followed by SNK or Dunnett´s post-hoc test. δ $p<0.05$ *vs* T0; ϕ $p<0.05$ *vs* T4; φ $p<0.05$ *vs* T8; λ $p<0.05$ *vs* T12 of the same group.

FIGURE 5

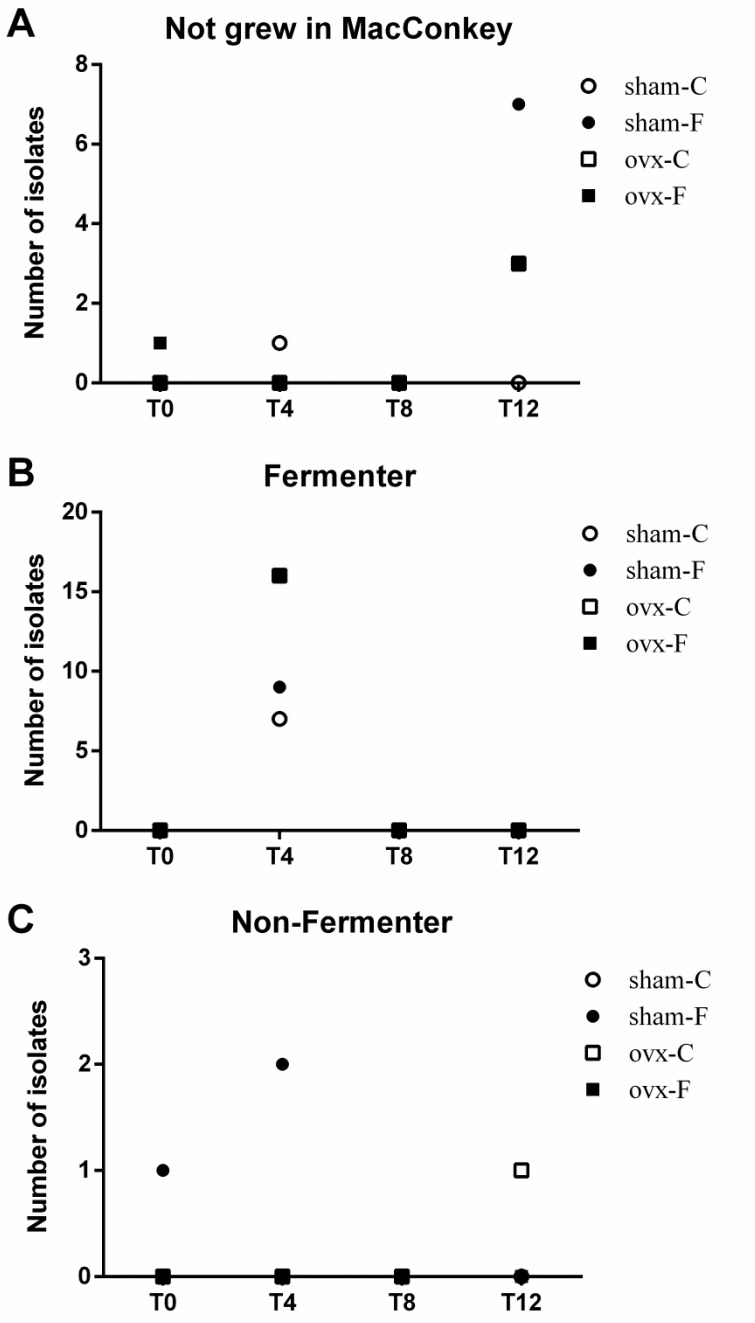

Figura 5 – Total Gram-negative bacilli (GNB) recovered from faeces samples of intact (sham) or ovariectomized (ovx) *Wistar* rats fed chronically fructose solution (-F) or tap water (-C) and cultured on MacConkey agar for fermentation test. In (A): total number of isolates that did not grow in MacConkey; (B) total number of isolates that fermented on MacConkey; (C): total number of isolates that did not fermenter in MacConkey. T0 = data obtained immediately before surgery (baseline); T4 = after 4 weeks; T8 = after 8 weeks; and T12 = after 12 weeks of surgery and fructose intakeDifferences between groups were analyzed by Two-way ANOVA followed by Student Newman-Keuls (SNK) or Kruskal-Wallis One-way ANOVA on Ranks followed by Dunn's post hoc; differences inside a group through time were analyzed by One-way ANOVA repeated measures (RM) or Friedman RM ANOVA on Ranks followed by SNK or Dunnett´s post-hoc test.

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