Universidade Estadual Paulista

“Júlio de Mesquita Filho”

Faculdade de Ciências Farmacêuticas

Efeito do consumo diário de suco de laranja sobre a microbiota intestinal, composição corporal e perfil lipídico e glicídico de mulheres

Melaine Priscila Fidélix

Tese apresentada ao Programa de Pós-graduação em Alimentos e Nutrição para obtenção do título de Doutor em Alimentos e Nutrição.

Área de Concentração: Ciências Nutricionais

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TÍTULO DA TESE: Influência do efeito prebiótico do suco de laranja na excreção urinária das flavanonas após ingestão crônica de suco de laranja

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Dedico

Àqueles que me inspiraram a fazer esta tese,

mas que jamais a lerão.

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As facilidades nos impedem de caminhar. Mesmo as críticas nos auxiliam muito"

Chico Xavier

Resumo

Introdução: Aos compostos do suco de laranja 100% puro (SL) são atribuídos várias propriedades biológicas, como atividade antioxidante, anti-hipertensiva, redutora do colesterol e glicemia sanguíneos, e atualmente estudos *in vitro* têm mostrado que o suco de laranja pode ter ação seletiva sob a microbiota intestinal. **Objetivo**: Investigar o impacto do consumo diário de suco de laranja sobre a microbiota intestinal e no perfil glicídico e lipídico em mulheres. **Métodos:** Foram avaliadas 10 mulheres por período de 120 dias, distribuídos em três etapas: (1ª): dieta livre de citrus 30º dia (30 dias); (2ª) ingestão de 300 mL/dia de SL 90º dia (60 dias); (3ª) dieta livre de citrus 120º dia (30 dias). Avaliação dietética e antropometria foram realizadas quinzenalmente durante o período experimental. Foram colhidas amostras de sangue, urina e fezes, no início do estudo e a cada 30 dias, até o final do experimento. Foi realizado: analise dos parâmetros bioquímicos; identificação dos metabólitos urinários por UHPLC-UV-VIS; e análise da composição da microbiota por sequenciamento de gene RNAr 16S. **Resultados:** A avaliação da composição corporal e a excreção urinária não mostraram alterações durante as etapas experimentais. Os parâmetros bioquímicos mostraram diminuição da glicose (-6,25%), da insulina (-32,7%) e da resistência à insulina (-43,8%), além do colesterol total (-13,9%), LDL (-16%) e triglicerídeos (-30%) com a ingestão de SL (90º dia). Após a parada do consumo de SL (120º dia), os parâmetros bioquímicos retornaram aos valores inicias. Foi observado um aumento dos filos Actinobacteria, bem como diminuição dos Firmicutes, Bacteroidetes e Proteobacteria após o consumo de SL (90º dia). As famílias *Bifidobacterium*, *Lactobacillaceae* e *Streptococcaceae* apresentaram aumento após o consumo de SL. Houve correlação positiva entre *Bacteroidaceae, Porphyromonadaceae, Prevotellaceae, Ruminococcaceae* e *Enterobacteriaceae* com glicose, colesterol total, triglicérides, LDL-C, insulina e HOMA-IR, e uma correlação negativa com o HDL-C. *Bifidobacterium, Coriobacteriaceae, Lachnospiraceae e Lactobacillaceae* tiverão correlação negativa com glicose, colesterol total, triglicerídeos, LDL-C, insulina e HOMA-IR, e correlação positiva com HDL-C. **Conclusão:** O suco de laranja mostrou efeito modulador positivo sobre a microbiota intestinal e no perfil glicídico e lipídico de mulheres.

Palavras-chave: Prebioticos, Microbiota Intestinal, Suco de Laranja 100% puro, Marcadores Metabólicos, Composição Corporal.

Abstract

Introduction: 100% pure orange juice (OJ) compounds are assigned to various biological properties, such as antioxidant, antihypertensive, blood cholesterol lowering and glycemia activity. Currently, in vitro studies have shown that orange juice can present selective action of intestinal microbiota. **Objective:** To investigate the impact of daily consumption of orange juice on the intestinal microbiota and on the lipid and glucose profile in women. **Methods:** Ten women were evaluated for 120 days at three differen stages: (1st) citrus-free diet 30th day (30 days); (2nd) intake of 300 mL / day SL 90th day (60 days); (3rd) citrus-free diet 120th day (30 days). Dietary assessment and anthropometry were performed biweekly during the experimental stages. Samples of blood, urine and feces were taken at the beginning of the study, and every 30 days until the end of the experiment. It was carried out: analysis of the biochemical parameters; identification of urinary metabolites by UHPLC-UV-VIS; and analysis of the composition of the microbiota by 16S rRNA gene sequencing. **Results:** Changes during the experimental stages were not found in evaluation of body composition and urinary excretion. Biochemical parameters showed a decrease in glucose (-6.25%), insulin (-32.7%) and insulin resistance (-43.8%), total cholesterol (-13.9%), LDL (-16%) and triglycerides (-30%) with ingestion of OJ (90th day). After stopping OJ intake (120th day), the biochemical parameters returned to the initial values. An increase of Actinobacteria phyla, as well as a decrease of Firmicutes, Bacteroidetes and Proteobacteria after consumption of OJ (90th day) were observed. The families *Bifidobacterium, Lactobacillaceae* and *Streptococcaceae* showed an increase after consumption of OJ. There was a positive correlation between *Bacteroidaceae, Porphyromonadaceae, Prevotellaceae, Ruminococcaceae* and *Enterobacteriaceae* with glucose, total cholesterol, triglycerides, LDL-C, insulin and HOMA-IR, and a negative correlation with HDL-C. *Bifidobacterium, Coriobacteriaceae, Lachnospiraceae* and *Lactobacillaceae* will have negative correlation with glucose, total cholesterol, triglycerides, LDL-C, insulin and HOMA-IR, and a positive correlation with HDL-C. **Conclusion:** Orange juice showed a positive modulating effect on the intestinal microbiota and on glucose and lipid profile of women.

Key words: Prebiotic, Intestinal Microbiota, 100% pure Orange Juice, Metabolic biomarkers, Body Composition

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Microbiota

O corpo humano é um ecossistema que sustenta trilhões de microrganismos que vivem principalmente, embora não exclusivamente, dentro do trato gastrointestinal[1]. O trato gastrointestinal humano é um ecossistema muito complexo, no qual a microbiota intestinal interage com células hospedeiras e componentes alimentares[2]. O estômago e o intestino delgado apresentam um ambiente desfavorável para a colonização de bactérias devido a ação bactericida do suco gástrico. Enquanto isso, na região do cólon as bactérias encontram condições favoráveis para a sua proliferação, decorrente do abundante suprimento nutricional e ausência de secreções[3,4].

Estima-se que o trato gastrointestinal seja habitado por 10^{11} células por mL de conteúdo luminal, compreendendo cerca de 400 a 500 espécies bacterianas[5]. Nos indivíduos saudáveis as bactérias são encontradas de forma heterogênea em todo o trato gastrointestinal. Este é composto por bactérias anaeróbias estritas, que superam as bactérias anaeróbias e aeróbicas facultativas[6]. Embora mais de 100 filos bacterianos tenham sido descritos, os principais filos encontrados são Bacteroidetes, Firmicutes, Proteobacteria e Actinobacteria[7]. Entretanto, a microbiota intestinal do adulto é dominada por dois filos: Bacteroidetes e Firmicutes[2,8].

Durante muitos anos acreditou-se que o bebê era estéril ao nascimento e que a colonização microbiana do intestino começava imediatamente após o nascimento[9]. No entanto, estudos recentes mostram que a colonização pode começar antes do nascimento com colonização microbiana na placenta, no

fluido amniótico e no cordão umbilical[10]. Além disso, outros fatores contribuem para a colonização intestinal precoce, como a idade gestacional ao nascimento e o tipo de parto[9,10]. No parto vaginal normal, a microbiota vaginal desempenha um papel fundamental na colonização do bebê ao nascer[11], no qual anaeróbios facultativos, como enterobactérias, enterococos e lactobacilos são os primeiros colonizadores[12]. Outra grande influência na microbiota neonatal é a dieta infantil, pois lactentes alimentados no peito têm microbiota enriquecida em *Lactobacillus , Staphylococcus* e *Bifidobacterium*, em contrapartida, os bebês alimentados com fórmula têm microbiota dominada por *Roseburia , Clostridium ,* e *Anaerostipes*[10]*.* Alguns estudos sugerem que a microbiota intestinal atinge composição relativamente estável e semelhante aos adultos, por volta dos 3 anos de idade[12-14], enquanto outros estudos demonstram que o desenvolvimento é contínuo desde a infância até a adolescência[15-17]. Além disso, novas alterações aparecem na senescência, diferindo a microbiota de idosos da microbiota central e dos níveis de diversidade de adultos mais jovens[13,14].

A composição da microbiota intestinal é influenciada por fatores intrínsecos (fatores genéticos e imunológicos) e extrínsecos (uso medicamentos, dieta e estilo de vida)[5,6]. A microbiota é responsável por proteger a mucosa intestinal contra microrganismos patógenos, modular o sistema imunológico, sintetizar algumas vitaminas, degradar os componentes não digeríveis da dieta e produzir metabólitos, tais como os ácidos graxos de cadeia curta, que são as principais fontes de energia para os colonócitos[18-21]. Para assegurar esses benefícios à saúde do hospedeiro, a microbiota deve

permanecer em homeostase, ou seja, em equilíbrio entre as bactérias comensais e patogênicas[22].

Como já citado, o metabolismo e a composição da microbiota podem ser influenciados pela dieta[5,18-20,22-24]. A ingestão de microrganismos probióticos, ingredientes prebioticos ou combinações simbióticas está fortemente relacionada com a modulação da microbiota intestinal[5,18-20,22-25]. Como prebioticos entende-se como um substrato que é utilizado seletivamente por microrganismos hospedeiros conferindo benefício à saúde. A seletividade não significa necessariamente efeitos em apenas um grupo microbiano; pode se estender a vários grupos microbianos, mas não a todos. Além disso, os microrganismos afetados e os metabólitos produzidos estão associados à efeitos fisiológicos, tais como inibição de patógenos, estimulação imunológica, redução dos níveis lipídicos no sangue, produção de metabolitos que influenciam a função cerebral, energética e cognição, biodisponibilidade mineral, entre outros[26].

Um dos componentes dietéticos prebióticos, capazes de modificar microbiota intestinal, são as fibras alimentares. Isso porque, as fibras provenientes da dieta são compostas, principalmente, por polissacarídeos e em menor parte por oligossacarídeos que não são digeridos pelas enzimas intrínsecas do estômago e do intestino humano. Por isso, grande parte das fibras que passam pelo trato gastrointestinal é degradada pela microbiota intestinal, ocorrendo a formação e liberação de ácidos graxos de cadeia curta (ácido acético, ácido propiônico e ácido butírico) como produto do metabolismo microbiano[26,27].

Ainda, estudos sugerem que alimentos ricos em compostos fenólicos também podem atender aos critérios de prebióticos, tendo um impacto significativo sobre a microbiota intestinal, uma vez que grande parte (90-95%) dos polifenóis da dieta não são absorvidos no intestino delgado[26,28-30]. As bactérias presentes no cólon atuam na bioconversão destes compostos, dando origem a uma variedade de metabólitos bioativos, entre estes, os ácidos graxos de cadeia curta, que promovem a manutenção da homeostase intestinal[31].

Os ácidos graxos de cadeia curta exercem funções no metabolismo humano. Estão relacionados à síntese dos ácidos graxos de cadeia longa (lipogênese), cetogênese (síntese de corpos cetônicos), inibição da síntese de colesterol e atuam como substrato para a via de formação do piruvato da gliconeogênese. Além de serem uma das principais fontes de energia para os colonócitos (mucosa intestinal), os quais estimulam a proliferação celular do epitélio e intensificam a absorção de sódio e água[26,32-36].

Compostos Fenólicos

Compostos fenólicos são substâncias amplamente distribuídas na natureza com mais de 8.000 compostos polifenólicos já identificados em plantas, incluindo flavonoides, álcoois fenólicos, lignanas, estilbenos e ácidos fenólicos[37,38]. Podem ser pigmentos, que dão a aparência colorida aos alimentos, ou produtos do metabolismo secundário, normalmente derivados de reações de defesa das plantas contra agressões por radiação ultravioleta, radicais livres e agentes patogênicos[37,39,40]. De acordo com estudos epidemiológicos quando ingeridos, esses compostos exercem proteção ao organismo humano contra algumas doenças[39,41].

Os principais polifenóis encontrados na dieta humana são os ácidos fenólicos e os flavonoides, os quais representam aproximadamente 2/3 e 1/3 do total da ingestão diária de polifenóis, respectivamente[42,43]. Os flavonoides compõem um grupo de polifenóis com estrutura básica comum compreendendo 15 átomos de carbono (C6-C3-C6) no seu núcleo fundamental, com dois anéis aromáticos (anel A e B) unidos por uma cadeia de três átomos de carbono, que pode ou não formar um terceiro anel (anel C) (Figura 1)[44-46].

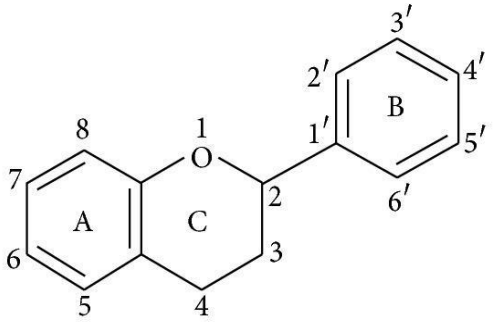

Figura 1**.** Estrutura básica dos Flavonoides[46]

Uma grande diversidade estrutural ocorre nos flavonoides em função das pequenas modificações químicas ocorridas na estrutura básica destes compostos, que podem ser por meio de hidroxilação, metilação, acilação, glicosilação, hidrogenação, malonilações e sulfatações[47]. Com essas derivações os flavonoides dietéticos são classificados em 6 subclasses: flavonóis, flavonas, flavanonas, catequinas, isoflavonas e antocianidinas (Figura 2)[45].

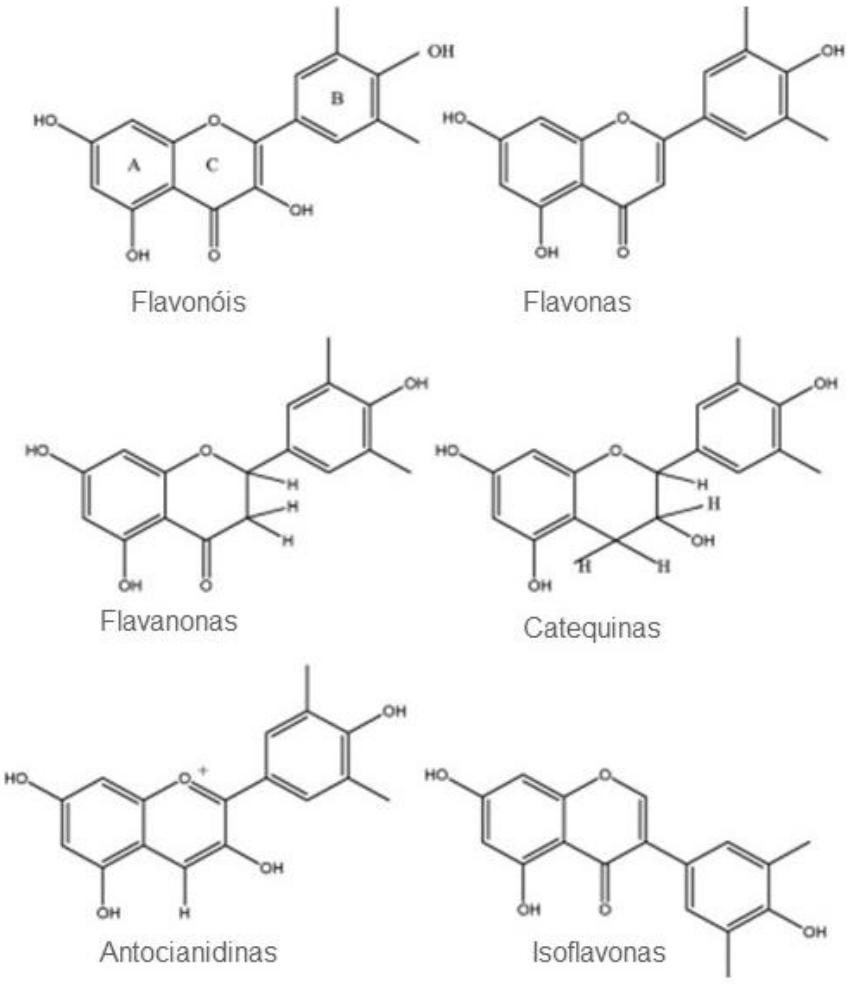

Figura 2. Estrutura molecular dos flavonoides[45]

Nas frutas cítricas, as flavanonas são a classe mais comum de flavonoides, sendo aproximadamente 95% dos flavonoides totais das frutas. Na laranja as flavanonas estão presentes predominantemente nas formas glicosídicas, naringina e hesperidina; mas também são encontradas nas formas agliconas, naringenina e hesperitina[38,48]. A forma glicosídica é caracterizada pela presença de uma cadeia de três carbonos saturados e um átomo de oxigênio no C4, sendo geralmente glicosiladas por um dissacarídeo na posição C7[46]. Este dissacarídeo pode ser uma neohesperidose, que confere o sabor

amargo, encontrada na naringina em *grapefruit*, ou uma rutinose, insípida, encontrada na hesperidina em laranjas[43].

As flavanonas contém vários glicosídeos, sendo os principais nas laranjas, da hesperidina: hesperitina-7-O-rutinosídeo, hesiperitina-7-O-glicuronídeo; e da naringina: naringenina-7-O-rutinosídeo, naringenina-7-O-glicuronídeo, naringenina-4-O-glicuronídeo[46,49,50] (Figura 3).

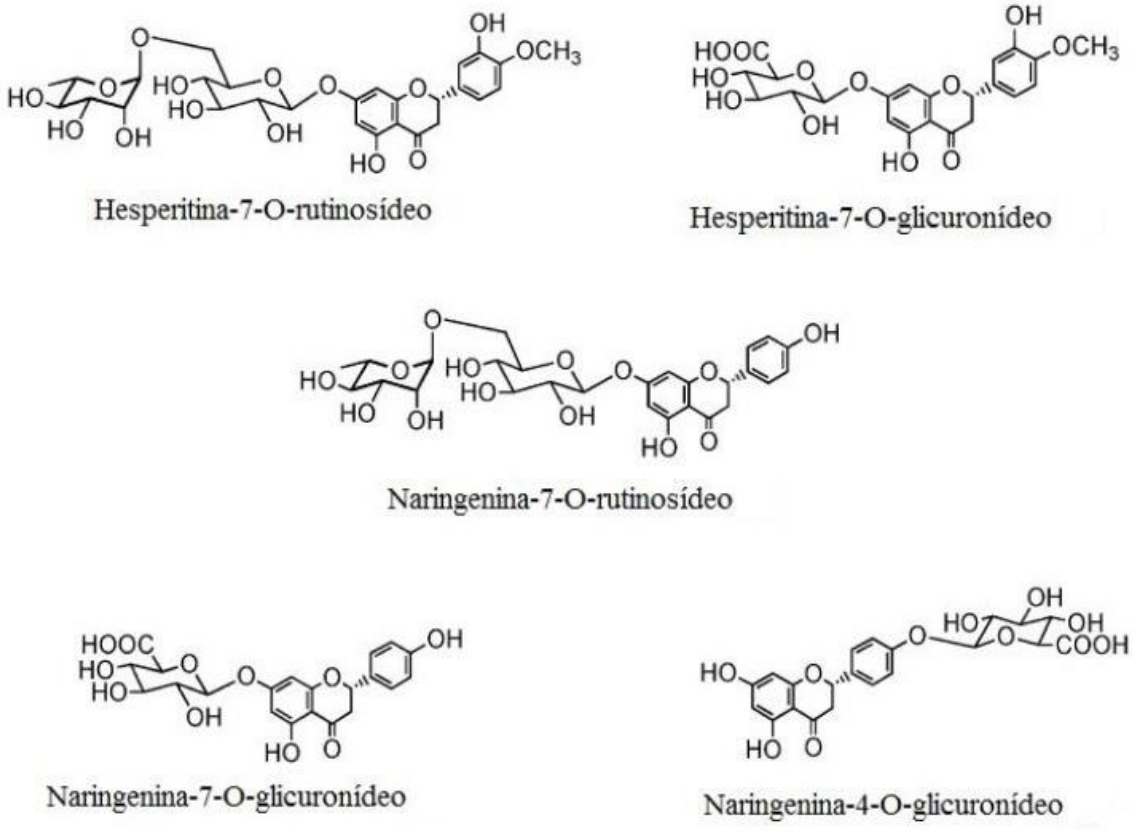

Figura 3. Estruturas glicosiladas das flavanonas[50]

A composição das flavanonas na laranja varia de acordo com a parte do fruto, sendo que as partes sólidas da fruta, como o albedo (parte branca) e as membranas que separam os segmentos, são os mais ricos em flavanonas, em comparação com as vesículas de suco (polpa). Além disso, os estágios de maturidade e pós-colheita também afeta os níveis de flavonoides cítricos[50-52].

Metabolismo e Biodisponibilidade das Flavanonas

A garantia da eficácia biológica das flavanonas depende de serem absorvidas e metabolizadas, e terem os metabólitos bioativos distribuídos aos tecidos sensíveis à sua ação[53]. Do ponto de vista nutricional, a biodisponibilidade representa os efeitos gerais da absorção, distribuição, metabolismo e excreção de um nutriente presente nos alimentos[52].

As flavanonas são resistentes ao suco gástrico do estômago, sendo absorvidas no trato gastrointestinal após extensiva metabolização por enzimas intestinais e hepáticas e deglicosilação por enzimas de bactérias intestinais, desde o intestino delgado e terminando no intestino grosso[39,54,55]. Além disso, alguns fatores podem interferir na absorção das flavanonas, entre os quais está a composição química, pois a molécula de glicose é um dos principais determinantes do local de absorção e da biodisponibilidade dos flavonoides, sendo reconhecido que a biodisponibilidade dos monoglicosídeos de flavonoides é muito maior do que os rutinosídeos[56-58]. Este comportamento se deve ao intestino delgado humano conter β-glicosidases capazes de hidrolisar glicosídeos de flavanonas, mas não rutinosídeos (ramnoglucósidos), que precisam alcançar o cólon e serem expostos a α-ramnosidase provenientes de algumas das bactérias residentes[59].

Primeiramente, após a ingestão do suco de laranja, por exemplo, os monoglicosídeos de flavanonas são absorvidos no intestino delgado após hidrólise pela lactase-florizina hidrolase, e então, a aglicona livre, por ser hidrofóbica, difunde-se através das células epiteliais por transporte passivo ou por difusão facilitada[60,61]. Porém, algumas flavanonas conjugadas à glicose podem ser absorvidas através do transportador de glicose sódio dependende

(SGLT1) para o interior do enterócito e deglicosiladas pelas β-glicosidases presentes intracelularmente. Ambas as vias de absorção originam agliconas intracelulares que, posteriormente, são metabolizadas em conjugados glicurônicos ou sulfatados e liberados na corrente sanguínea[58,60]. Entretanto, a maior parte dos glicosídeos não são hidrolisados ou não apresentam afinidade pela SGLT1, tais como os rutinosídeos. Estes são transportados na forma intacta em direção ao cólon, onde são hidrolisados pela α-ramnosidase, produzida pela microbiota do local, liberando a forma aglicona, que pode então ser absorvida pelo intestino[28,58,61,62].

No intestino delgado e no epitélio do cólon, os flavonoides poderão sofrer reações de conjugação, como a glicuronidação e a metilação[28,63,64]. Uma vez absorvidas, as flavanonas são conduzidas ao fígado pela circulação entero-hepática ligadas à albumina ou via linfática, onde sofrerão outras biotransformações envolvendo reações de fase I (oxidação, redução, hidrólise) e, principalmente, reações de fase II (metilação, sulfatação e glicuronidação), formando metabólitos com ácidos glicurônicos através da UDP-glicuronosiltransferases (UGTs) e grupos sulfatos por meio das sulfotransferases (SULTs)[28,38,63]. A glicuronidação e a sulfatação são especialmente importantes porque aumentam substancialmente o peso molecular e a solubilidade do composto original para que possa ser eliminado através da urina ou da bile[24,65-60].

Por fim, esses metabólitos conjugados aos glicuronídeos e sulfoglicuronídeos tanto irão para a corrente sanguínea e exercer a função sistêmica, quanto serão excretados pela bile e/ou urina, dependendo do metabólito formado (Figura 4)[28,69]. Os compostos não absorvidos, bem como

aqueles excretados juntamente com a bile no lúmen intestinal, seguem para o cólon onde serão catabolizados pela microbiota, produzindo uma variedade de compostos fenólicos de baixo peso molecular[70-72].

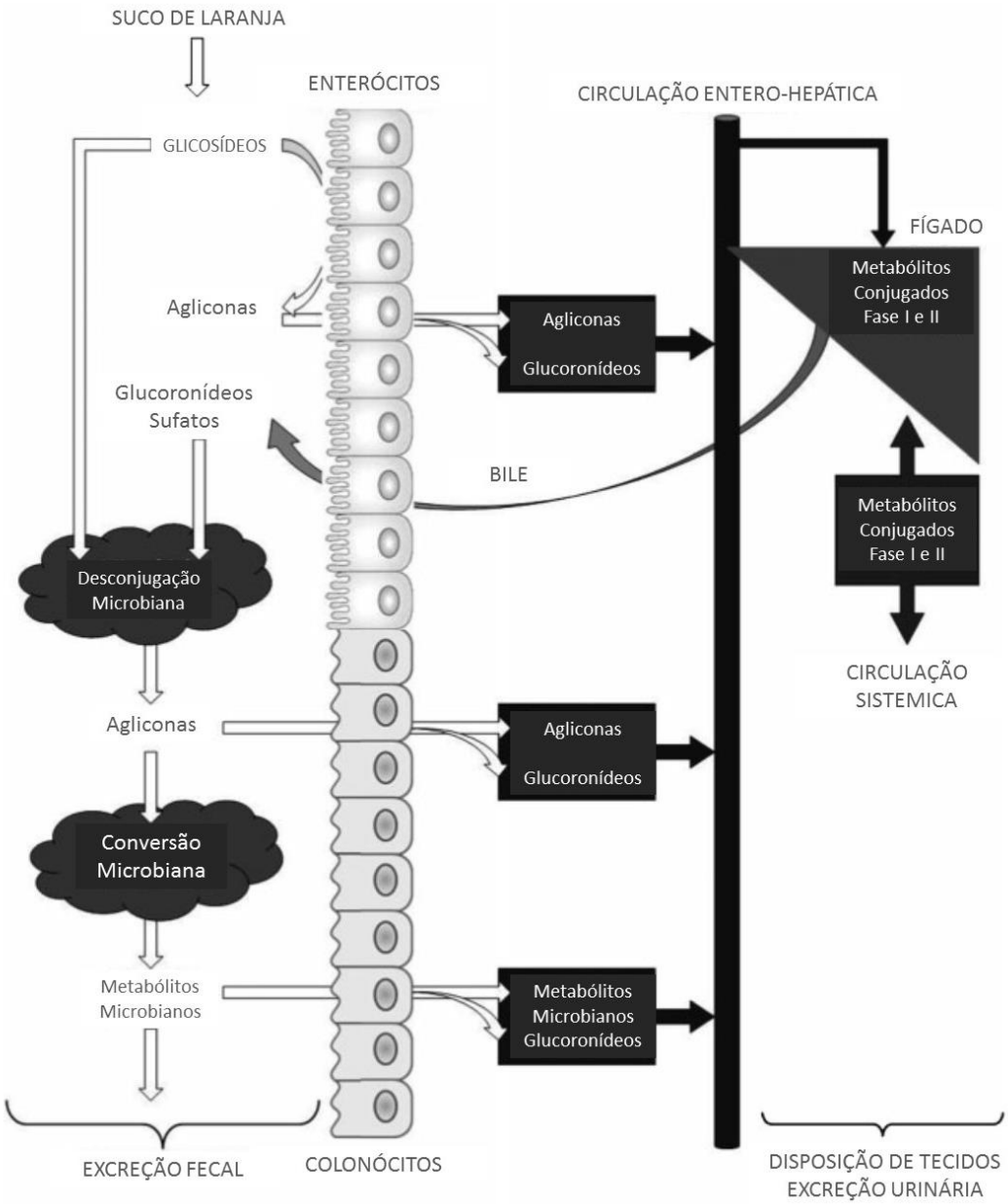

Figura 4. Metabolismo das flavanonas adaptado[28]

Devido a via de absorção distinta das flavanonas, a meia vida desses compostos também é diferente. A concentração sanguínea máxima dos compostos produzidos no cólon tem sido captada em média 6 h após a ingestão do suco de laranja[58,69,73]. Em contraste, os compostos absorvidos no intestino delgado, local onde a absorção ocorre mais rapidamente, a meia vida é de aproximadamente 1 h[58]. A excreção urinária das flavanonas ocorre principalmente durante as 24 h após a ingestão com um pico entre 6 a 12 h. Estudos anteriores sugerem, no entanto, que é suficiente a coleta de urina em um único dia, porque a variação diária no nível individual é limitada[74]. Após o consumo de suco de laranja, a excreção relativa urinária de hesperitina pode variar de 1,7 até 8,1%[50,59,70,73,75-79]. A excreção de naringenina após o consumo de suco de laranja é encontrada entre 0,7 até 20,3% da ingestão[50,59,70,73,75-79]. A excreção fecal das flavanonas é bem citada em estudos experimentais e *in vitro*. Parte das flavanonas são excretadas na forma inalterada, sendo estimado 33% para hesperitina e 15% para hesperidina. Além disso, também é encontrado nas fezes a naringina, naringenina e naringenina glicuronídeo. Adicionalmente, nas fezes humanas, devido a extensa metabolização da microbiota, são encontrados os catabólitos fenólicos capazes de elevar a recuperação das flavanonas a 100%[70,71,78,80].

Laranja e Suco de Laranja

A laranja é uma das frutas mais cultivadas em todo o mundo, produzida pela laranjeira (Citrus x sinensis), uma árvore da família Rutaceae de porte médio e copa densa, arredondada e perene. Essencialmente, a laranja é composta por diversas vesículas de suco protegidas por uma película de cera,

a casca (Figura 5). É na casca (flavedo) que estão as substâncias responsáveis pelo aroma e pela cor da fruta. Já a parte comestível é composta por segmentos que possuem vesículas de suco e pela parte sólida que é fonte de fibras e compostos bioativos (membranas e albedo), além de sementes[81,82]. Existem mais de 100 variedades de laranjas cultivadas pelo mundo. No Brasil, as mais comuns são as laranjas Bahia, Pêra, Rubi e Valência[81].

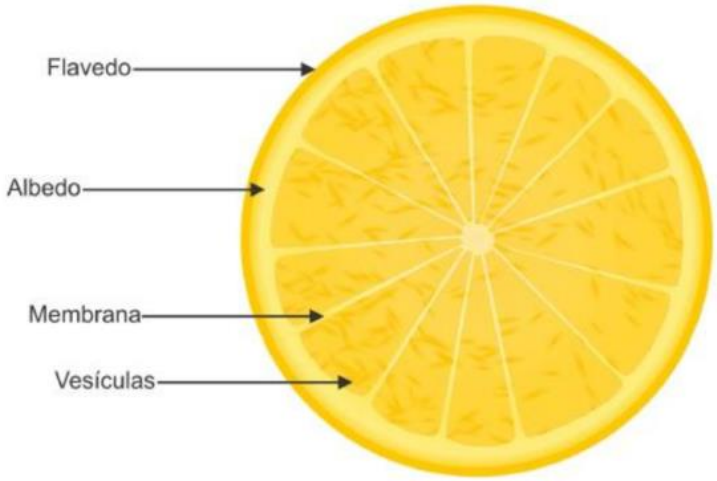

Figura 5. Partes que compõem a laranja[82]

Originada na Ásia, provavelmente na China, por volta de 4.000 anos, a laranja foi trazida para as Américas pelos exploradores no final dos anos 1400 e início de 1500. No Brasil, as mudas de laranjeiras foram introduzidas durante o período da colonização e com o tempo, a citricultura destacou-se em vários estados. Posteriormente, em Araraquara no ano de 1950, estendendo-se ao norte e noroeste do Estado de São Paulo[83].

Atualmente, o país responde por 34% da laranja e 56% do suco produzido em todo o mundo, considerando a média das últimas cinco safras, sendo 97% dessa produção destinada à exportação. Ainda, o Brasil é responsável por 76% de participação no comércio mundial de suco de laranja, consolidando-se como o mais importante fornecedor global desse produto[84]. Entretanto o consumo de laranja no Brasil, identificado pela POF 2008-2009[85], é bem menos que uma laranja por dia. Enquanto uma laranja média tem 180 g[86], o consumo médio encontrado foi de 20 g/d[85]. Em adição, o consumo de suco de laranja no Brasil, saiu de 42 mil toneladas de Suco Concentrado Congelado (FCOJ, sigla em inglês), equivalente a 66º Brix em 2003, para 69 mil toneladas em 2016[84]. Porém, nosso consumo ainda é baixo se comparado, por exemplo, com os Estados Unidos, que apresentou um consumo de 624 mil toneladas de FCOJ equivalente a 66º Brix em 2016[84].

Na fabricação do suco de laranja existem diferentes processos de extração e processamento do suco, e estes influenciam na concentração dos nutrientes e nas quantidades dos flavonoides[51,75]. Entre os sucos de laranja prontos para o consumo encontra-se o NFC, do termo inglês “non-frozen concentrated”. É um suco pasteurizado que não passou pelo processo de concentração ou diluição durante a produção, e ainda, não é acrescido de açúcares. Além disso, em termos de sabor é o que mais se assemelha ao suco fresco, segundo o consumidor brasileiro[81].

O suco de laranja fresco apresenta maiores quantidades de vitamina C e ácido fólico, aproximadamente 125 mg e 76 µg, respectivamente, enquanto que os sucos pasteurizados apresentaram 86 mg de vitamina C e 46 µg de folato. Por outro lado, os sucos integrais e industrialmente pasteurizados apresentam

aproximadamente o dobro da quantidade de flavonoides cítricos quando comparados ao suco fresco[51]. Isso porque, no processo de extração do suco, são usadas também as partes sólidas das laranjas, tendo maior aproveitamento do fruto[81].

Efeitos Benéficos do Suco de Laranja

Estudos anteriores de suplementação com suco de laranja, ou com flavanonas na forma isolada, tem demonstrado várias propriedades de proteção à saúde. As ações são relatadas como: antioxidantes, hipolipidêmicas, anti-hipertensivas e anti-inflamatórias[87-91].

Estudo *crossover* com 16 indivíduos (homens e mulheres) mostrou que placebo com hesperidina e suco de laranja, aumentou significativamente a capacidade antioxidante no soro sanguíneo. Evidenciando que os compostos fenólicos da laranja contribuem diretamente para a proteção oxidativa pós-prandial, que é um importante contribuinte para o desenvolvimento de doenças crônicas[88].

Pesquisa que comparou o perfil lipídico de homens e mulheres que consumiam suco de laranja por pelo menos 12 meses a indivíduos não consumidores, encontrou concentrações de colesterol total, LDL-c, ApoB e a razão LDL/HDL significativamente menores nos consumidores de suco de laranja do que nos indivíduos não consumidores. Concluindo, que o consumo de suco de laranja ao longo tempo está associado a baixos fatores de risco para doença cardiovascular[87]. Além disso, um estudo com consumo menor (4 semanas) em homens de meia-idade e com excesso de peso, evidenciou a diminuição da pressão arterial diastólica[89].

Outro estudo demonstrou que o consumo oral diário durante três semanas de hesperidina melhora a função endotelial, reduz a circulação de biomarcadores da inflamação e altera favoravelmente o perfil lipídico em indivíduos com síndrome metabólica, mostrando o efeito anti-inflamatório da hesperidina isolada[90].

Pacientes com hepatite C sob terapia antiviral que beberam 500 mL de suco de laranja diariamente por 8 semanas tiveram uma melhora da capacidade antioxidante, perfil lipídico, marcadores inflamatórios do fígado e nível de aspartato transaminases, quando comparados aos pacientes nas mesmas condições, mas que não consumiam suco de laranja. Os resultados indicam que o suco de laranja é um alimento adequado para pacientes com hepatite C crônica, com a vantagem da falta de toxicidade e alta oferta de antioxidante dietético[91].

Interações entre Suco de Laranja e Microbiota Intestinal

Evidências de estudos em humanos tem mostrado que alguns compostos fenólicos podem contribuir para a manutenção da saúde intestinal, preservando o equilíbrio microbiano através da estimulação do crescimento de bactérias benéficas e inibição de bactérias patogênicas, exercendo efeitos prebióticos[28,29,92].

Estudo *in vitro* mostrou o potencial envolvimento da microbiota colônica na biodisponibilidade global dos flavonoides do suco de laranja. Foi observado aumento na quantidade de catabólitos através da produção de ácidos fenilpropiônicos e subsequentes conversões hepáticas que levam ao ácido hipúrico e os análogos hidroxilados[93]. Além disso, já foi demonstrado

o potencial enzimático de *Bifidobacterium longum* e *Lactobacillus rhamnosus*. Ambas podem estar envolvidas no catabolismo do cólon das flavononas presente no suco de laranja[94]. Ainda, estudo *in vitro*, demonstrou em Simulador do Ecossistema Microbiano Intestinal Humano (SHIME®) que o suco de laranja teve influência positiva na microbiota humana, sugerindo um efeito prebiótico seletivo deste alimento na mucosa intestinal[95].

Estudo *in vivo* identificou metabólitos da naringina na urina e fezes de ratos e cachorros, além da identificação de ácidos graxos de cadeia curta decorrente da biotransformação da naringina[96]. Após suplementação de hesperitina em ratos foi identificado aumento da excreção de ácidos graxos de cadeia curta, além da diminuição da proporção de *Clostridium* XIVa nas fezes[72].

Em humanos, estudo examinou os efeitos de suplementação da *Bifidobacterium longum*, administrada por via oral e biodisponibilidade de flavanonas. A suplementação do probiótico mostrou efeito positivo, aumentando a biodisponibilidade de flavanonas do suco de laranja[97]. Um ensaio clínico recente, com mulheres que consumiram suco de laranja por dois meses, apresentou melhorou do colesterol, da glicose e da sensibilidade à insulina. Além disso, foi observada uma modulação positiva na composição da microbiota e na atividade metabólica, com o aumento de Bifidobacterium spp. e Lactobacillus spp e produção de ácido graxos de cadeia curta[98].

Entretanto, as informações disponíveis ainda são limitadas sobre quanto os componentes dietéticos do suco de laranja, podem influenciar a microbiota intestinal. Até o momento, poucos estudos se concentraram nas interações entre ingestão do suco de laranja e microbiota.

Dado estas evidências, formulou-se a hipótese de que o consumo de suco de laranja pode melhorar a colonização microbiana, modulando-a beneficamente, além de controlar parâmetros antropométricos e bioquímicos. Com isso, este estudo teve o objetivo de explorar a associação entre a ingestão diária de suco de laranja e microbiota intestinal. Para isso foi avaliado a composição da microbiota intestinal, recuperação urinária das flavanonas e parâmetros nutricionais e bioquímicos, após 60 dias de consumo de suco de laranja em mulheres.

Capítulo 1.

Daily consumption of orange juice modulated intestinal microbiota and improved glucose and lipids metabolism in women: a controlled clinical trial

Daily consumption of orange juice modulated intestinal microbiota and improved glucose and lipids metabolism in women: a controlled clinical trial

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ABSTRACT

Orange juice is a natural source of sugars, vitamins, minerals and flavanones and is associated with health benefits. In addition, orange juice has selective activity on intestinal microbiota in vitro improving its quality. This study investigated the impact of habitual consumption of orange juice 100% pure (OJ) on the intestinal microbiota and metabolic outcomes in a controlled clinical trial. Biochemical, anthropometric and dietary parameters of ten women were monitored for 120 days: (1) Diet citrus-free period: regular diet free of OJ for 30 days (1st to 30th day); (2) Orange Juice period: regular diet supplemented with orange juice for 60 days (31st to 90th day); (3) Washout period: regular diet free of orange juice for 30 days (91st to 120th day). Samples of blood, urine and faeces were taken at the beginning and every 30 days. Urinary metabolites were identified by UHPLC and the microbiota composition was evaluated by RNAr 16S. Analysis of the biochemical parameters at the end of the orange juice period showed a decrease in glucose (-6.5%), insulin (-33%) and insulin resistance (-44%). There was also reduction of total cholesterol (-14%), LDL (-16%) and triglycerides (-30%). After washout period (120th day) all biochemical parameters returned to the initial values. It was no changes on body weight or body fat during the experiment time. Intestinal bacteria *Bifidobacterium*, *Lactobacillaceae* and *Streptococcaceae* increased with daily consumption of orange juice (90th day), and it was detected negative correlations among *Bifidobacterium*, *Coriobacteriaceae*,

Lachnospiraceae and *Lactobacillaceae* and blood serum glucose, triglycerides, cholesterol, LDL-C, insulin and HOMA-IR, and a positive correlation with HDL-C. In conclusion, orange juice showed a prebiotic effect by positively modulate the intestinal microbiota and the glucose and lipid metabolic profile in healthy young women.

Keywords: Prebiotic, Intestinal Microbiota, Orange Juice100% pure, Metabolic Biomarkers, Body Composition

INTRODUCTION

A significant number of recent epidemiological studies have shown that consumption of pure fruit juice is not associated with body weight or fat gain. In addition, fruit juices that contribute with free sugars in the diet, add valuable vitamins, minerals and bioactive compounds of great impact on maintaining health (Hyson, 2015; Rampersaud & Valim, 2017; Bellisle et al, 2018). Similarly, observational studies have shown that fruit juices are not contribute to development of chronic diseases such as diabetes e cardiovascular diseases (Aptekmann & Cesar, 2013; Khan et al, 2018). A recent update has shown that the phenolic compounds and flavonoids of fruits confer the anticancer and cardiovascular protection, and this is due to the antioxidant and anti-inflammatory activity of these compounds (Yi et al, 2017).

Flavanones are the predominant flavonoids of citrus fruits, and orange juice is the main dietary source of hesperidin (Kawaii et al, 1999; Brett et al, 2009). Numerous investigations have shown that these compounds in orange juice 100% pure (OJ) improved the antioxidant and anti-inflammatory status, glucose and lipid metabolism in obese patients and patients with metabolic syndrome, and also promote maintenance of body composition (Dourado & Cesar, 2015; Silveira et al, 2015; Yi et al, 2017; Aptekmann et al, 2010,).

Orange juice flavanones are found as glycosylated molecules (hesperidin and naringin) that are degraded in aglycones (hesperetin and naringenin) by intestinal bacteria (Kanaze et al, 2007; Actis-Goretta et al, 2015). Flavanones have been shown to be poorly bioavailable with recoveries in relatively small amounts related to the dose ingested by systemic circulation, as glucuronides and sulfated metabolites (Manach et al, 2003; Mullen et al, 2008; Borges et al, 2013; Roowi et al, 2009; Pereira-Caro et al,

2014). However, in the large intestine both hesperitin and naringenin are broken down by microbiota into compounds, such as short chain fatty acids (SCFA) that perform various biological functions (Unno et al, 2015). SCFA may promote the maintenance of intestinal homeostasis and exert essential functions in human metabolism (Duñas et al, 2015; Mosele et al, 2015; Parkar et al, 2013).

It is estimated that the gastrointestinal tract is inhabited by 10^{11} cells per mL of luminal contents, comprising of 400 to 500 bacterial species (Scott et al, 2013). The main microbiota phyla are Bacteroidetes, Firmicutes, Proteobacteria and Actinobacteria (Barbosa et al, 2010). The microbiota is responsible for protecting the intestinal mucosa against pathogenic microorganisms, modulate the immune system and synthesize some vitamins (Lourens-Hattingh et al, 2001; Saad, 2006; Ríos-Covián et al, 2016). Therefore, it is important to maintain the homeostasis of the microbiota, since it can destabilize through stress, drug treatments and unbalanced diets (Lourens-Hattingh et al, 2001; Saad, 2006; Scott et al, 2013; Walsh et al, 2014; Ríos-Covián et al, 2016).

It is known that the metabolism and intestinal microbiota composition can be influenced by dietary standard, probiotic and prebiotic ingredients, and by bioactive compounds (Lourens-Hattingh et al, 2001; Saad, 2006; Scott et al, 2013; Walsh et al, 2014; Ríos-Covián et al, 2016). Evidence from human studies has shown that some polyphenols may contribute to maintain the intestinal health, preserving microbial balance by stimulating the growth of beneficial bacteria and inhibiting pathogenic bacteria (Kemperman et al, 2013; Wan et al, 2018).

Previous study showed in the Human Intestinal Microbial Ecosystem Simulator (SHIME®) that orange juice positively influenced the human microbiota, suggesting a selective prebiotic effect of this food on the intestinal mucosa (Duque et al, 2016). A recent clinical trial, made in our laboratory, showed that the administration of orange juice for two months improved cholesterol, glucose and insulin sensitivity; Also, it was detected a positive modulation on the microbiota composition and metabolic activity, with increasing of *Bifidobacterium* spp. and *Lactobacillus* spp and SCFA production. These results evidenced that the consumption of orange juice improved the microbial colonization, with positive consequences on anthropometric and biochemical parameters (Lima et al, 2019).

Based on the evidences, it was hypothesized that consumption of orange juice may improve microbial colonization, in addition to controlling changes in glucose and

lipid metabolism. This study aimed to explore the impact of daily intake of orange juice on the intestinal microbiota and biochemical markers in women.

MATERIALS AND METHODS

Subjects

Ten healthy female volunteers were selected among students of Pharmacy School, UNESP, in Araraquara, Brazil. Inclusion criteria were women, 20-50 years, non-smokers and not vegetarians, without food allergy or intolerance, they were not taking hormones, dietary supplements, and medication for any gastrointestinal or metabolic disease, no probiotics or prebiotics in the last 3 months, and no antibiotics in the last 6 months. They did not regularly drink alcohol or perform intensive physical exercise. The study was approved by the Ethical Board of the Pharmacy School, UNESP (protocol #1,644,906), and the enrolled in the Clinical Trial Protocol Registration and Results System (ID: NCT03032861).

Orange juice

Commercial orange juice 100% pure was provided by a local producer (Citrosuco S.A., Matao-SP, Brazil). The juice stored in 2 L PET bottles at -20°C, and distributed weekly to volunteers, without label identification, and thawed under refrigeration before consume of 300 mL daily.

Physicochemical characteristics of the orange juice were determined according to AOAC International (1995). Flavanones were extracted from thawed and homogenized orange juice, and triplicate samples (5 mL) were centrifuged at 8583 x g at 12ºC for 30 min. Supernatants were collected and was spiked internal standard 7-

crystalline ethoxycoumarin (IS) and analyzed without further processing. The pellets were extracted with 10 mL of 80% DMSO, under stirring for 1 minute in the vortex. This solution was placed in an ultrasonic bath for 15 minutes and centrifuged at 8583 x g at 12ºC for 30 min. The supernatant was filtered and collected in a volumetric flask of 25 mL and the precipitate was again extracted. The supernatants were combined and the flask was completed with 80% (v/v) DMSO. After that, 10 mL of sample were cleared passing through C18-E Sep Pac cartridge Strata® (50 mg/1 mL) preconditioned with 10 mL of DMSO and 20 mL of milli-Q water. The flavanones were eluted from the cartridge with 20 mL of DMSO solution (400 µL) was filtered of through a syringe filter of 0.22 µm (Allcrom, São Paulo, BR) for UHPLC-UV-VIS analysis.

Study design

A controlled clinical study with temporal series intergroup design, which includes: (1) Diet citrus-free: 30th day, 30 days which included similar food pattern for all participants, without restriction of energy, avoiding rich sources of flavonoids, probiotics and prebiotics, and alcoholic beverages (1st to 30th day); (2) Trial with orange juice: 90th day, intervention of 60 days consuming 300 mL/d of orange juice (31st to 90th day); and (3) Trial without orange juice: 120th day, 30 days without orange juice (91st to 120th day) (Figure 1). All participants maintained the standardized diet and dietary restrictions during the 120 days. Data of nutritional assessment (anthropometric and dietetic history), sampling of blood serum were collected before the start and throughout the experimental period in total of 24 hours urine and feces.

Sample size

The sample size was calculated based on previous data from urine hesperitin after intake 100% orange juice (Tomás-Navarro et al, 2014). A minimum sample size of 9 participants per group was calculated with a confidence level of 95% ($\alpha = 5\%$) and a power of 80%. Considering the 10% dropout, the sampling was increased to 10 participants.

Nutritional assessment

Body composition

Body weight (kg), height (m), muscle mass (kg), fat mass (kg) and body fat percentage (%) were measured with bioimpedance (Inbody 720®), collected at Basal period and every 30 days until the experiment finishing. The body mass index (BMI) was interpreted according to World Health Organization (2000).

Dietary assessment

Food intake was estimated using 3-day food records, days non-consecutive, applied on the eve of experiment beginning (Basal period) and subsequently every 15 days until the experiment finishing. A food frequency questionnaire was also applied before consumption of orange juice.

The analysis of the intake data were calculated using the NUTRILIFE® Software based on Brazilian Food Composition Table (NEPA, 2011).

Blood collection, evaluation of metabolic biomarkers and antioxidant action

Volunteers fasting blood samples were made before Basal period, in 30^{th}, 60^{th}, 90^{th} and 120^{th} day of the experimental schedule. All samples were collected at local

clinical laboratory and analyzed for total cholesterol, HDL-C, LDL-C, triglycerides, glucose and insulin. Glycemic and insulin curves were performed after glucose load at 0, 30, 60, 90 and 180 min time schedule twice: at 30th day and after 90th day. Insulin resistance was estimated by HOMA-IR equation: HOMA1-IR = glucose (mmol/L) x 0.0555 x insulin (μU/mL)/22.5 (Mathews, 1985).

Lipid peroxidation was assessed by thiobarbituric acid reactive substances assay (Yagi, 1998) and total antioxidant capacity (TAC) by radical 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) assay (Re, 19999).

Urine collection

Sampling of total 24 hours urine were collected on before Basal eve period, 30th, 60th, 90th and 120th day of the experimental schedule. Urine was collected in a suitable container and kept at 4 °C until delivery to the laboratory. Total volume was recorded, the samples were homogenized, and followed by storage in a sterile plastic tube (10 mL) kept at -80°C until analysis.

For the metabolites extraction of flavanones in the urine, sample of each individual was adjusted to pH 3 with 5% formic acid. It was cleared passing through of C18-E Sep Pac cartridge Strata® (50 mg/1 mL) preconditioned with 10 mL of methanol and 20 mL of milli-Q water. Metabolites were eluted from the cartridge with 20 mL of methanol. The methanolic extract was filtered, centrifuged at 6860 x g for 20 min at 12ºC and the supernatant was collected. The whole procedure was repeated twice and the organic phases containing the metabolites were combined and dried under vacuum using centrifugal evaporator at 40ºC (SpeedVac SVC 200H, Savant, Holbrook, NY, USA). The resulting extracts were redissolved in 400 μL of DMSO (containing IS) and filtered through a syringe filter of (0.22 μm) for UHPLC-UV-VIS analysis.

Stool collection

Total 24 hours feces were collected in an appropriate container and kept at 4°C until delivery. The samples were performed on before Basal eve period, 30^{th}, 60^{th}, 90^{th} and 120^{th} days of the experimental schedule. The feces were homogenized, and a sample was stored in a sterile plastic tube (10 g) and kept at -80°C until analysis. Subsequently, the samples were lyophilized and sent for microbiological analysis employing 16S rRNA gene sequencing.

To start the DNA isolation, 700 μL of bead solution was added to the freeze-dried sample, and the next steps were performed according to the kit's manual. DNA isolation of each sample was performed using the "PowerLyzer@PowerSoil DNA Isolation Kit" (Qiagen, Valencia, USA).

After DNA isolation, the polymerase chain reaction I (PCR I) was conducted. V3 region (~ 190 bp) of the 16S rRNA gene was amplified using primers compatible with a Nextera Index Kit (Illumina) (NXt_388_F: 5′-TCGT C G GCAGCGTCAGATGTGTATAAGAGACAGACWCCTACGGGWGGCAGCAG-3′ and NXt_518_R: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATTA CCGCGGCTGCTGG-3′). PCR was performed using 12 μL of AccuPrime SuperMix II (Life Technologies, Camarillo, USA), 5 μL of genomic DNA (~ 20 ng/μl), and 0.5 μL of each primer (10 μM). Nuclease-free water was added to complete the volume to 20 μL. The following setup was used: 95°C for 2 min of initial denaturation, followed by 33 cycles of denaturation at 95°C for 15 s, annealed at 55°C for 15 s, followed by elongation at 68 °C for 30 s, final extension at 68°C for 4 min, and final cooling to 4°C (Williams, 2017).

To incorporate primers with adapters and indexes, a new PCR was performed (PCR II). PCR II reactions were performed using 2.0 μL of primers P5 and P7 (Nextera

Index Kit), 12 μL Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Tewksbury, USA), 2 μL PCR I product, and nuclease-free water for a total volume of 25 μL. The following setup was used: initial denaturation at 98°C for 1 min, followed by 13 cycles of 98°C for 10 s, annealing at 55°C for 20 s and elongation at 72°C for 20 s, final extension at 72°C for 5 min, and cooling to 4°C. After PCR II, the purification of the amplified fragments, along with adapters and tags was conducted through AMPure XP beats (Beckman Coulter Genomic, Indianapolis, USA) (Williams, 2017).

The sequencing was performed on the Illumina NextSeq instrument as part of flowcell using a 2 × 150-cycle MID output kit V2 (Illumina, San Diego, USA). The raw dataset of pair-ended reads and respective quality scores were merged and trimmed with settings, according to Williams et al (2017). Subsequent analysis steps were conducted using the Quantitative Insight Into Microbial Ecology (QIIME) opensource software package (1.7.0 and 1.8.0) (Caporaso, 2011). UPARSE pipeline was employed in order to purge the dataset of chimeric reads, as well as to construct de novo operational taxonomic units (OTUs). As a reference database, the green genes (13.8) 16S rRNA gene collection was used, as previously described by McDonald et al (2012). To normalize different depths of sequencing samples, the matrix abundance of taxonomic units of each sample were divided by the total number of pairings after cutting. For rarefied OTU tables (23,000 reads/sample), alpha diversity measures expressed with an observed species (sequence similarity 97% OTUs) value were computed. For this purpose, the alpha rarefaction workflow was employed.

UHPLC-UV-VIS-PDA analysis

Analyses of the orange juice flavonoids (hesperidin and naringin) and the metabolites of hesperitin and naringenin (hesperetin-7-glucoronide and hesperetin-3-

glucuronide and naringenin-7-O-glucuronide) in urine were analyzed by an adapted UHPLC method for maximum flavanone sensitivity (Silveira et al, 2014), using a Shimadzu® Ultra High Performance Liquid Chromatograph-Nexera X2 series system connected in parallel with UV-VIS SPD-M20A PDA detector. Compound separations were achieved with a Shim-pack XR-ODS III C18 column (2.0 x 200 mm) using linear gradients of acetonitrile/0.5% formic acid, initially composed of 10:90 (v/v), and increased in acetonitrile content to 30:70, 55:45, and then decreased to 10:90 (v/v) at 10, 17:30 and 20 min, respectively, at a flow rate of 0.40 mL/min and 0.10 mL injected sample. Data handling was done by LabSolutions software (Shimadzu Corporation, Japan). Detection and identifications of the metabolites were based on their characteristic UV spectra (285 nm). Flavonoids quantifications from orange juice were done by external calibration curves obtained by injecting different amounts of stock solution, containing the internal standard and all the compounds of interest. Metabolites quantifications from urine were made by peak area (PA)/μg conversion factors of authentic metabolite standards using integrated peak areas, obtained either in the scan mode for urine samples. Different amounts of external standards (hesperetin-7-glucoronide and hesperetin-3-glucuronide and naringenin-7-O-glucuronide) and internal standard 7-crystalline ethoxycoumarin were dissolved in DMSO to build external calibration curves.

Statistical analysis

Clinical characteristics were documented by descriptive statistics. All results are expressed as mean ± SD or SEM. The data distributions were tested for normality, and subsequently, analyzed by One Way Repeated Measures Analysis of Variance when normal or Friedman Repeated Measures Analysis of Variance when no normal, with

significance level of $p<0.05$, using Sigma Stat version 3.11 (Systat Software Inc., USA). A simple correspondence analysis was used to test the correlation between periods with and without orange juice and the microbiota composition using the Minitab Software (State College, USA) (Minitab 2010). Correlation analyses were made to correlate the changes of metabolic biomarkers with specific groups of bacteria using the Spearman correlation test. Spearman correlation test was conducted using the open-source RStudio software program (RStudio 2017). This program was also used to create a heatmap based on the relative abundances of different genera.

RESULTS

Subjects

The women's baseline characteristics were 28.5 ± 8.4 y, weight 63.4 ± 9.6 kg, and body mass index 24.1 ± 3.3 kg/m^2. Participants started the experiment with average BMI in the eutrophic group, although two participants were overweight (Table 1). Food intake was balanced, with the exception of vitamin C that was above recommendation (DRI, 2006). In addition, in the food frequency questionnaire was identified that the main sources of vitamin C were: orange in nature (5.4 times/week); orange juice (4.8 times/week); papaya (2.7 times/week) and broccoli (1.4 times/week). Among these sources of flavanones (Bhagwt et al, 2013), the main source cited was: orange in nature (5.4 times/week); orange juice (4.8 times/week); lemon - such as seasoning (4.3 times/week); tomatoes (3.3 times/week) and wine red (0.5 times/week). Regarding about biochemical tests, in all cases the results were within the desirable values (Table 1).

Orange juice composition

Nutritional composition was obtained from USDA (2018). Physicochemical characteristics found were within the stipulated standards for OJ (Table 2A).

The average of two flavanones (hesperidin and naringin) used in this study and the average flavanones content of pellets and supernatants, along with percent averages are listed in Table 2B. The flavanone glycosides, hesperidin and naringin, were identified by their elution times, UV characteristic and compared to authentic standards.

Nutritional assessment

Body composition throughout the experimental period are presented in Table 3. According the body mass index establish by WHO (2000), the participants presented on average eutrophic nutritional status. No differences were found between the variables of body composition throughout the experiment.

Dietary assessment showed that macronutrient (carbohydrate, protein and lipid) intake was close to macronutrient distribution recommendations (DRI, 2006), as well such as fiber consumption. Trial with orange juice: 90th day showed increase in vitamin C intake by 75% in relation to Diet citrus-free: 30th day. In Diet citrus-free: 120th day there was a reduction of 39% of vitamin C (Table 4).

Evaluation of metabolic biomarkers and antioxidant action

All subjects showed blood serum biochemical measures within the reference values for all periods of the experiment, compatible with apparent health. In 90th day there was reduction of glycemia (-6.3%), insulin (-33%), HOMA-IR (-44%), triglycerides (-30%), total cholesterol (-14%) and LDL-C (-16%) compared to the 30th day values (Table 3). After 30 days of washout (120th day) there was an increase on

glycemia (6.5%), insulin (38%), and HOMA-IR (51%), triglycerides (21%), total cholesterol (12%) and LDL-C (4,9%). In relation to antioxidant capacity there was increase of 0.5%, and increased by 14% to lipid peroxidation in 90th day compared to values of 30th day. The area under the curve (AUC) and the maximum concentration (Cmax) of the glycemic and insulin curves are presented in Figure 2. The AUC and Cmax of insulin decreased by 16% ($p < 0.001$) in 90th day (Figure 2).

Urine Metabolites

In the present study, analyses of the flavanones metabolites were evaluating by UPLC analyses of extracts of urine samples obtained over 24 h period for each human subject. Urine samples showed similar amounts of hesperetin-3'-O-glucoronide, hesperetin-7-O-glucuronide and naringenin-7-O-glucuronide at the Basal period, 60th and 90th day of the experiment. But after volunteers have stopped to eat or drink any source of citrus fruits, it was no detected metabolites in the urine for any subjects (Table 5). Metabolites were identified as hesperetin-7-0-glucuronide and hesperetin-3'-0-glucuronide respectively, by comparisons with authentic standards. The percentages of excretion/absorption estimated were within the values found in the literature.

Bioavailability was estimated as the ratio of 24-hour urinary excretion to the oral dose, expressed as a percentage of the oral dose of parental flavanones (Kanaze et al, 2007).

Microbiota composition

Comparing the microbiota of healthy women before and after consumption of OJ showed a separation into two different clusters. Orange juice change the intestinal bacterial community (Figure 3).

Figure 4 shows the main bacterial phylum determined in the microbiota from women before and after consumption of orange juice. A relative abundance high of Firmicutes, followed by Actinobacteria, and Bacteroidetes was observed in 30th day. The OJ consumption stimulated the increase in Actinobacteria as well as a decrease in Firmicutes, Bacteroidetes and Proteobacteria.

As observed in Figura 5, the increase in Actinobacteria in 90th day was mostly due to the abundance of the *Bifidobacterium*. There was a decrease in Bacteroidetes, after the ingestion of orange juice, with greater contribution of the *Bacteroidaceae* and *Prevotellaceae*. In Firmicutes the most present families in the microbiota were *Lachnospiraceae, Lactobacillaceae, Ruminococcaceae* and *Streptococcaceae. Lachnospiraceae* was predominant before and after consumption of orange juice (Figure 6). There was a small increase in the *Lactobacillaceae* and *Streptococcaceae*. Proteobacteria decreased after the OJ (90th day), by decrease of their only family, *Enterobacteriaceae*.

Correlation analysis was performed to identify the bacteria related to changes biochemical parameters (Figure 7). The relative abundance of *Bacteroidaceae, Porphyromonadaceae, Prevotellaceae, Ruminococcaceae* and *Enterobacteriaceae* showed positive correlations with glucose, total cholesterol, triglycerides, LDL-C, insulin and HOMA-IR, and a negative correlation with HDL-C. *Bifidobacterium, Coriobacteriaceae, Lachnospiraceae* and *Lactobacillaceae* indicated a negative correlation with glucose, total cholesterol, triglycerides, LDL-C, insulin and HOMA-IR, and a positive correlation with HDL-C.

DISCUSSION

In the present study, daily consumption of OJ not influenced the body weight or body composition of healthy women, but improved glycides and lipids metabolism, associated with increase of antioxidant status. Orange juice daily consumption increased the intake of vitamin C, but not changed the energy balance or macronutrients intake in the diet. Supplementation of OJ contribute to the appearance of citrus flavanones on the urinary excretion, and increased the bioavailability of these compounds, which are associated with the benefits on the lipids and glycides metabolism. The microbiota pattern changed after the consumption of orange juice, showing an increase in the phylum Actinobacteria and decrease of Firmicutes, Bacteroidetes and Proteobacteria. The study of association between metabolic biomarkers and microbiota has showed that glucose, insulin, HOMA-IR, triglycerides, total cholesterol and LDL-C are positively correlated with the families of *Bacteroidaceae*, *Porphyromonadaceae*, *Prevotellaceae*, *Ruminococcaceae* and *Enterobacteriaceae* and negatively with the families of *Bifidobacterium*, *Coriobacteriaceae*, *Lachnospiraceae* and *Lactobacillaceae*. These last findings showed that orange juice had a prebiotic effect on the volunteers' microbiota, improving markers of lipid and glucose metabolism.

The dietary intake of the women from this study was energy and nutrients balanced and included good amounts of fruits and vegetables. Therefore, no advice was needed to improve dietary pattern. After 12 weeks of follow-up, no changes were observed in proteins, carbohydrates, total lipids, cholesterol, saturated fatty acids and fiber intake. As expected, consumption of orange juice increased vitamin C intake 1.7 times, and 3.4 times than the daily recommendation (RDA), without exceed the upper limit of the dietary intake (DRI, 2006). Others has been shown that 100% orange juice was associated with improved diet quality, does not replace whole fruits from the diet and helps to achieve and maintain balance caloric (O'Neil et al, 2012; Rampersaud & Valim, 2015;)

An improvement of insulin sensitivity with reduction in blood glycemia, after 90^{th} day was observed in this controlled clinical trial. After 30 days of washout (120^{th} day), the blood serum glycemia and insulin, and HOMA-IR, returning to previous values at Citrus-free period 30^{th} day. These effects on metabolic parameters were presumably derived from orange juice compounds, such as flavanones, pectin, or by the

beneficial changes in the intestinal microbiota (Jung et al, 2006; Bahadoran et al, 2013; Pedersen et al, 2016; de Paiva et al, 2019).

It was previously shown in diabetic rats that hesperidin and naringenin attenuated gluconeogenesis, raising insulin sensitivity and improving glycemic control (Jung et al, 2006; Bahadoran et al, 2013). Naringenin also showed antihyperglycemic effect in vivo inhibiting digestion and absorption, and delaying glucose uptake by the intestinal brush border membrane (Hanhineva et al, 2010; Williamson 2013).

Blood glucose levels may also be affected by soluble dietary fibers, such as pectin from orange juice, which may decrease intestinal transit time, leading to more gradual absorption of nutrients and prolonged satiety (Wanders et al, 2011). A clinical study from our group found that although amount of pectin is low in orange juice, it helps lower the glycemic peak compared to an isocaloric drink, showing a lower blood glucose post-prandial effect (de Paiva et al, 2019). In addition, intestinal fermentation of pectin or metabolism of flavanones stimulate the growth of microbiota increasing production of short-chain fatty acids (propionate, butyrate and acetate), which can improve liver and muscle insulin sensitivity and the uptake of glucose into the muscle (Assini et al, 2013; Unno et al, 2015).

In the present work it was verified reduction in the blood concentrations of total cholesterol, triglycerides and LDL-C after the consumption of orange juice. Moreover, in 120th day (washout period) an increase in all these variables was observed. Indeed, the lipid-lowering effect of orange juice consumption has been demonstrated in humans (Aptekmann & Cesar, 2010; Dourado & Cesar, 2015; Silveira et al, 2015), and several mechanisms have been attributed to citrus flavonoids to explain the decrease in plasma lipids.

An experimental study with mice model of metabolic syndrome showed that supplementation of naringin activated hepatic AMPK leading to increase of PPARα gene expression. In turn, PPARα reduced the synthesis of fatty acids and glucose, the production of apoB and VLDL and also inhibited the hepatic activity of HMG-CoA reductase (Pu et al, 2012). In hepatocytes, hesperidin and naringin decreased the monoacylglycerol transfer protein (MTTP) and the enzyme acyl-CoA cholesterol acyltransferase (ACAT), which work together to assemble VLDLs (Mulvihill et al, 2009). In addition, naringenin increased the expression and activity of the LDL receptor (LDL-R), involved in the clearance of plasma lipoproteins. All these mechanisms

attributed to flavanones reduce the hepatic volume of triglycerides, decreasing the secretion of VLDL and, consequently, the amount of circulating LDL-C (Chanet et al, 2012).

In the present study, it was a gradual enhance of antioxidant status in the volunteer's blood, reaching a significant effect two months after the daily intake of orange juice (90^{th} day of experiment). Indeed, we have previously shown a positive effect on the antioxidant capacity after regular ingestion of orange juice (Dourado & Cesar, 2015; Silveira et al, 2015). These effects have been attribute to high concentration of vitamin C and hesperidin in the juice that exerts direct elimination of free radicals and increases the Nrf2-ARE way, which is related to the control of antioxidant genes (Parhiz et al, 2015). On the other hand, naringenin can reduce free radicals increasing the antioxidant activity of superoxide dismutase, catalase and glutathione (Zaidun et al, 2018).

Commercial processing of orange juice influences the concentration and solubility of citrus flavonoids. In the processed juice, some of the extracted flavanones remain soluble, while others are precipitates becoming part of the cloud in the juice. In vitro studies have shown that soluble fraction flavanones are readily available to be absorbed. However, flavanones precipitated in the cloud require enzymatic actions in the gastrointestinal tract to be absorbed (Gil-Izquierdo et al, 2001; Gil, 2002; Gil-Izquierdo et al, 2003; Nielsen et al, 2006; Vallejo et al, 2010;). The chemical analysis of orange juice performed in this study showed a higher percentage of hesperidin (71.5%) in the cloud, and a higher percentage of naringin in the supernatant (62.5%) or soluble fraction.

An increase in urinary flavanones was also observed after 60 and 90 days of consumption of orange juice; in contrast, no flavanone was detected prior to consumption or after volunteers stopped drinking orange juice. These findings agree with previous studies showing that levels of flavanone metabolites in the bloodstream and urine are related to the concentration of flavanones in the diet, which accompanied consumption of orange juice (Aschoff et al, 2015, Aschoff et al., 2016).

Urinary recovery of metabolites of hesperetin and naringenin, at basal period, during orange juice supplementation and afterwards, are in agreement with the literature (Erlund et al, 2001; Manach et al, 2003; Mullen et al, 2008; Brett et al, 2009; Tomás-Navarro et al, 2014; Silveira et al, 2014; Vallejo et al, 2010; Aschoff et al, 2016;

Pereira-Caro et al, 2017). In our study, the bioavailability of hesperitin was estimated at 7%, while naringenin was 1.8%. The total bioavailability for both flavanones was 8.8% regarding the ingested dose.

Considering that OJ has a hesperidin to naringin ratio of 5 to 1, and the bioavailability ratio was 3.8 to 1 among these compounds, a higher bioavailability of naringenin to hesperidin is suggested. This assumption is consistent with higher amount of naringenin on soluble fraction of orange juice than in the precipitate, and otherwise for hesperidin. These results reflect the specific variations on the flavanones bioavailability and metabolism, and some of the differences on the colon microbiota, which are essential for the absorption of flavanones (Manach et al, 2005; Vallejo et al, 2010; Tomáz-Navarro et al, 2014).

Most OJ glycoside hesperidin and naringin are deconjugated by the microbiota to hesperitin and naringenin before it is absorbed into the distal colon. Within the enterocyte cell, aglycones compounds are conjugated to glucuronides and secreted into the portal-hepatic circulation. In the liver, glucuronide metabolites will be conjugated to phase II metabolites by glucuronidation, sulfation or methylation and excreted in the systemic circulation. Those metabolites will be circulating 4 to 12 hours, reaching various organs, such as heart, pancreas, liver, spleen, brain, adipose tissue, muscle, and finally kidneys. Of the kidneys, they are excreted by urine (Erlund et al, 2001; Kanaze et al, 2007; Bredsdorff et al, 2010; Kemperman et al, 2010).

In addition to the deglycosylation of flavanones by rhamnosidase and beta-glucosidase synthetized by the *Lactobacillus* and *Bifidobacterium* strains, the colonic microbiota is responsible for the fission of aromatic rings of polyphenols, producing short chain fatty acids and promoting to a large extent the absorption of acetate, propionate and butyrate. In summary, the addition of 16% of the intact aglycones to the 88% of the biotransformed flavonoids in short chain fatty acids reaches almost 100% of the ingested dose, showing a high absorption of the flavonoids. (Erlund et al, 2001; Brett et al, 2009; Pereira-Caro et al, 2014; Tomás-Navarro et al, 2014; Actis-Goretta et al, 2015; Aschoff et al, 2016; Requena et al, 2018).

In the present study, visualization, clustering, and modeling of the diversity in microbiota results showed that the OJ supplementation changed microbiota profile. Recently, it has been reported that the proportion of intestinal bacteria species may change in response to the addition of novel diet foods, although this may not constitute

a change in enterotypes (Wu et al, 2011; Lombardi et al, 2018; Wang et al, 2018). Enterotypes were associated with long-term diets, as Bacteroides with protein and animal fat, and Prevotella with carbohydrates. A controlled-feeding study of 10 subjects showed that microbiome composition changed detectably within 24 hours of initiating a high-fat/low-fiber or low-fat/high-fiber diet, but that enterotype identity remained stable during the 10-day study. Showing that the enterotypes need a longer period to present changes (Wu et al, 2011).

After consumption of OJ (90^{th} day) was observed an increase in Actinobacteria and decrease of Firmicutes, Bacteroidetes, and Proteobacteria. Relative abundance of *Bifidobacterium* (Actinobacteria) was higher in 90^{th} day. *Bifidobacterium* produces acetate and lactate as major fermentation products from sugars, therefore several strains of *Bifidobacterium* are commercially applied as probiotics (Rajilic-Stojanovic & de Vos, 2014). Acetate plays a role in central appetite regulation, and increasing production in the distal colon promote fat oxidation, improved glucose homeostasis and inflammatory status (Chambers et al, 2018)

Lactobacillus spp. (Firmicutes) produces lactic acid as the main production of fermentation, and supplementation of this probiotic preserves the diversity of the gut microbiota in adverse situations (Rajilic-Stojanovic & de Vos, 2014; Kato-Kataoka et al, 2016). It has been shown that *Lactobacillus* spp. improved in a short- and long-term fasting and postprandial glycemia, glycated hemoglobin (HbA1c), serum insulin concentrations, and insulin resistance (Razmpoosh et al, 2015). On the other hand, *Lachnospiraceae* (Firmicutes) remained stable before and after consumption of orange juice. Some members of this family are associated with the production of butyrate, which is used by intestinal epithelial cells as a source of energy contributing to energy homeostasis; reduces cholesterol synthesis, and has anticancer and anti-inflammatory properties (Hamer et al., 2008, de Vadder et al, 2014).

In the present study, a negative correlation was detected between Actinobacteria (*Bifidobacterium* and *Coriobacteriaceae)* and Firmicutes (*Lachnospiraceae* and *Lactobacillaceae*) with biochemical parameters such as glucose, insulin, HOMA-IR, triglycerides, total cholesterol and LDL-C. The significance of this correlation and its health benefits can be understood as a combination of two main factors: (1) the effect of the increase of short chain fatty acids produced by microorganisms, which was increased by the supplementation of orange juice (Assini et al, 2013; Unno et al, 2015)

and (2) the effect of flavanone metabolites of orange juice which reduced cholesterol and triglycerides by inhibition of synthesis or by increased catabolism; and due to increased insulin sensitivity (Jung et al, 2006; Mulvihill et al, 2009; Aptekmann & Cesar, 2010; Hanhineva et al, 2010; Wanders et al, 2011; Chanet et al, 2012; Pu et al, 2012; Bahadoran et al, 2013; Williamson 2013; Dourado & Cesar, 2015; Silveira et al, 2015; de Paiva et al, 2019).

In addition, it was detected a positive correlation between *Bacteroidacea*, *Porphyromonadaceae* and *Prevotellaceae* (Bacteroidetes), *Ruminococcaceae* (Firmicutes) and *Enterobacteriaceae* (Proteobacteria) with the glucose and lipid metabolism. Previous study have shown an associated positive between *Ruminococcaceae*, with levels of triglycerides in the blood serum (Lahti et al, 2013). This correlation shows that the changes in the biochemical markers were a reflection of the decrease of these microorganisms, which were diminished by the supplementation of orange juice. These results showed that the orange juice intake stimulated beneficial microorganisms that by its turn improved biochemical parameters.

This study showed several strong aspects, including a very homogeneous group of volunteers and highly qualified and trained to follow dietary restrictions; and assessment of food intake with multiple repetitions over time and high adherence of the volunteers. However, some limitations were also observed, such as the duration of the study (12 weeks) and the lack of interpretation of the correlations between the biochemical parameters and some microorganisms due to insufficient evidence on the biological causality between them.

In conclusion, the dynamic view between biochemical parameters and the microbiome provided relevant insights into the interaction between orange juice consumption and intestinal health. These results indicate a prebiotic effect of orange juice, with a positive influence on the intestinal microbiota and metabolic biomarkers of young women. It is also suggested that, to obtain such benefits, orange juice should be consumed regularly and in moderate amounts along with a balanced diet.

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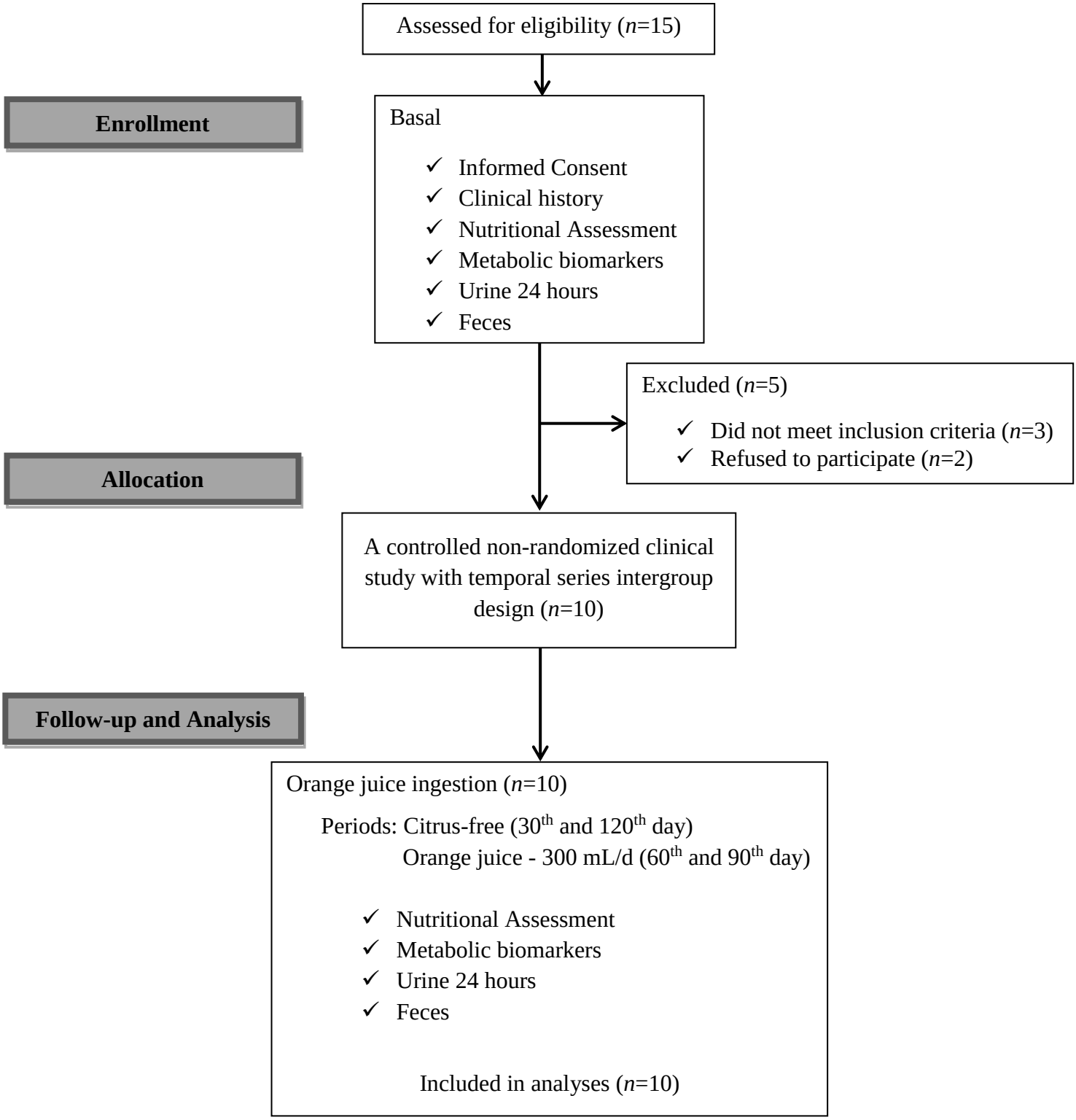


Figure 1. Trial design

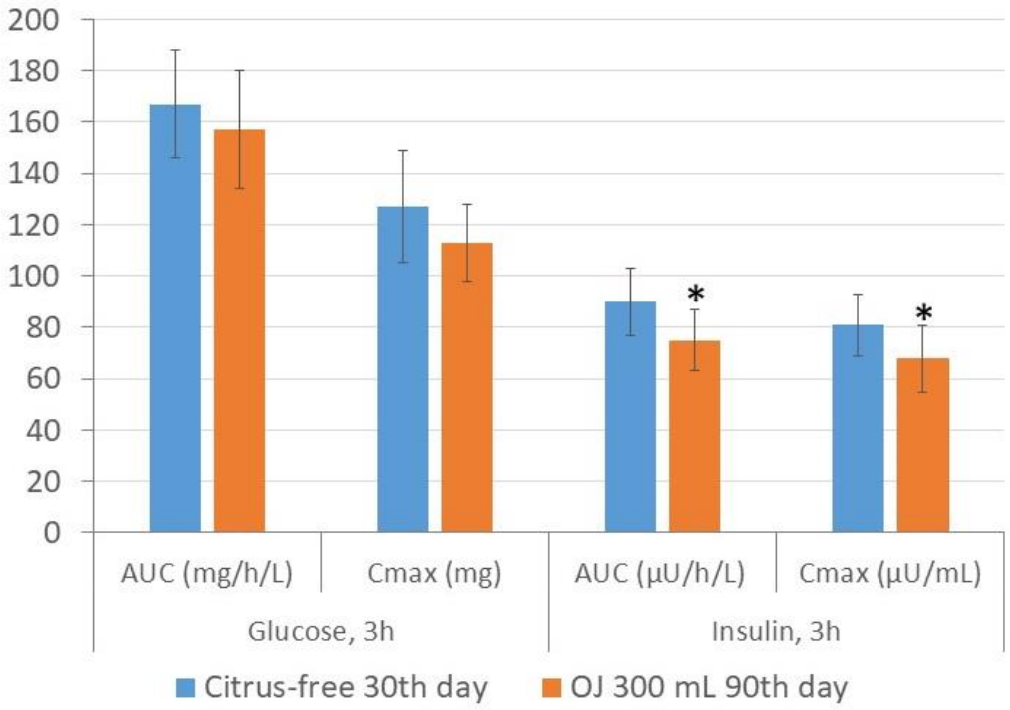


Figure 2: Area under the curve (AUC) and maximum concentration (Cmax) of the glycemic and insulin curves at before and after 60 days of consumption of 100% orange juice pure (300 mL/d) * p 0.01 (Citrus-free: 30th x Trial with Orange Juice: 90th) (n=10).

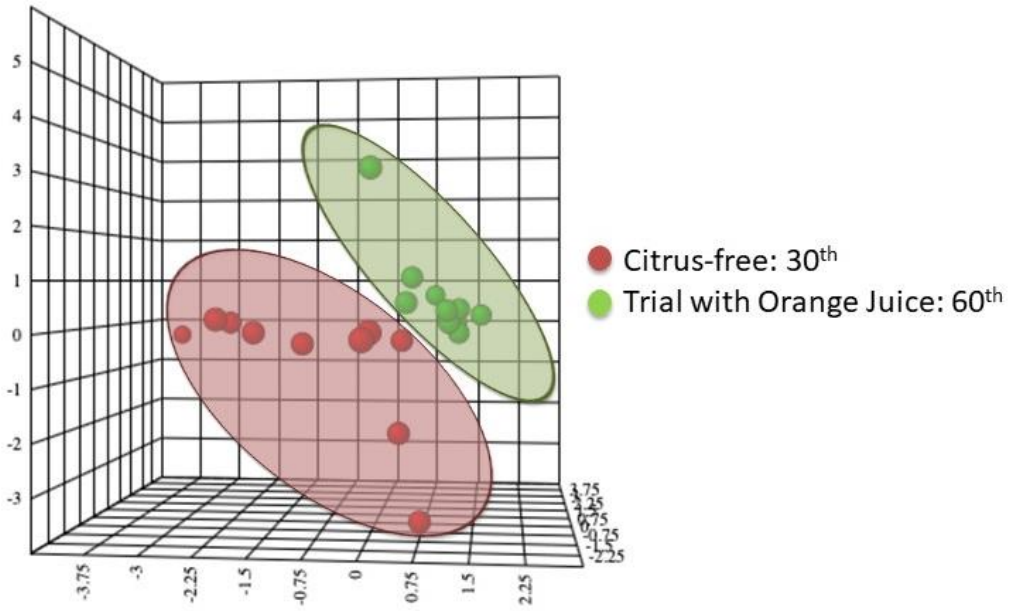


Figure 3: Principle coordinate analyses of the intestinal microbiota of healthy women at before and after 60 days of consumption of 100% pure orange juice (300 mL/d) (Citrus-free: 30th x Trial with Orange Juice: 90th) (n=10).

Figure 4: The main bacterial phylum determined in the microbiota from health women at before and after 60 days of consumption of 100% pure orange juice (300 mL/d) (Citrus-free: 30th x Trial with Orange Juice: 90th) (n=10).

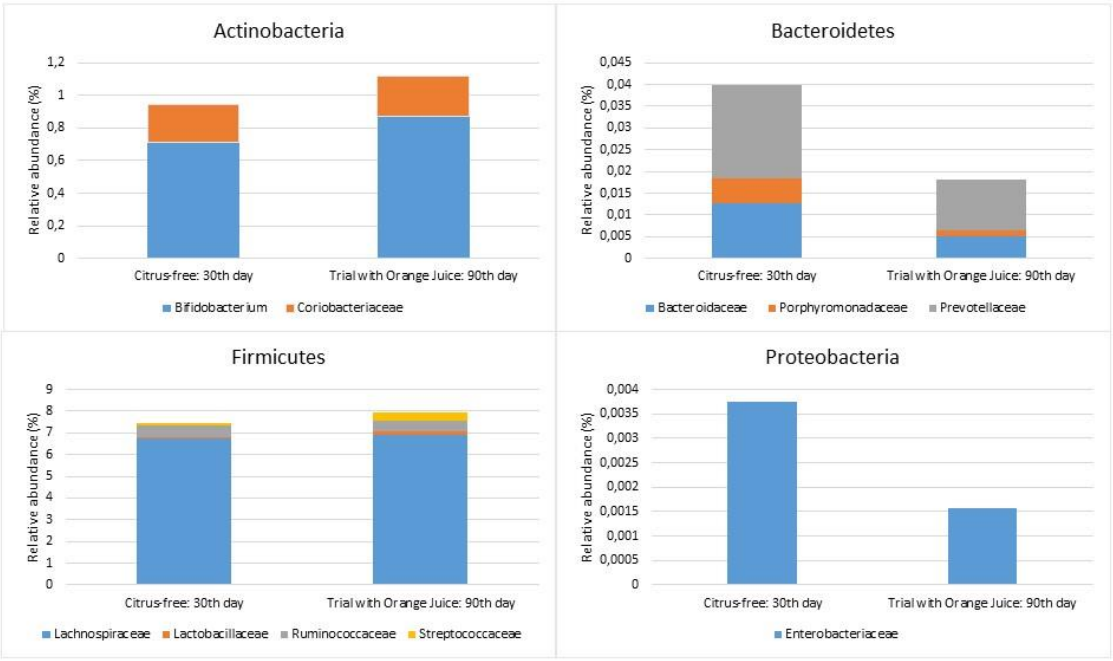


Figure 5: Microbiota composition at a family level before and after 60 days of consumption of 100% pure orange juice (300 mL/d) (Citrus-free: 30th x Trial with Orange Juice: 90th) (n=10).

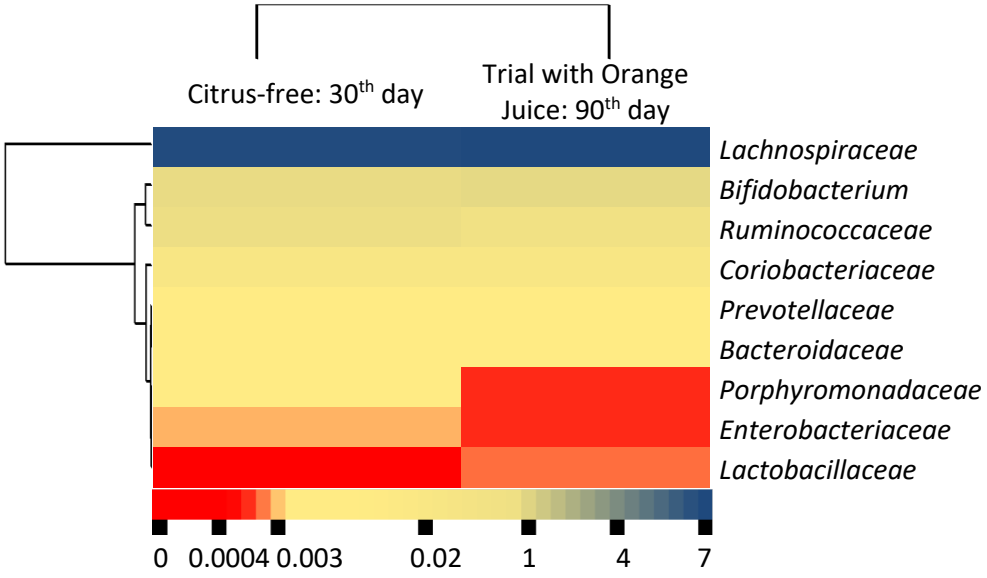


Figure 6: Relative abundance of bacterial genera in the women microbiota before and after 60 days of consumption of 100% pure orange juice (300 mL/d) (Citrus-free: 30th x Trial with Orange Juice: 90th) (n=10).

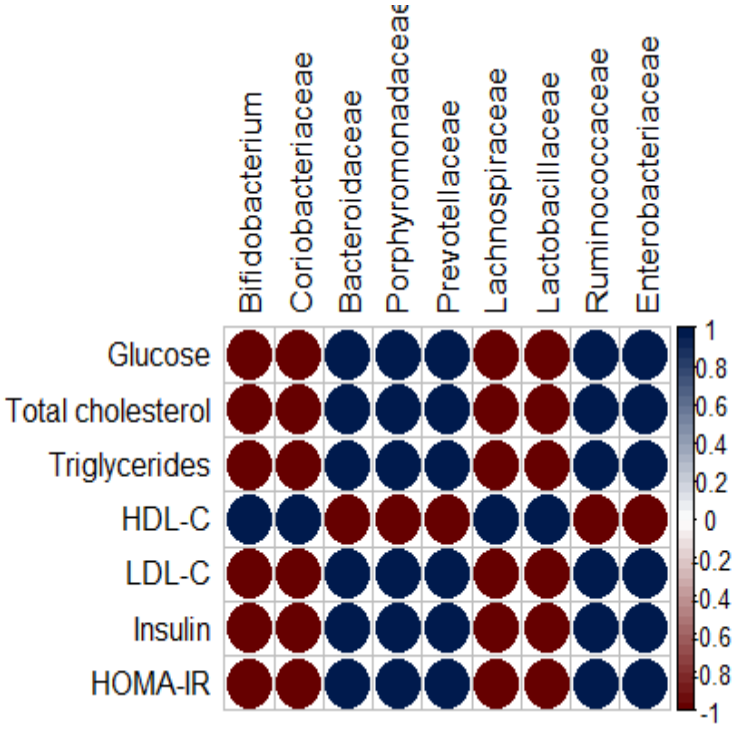


Figure 7: Correlation between biochemical parameters and bacterial genera. Significant correlations are indicated by asterisk ($p < 0.05$) (Spearman correlation) (n=10).

Table 1. Baseline characteristics of female volunteers: body composition, metabolic biomarkers, and urinary flavanones (n=10).

Variables	**Basal (0 day)**
A) Body composition	
Weight (kg)	63.0 ± 9.13
BMI (kg/m^2)	23.9 ± 3.13
Lean muscle mass (kg)	38.4 ± 4.03
Body fat mass (kg)	22.9 ± 4.96
% Body Fat	34.8 ± 5.67
B) Metabolic biomarkers	
Glucose (mg/dL)	79.1 ± 6.28
Insulin (µU/mL)	11.5 ± 1.87
HOMA-IR	2.41 ± 0.37
Triglycerides (mg/dL)	125 ± 38.9
Total cholesterol (mg/dL)	198 ± 27.8
HDL-C (mg/dL)	59.6 ± 7.70
LDL-C (mg/dL)	114 ± 25.5
Antioxidant capacity (mM)	1.94 ± 0.02
Lipid peroxidation (mM)	1.3 ± 0.7
C) Urinary metabolites	
Hesperetin-3-O-Glucoronide (mg)	2.68 ± 0 .31
Hesperetin-7-O-Glucoronide (mg)	2.35 ± 0.29
Naringenin-7-O-Glucuronide (mg)	0.23 ± 0.06
D) **Bioavailability %**	
HSP in 24h (dose 72.9 mg)	6.89 ± 0.82
NRG in 24h (dose 14.6 mg)	1.57 ± 0.42

Table 2A. Nutritional and physicochemical composition in 300 mL of commercial processed orange juice.

Nutritional Composition of Orange Juice*	
Energy (kcal)	144.0
Total Carbohydrate (g)	33.9
Fiber, total dietary	0.84
Protein (g)	2.0
Lipids (g)	0.4
Ascorbic acid (mg)	98.7
Physicochemical Composition	
pH	4.4
Total soluble solids (°Brix)	14.4
Titratable acidity (g citric acid/100 mL)	1.8
Ratio (soluble solids/titratable acidity)	19.8
Total antioxidant capacity (μmol Trolox 100 mL^{-1})	4.3 ± 0.4

*USDA National Nutrient Database for Standard Reference

Table 2B. Flavanones in the pellets and supernatant fractions in 300 mL of commercial processed orange juice measured by UPLC-ES.

Orange Juice	**Pellet**		**Supernatant**		**Total**	
	mg	**%**	**mg**	**%**	**mg**	**%**
Hesperidin	52.1 ± 1.2	71.5	20.8 ± 0.9	28.5	72.9 ± 1.6	100
Naringin	5.5 ± 0.2	37.5	9.1 ± 0.5	62.5	14.6 ± 0.5	100
Total Flavanones	57.6 ± 0.8	65.8	29.9 ± 0.7	34.2	87.5 ± 1.0	100

Table 3. Body composition and metabolic biomarkers of women during clinical trial with citrus-free diet and orange juice supplemented diet (n=10).

Variables *Diet:*	Citrus-free	+ Orange Juice (300 mL/d)		Citrus-free
Timeline:	30th day	60th day	90th day	120th day
A) Body composition				
Weight (kg)	63.4 ± 9.64	63.5 ± 9.23	63.6 ± 9.51	63.7 ± 9.01
BMI (kg/m^2)	24.1 ± 3.29	24.1 ± 3.13	24.1 ± 3.26	24.2 ± 3.05
Lean muscle mass (kg)	38.7 ± 4.31	39.3 ± 4.45	38.8 ± 4.52	39.6 ± 4.65
Body fat mass (kg)	23.0 ± 5.09	22.7 ± 5.03	23.3 ± 5.23	22.4 ± 4.85
% Body Fat	34.5 ± 5.13	33.9 ± 4.75	34.6 ± 5.13	33.5± 5.25
B) Metabolic biomarkers				
Glucose (mg/dL)	80.1 ± 4.77^a	76.7 ± 7.30^{ab}	75.0 ± 8.01^b	79.9 ± 6.92^a
Insulin (µU/mL)	11.9 ± 2.17^a	10.5 ± 2.82^{ab}	8.07 ± 1.96^b	11.2 ± 2.69^{ab}
HOMA-IR	2.42 ± 0.55^a	1.88 ± 0.50^{ab}	1.36 ± 0.30^b	2.06 ± 0.33^a
Triglycerides (mg/dL)	128 ± 30.7^a	115 ± 28.8^{ab}	88.9 ± 20.7^b	108 ± 25.6^{ab}
Total cholesterol (mg/dL)	201 ± 28.4^a	182 ± 24.4^{ab}	173 ± 28.8^b	194 ± 22.1^a
HDL-C (mg/dL)	55.5 ± 9.40	60.1 ± 10.3	59.8 ± 12.9	58.7 ± 13.6
LDL-C (mg/dL)	114 ± 27.9^a	97.1± 24.1^b	95.4 ± 19.6^b	100 ± 24.1^{ab}
Antioxidant capacity (mM)	1.93 ± 0.02^b	1.93 ± 0.02^b	1.94 ± 0.06^a	1.93 ± 0.03^b
Lipid peroxidation (mM)	1.4 ± 0.7^b	1.3 ± 0.6^b	1.2 ± 0.6^a	1.3 ± 0.7^b

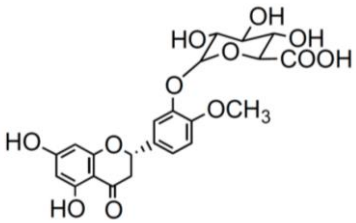
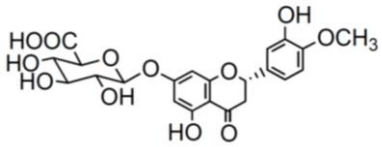
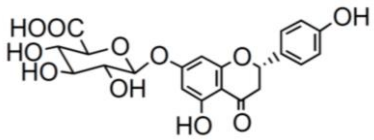
Different letters represent significant difference ($p < 0.05$) between periods.

Table 4. Intake of energy, macronutrients and micronutrients of women during clinical trial with citrus-free diet and orange juice supplemented diet (n=10).

Nutrients / ***Diet:***	**Citrus-free: 30th day**			**+ Orange Juice (300 mL/d): 90th day**				**Citrus-free: 120th day**	
Days:	0	15	30	15	30	45	60	15	30
Energy (kcal)	1937 ± 193	1920 ± 273	2048 ± 156	2046 ± 340	1993 ± 253	1887 ± 109	2046 ± 350	2046 ± 350	2033 ± 250
Carbohydrate (g)	254 ± 29.5	265 ± 36.0	266 ± 30.6	271 ± 39.5	260 ± 38	265 ± 30.9	264 ± 35.6	274 ± 35.3	275 ± 37.5
Fiber (g)	27.6 ± 6.8	24.3 ± 4.5	22.9 ± 5.24	24.8 ± 4.7	22.6 ± 5.6	25.8 ± 4.8	25.9 ± 5.6	26.8 ± 2.99	25.1 ± 6.1
Protein (g)	96.7 ± 19.1	94.3 ± 12.1	95.3 ± 14.3	92.4 ± 16.1	92.1 ± 22.9	92.9 ± 20.9	92.2 ± 22.8	93.6 ± 6.5	89.8 ± 20.3
Total fat (g)	65.4 ± 4.5	66.5 ± 2.8	64.6 ± 5.5	66.6 ± 3.6	66.3 ± 3.6	65.6 ± 4.3	65.8 ± 6.5	66.7 ± 3.9	65.9 ± 5.0
Cholesterol (mg)	253 ± 61.93	242 ± 38.3	238 ± 59.3	249 ± 58.4	236 ± 54	266 ± 31.3	236 ± 58.2	247.8 ± 58.2	268 ± 51.1
Saturated FA (g)	16.18 ± 3.8	17.2 ± 3.4	17.8 ± 4.1	17.7 ± 2.0	16.5 ± 4.3	16.8 ± 3.6	16.5 ± 2.8	18.4 ± 1.9	17.86 ± 1.92
Vitamin C (mg)	148 ± 36[a]	146 ± 26.3[a]	145 ± 28.5[a]	250 ± 39.9[b]	252 ± 29.9[b]	256 ± 32.9[b]	254 ± 35.5[b]	151 ± 23.9[a]	155 ± 36.9[a]

Saturated FA, saturated fatty acids. Different letters represent significant difference ($p < 0.05$) between periods.

Table 5. Metabolites of hesperitin (HSPT) and naringenin (NRGN) in the urine 24 h of women during clinical trial with citrus-free diet and orange juice supplemented diet (n=10).

Urinary metabolites	***Diet:***	**Citrus-free**	**+ Orange Juice (300 mL/d)**		**Citrus-free**
	Timeline:	**30th day**	**60th day**	**90th day**	**120th day**
HSPT-3-O-Glucoronide (mg) 		n.d.[a]	2.68 ± 0.19	2.73 ± 0.29	n.d.
HSPT-7-O-Glucoronide (mg) 		n.d.	2.39 ± 0.24	2.41 ± 0.28	n.d.
Total (mg)		n.d.	5.07 ± 0.29	5.13 ± 0.42	n.d.
[b] Bioavailability HSPT (%) (HSPT oral dose = 72.9 mg)		n.d.	6.95 ± 0.39	7.04 ± 0.57	n.d.
NGRN-7-O-Glucuronide (mg) 		n.d.	0.24 ± 0.11	0.29 ± 0.10	n.d.
[b] Bioavailability NGRN (%) (NGRN oral dose = 14.6 mg)		n.d.	1.65 ± 0.73	2.00 ± 0.68	n.d.

[a] n.d., not detected

[b] Bioavailability = ($AUC_{0\text{-}24h}$ urine / oral dose) x 100

Considerações Finais

O consumo de 60 dias seguidos de suco de suco de laranja apresentou efeito prebiotico, modulando positivamente a microbiota intestinal. Além de promover uma ação benéfica no controle do perfil glicêmico e lipídico de mulheres.

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APÊNDICE A - TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Eu _____,RG _____, Estado Civil _____, Idade _____ anos, Residente na _____, nº _____, Bairro _____, Cidade _____, Telefone _____.

Declaro ter sido esclarecido sobre os seguintes pontos:

1. O trabalho “Influência do efeito prebiótico do suco de laranja na biodisponibilidade das flavanonas após ingestão crônica de suco de laranja” tem por finalidade investigar a biodisponibilidade das flavanonas (compostos bioativos) e seus metabólitos nas fezes, sangue e urina, após a ingestão crônica do suco de laranja;
2. Ao participar desse trabalho ajudarei na identificação de um alimento altamente acessível com efeito prebiótico;
3. Doarei para a realização dessa pesquisa, os seguintes materiais biológicos: sangue, urina e fezes. Será coletado 30 mL de sangue depois de jejum de 12 horas e depois de 2, 4, 8, 24 horas. Isso se repetirá 5 vezes durante o projeto, com intervalo de 30 dias. A coleta de todas as amostras será realizada no Laboratório de Análises Clínicas São Lucas de Araraquara. A urina de 24 horas será coletada, na véspera do início do experimento e a cada 30 dias até a data final. Será fornecido aos voluntários recipientes apropriados para coleta. As fezes totais das últimas 24 horas serão coletadas na véspera do início do experimento e a cada 30 dias até a data final. Sera fornecido aos voluntários recepiente apropriado para coleta. Todos os materiais serão utilizados exclusivamente para essa pesquisa, não podendo ser reutilizado em pesquisa posterior;
4. A minha participação como voluntário terá a duração de 120 dias (4 meses);
5. Ao participar dessa pesquisa poderei ter o incomôdo das coletas de sangue. As coletas de urina e fezes não são invasivas e podem causar algum desconforto;
6. Todas as vezes que houver necessidade de retorno, voltarei ao Laboratório de Análises Clínicas São Lucas de Araraquara para coletas de sangue e no

Laboratório de Nutrição (FCFAR – UNESP- Araraquara) para entrega da urina e fezes e retirada do suco;

7. Não terei nenhuma despesa com os exames ou para receber o suco ao participar desse estudo. Receberei lanches e almoço no dia da coleta de sangue e serei ressarcida com o valor do transporte até o laboratório;
8. Meu nome será mantido em sigilo, assegurando assim a minha privacidade e se desejar, serei informado sobre os resultados dessa pesquisa (pela instituição ou profissional competente);
9. Estou ciente de que o material a ser doado será utilizado exclusivamente nesta pesquisa, não podendo ser armazenado para uso posterior sem o meu consentimento;
10. Poderei me recusar a participar ou mesmo retirar meu consentimento a qualquer momento da realização dessa pesquisa, sem nenhum prejuízo ou penalização (isto é, sem interrupção do meu tratamento, quando for o caso);
11. Qualquer dúvida ou solicitação de esclarecimentos, poderei entrar em contato com a equipe científica do projeto: pesquisadora Melaine Priscila Fidélix pelo telefone (0XX16) 3301-6766/99660-9322 no Laboratório de Nutrição da FCFar – UNESP Araraquara;
12. Para notificação de qualquer situação, relacionada com a ética, que não puder ser resolvida pelos pesquisadores deverei entrar em contato com o Comitê de Ética em Pesquisa da Faculdade de Ciências Farmacêuticas do Câmpus de Araraquara da UNESP, pelo telefone (0XX16) 3301-6897;
13. Este documento será assinado em duas vias, na qual serei portador de uma via e a outra ficará com o pesquisador.

Diante dos esclarecimentos prestados, concordo em participar, como voluntária(o), do estudo "Influência do efeito prebiótico do suco de laranja na biodisponibilidade das flavanonas após ingestão crônica de suco de laranja".

Araraquara, _____

_____ _____

Assinatura do Voluntário Assinatura do Pesquisador

APÊNDICE B - Questionário de seleção

Data: ____/____/_____

Nome: _____

Telefone: _____ celular: _____ Sexo: M ( ) F( )

Profissão: _____ DN _____/_____/_____ Idade: _____

e-mail: _____

I–Antropometria

Peso : _____ Altura _____ IMC_____

Obs_____

II- História Clínica

1 – Você tem ou teve alguma destas doenças citadas abaixo:
- ( ) Ataque cardíaco
- ( ) Derrame
- ( ) Diabetes
- ( ) Hipertensão
- ( ) Câncer
- ( ) Outras: _____

2- Alguém da sua família (pai, mãe, irmãos, avós) já teve algumas destas doenças abaixo:
- ( ) Ataque cardíaco ( ) Hipertensão
- ( ) Derrame ( ) Câncer
- ( ) Diabetes
- ( ) Outras: _____

3 – Você está atualmente grávida ou amamentando?
- ( ) Não
- ( ) Sim (grávida) ( ) Sim (amamentando)

4 - Você faz uso de algum remédio?
- ( ) Não
- ( ) Sim. Qual (s): _____

5 - Você fez uso de algum antibiótico nos últimos 6 meses?
- ( ) Não
- ( ) Sim. Qual (s): _____

6 – Você fuma ?
- ( ) Não
- ( ) Sim. Qual (s): _____
- Quantos cigarros (ou outro tipo de fumo) por dia? _____

7 – Você consome álcool?

( ) Não
( ) Sim. Qual (s): _____
Quantidade por semana: _____

8 - Como é o seu funcionamento intestinal?
( )regular ( )irregular
Quantidade por dia/semana: _____

II – Informações Dietéticas

8 – Quantos copos de água você bebe diariamente?
_____

9 – Você está atualmente seguindo alguma dieta?
( ) Não
( ) Sim. Qual tipo: _____
( ) prescrita por profissional de nutrição ou médico
( ) prescrita por você mesmo

10 – Você utiliza alguma forma de suplemento alimentar? (ex: vitaminas, minerais, proteínas, etc.)
( ) Não
( ) Sim. Qual (s):
_____
_____

11 – Você utiliza ou utilizou probiótico ou prebiótico nos últimos 3 meses?
( ) Não
( ) Sim. Qual (s):
_____

12- Outras observações importantes sobre sua alimentação.

_____
_____
_____
_____
_____
_____

APÊNDICE C - Modelo do registro alimentar

Dia [1] [2] [3] – Registro Alimentar 24h

Data: ___/___/____ Dia semana: _____

Nome:_____

HORARIO/LOCAL	REFEIÇÕES	ALIMENTOS/QUANTIDADES

INSTRUÇÕES PARA REGISTRO ALIMENTAR DE 3 DIAS

As instruções abaixo irão auxiliar no procedimento do seu registro alimentar. Siga-as corretamente e releia-as sempre que tiver alguma dúvida.

1. Anote tudo o que você comer em três dias não consecutivos sendo dois dias da semana (exemplo: terça e quinta-feira) e um dia do final de semana (sábado ou domingo);
2. Quando você relatar um alimento ou bebida, seja o mais claro e preciso possível. Anote tudo no momento em que estiver comendo. Evite reconstruir as refeições de memorias, não deixe para anotar depois que tiver acabado de comer. Coloque a marca do produto se for o caso;
3. Registre todos os alimentos que ingerir durante o dia inteiro, até mesmo uma bala, chicletes, etc. exceto água;
4. Forneça o máximo de informações como, por exemplo, se na preparação foi utilizado molho (creme de leite, molho de tomate, molho branco, etc), se a carne é a milanesa, assada ou frita. Indique se os legumes e verduras são cozidos ou crus, servidos com margarina, manteiga, azeite, etc;
5. Informe com precisão, sempre que possível, o volume dos líquidos e peso de alimentos. Informe a colher utilizada nas medidas: café, chá, sobremesa, sopa, de servir, escumadeira e concha e se estas eram cheias ou rasas. Tente descrever bem as porções:
    a. - 1 coxa média de frango, fruta com pele.
    b. - 4 colheres de sopa de cenoura crua ralada.
6. Coloque o tamanho dos alimentos caso não saiba o peso (pequeno, médio, grande). Por exemplo: uma maçã pequena, uma pera grande, umafatia média de abacaxi.
7. Informe o tipo de pão que você costuma comer (forma, francês, branco, integral, de leite etc). Anote tudo o que foi utilizado no pão (manteiga, margarina, geléia, requeijão, queijo, etc.).
8. Anote todos os ingredientes utilizados para adoçar alguma refeição como, por exemplo, açúcar ou adoçante no café.
9. Informe o tipo de leite que você costuma comer (integral, desnatado, semidesnatado, de soja, etc.).

APÊNDICE D – Questionário de frequência alimentar

Produtos	Quantas vezes você come (de 0 a 10)	Unidade (Dia, Semana, Mês)	Porção Média (M)	Sua porção P M G GG
Leites e derivados				
Leite desnatado			½ copo de requeijão – 125 ml	
Leite semidesnatado			½ copo de requeijão – 125 ml	
Leite integral			½ copo de requeijão – 125 ml	
Iogurte Natural			1 unidade pequena – 140 g	
Iogurte com Frutas			1 unidade pequena – 140 g	
Queijo branco (minas/ frescal/ricota)			1 fatia média - 30 g	
Queijo amarelo (prato/mussarela/provolone/parmesão)			1,5 fatias grossas – 30 g	
Carnes e peixes				
Carne de boi (bife,cozida,assada), miúdos,vísceras.			1 bife médio ou 2 pedaços (100 g)	
Carne de porco (lombo,bisteca)			fatia média (100g)	
Carne seca, carne de sol, bacon			2 pedaços pequenos (40 g)	
Linguiça			1 gomo médio (60g	
Frango (cozido, frito, grelhado, assado)			1 pedaço ou 1 filé pequeno (60g)	
Peixe (cozido,frito,assado), frutos do mar			1 filé peq ou 1 posta peq (100 g)	
Embutidos (salsicha, salame, presunto, mortadela)			2 fatias médias (30g)	
Hamburguer,nuggets, almondega			1 unidade média (60g)	
Molhos e temperos				
Azeite, óleo ou vinagre para tempero de salada			1 fio (5 ml)	
Maionese,molho para salada, patê e chantilly			1 colher de chá (0,35 g)	
Sal para tempero de salada e condimentos			1 pitada (0,35 g)	
Leguminosas e ovos				
Ovo (cozido,frito)			1 unidade (50g)	
Feijão (carioca,roxo,preto,verde)			1 concha média (86g)	
Lentilha,ervilha seca, grão de bico, soja			1 colher de servir (35g)	
Feijoada, feijão tropeiro			1 concha média (210g)	

Frutas/Verduras/Legumes				
Alface			3 folhas médias (30g)	
Tomate			3 fatias médias (40g)	
Cenoura			1 colher de sopa (25)	
Outros legumes (abobrinha,berinjela,chuchu,pepino)			1 colher de sopa cheias (30g)	
Outras verduras cruas (acelga,rúcula,agrião)			1 prato de sobremesa (38g)	
Outras verduras cozidas (acelga,espinafre,escarola, couve)			1 colher de servir (30g)	
Brócolis			1 ramo ou 2 colheres de sopa (30g)	
Couve-flor,repolho			1 ramo ou 2 colheres de sopa (30g)	
Mexerica, abacaxi			1 unidade média ou 1 fatia grande (180g)	
Laranja			1 unidade media (180g)	
Limão (para tempero)			½ unidade (3 mL)	
Banana			1 unidade média (86g)	
Maçã, pera			1 unidade média (110g)	
Melão, melancia			1 fatia média (150g)	
Mamão			1 fatia média ou ½ unidade média (160g)	
Goiaba			1 unidade grande (225g)	
Abacate			2 colheres de sopa cheias (50 g)	
Arroz e Tubérculos				
Arroz branco ou integral cozido com óleo temperos			2 escumadeiras médias (120 g)	
Batata frita ou mandioca frita			2 colheres de servir cheias (100g)	
Batata,mandioca,inhame (cozida ou assada), purê			1 escumadeira cheia (90g)	
Salada de maionese com legumes			3 colheres de sopa (90g)	
Farinha de mandioca, farofa,cuscuz,aveia,tapioca			3 colheres de sopa (40g)	
Pães e biscoitos				
Pão francês,pão de forma,integral,pão doce,torrada			1 unidade ou 2 fatias (50g)	
Biscoito sem recheio (doce, salgado)			4 unidades (24g)	
Biscoito recheado, wafer, amanteigado			3 unidades (41g)	

Bolo (simples, recheado)			1 fatia média (60g)	
Manteiga ou margarina passada no pão comum			3 pontas de faca (15g)	
Manteiga ou margarina passada no pão light			3 pontas de faca (15g	
Sanduiche (cachorro quente, hambúrguer)			2 unidades simples (220g)	
Sopas e Massas				
Sopas (de legumes,canja,creme,etc)			1 concha média (150g)	
Salgados fritos (pastel,coxinha,rissoles,bolinho)			1 unidade grande (80g)	
Salgados assados(esfiha,bauruzinho,torta)			2 unidades ou 2 Pedaços médias (140g)	
Macarrão com molho, sem carne			1 prato raso (200g	
Macarrão com molho com carne, lasanha,nhoque			1 escumadeira ou 1 pedaço pequeno (110g)	
Pizza, panqueca			2 fatias peq ou 2 unidades (180g)	
Polenta cozida ou frita			2 colheres de sopa ou 2 fatias pequenas (70g)	
Sobremesas e Doces				
Açúcar, mel, geleia			1/2 colher de sopa (6g)	
Chocolates,bombom, brigadeiro			1 barra pequena (25g)	
Achocolatado em pó (adicionado ao leite)			2 colheres de sopa (25g)	
Sobremesas, doces, tortas e pudins			1 pedaço ou 1 fatia média (60g)	
Bebidas				
Café ou chá com açúcar			2 xícaras de café (50 ml)	
Café ou chá sem açúcar			2 xícaras de café (50 ml)	
Suco natural com açúcar			1/2 copo americano (80ml)	
Suco natural sem açúcar			1/2 copo americano (80ml)	
Suco de laranja puro sem açúcar			1/2 copo americano (80ml)	
Suco artificial			1 copo de requeijão (240 ml)	
Refrigerante comum			1 copo de requeijão (240ml)	
Refrigerante diet/light			1 copo de requeijão (240ml)	
Cerveja			2 latas (700ml)	
Vinho			1 taça (150 mL)	

ANEXO A – Parecer de aprovação do Comitê de Ética

UNESP - FACULDADES DE CIÊNCIAS FARMACÊUTICAS DO CAMPUS DE

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: INFLUÊNCIA DO EFEITO PREBIÓTICO DO SUCO DE LARANJA NA BIODISPONIBILIDADE DAS FLAVANONAS APÓS INGESTÃO CRÔNICA DE SUCO DE LARANJA

Pesquisador: Melaine Priscila Fidélix

Área Temática:

Versão: 3

CAAE: 53228116.9.0000.5426

Instituição Proponente: Faculdade de Ciências Farmacêuticas do Câmpus de Araraquara da UNESP

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.644.906

Apresentação do Projeto:

O projeto está bem redigido e as revisões solicitadas já foram executadas.

Objetivo da Pesquisa:

O objetivo está claramente apresentado e não há observações a serem adicionadas.

Avaliação dos Riscos e Benefícios:

Foram realizadas de acordo com a legislação vigente e as solicitações do Comitê de Ética em Pesquisa.

Comentários e Considerações sobre a Pesquisa:

A pesquisa é interessante e pode trazer benefícios para a comunidade caso os resultados estejam de acordo com o esperado.

Considerações sobre os Termos de apresentação obrigatória:

Os termos obrigatórios foram apresentados e estão em conformidade com o que pede este Comitê.

Conclusões ou Pendências e Lista de Inadequações:

Aprovo, "ad referendum" do Comitê de Ética em Pesquisa, o projeto de pesquisa ora apresentado.

Endereço: Rodovia Araraquara Jaú, km 1
Bairro: Campus Universitário **CEP:** 14.801-902
UF: SP **Município:** ARARAQUARA
Telefone: (16)3301-6897 **E-mail:** sta@fcfar.unesp.br

ANEXO B – Parecer *ClinicalTrials*

ClinicalTrials.gov PRS
Protocol Registration and Results System

ClinicalTrials.gov PRS DRAFT Receipt (Working Version)
Last Update: 01/24/2017 10:06

ClinicalTrials.gov ID: NCT03032861

Study Identification

Unique Protocol ID:	SaoPSU2
Brief Title:	The Prebiotic Effect of Daily Intake of Orange Juice Affects the Bioavailability of Flavanones?
Official Title:	Influence of the Prebiotic Effect of Orange Juice on the Bioavailability of Flavanones After Chronic Intake of Orange Juice.
Secondary IDs:	

Study Status

Record Verification:	January 2017
Overall Status:	Active, not recruiting
Study Start:	September 2016 []
Primary Completion:	October 2016 [Actual]
Study Completion:	February 2017 [Anticipated]

Sponsor/Collaborators

Sponsor:	São Paulo State University
Responsible Party:	Principal Investigator Investigator: Thais Cesar [thaiscesar] Official Title: PhD Affiliation: São Paulo State University
Collaborators:	

Oversight

U.S. FDA-regulated Drug:	
U.S. FDA-regulated Device:	
IND/IDE Protocol:	No
Human Subjects Review:	Board Status: Approved Approval Number: 1.644.906 Board Name: Ethics Research Committee of Pharmacy School, UNESP Board Affiliation: Sao Paulo State University Phone: 55 16 33014657 Email: sta@fcfar.unesp.br
Data Monitoring:	Yes