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Dietary whey proteins shield murine cecal microbiota from extensive disarray caused by a high-fat diet

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ABSTRACT

High-fat diets are used to induce adverse alterations in the intestinal microbiota, or dysbiosis, generalized inflammation and metabolic stress, which ultimately may lead to obesity. The influence of dietary whey proteins, whether intact or hydrolyzed, has been reported to improve glucose homeostasis and reduce stress. Therefore, the purpose of this work was to test if dietary milk-whey proteins, both in the intact form and hydrolyzed, could have an effect on the compositional changes of the cecal microbiota that can be induced in mice when receiving a high-fat diet in combination with the standard casein. Male C57BL/6 mice were fed a control casein diet (AIN 93-G); high-fat-casein (HFCAS); high-fat-whey protein concentrate (HFWPC) and high-fat whey-protein hydrolysate (HFWPH) for 9 weeks. The intestinal microbiota composition was analyzed by 16S-rRNA of the invariant (V1–V3) gene, potentially endotoxemic lipopolysaccharide (LPS) release was determined colorimetrically, and liver fat infiltration assessed by light microscopy. The high-fat diet proved to induce dysbiosis in the animals by inverting the dominance of the phylum *Firmicutes* over *Bacteroidetes*, promoted the increase of LPS and resulted in liver fat infiltration. The whey proteins, whether intact or hydrolyzed, resisted the installation of dysbiosis, prevented the surge of circulating LPS and prevented fat infiltration in the liver. It is concluded that dietary whey proteins exert metabolic actions that tend to preserve the normal microbiota profile, while mitigating liver fat deposition in mice consuming a high-fat diet for nine weeks. Such beneficial effects were not seen when casein was the dietary protein. The hydrolyzed whey protein still differed from the normal whey protein by selectively protecting the *Bacteroidetes* phylum.

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1. Introduction

One of the first adverse effects of excessive saturated fats delivered by the diet is the impact on the intestinal microbiota. Habitual ingestion of excessive amounts of saturated fats will set off a series of adverse consequences that begin with an intestinal microbial imbalance, inflammation and damage of the intestinal epithelium (Anhê et al., 2015; Hattori & Taylor, 2009; Hildebrandt et al., 2009; Kim, Gu, Lee, Joh, & Kim, 2012; Shen et al., 2006).

This type of inflammation is known to result mainly from lipopolysaccharides (LPS) derived mostly from the cell wall of gram-negative bacteria as they are released into the bloodstream (Anhê et al., 2015; Cani et al., 2007). Should the excessive ingestion of saturated fats be extended over time, inflammation will become chronic and eventually lead to dyslipidemia, fat deposition in the liver and most other tissues, with additional consequences such as increased levels of C-reactive protein and insulin resistance (Monteiro & Azevedo, 2010).

While the impact of dietary fats on the microbiota has been the object of a number of studies, the role of dietary protein has received little attention, except for the milk whey proteins. Tranberg, Madsen, Hansen, and Hellgren (2014) compared the effect of casein to that of a whey protein isolate in C57BL/6J mice receiving a high-fat diet and, although there was an initial weight reduction due to an adaptation to whey, no effect of the different proteins on the microbiota was detected. Other findings on the molecular mechanisms by which the complete set of milk whey proteins and peptides contained therein can exert such specific benefits indicate that some constituting peptides can promote translocation of GLUT4 to the plasma membrane thus increasing glucose uptake and glycogen synthesis in Wistar rats (Morato et al., 2013). In turn, the hydrolysate of the complete set of whey proteins has been reported to also grant additional protection to sensitive body proteins by means of the heat-shock proteins (HSPs) (Moura et al., 2014).

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Owing to the abundance of specific sequences in the whey proteins that generate bioactive peptides responsible for the regulation of energy expenditure and various other cell-protecting features (Shi et al., 2011), we wished to investigate the milk whey proteins further in comparison to casein and the hydrolyzed whey protein. Therefore, this study was designed to compare the role of the bovine milk whey proteins and casein on the potentially damaging consequences produced by the experimental high-fat-diet model on male C57BL/6 mice.

2. Experimental

2.1. Animals and experimental protocol

Animals used in the biological assay were purchased from the Multidisciplinary Center for Biological Research of the University of Campinas (CEMIB). The experiments were approved by the Ethics Committee on the use of Animals (CEUA/UNICAMP) (3015-1 protocol), in accord with the ethical principles for animal experimentation adopted by the Brazilian Society of Laboratory Animal Science (SBCAL). Thirty-four C57BL/6 young adult male mice were kept in individual cages with food and water ad libitum, under an inverted 12-hour light/dark cycle (07:00 to 19:00 dark/19:00 to 7:00 a.m. light). Room temperature was maintained at 22 ± 2 °C and relative humidity at 55 ± 10%. The animals were divided into 4 groups according to the diet: Control (AIN 93G; n = 10), Hyperlipidic control (HFCAS; n = 8), Group treated with the hyperlipidic diet containing only whey protein (HFWPC; n = 8), Group treated with the hiperlipidic diet containing only hydrolyzed whey protein (HFWPH; n = 8).

The diets were further described as follows: a standard normolipidic diet (AIN 93-G) modified to contain 14% protein, and the above, modified by adding lard to 38% lipids (11.4% of plant-derived lipids (soybean oil) and 88.6% of lard Cintra et al., 2012). The latter was called the "casein-high-fat diet" (Table 1). Other than that, all formulations followed the recommendations of the American Institute of Nutrition (AIN 93-G) (Reeves, Nielsen, & Fahey, 1993). After 9 weeks on the experimental protocol, the animals were anesthetized intraperitoneally using a mixture of ketamine and xylazine at a ratio of 10 mg/kg and 150 mg/kg, respectively.

Table 1
Formulation and proximate composition of the experimental diets (g/100 g).

Components	AIN 93G	HFCAS	HFWPC	HFWPH
Corn starch	39.75	13.81	13.74	13.52
Dextrinized starch	13.20	13.20	13.20	13.20
Casein	20.0	17.74	0	0
WPC	0	0	17.81	0
WPH	0	0	0	18.03
Fiber (cellulose)	5.00	5.00	5.00	5.00
Sucrose	10.00	10.00	10.00	10.00
Soybean oil	7.00	4.00	4.00	4.00
Lard	0	31.20	31.20	31.20
Vitamin mixture	1.00	1.00	1.00	1.00
Mineral mixture	3.50	3.50	3.50	3.50
L-Cystine	0.30	0.30	0.30	0.30
Choline bitartrate	2.5	2.5	2.5	2.5
t-Butylhydroquinone	0.0014	0.0014	0.0014	0.0014
Protein	13.99 ± 0.28^{a}	14.23 ± 0.18^{a}	14.70 ± 0.16^{a}	14.50 ± 0.31^{a}
Lipid	7.49 ± 0.01^{a}	37.92 ± 0.37^{b}	38.54 ± 0.06^{b}	37.50 ± 0.26^{c}
Carbohydrate*	78.52 ± 0.18^{a}	47.85 ± 0.28^{b}	46.76 ± 0.21^{cb}	48.00 ± 0.21^{b}
Moisture	6.79 ± 0.14^{a}	4.15 ± 0.34^{b}	4.19 ± 0.25^{b}	4.63 ± 0.17^{b}
Ash	2.48 ± 0.01^{b}	1.99 ± 0.01^{c}	2.63 ± 0.16^{b}	2.98 ± 0.00^{a}
Energy‡	437.45	589.60	592.70	587.50

WPC = Whey protein concentrate; WPH = Whey protein hydrolyzate.
Tukey test at level of 5%. Means ± SEM followed by the same superscript letter do not differ statistically along the same line. *Carbohydrates calculated by difference. ‡Energy (kcal/100 g) = (lipids × 9) + (proteins × 4) + (carbohydrates × 4).

2.2. Sequencing the intestinal microbiota

Total DNA was extracted from the gut with the QIAmp DNA Stool Kit. Every two animals of each group were pooled to generate four replicates per group. The library construction and sequencing were performed as recommended by the manufacturer (Illumina, San Diego, CA, USA). The gene for the 16S rRNA were generated using degenerate primers targeting the V3 and V4 hypervariable region of the bacterial 16S rRNA gene and Nextera XT index kit (Illumina, San Diego, CA, USA). In this sequence 5′-*TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG***CCTACGGGNGGCWGCAG**-3′ the portion in italics corresponds to the adapter Nextera®, and the sequence in bold is the initiator widely conserved V3. The reverse initiator used was 5′-*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG***GACTACHVGGGTATCTAATCC**-3′, the portion in italics corresponding to the adapter Nextera and the sequence in bold is the initiator of ample utilization V4. For the preparation of the libraries, the kit "Illumina Truseq DNA Sample Preparation v2" was used, each sample being labeled with a bar code. The final pool, at a concentration after dilution 12 nM, was used for sequencing. The PhiX Control v3 (Illumina) was added to the pool at 35% of the final concentration as described in the Illumina procedure. Sequencing was performed on an Illumina Hiseq 2500 sequencer at the Laboratório Central de Tecnologias de Alto Desempenho em Ciências da Vida (LaCTAD-Unicamp, Campinas, Brazil).

2.2.1. Phylotyping, characterization and taxonomy

The identity of sequences was calculated using the megablast technique. The taxonomy was filed seeking the best megablast against Green Genes. The algorithm is a high-performance implementation of the Ribosomal Database Project (RDP) Classifier described in Wang, Garrity, Tiedje, and Cole (2007).

2.3. Western blotting

Extracts were applied onto a polyacrylamide gel, separated by electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Bio-Rad transfer apparatus). After transfer, the membranes were blocked for 2 h with a 5% solution of skim milk in Tris-saline (base) solution, and then washed with the basal solution in three 10-min steps. Then, the membranes were incubated overnight with anti-TLR4 antibody (SC 30002; α-Tubulin (Cell 2144), Cell Signaling Technology, Inc., Danvers, MA, USA) under continuous stirring at 4 °C, and washed till the next day with the basal solution three times again. Subsequently, the membrane was incubated with the secondary antibody in blocking solution for 2 h, washed with baseline solution for 2 h and, lastly, receiving a chemiluminescent solution. The method identified and quantified the immunoreactive bands by densitometry, followed by image capturing and quantification in a Bio-Rad photodocumenter. The generated files were analyzed using the Un-Scan-It Gel 6.1® program.

2.4. Blood for LPS and standard biochemical analyses

After anesthesia, blood was collected by vein puncture of the aorta, the blood samples placed in sterile tubes in order to separate the serum by centrifugation (1000 ×*g* for 10 min at 4 °C), and the sera frozen at −80 °C until analysis. Serum concentrations of endotoxins were measured using the colorimetric quantitative method LAL (Lyzate Amebocyte from *Limulus*). Kinetic Chromogenic LAL commercial kits were from Lonza (Lonza, Inc. Allendale, N.J., USA; cat n° 50–647 U). Briefly, the procedure involved the dilution of 20 μL at 1:5 in 80 μL, reagent:water (Lonza). Following inactivation at 70 °C for 10 min, the diluted samples (25 μL) were pipetted onto a 96-well sterile plate (LAL). Then, 50 μL of the commercial lysate solution were added to each well and incubated for 10 min at 37 ± 1 °C. Passed this time, 100 μL of the substrate reaction solution were pipetted, and the reaction

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