Dietary additive octyl and decyl glycerate modulates metabolism and inflammation under different dietary patterns with the contribution of gut microbiota

Junhui Zhang[1, 3, 4, a], Huilin Yu[1, 3, 4, a], Qianqian Wang[1, 3, 4], Haiying Cai[1, 2], Fei Shen[1, 3, 4], Shengyue Ruan[1, 3, 4], Yue Wu[1, 3, 4], Tao Liu[1, 3, 4], Fengqin Feng[1, 3, 4], and Minjie Zhao[1, 3, 4]*

1. College of Biosystems Engineering and Food Science, Zhejiang University, Hangzhou, 310058, China;

2. School of Biological & Chemical Engineering, Zhejiang University of Science &Technology, Hangzhou, 310023, China;

3. National Engineering Laboratory of Intelligent Food Technology and Equipment, Zhejiang University, Hangzhou, 310058, China;

4. Key Laboratory for Agro-Products Nutritional Evaluation of Ministry of Agriculture, Zhejiang University, Hangzhou, 310058, China.

* Correspondence:

Professor Minjie Zhao

Zhejiang University, 866 Yuhangtang Road, Hangzhou, 310058, China

E-mail: minjiezhao@zju.edu.cn

[a] Junhui Zhang and Huilin Yu contributed equally to this work

Supplementary methods:

2.3 Biochemical analysis

Using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) and following the manufacturer's instructions to measure serum levels of high-density lipoprotein cholesterol (HDL cholesterol), low-density lipoprotein cholesterol (LDL cholesterol), triglycerides, total cholesterol, and glucose. Free fatty acid (FFA), insulin, lipopolysaccharide binding protein (LBP), lipopolysaccharide (LPS), interleukin 1β (IL-1β), interleukin 6 (IL-6), tumor necrosis factor (TNF), and monocyte chemotactic protein 1 (MCP-1) were all measured using ELISA kits from Wuhan ColorfulGene Biological Technology Co., Ltd. (Wuhan, Hubei, China). The method used to calculate the homeostasis model assessment of insulin resistance (HOMA-IR) was serum insulin (mU L−1) × serum glucose (mmol L−1)/22.5. The specific experimental methods of ELISA kits are as follows:

1. Dilution of Standards

Ten wells are set for standards in a Microelisa stripplate. In Well 1 and Well 2, 100 μL

Standard solution and 50 μL Standard Dilution buffer are added and mixed well. In Well 3 and Well 4, 100 μL solution from Well 1 and Well 2 are added respectively. Then 50 μL Standard Dilution buffer are added and mixed well. 50 μL solution is discarded from Well 3 and Well 4. In Well 5 and Well 6, 50 μL solution from Well 3 and Well 4 are added respectively. Then 50 μL Standard Dilution buffer are added and mixed well. In Well 7 and Well 8, 50 μL solution from Well 5 and Well 6 are added respectively. Then 50 μL Standard Dilution buffer are added and mixed well. In Well 9 and Well 10, 50 μL solution from Well 7 and Well 8 are added respectively. Then 50μl Standard Dilution buffer are added and mixed well. 50 μL solution is discarded from Well 9 and Well 10. After dilution, the total volume in all the wells are 50 μL and the concentrations are 120 pg/mL, 80pg/mL, 40pg/mL, 20pg/mL and 10pg/mL, respectively.

2. In the Microelisa stripplate, leave a well empty as blank control. In sample wells, 40 μL sample dilution buffer and 10 μL sample are added (dilution factor is 5).

Samples should be loaded onto the bottom without touching the well wall. Mix well with gentle shaking.

3. Add 50 μL HRP-Conjugate reagent to each well except the blank control well.

4. Incubation: incubate for 30 min at 37℃ after being sealed with Closure plate membrane.

5. Dilution: dilute the concentrated washing buffer with distilled water (30 times for 96T and 20 times for 48T).

6. Washing: carefully peel off the Closure plate membrane, aspirate and refill with the wash solution. Discard the wash solution after resting for 30 seconds. Repeat the washing procedure 5 times.

7. Coloring: Add 50 μL Chromogen Solution A and 50 μL Chromogen Solution B to each well, mix with gently shaking and incubate at 37℃ for 10 minutes. Please avoid light during coloring.

8. Termination: add 50 μL stop solution to each well to terminate the reaction. The color of the well should change from blue to yellow.

9. Read absorbance O.D. at 450 nm using a Microtiter Plate Reader. The OD value of the blank control well is set as zero. The assay should be carried out within 15 minutes after adding the stop solution.

2.4 Histology analysis

The tissues were taken out of paraformaldehyde, alcohol-dehydrated, embedded in paraffin wax, and cut into 4 μm thickness. The epididymal fat was stained with hematoxylin and eosin (H&E) in Experiment 1 and Experiment 2. The specific experimental methods of H&E are as follows:

1. Dewaxing: Put the paraffin section sample baked on the dyeing rack in the oven into xylene I for 20min. The sample was transferred to xylene II and soaked for 20min. After the wax on the sample dissolved, the sample was transferred to anhydrous ethanol I to soak for 5min. Transfer the sample to anhydrous ethanol II for 5min. Scrub the 20s with anhydrous ethanol. Transfer the 20S to the basin and rinse the alcohol on the sample with tap water.

2. Dyeing and dehydration: Slice into HE dye solution 1 in the dyeing tank for 3-5min. Take out the sections and wash them with a dyeing cup until the sections are colorless. Slice into HE staining solution 2 in the dyeing tank for 3-5s and wash quickly. Slice into HE staining solution 3 for 3-5s in the dyeing tank and wash quickly. Slice into 85% ethanol, 95% ethanol, HE staining solution 4, anhydrous ethanol I anhydrous ethanol II, anhydrous ethanol III, n-butanol, xylene I, and xylene II and soak in each cylinder for 3-5min.

3. Sealing plate: Remove the slices and put them in the tuyere to dry quickly. Seal the slices with neutral gum.

The liver was stained with oil red O in Experiment 2. The stained slices were examined using the Leica Application Suite v4 (Wetzlar, Germany). The specific experimental methods of oil red O are as follows:

1. Reheat and dry the frozen slices, then fix them in the fixative solution for 15 minutes, wash them with tap water, and dry them.

2. Stain sections with Oil Red solution for 8- 10 min in the dark, and cover it with a lid during dyeing.

3. Take out the slices, stay for 3s and then immerse them in two cups of 60% isopropanol for differentiation, in turn, 3s and 5s respectively. The slices were immersed in 2 cups of pure water in turn for 10s each.

4. Take out the slices, immerse in hematoxylin for 3-5 min after 3s, and then rinse in 3 cups of pure water for 5s, 10s, and 30s in turn. Treat it with a differentiation solution (60% alcohol as solvent) for 2-8s, 2 cups of distilled water for 10s each, and Scott Tap Bluing for 1s. Then lightly dip the slices in 2 cylinders of tap water for 5s and 10s in turn, and check the staining effect by microscope.

5. Seal the slices with glycerin gelatin.

6. Observe microscope inspection, image acquisition, and analysis.

The size and frequency of the epididymal fat and the fat region of the liver were determined using Image-Pro Plus 6.1 (Media Cybernetics, Inc., Rockville, MD, USA).

Supplementary Table 1

Compositions of experimental diets

Ingredients (g / 100 g diet)	**Normal chow diet**	**High fat diet**
Casein	18.96	23.31
L-Cystine	0.28	0.35
Corn Starch	29.86	8.48
Maltodextrin	3.32	11.65
Sucrose	33.17	20.14
Cellulose	4.74	5.83
Soybean Oil	2.37	2.91
Lard	1.90	20.68
Mineral Mix	2.68	3.31
Potassium Citrate, 1 H_2O	1.56	1.92
Vitamin Mix	0.95	1.16
Choline Bitartrate	0.19	0.23
Calories supplementation (kcal %)		
Proteins	20	20
Carbohydrates	70	35
Fats	10	45
Total calories (kcal / 100 g diet)	385	473

Supplementary Table 2

Primer Sequences Used for qRT-PCR Analysis

	Forward Primer (5'-3')	Reverse Primer (5'-3')
PPARα	GTCCTCAGTGCTTCCAGAGG	GGTCACCTACGAGTGGCATT
PPARg	GCATTTCTGCTCCACACTATGA	TCGCACTTTGGTATTCTTGG
SCD1	CCGAAGAGGCAGGTGTAGAG	TTCTTACACGACCACCACCA
SREBP-1C	GATCAAAGAGGAGCCAGTGC	TAGATGGTGGCTGCTGAGTG
HMGCR	GGACCAACCTTCTACCTC	CCATCACAGTGCCACATAC
LDLR	CAGCTCTGTGTGAACCTGGA	TTCTTCAGGTTCGGGATCAG
VLDLR	TACCCTAGACGGAGCCAAGA	GTAAACAAAGCCCGACAACG
G6PC	TCTGTCCCGGATCTACCTTG	GTAGAATCCAAGCGCGAAAC
PEPCK	TAGGAGCAGCCATGAGAT	CGAAGTTGTAGCCGAAGA
TNF	AGGCACTCCCCCAAAAGAT	CAGTAGACAGAAGAGCGTGGTG
CCL2	GTGCTGACCCCAAGAAGGAA	GTGCTGAAGACCTTAGGGCA
TLR4	TATCCAGGTGTGAAATTGAAACA ATT	GGGTTTCCTGTCAGTATCAAGTTT G
IL-6	CTCCCAACAGACCTGTCTATAC	CCATTGCACAACTCTTTTCTCA
FXR	CCAGACAGACAATACATCAAG	TTCACTCTCCAAGACATCAG
SHP	GGAGTATGCGTACCTGAAG	TGCCTGGAATGTTCTTGAG
YWHAZ	TTCTTGATCCCCAATGCTTC	TTCTTGTCATCACCAGCAGC