**Supplemental Material**

### Gut lactate increases circulating L-Lac-Phe and key metabolites linked to human health

Emaan Ghias 1, Mette Glavind Bülow Pedersen 2,3,4, Camilla Bak Nielsen 1, Louise Vase Bech 1, Ida Marie Modvig 5, Natasa Brkovic Zubanovic 2,4, Jacob Marthinsen Seefeldt 6, Roni R. Nielsen 6, Esben Søndergaard 2,3,4, Kim Frisch 1, Jakob Hansen 1, Jens Juul Holst 5, Niels Møller 2,3, Nikolaj Rittig 2,3,4\*, Mogens Johannsen 1\*

*1 Department of Forensic Medicine, Aarhus University, Aarhus, Denmark; 2 Department of Clinical Medicine, Aarhus University, Aarhus, Denmark; 3 Department of Endocrinology and Metabolism, Aarhus University Hospital, Aarhus, Denmark; 4 Steno Diabetes Center Aarhus, Aarhus University Hospital, Aarhus, Denmark; 5 Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark; 6 Department of Cardiology, Aarhus University Hospital, Aarhus, Denmark*

### This file includes:

Supplementary Methods

Supplemental Table S1

Supplemental Figure S1 to S3

### Methods

## Reagents

Acetonitrile (hyper grade), methanol (hyper grade), and formic acid (LC-MS grade) were obtained from VWR International, LLC (Radnor, USA). L- and D-Lac-Phe were synthesized in-house. Authentic standards of L-Lac-Val (((S)-2-hydroxypropanoyl)-L-valine) was purchased from Enamine Ltd. (Kyiv, Ukraine) and d5-Lac-Phe (N-lactoyl-phenylalanine-d5) was purchased from Hello Bio Limited (Dunshaughlin, Ireland).

## Plasma preparation for targeted analysis of Lac-AAs

To prepare the samples, 1500 μL of 100% ice-cold methanol was added to 200 μL of plasma for protein precipitation. The extraction suspension was shaken at room temperature for 5 min at 1000 RPM and then centrifuged (12 x g, 10 min, room temperature). The supernatant was transferred to glass tubes and dried at 40°C using a SpeedVac™ SPD1030 (Thermo Fisher Scientific, Waltham, MA, USA). Dried samples were reconstituted in 75 μL of 0.1% formic acid and centrifuged (12 x g, 5 min, room temperature). Lastly, supernatants were transferred to MS vials and stored at -20 °C until analysis.

## Animals and ethical considerations

6 male Wistar rats (~230 g) were obtained from Janvier (Le Genest-Saint-Isle, France) and housed two to four rats per cage. Rats were allowed one week of acclimatization and kept on a 12:12 h light/dark cycle with ad libitum access to water and standard feed.

Experiments were conducted with permission from the Danish Animal Experiments Inspectorate (2023-15-0201-01408) and the local ethical committee (EMED P23-262) in accordance with the EU Directive 2010/63/EU, Danish legislation governing animal experimentation (1987), and the National Institute of Health.

## Isolated rat small intestine perfusion protocol

Non-fasted rats were anesthetized using Hypnorm/midazolam (0.079 mg fentanyl citrate + 2.5 mg fluanisone + 1.25 mg midazolam) via subcutaneous injection. The intestines were isolated and perfused in situ as described previously (1–3).

The intestinal lumen was flushed with isotonic (0.9%; 0.31 Osmol/L) NaCl at room temperature, to flush out any intestinal contents. A catheter was inserted into the superior mesenteric artery and used to vascularly perfuse the intestine with perfusion buffer (Krebs- Ringer bicarbonate buffer fermented with 0.1% BSA, 5% dextran T-70 (Pharmacosmos, Holbaek, Denmark) containing 3.5 mM of glucose) at a flow rate of 7.5 mL/min. The perfusion buffer was warmed up to 37°C and oxygenated (95% O2 and 5% CO2) by passing it through a Uniper UP-100 perfusion system (Hugo Sachs; Harvard Apparatus, March-Hugstetten, Germany), and perfusion pressure was measured continuously throughout each experiment.

A metal catheter was inserted into the hepatic portal vein to collect the venous effluent – perfusate. Rats were euthanized by perforation of the diaphragm when proper flow was achieved. The isolated intestines were perfused for 25 minutes for equilibration of the system before the experimental protocol was started.

Each protocol started with a 15-minute baseline infusion followed by addition of lactate solutions through the luminal tube. The intestines were luminally stimulated using two different concentrations of lactate, 36 mg/mL for 10 minutes and then 72 mg/mL for 15 minutes. The intestines were then vascularly stimulated with a 1.8 mg/mL lactate solution at an infusion rate of 0.375 mL/min. To measure Lac-AA levels, approximately 3 mL of perfusate was collected every 5 minutes for 115 minutes through the draining catheter. Samples were immediately placed on ice and stored at -20°C until further analysis. Lac-AAs were measured as detailed below.

The model was luminally perfused with NaCl at a flow rate of 0.250 mL/min between each intervention and a 5-minute Bombesin (BBS) vascular stimulation was used as a positive control for GLP-1 at the end of each experiment.

To measure GLP-1 output, perfusate was collected every 1 min through the draining catheter. Samples were immediately placed on ice and stored at -20°C until further analysis. Total GLP-1 was measured with an in-house radioimmunoassay using a C-terminal specific antibody targeting amidated forms of GLP-1 (code no. 89390) (4).

## Perfusate preparation for targeted analysis of Lac-AAs

To prepare the samples, 1400 μL of 100% ice-cold methanol and 100 μL of the d5-Lac-Phe internal standard in methanol were added to 500 μL of perfusate for protein precipitation. The extraction suspension was shaken at room temperature for 5 min at 1000 RPM and then centrifuged (12 x g, 10 min, room temperature). The supernatant was transferred to glass centrifuge tubes and dried down at 40°C using a TurboVap® LV (Biotage, Uppsala, Sweden) nitrogen evaporator. Dried samples were reconstituted in 75 μL of 0.1% formic acid and centrifuged (12 x g, 5 min, room temperature). Lastly, supernatants were transferred to MS vials and stored at -20°C until analysis.

## Targeted analysis of plasma and perfused intestine samples for Lac-AAs

Targeted analysis of all samples was performed on a Sciex Exion LCTM (SCIEX, Framingham, MA, USA) on an ACQUITY UPLC HSS T3 C18 column (2.1 mm x 100 mm, 1.8 µm; Waters, Dublin, Ireland). Multiple reaction monitoring (MRM) was performed using a Sciex QTRAP® 6500+ (SCIEX, Framingham, MA, USA) in ESI- mode. The mobile phase consisted of H2O with 0.1% FA (v/v) (A) and a mixture of MeOH/ACN (1:1 v/v), 0.1% FA (v/v) (B).

The autosampler was set at 10°C with 10 μL injection volume per sample. The flow rate was set to 0.35 mL/min at a column temperature of 45°C with the following 15 min gradient: 0% B (0-2 min), 0-40% B (2-6 min), 40-60% B (6-9 min), 60-88% B (9-10 min), 88-100% (10-11 min), 100% B (11-12 min), 100-0% (12-13 min), 0% B (13-15 min).

The Sciex QTRAP-MS/MS, equipped with an electrospray ionization source (ESI), was operated in negative mode (ESI−), using an IonSpray voltage of -4.0 kV. Collision gas was set to medium and dwell time was 0.025 seconds. Collision energies, declustering potential, and entrance potential for all MRM transitions were optimized by tuning authentic standards of target compounds into the mass spectrometer. Fragments tuned in for MRM analysis along with their respective MRM settings are outlined below:

**Table S1.** Acquisition parameters of target analytes and the internal standard, d5-Lac-Phe.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Compound** | **MRM transitions (m/z)** | **Collision energy (eV)** | **Declustering potential (V)** | **Entrance potential (V)** |
| L/D-Lac-Phe | 236 → 88 | -20 | -40 | -10 |
| L/D-Lac-Phe | 236 → 91 | -27 | -40 | -10 |
| L/D-Lac-Phe | 236 → 146.1 | -20 | -40 | -10 |
| L-Lac-Val | 188 → 43 | -16 | -59 | -6 |
| L-Lac-Val | 188 → 116 | -20 | -59 | -6 |
| L-Lac-Val | 188 → 144 | -34 | -59 | -6 |
| d5-Lac-Phe | 241.1 → 88.1 | -21 | -75 | -6 |
| d5-Lac-Phe | 241.1 → 96.1 | -29 | -75 | -6 |
| d5-Lac-Phe | 241.1 → 151.8 | -21 | -75 | -6 |

## Data Processing and Analysis

Data acquisition was carried out using Analyst 1.5 Software (AB Sciex, Concord, Ontario, Canada) and MultiQuant TM 3.0 Software (AB Sciex, Concord, Ontario, Canada) was used to integrate chromatographic peaks and visually inspect the data. Analyte levels were measured using peak areas that were normalized by the d5-Lac-Phe internal standard. Analyte concentrations were calculated using calibration curves by linear regression, where the peak area ratio of one analyte product ion and the product ion of the internal standard was plotted against the following theoretical concentrations.

For the plasma samples, seven-point calibration curves for L- and D-Lac-Phe and L-Lac-Val were generated in water at 1, 5, 10, 50, 200, and 500 nM. For the perfusate samples, six-point calibration curves for L-Lac-Phe and L-Lac-Val were generated in Krebs- Ringer bicarbonate buffer (purchased from Merck (Darmstadt, Germany)) at 0.2, 0.5, 1, 5, 10, and 50 nM. All standard curve samples were generated using synthesized standards of L- and D-Lac-Phe, and reference standards of L-Lac-Val and d5-Lac-Phe.

## Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 10.2.3). P values <0.05 were considered statistically significant. One-way ANOVA (*Šídák*) was used to test for statistical significance of GLP-1 concentrations (presented as means ± SEM); mean values from the stimulation period (20 consecutive minutes; minute 11-30 and minute 46-55) were compared with mean values from the baseline period (10 min before administration of lactate; minute 1-10 and minute 35-44).

## References

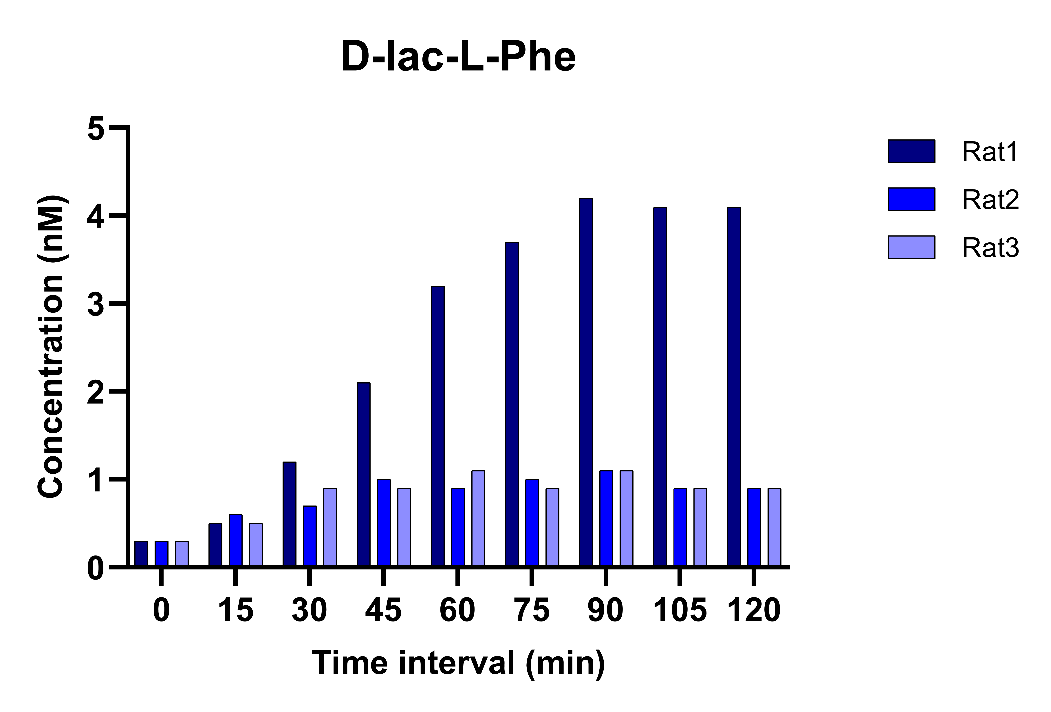
1. **Modvig IM**, **Kuhre RE**, **Jepsen SL**, **Xu SFS**, **Engelstoft MS**, **Egerod KL**, **Schwartz TW**, **Ørskov C**, **Rosenkilde MM**, **Holst JJ**. Amino acids differ in their capacity to stimulate GLP-1 release from the perfused rat small intestine and stimulate secretion by different sensing mechanisms. *Am J Physiol Endocrinol Metab* 320: E874–E885, 2021. doi: 10.1152/ajpendo.00026.2021.

2. **Modvig IM**, **Kuhre RE**, **Holst JJ**. Peptone-mediated glucagon-like peptide-1 secretion depends on intestinal absorption and activation of basolaterally located Calcium-Sensing Receptors. *Physiol Rep* 7: e14056, 2019. doi: 10.14814/phy2.14056.

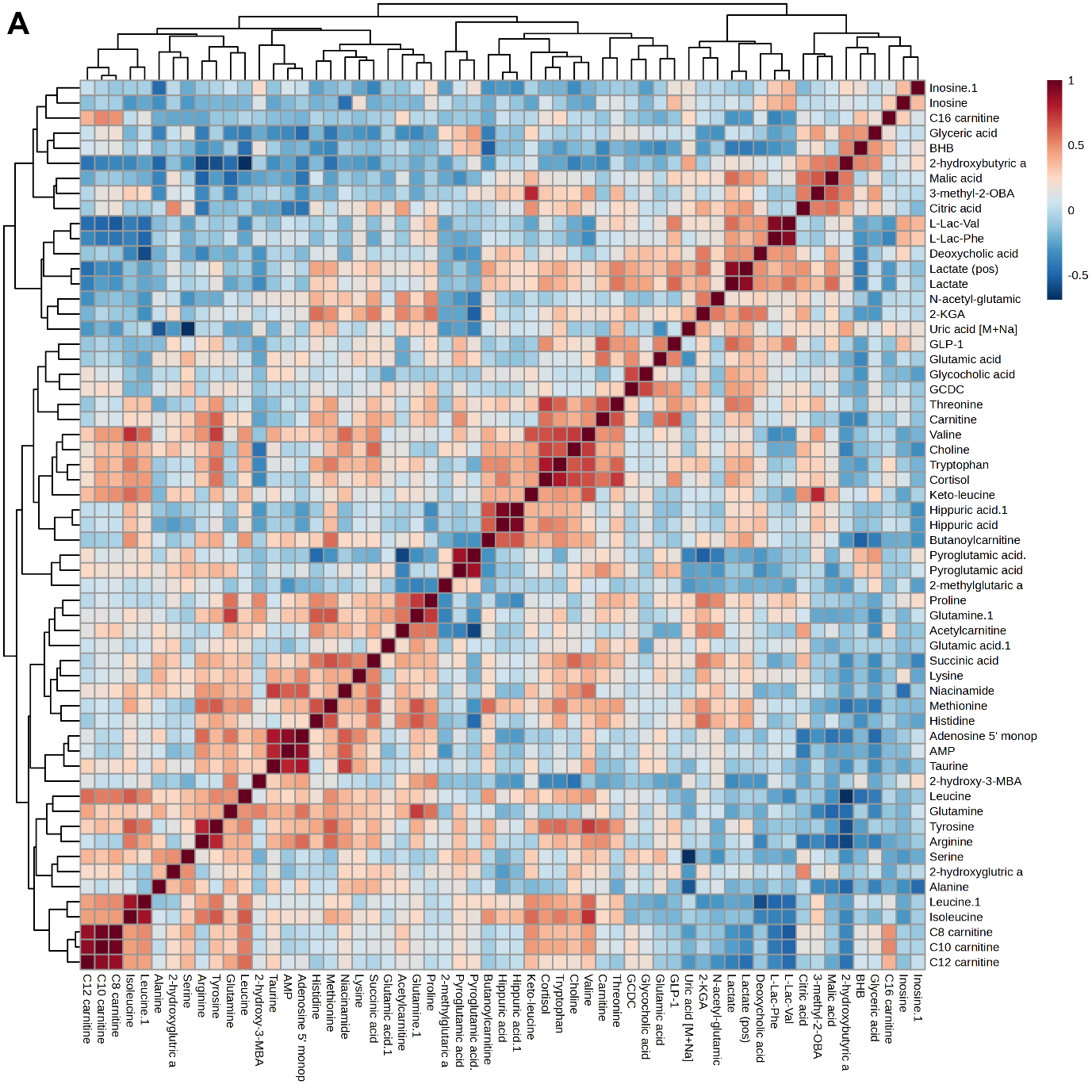
3. **Kuhre RE**, **Holst JJ**. Mechanisms Underlying Gut Hormone Secretion Using the Isolated Perfused Rat Small Intestine. *J Vis Exp* 2019: e58533, 2019. doi: 10.3791/58533.

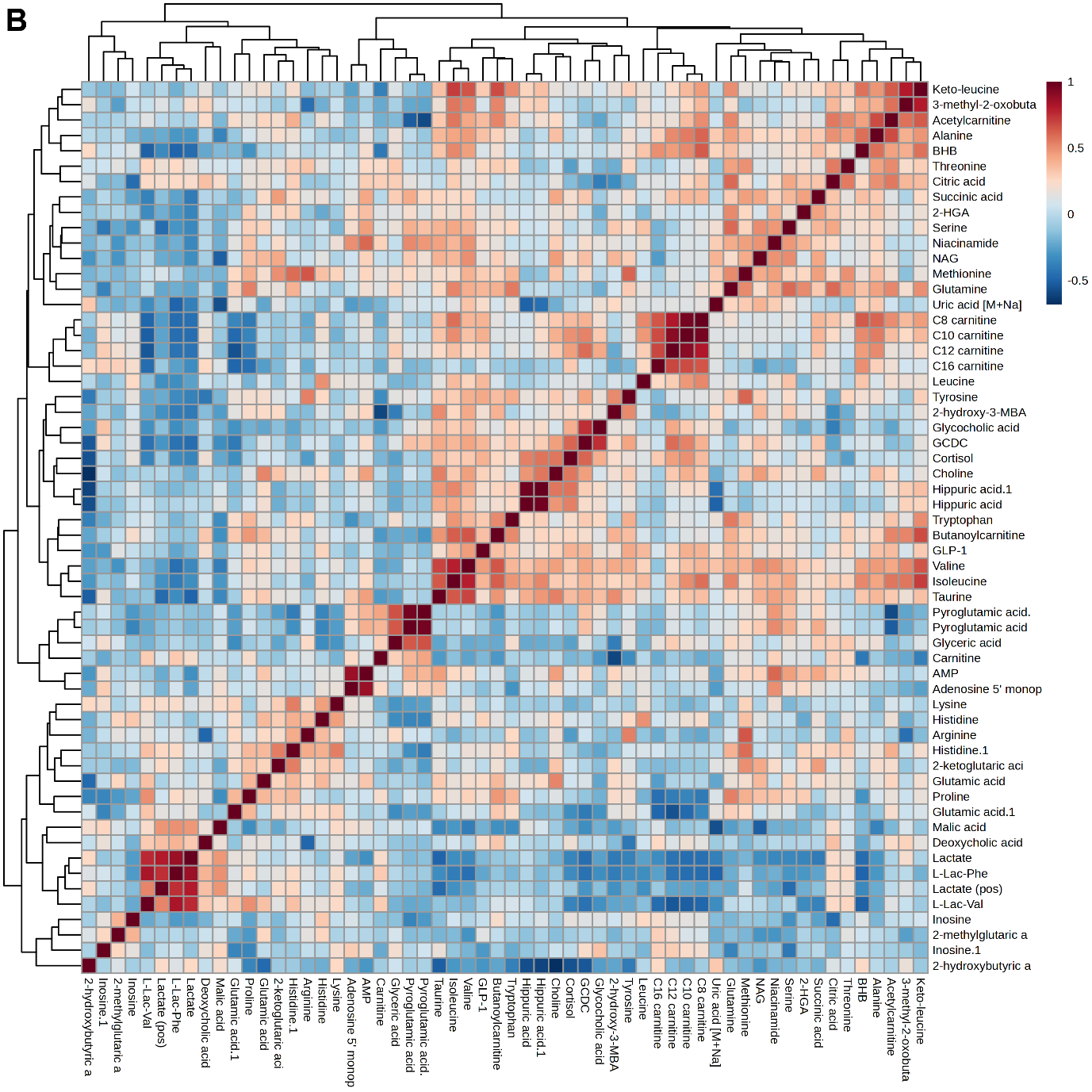
4. **Ørskov C**, **Rabenhøj L**, **Wettergren A**, **Kofod H**, **Holst JJ**. Tissue and Plasma Concentrations of Amidated and Glycine-Extended Glucagon-Like Peptide I in Humans. *Diabetes* 43: 535–539, 1994. doi: 10.2337/diab.43.4.535.

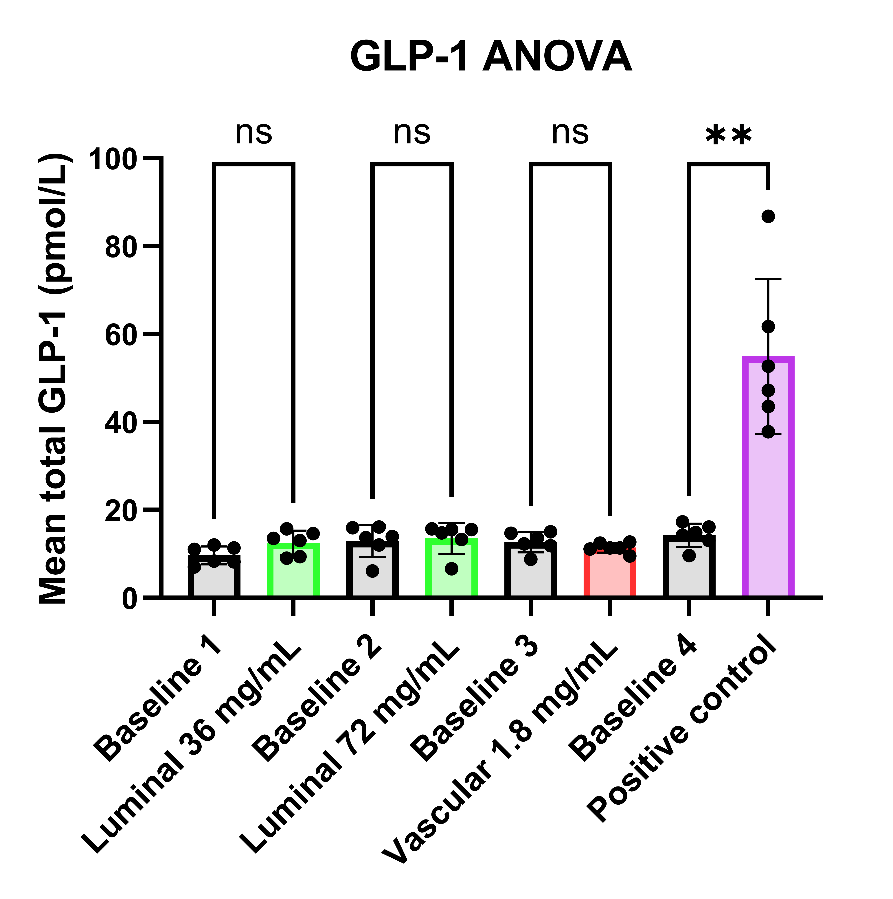
**Supplemental Figures**



**Figure S1.** Plasma concentrations of D-lac-L-Phe measured in rats after oral administration of 3200 mg/kg D-lactate.

**Figure S2A.** Hierarchical clustering analysis. The heatmap was generated using MetaboAnalyst 6.0 and shows Pearson correlations of annotated metabolites after oral (*A*) administration of D/L-Na lactate. Each unit represents a metabolite colored by its intensity on a normalized scale from blue (decreased) to red (increased).

**Figure S2B.** Hierarchical clustering analysis. The heatmap was generated using MetaboAnalyst 6.0 and shows Pearson correlations of annotated metabolites after intravenous (*B*) administration of D/L-Na-lactate. Each unit represents a metabolite colored by its intensity on a normalized scale from blue (decreased) to red (increased).



**Figure S3.** Statistical testing of GLP-1 output measured across the perfused rat intestine experiments (n = 6). Data are presented as mean ± SEM. ns, nonsignificant, p > 0.05. \*p < 0.05, \*\*p < 0.021, \*\*\*p < 0.002, \*\*\*\*p < 0.0001 by a One-way ANOVA with correction for multiple testing (*Šídák*).