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Effect of *Lactobacillus casei* Shirota supplementation on trimethylamine-*N*-oxide levels in patients with metabolic syndrome: An open-label, randomized study

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ABSTRACT

Background: Recent studies in animal models have shown a link between ingestion of dietary phosphatidylcholine (PC), choline, L-carnitine and cardiovascular risk. Intestinal microbiota-dependent metabolism of PC and L-carnitine is involved in formation of trimethylamine (TMA), which is further metabolized to the proatherogenic compound trimethylamine-*N*-oxide (TMAO). It has been suggested that changes in gut microbiota by supplementation of probiotic drinks might alter TMAO levels. Hence, the aim of this analysis was to investigate the impact of *Lactobacillus casei* Shirota (*Lc*S) on formation of TMAO in subjects with metabolic syndrome.

Methods: In a single-center, prospective, randomized-controlled study 30 subjects with metabolic syndrome were randomized to receive either 3 times daily 6.5×10^9 CFU (colony-forming units) *Lc*S (probiotic group) or not (standard therapy group) for 12 weeks. TMAO plasma levels were quantified by means of liquid chromatography and tandem mass spectrometry.

Results: Thirteen patients in the probiotic group and 15 in the standard therapy group finished the study. Mean age was 52 ± 11 and 55 ± 9 years, respectively. TMAO levels decreased during the intervention period in both groups (from 4.66 ± 2.66 μM to 4.31 ± 2.04 μM in the probiotic group and from 4.64 ± 2.75 μM to 4.40 ± 2.14 μM in the control group). Changes in TMAO between subjects receiving *Lc*S (−0.25 ± 2.39 μM) and controls (−0.34 ± 2.23 μM) were not significantly different ($p = 0.510$).

Conclusion: In conclusion, intake of *Lc*S for 12 weeks did not affect levels of TMAO in patients with metabolic syndrome.

1. Introduction

Although the mortality rates of cardiovascular disease (CVD) decreased substantially over the last decade, still almost half of all deaths in Europe are attributable to CVD [1]. Recently, there is growing evidence that the intestinal microbial organisms interfere in the development of complex metabolic phenotypes including obesity, insulin resistance and cardiovascular disease [2–5]. Several animal models and human clinical studies identified a direct mechanistic link between intestinal microbiota-dependent metabolism of the choline fraction in dietary phosphatidylcholine

Abbreviations: TMA, trimethylamine; TMAO, trimethylamine-*N*-oxide; *Lc*S, *Lactobacillus casei* Shirota; PC, phosphatidylcholine; C, choline; CVD, cardiovascular disease; MetS, metabolic syndrome; FMO, flavin monooxygenase; sVCAM-1, soluble vascular cell adhesion molecule 1; sICAM-1, soluble intercellular adhesion molecule 1; hsCRP, high-sensitivity C-reactive Protein; HOMA-IR, Homeostasis Model Assessment for Insulin Resistance; LAC/MAN-Ratio, lactulose/mannitol ratio; DAO, diaminooxidase; CFU, colony-forming unit.

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(lecithin) and cardiovascular disease through the production of trimethylamine-*N*-oxide (TMAO) [6–8]. In a first step, the choline component of phosphatidylcholine (PC) and L-carnitine are metabolized by gut microbiota to trimethylamine (TMA). In a second step, TMA is then converted by hepatic flavin monooxygenases into TMAO in the liver. The meta-organismal pathway from PC to TMAO is shown in Fig. 1. The major dietary sources for choline, and hence the TMAO production include eggs, milk, liver, red meat, shell fish, poultry and fish [9].

Although TMAO was suggested to be proatherogenic [10], the detailed mechanism by which TMAO might be causally linked to accelerated cardiovascular disease and what role specific microbial species play is currently unclear.

Therefore it was hypothesized, that changes in gut microbiota by the supplementation of probiotic drinks might reduce the metabolism of choline or L-carnitine into TMA, but interventional data in humans are lacking so far.

The aim of the current analysis was to investigate whether *Lactobacillus casei* Shirota (*Lc*S) supplementation over 12 weeks has an impact on TMAO formation in subjects with metabolic syndrome.

2. Materials and methods

2.1. Subjects

Adult metabolic syndrome patients were identified from the outpatient clinic at the Division of Endocrinology and Metabolism at the Medical University of Graz. 30 patients with metabolic syndrome (MetS) were randomized to receive either food supplementation with a milk drink containing *Lc*S (3 bottles a day, á 65 ml, containing *Lc*S at a concentration of 10^8/ml, Yakult light®, Yakult Austria, Vienna) for twelve weeks or served as controls without the supplementation of the milk drink. MetS was defined using the modified NCEP-ATP-III-guidelines [11]. Patients treated with antibiotics within the previous 7 days, any immunomodulatory therapy 1 month prior to study entry, concomitant use of supplements (pre-, pro-, or synbiotics), inflammatory bowel or celiac disease or subjects with clinical signs of infectious diseases were excluded from participation. Written informed consent was provided by all subjects, and the study protocol was approved by the regional ethics committee in Graz (20-037 ex 08/09) and performed according to the Declaration of Helsinki.

2.2. Study design

Details about the study design have been published previously [12]. In brief, we performed a single-center, prospective, randomized, controlled 12 weeks clinical trial. Patients were advised to consume no other probiotic supplements during the study period.

2.3. Assessments

Assessments were at baseline and study end (approximately 12 weeks after baseline). Biochemical and clinical assessment have been reported elsewhere [12].

2.4. Materials and determination of TMAO

D9-TMAO was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA). TMAO, acetonitrile and methanol were obtained from Sigma (St. Louis, MO). Plasma was immediately prepared from venous blood drawn into ethylenediaminetetraacetic acid tubes and then stored at −80 °C until analysis. 50 μl of human plasma were extracted with 405 μl acetonitrile and 45 μl methanol in the presence of an internal standard (4.5 nmol D9-TMAO). The mixture was vortexed, centrifuged and 200 μl of the supernatant were transferred into an autosampler vial. Chromatographic separation of TMAO was performed by an Accela HPLC (Thermo Scientific) on a Luna NH2 100 Å, 150 × 2 mm, 3 μm column. Solvent A was a water/acetonitrile (1:1, v/v) solution of 1% ammonium acetate (v/v) and 0.04% formic acid (v/v) and solvent B was acetonitrile/water (9:1, v/v). The gradient was run from 50% to 100% A in 6 min where it was held for another 2 min. The flow rate was 300 μl/min and 5 μl were injected from the autosampler vial. TMAO was determined by a TSQ Quantum Ultra (Thermo Scientific) triple quadrupole instrument in positive ESI mode. The spray voltage was set to 4000 V, capillary voltage to 35 V and the capillary temperature was at 270 °C. TMAO was detected in MRM mode at a transition from m/z 76 to m/z 58 and D9-TMAO at a transition from m/z 85 to m/z 67 respectively. Peak areas were calculated by QuanBrowser and TMAO was quantified by internal calibration based on a calibration curve.

2.5. Insulin resistance

The insulin resistance was calculated using the HOMA-IR (Homeostasis Model Assessment for Insulin Resistance) [13].

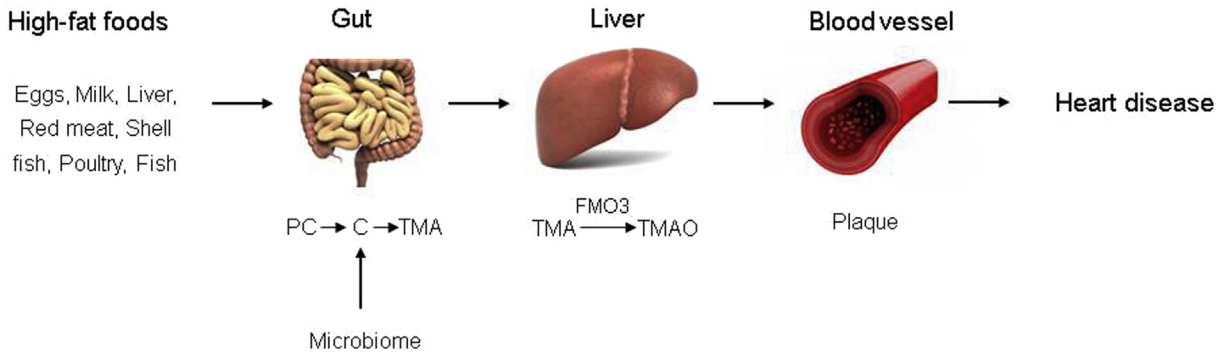

Fig. 1. TMAO metabolism and cardiovascular disease. In a meta-organismal pathway, the choline component of phosphatidylcholine (PC) and L-carnitine are metabolized by gut microbiota to trimethylamine (TMA). In the liver, the enzyme FMO3 processes TMA to TMAO. TMAO boosts the accumulation of cholesterol in macrophages, the accumulation of foam cells in artery walls, atherosclerosis and consequently it has been hypothesized that increased TMAO levels lead to heart attack, stroke, and cardiovascular death. TMA: trimethylamine; TMAO: trimethylamine-*N*-oxide; PC: phosphatidylcholine; C: choline; FMO: flavin monooxygenase. The images were kindly made available by Medicalgraphics.de (http://www.medicalgraphics.de/galerie.html).