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Avaliação da influência do vinho tinto sobre a microbiota intestinal e *N*-óxido de trimetilamina

Tese apresentada à Faculdade de Medicina da Universidade de São Paulo para obtenção do título de Doutor em Ciências

Programa de Cardiologia
Orientador: Prof. Dr. Francisco Rafael Martins Laurindo

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Evaluation of red wine influences on the gut microbiota and trimethylamine *N*-oxide

Thesis presented to the University of São Paulo School of Medicine for the degree of Doctor in Sciences

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Mentor: Prof. Dr. Francisco Rafael Martins Laurindo

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To Helena, Fernanda and Jaime.

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Abreviatura dos títulos e periódicos de acordo com List of Journals Index ed in Index Medicus.

Table of Contents

Lists

16S rRNA	high throughput sequencing targeting the 16S rRNA gene analysis
Abst	abstention
ACE	angiotensin-converting enzyme
ACTH	adrenocorticotropic hormone
ALT	alanine aminotransferase
AMG	amygdala
AMP	antimicrobial peptides
ANS	autonomic nervous system
ARB	angiotensin-receptor blocker
AST	aspartate aminotransferase
ASV	Amplicon Sequence Variants
BA	bile acid
BAT	brown adipose tissue
BCAA	branch-chain amino acid
CAD	coronary artery disease
CD 36	cluster of differentiation 36
CRF	corticotropin-releasing factor
CVD	cardiovascular disease
DBS	dried blood spots
DMB	3,3-dimethyl-1-butanol
ENS	enteric nervous system
ESI	electrospray ionization
FAD	flavin adenine dinucleotide
FGF15	fibroblast growth factor 15
FMN	flavin mononucleotide
FMO	flavin monooxygenase
FXR	farnesoid X receptor
GABA	gamma-aminobutyric acid
GEE	generalized estimating equation
GFR	glomerular filtration rate
GPCR	G protein-coupled receptor
HDAC	histone deacetylase
HDL	high-density lipoprotein cholesterol

HILIC	hydrophilic interaction
HIPP	hippocampus
HPA	hypothalamic pituitary adrenal
HYP	hypothalamus
ICC	intraclass Coefficient
IPA	indole propionate
LDL	low-density lipoprotein
LIMS	laboratory information management system
LPS	lipopolysaccharide
LPS	lipopolysaccharides
MAC	microbiota scessible carbohydrates
MACE	major cardiovascular events
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
PAMPs	pathogen-associated molecular patterns
PFPA	perfluoropentanoic acid
PGN	peptidoglycan
PPP	Pentose phosphate pathway
PRRs	pattern recognition receptors
PSA	polysaccharide
QC	quality control
RCT	reverse cholesterol transport
RP	reverse phase
rRNA	ribosomal RNA
RSD	relative standard deviation
RW	red wine
SCFA	short-chain fatty acids
sPLS-DA	sparse partial least squares regression - discriminant analysis
SR-A1	scavenger receptor A
TGR5	G-protein-coupled bile acid receptor 1
TMA	trimethylamine
TMAO	N-óxido de trimetilamina
TMAO	trimethylamine N-oxide

UPLC-MS/MS	ultra-performance liquid chromatography - tandem mass spectroscopy
VT	vinho tinto
WO	washout

Resumo

Haas EA. *Avaliação da influência do vinho tinto sobre a microbiota intestinal e N-óxido de trimetilamina* [tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2021.

Evidências recentes, experimentais e clínicas, revelam a importância da microbiota intestinal na fisiologia humana e em situações patológicas, especialmente em doenças cardiometabólicas. Diferentes perfis da microbiota intestinal são claramente relacionados à obesidade, diabetes mellitus tipo 2, esteatohepatite não alcoólica e doenças cardiovasculares. Mecanismos subjacentes responsáveis por esses efeitos deletérios incluem a ação de metabólitos dependentes da microbiota, como a N-óxido de trimetilamina (TMAO). TMAO é um metabólito plasmático, que tem como substrato dietético majoritariamente colina e carnitina, e se correlaciona fortemente com aterosclerose e eventos cardiovasculares maiores, como infarto do miocárdio e morte. Sendo assim, é um composto de grande interesse como instrumento diagnóstico e alvo terapêutico. Já o consumo moderado de vinho tinto (VT) é potencialmente cardioprotetor, através de mecanismos que podem envolver modulações do metabolismo redox e da microbiota intestinal. Além disso, o VT contém 3,3-dimetil-1-butanol, que reduz TMAO plasmática em animais. No entanto, os efeitos do VT na microbiota intestinal e na TMAO plasmática não são totalmente compreendidos. Conduzimos, portanto, um estudo randomizado, cruzado e controlado envolvendo 42 homens, com média de 60 anos e com doença arterial coronariana documentada. Comparamos o consumo de 250 mL de VT por dia, por 3 semanas, durante 5 dias na semana, com igual período de abstinência de álcool, com períodos de “washouts” entre as intervenções. Após o consumo de VT houve remodelamento significativo da microbiota intestinal, com diferença na beta diversidade e preponderância de *Parasuterella*, *Ruminococcaceae*, vários *Bacteroides* e *Prevotella*. A análise da metabolômica plasmática de 20 pacientes, escolhidos aleatoriamente, mostrou alterações após o VT consistentes com a modulação benéfica da homeostase redox, como o aumento nos precursores da riboflavina e do metabolismo do ascorbato. Entretanto, o TMAO plasmático não diferiu após a intervenção com VT. Além disto, os níveis de TMAO mostraram uma baixa concordância intra-individual ao longo do tempo. Desta forma, apontamos desafios para o uso de TMAO como biomarcador, dada sua alta variabilidade.

Em suma, a modulação da microbiota intestinal e da metabolômica plasmática podem contribuir para elucidar os possíveis benefícios cardiovasculares do consumo moderado de VT.

Descritores: Microbiota intestinal, Vinho, Metabolômica, *N*-óxido de Trimetilamina, Redox, Ribitol, Aminoácidos aromáticos, Indol Propionato, Endocanabinoides.

Abstract

Haas EA. *Evaluation of red wine influences on the gut microbiota and trimethylamine N-oxide* [thesis]. São Paulo: "Faculdade de Medicina, Universidade de São Paulo"; 2021.

Recent experimental and clinical evidence reveals the importance of the gut microbiota in human physiology and pathological situations, especially in cardiometabolic diseases. Different gut microbiota profiles closely relate to obesity, type 2 diabetes mellitus, non-alcoholic steatohepatitis and cardiovascular disease. Mechanisms for these deleterious outcomes include the effects of microbiota-dependent metabolites such as trimethylamine *N*-oxide (TMAO). TMAO, a plasma metabolite of dietary protein substrate - mainly choline and carnitine- strongly correlates with atherosclerosis and major cardiovascular events, such as myocardial infarction and death. Therefore, it is a highly studied compound as a diagnostic and therapeutic target. On the other hand, the moderate consumption of red wine (RW) is possibly cardioprotective through mechanisms that may involve redox metabolism and intestinal microbiota modulations. In addition, RW contains 3,3-dimethyl-1-butanol, which decreases plasma TMAO in animals. However, the effects of RW on the gut microbiota and plasma TMAO are not fully understood. Therefore, we conducted a randomized, crossover, and controlled study involving 42 male, on average 60 years old and with documented coronary artery disease. We compared the consumption of 250 mL of RW per day, for 3 weeks, for 5 days a week, with an equal period of abstinence from alcohol, given adequate washout periods between interventions. After RW consumption, there was a significant remodelling of the intestinal microbiota, with a difference in beta diversity and preponderance of *Parasuterella, Ruminococcaceae,* several *Bacteroides* and *Prevotella*. Analysis of the plasma metabolomics of 20 patients randomly selected showed, after RW, a beneficial modulation of redox homeostases, such as increased riboflavin precursors and ascorbate metabolism. However, plasma TMAO did not differ after intervention with RW. In addition, TMAO plasma levels showed a low intra-individual concordance over time. In this manner, we posed challenges for the use of TMAO as a biomarker, given its high intra-individual variability. Finally, the modulation of the gut microbiota and plasma metabolomics may contribute to the putative cardiovascular benefits of moderate RW consumption.

Descriptors: Gut microbiota; Wine; Metabolomics; trimethylamine *N*-oxide; Oxidation-reduction; Ribitol; Amino acids, aromatic; Idolepropionic acid;Endocannabinoids.

1. Introduction

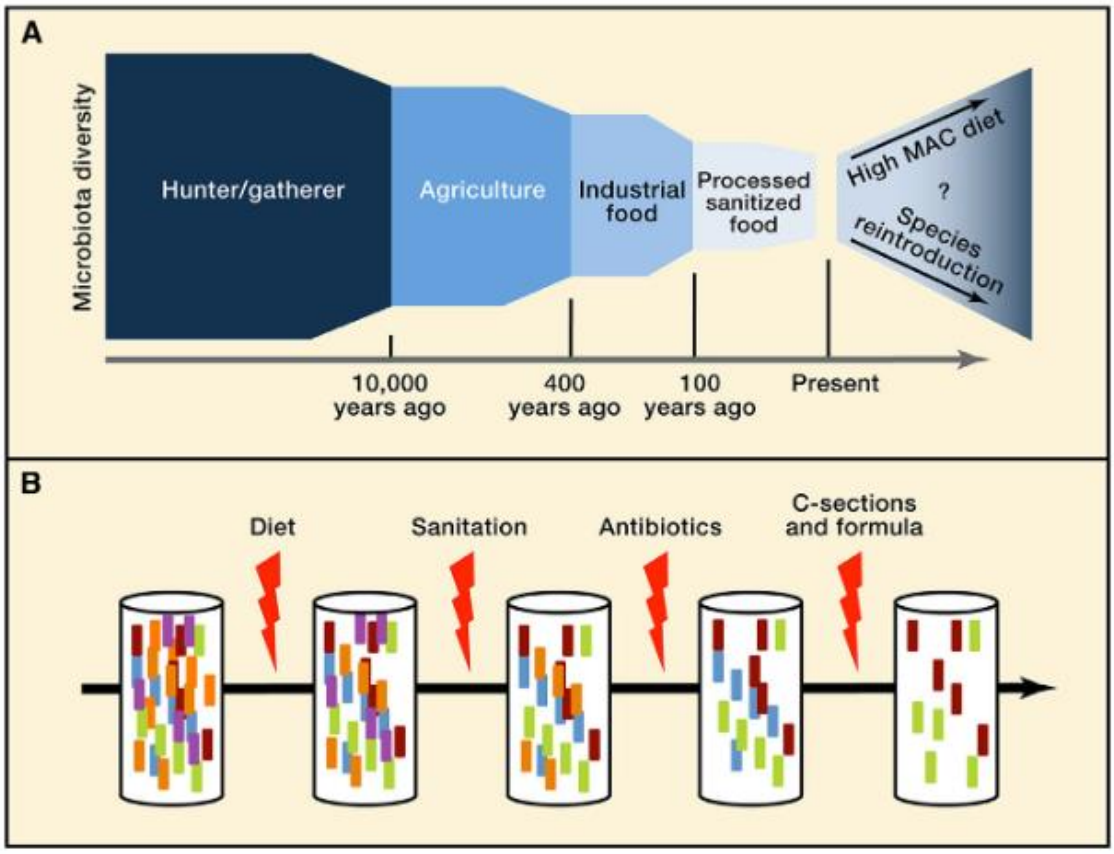
1.1. Gut microbiota: new insights of an ancient player

If we think of life on Earth as a timeframe of 24 hours, human beings arrived on the planet at the last minute. Since the first evidence of life on Earth ~ 4,5 billion years ago up to the upcoming of the first primates 70 million ago, a large part of the time the planet was mainly occupied by microbes. Human beings evolved side by side with bacteria and fungi long before we were humans, even before our ancestors were mammals. Therefore, it is not surprising that microorganisms inhabit and interact with all living beings, and the human body harbors a diverse ecosystem of trillions of microbial cells. It is estimated that bacteria cells that inhabit different organs and systems in humans possibly outnumber human cells in a ratio of 1: 1.3. There are also microorganisms in the intestine, such as fungi, archaea, and viruses, not yet precisely quantified.[1] However, solely in the gut, it is estimated that there are around 1000 bacterial species, with 2000 genes each. This supplants the number of human genes (~ 20,000) about 100 times. Approximately 40% of these bacterial genomes are found in the gut.[2] Although the human genes are stable and virtually do not change over a lifespan, the gut microbiome, i.e., the genes that compound the gut microbiota, are extremely modulable and, by several pathways, interacts with the host. The discovery of these essential microbial ecosystems inhabiting humans is a relatively new field in Sciences and now it is possible to map the human microbiome as never before. One of the factors enabling all these researches is the cost of genome sequencing, which is now 10 000 times cheaper than 15 years ago. And a million times cheaper than 20 years ago (https://www.genome.gov/sequencingcostsdata/).

These recent microbiome findings shed light on the traditional view that gut microbiota effects are limited to the host's digestive tract, or more recently extended to

metabolism and immune status, and fueled the conception that these microbes can widely impact diverse aspects of a host's physiology. Resembling an endocrine organ, the gut microbiota secretes metabolites that act like hormones, activating distant receptor systems in the human host. In the last decade, experimental and clinical evidence have increasingly shown the importance of the gut microbiota profile in health and disease states, especially cardiometabolic diseases.

There is ample evidence of gut microbiota signatures correlating with metabolic perturbances as obesity[3], insulin resistance and type 2 diabetes mellitus[4,5], nonalcoholic steatohepatitis[6], and cardiovascular disease (CVD)[7]. Interactions between resident microbes and host leading to immune dysregulation may explain several diseases that share inflammation as a common basis.[8] The decrease in microbiome diversity over time may influence gut microbiota pathological profiles that furnish inflammation (figure 1). Many circumstances changed in industrialized societies across time. For instance, diet and food processing radically changed in the last centuries since agriculture began ten thousand years ago. Although medical advances in the last two centuries clearly had a positive impact on life expectancy and overall populational health, for example, through vaccines, the use of antibiotics, as well as sanitary evolvements that controlled several infectious diseases, on the other hand, there was a rise of non-communicable diseases[9] (figure 2). Loss of microbiome diversity might interconnect these events.



A) Microbiota diversity was possibly altered at various steps of human evolution. As diversity and quantity of dietary fibers declined with agriculture, industrialized food production, and processed food, the image reflects data indicating a corresponding decline in microbiota diversity. Microbiota accessible carbohydrates, MAC. B) While diet is likely a key mediator of microbiota diversity, further technological and medical leaps while providing solutions for critical problems such as infectious disease have likely damaged the microbiota.

Figure 1- Human microbiota modifications in diversity over time in industrialized societies (from Sonnenburg, 2014).[8]

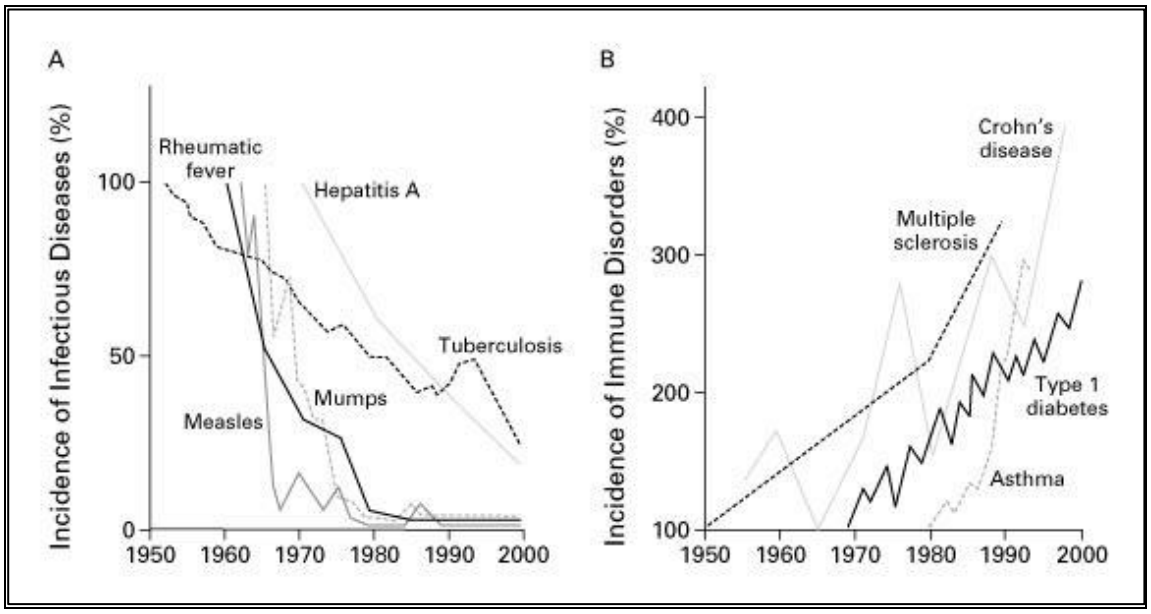
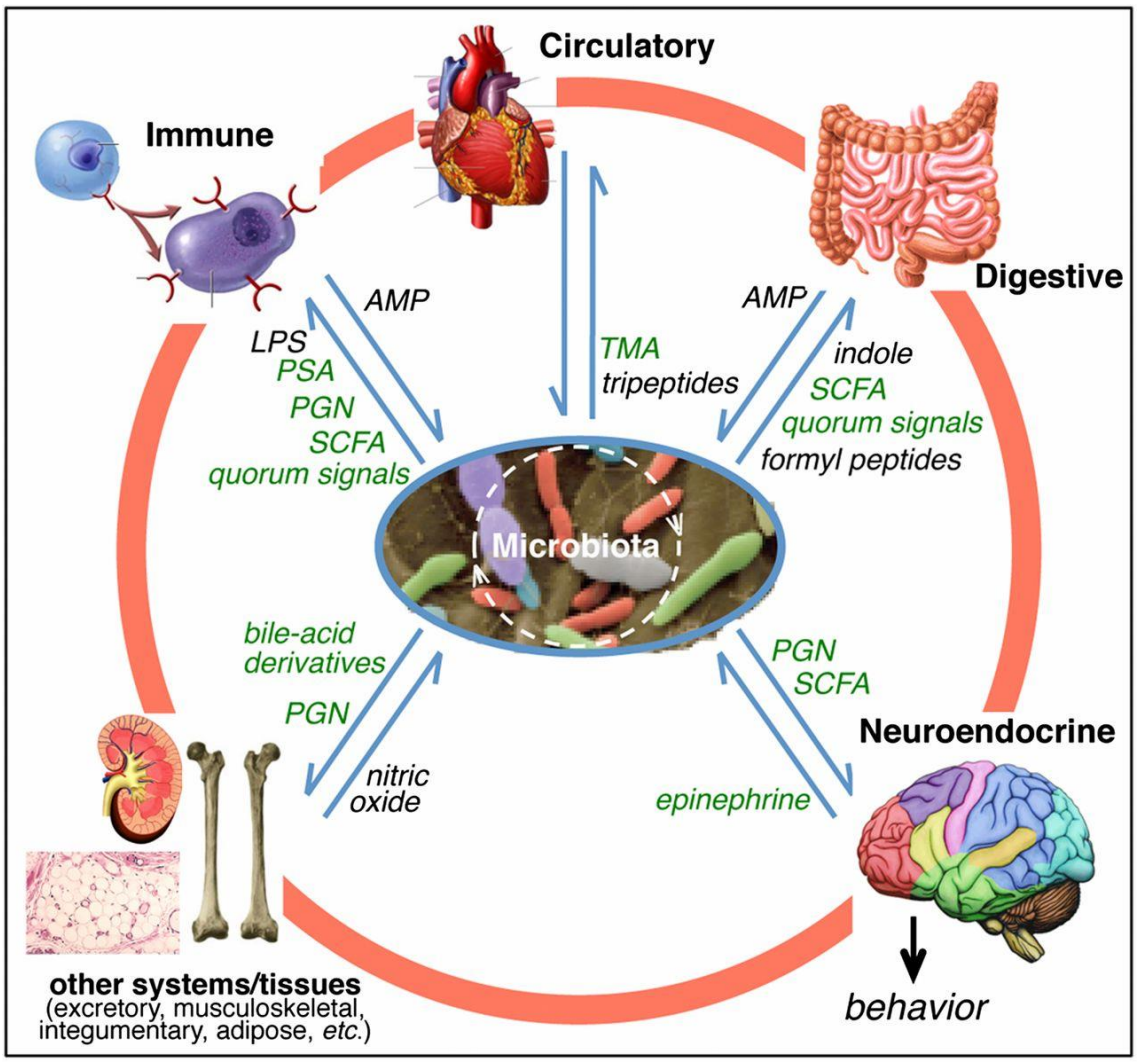


Figure 2- Inverse relation between the incidence of infectious diseases (panel A) and the incidence of immune disorders (panel B) from 1950 to 2000 (from Bach, 2002).[9]

1.2 Mechanisms by which gut microbiota interacts with the host

Several bidirectional pathways of interplays between the microbiota and host can influence several systems. Bacteria also have social behaviors, communicating with each other through chemical signaling (quorum signals). In addition to these signals, bacteria use cell surface-derived molecules to communicate with their hosts, affecting host processes both at the cellular level and at the system level organs (figure 3). On the other hand, host-derived signal molecules such as nitric oxide (NO) can be directly detected by microbes. The gut is probably the most dynamic and consequential bacterial signaling site that benefits animal hosts because of the number and diversity of its microbes and the inherent permeability and sensitivity of the gut epithelium.



Components of the microbiota, such as those in and on the gut, oral cavity, and skin, interact among themselves and exchange signals with the animal's organ systems, participating in the body's homeostasis. The microbiota also impacts animal behavior, creating a direct interface with other organisms. AMP, antimicrobial peptides; LPS, lipopolysaccharide; PGN, peptidoglycan; PSA, polysaccharide A; SCFA, short-chain fatty acids; TMA, trimethylamine oxide.

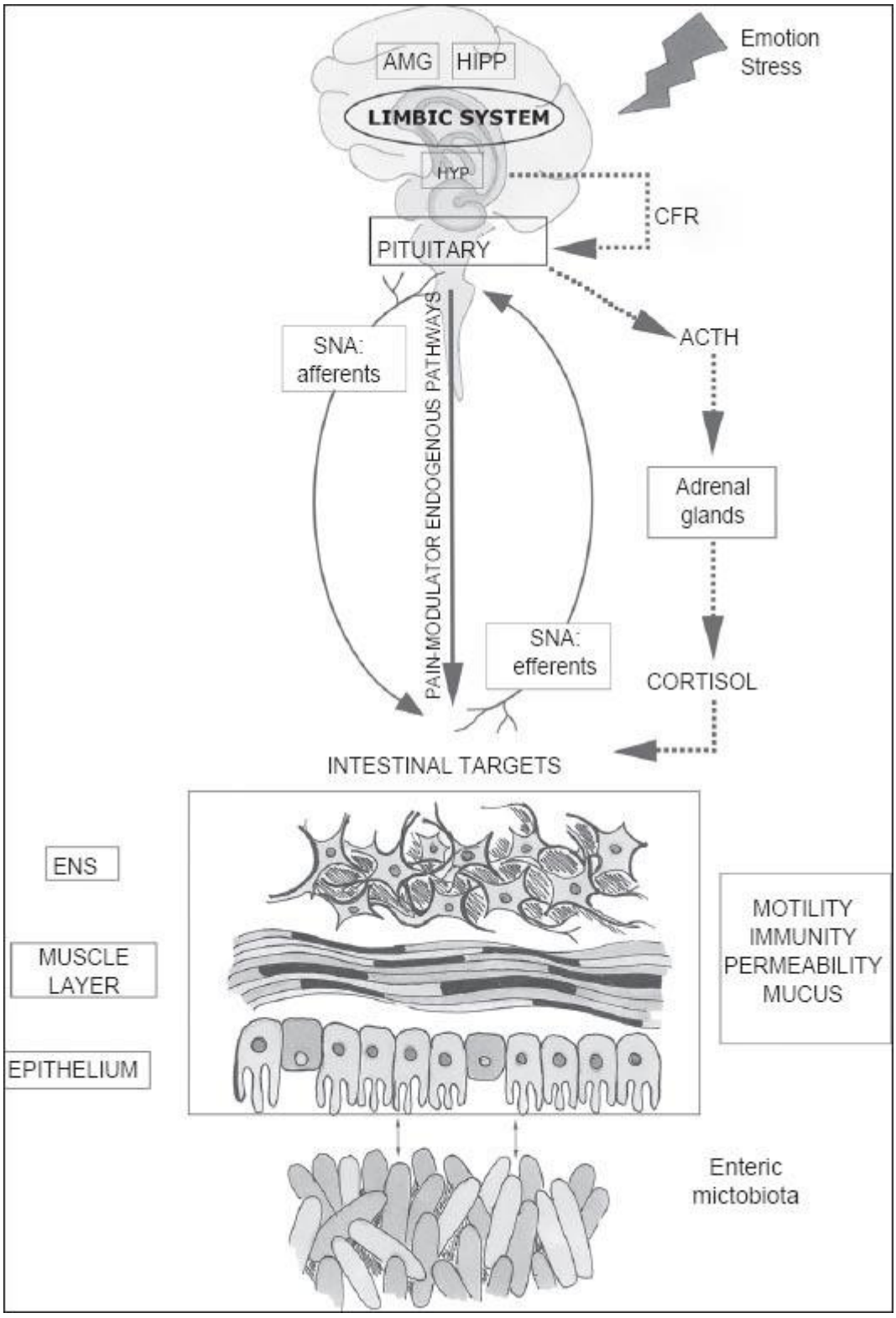
Figure 3- Signaling within and between the animal and its microbiota (from McFall-Ngai, 2013).[10]

An evolving understanding reveals that microorganisms present in the gut produce physiological modulators, such as short-chain fatty acids, tryptophan; gut hormones, e.g., gamma-aminobutyric acid (GABA), neuropeptide Y, dopamine; and pathogenic mediators, for example, trimethylamine *N*-oxide, that can modulate host

disease susceptibility. The gut microbiota interacts with the host through several complex pathways, including local and distant effects. [7,11]

Under physiological conditions, gut microbiota stimulates the immune system, mainly via intestinal-associated lymphoid cells. Thus, the gut microbiota is involved in activating and differentiating a broad range of T and B lymphocytes, as well as modulating the mucosal production of immunoglobulins, especially immunoglobulin A[7]. Additionally, gut bacteria are a source of pathogen-associated molecular patterns (PAMPs). PAMPs communicate with the host by eliciting responses involving membrane-bound pattern recognition receptors (PRRs), such as Toll-like receptors, or cytoplasmic PRRs such as NOD-like receptors or RIG- I-like receptors.[12]

There is also a bidirectional communication between the autonomic nervous system and the enteric nervous system in the gastrointestinal tract, that mainly occurs by way of the vagus nerve, which runs from the brain stem through the digestive tract and regulates almost every aspect of the passage of digested material through the intestines. (figure 4). Evidence shows that the gut microbiota also modulates excitatory and inhibitory neurotransmitters, e.g., serotonin, GABA and dopamine, and neurotransmitter-like substances, especially in response to physical and emotional stress. For instance, exacerbations on irritable bowel syndrome are frequently associated with emotional stress. [13] Microorganisms that colonize the digestive tract can be involved in regulating the sympatho- adrenomedullary and hypothalamus-pituitary-adrenal axes through the regulation or production of short-chain fatty acids (SCFA) and neurotransmitters such as GABA, dopamine and serotonin, as well as cytokines. The activation of these axes results in the release of catecholamines, as norepinephrine and epinephrine, and glucocorticoids into the circulatory system.



The central nervous system and in particular hypothalamic pituitary adrenal (HPA) axis (in dashed line) can be activated in response to environmental factors, such as emotion or stress. HPA is finalized to cortisol release and is driven by a complex interaction between amygdala (AMG), hippocampus (HIPP), and hypothalamus (HYP), constituting the limbic system. HYP secretion of the corticotropin-releasing factor (CRF) stimulates adrenocorticotropic hormone (ACTH) secretion from pituitary gland that, in turn, leads to cortisol release from the adrenal glands. In parallel, central nervous system communicate along

both afferent and efferent autonomic pathways (SNA) with different intestinal targets such as enteric nervous system (ENS), muscle layers and gut mucosa, modulating motility, immunity, permeability and secretion of mucus. The enteric microbiota has a bidirectional communication with these intestinal targets, modulating gastrointestinal functions and being itself modulated by brain-gut interactions.

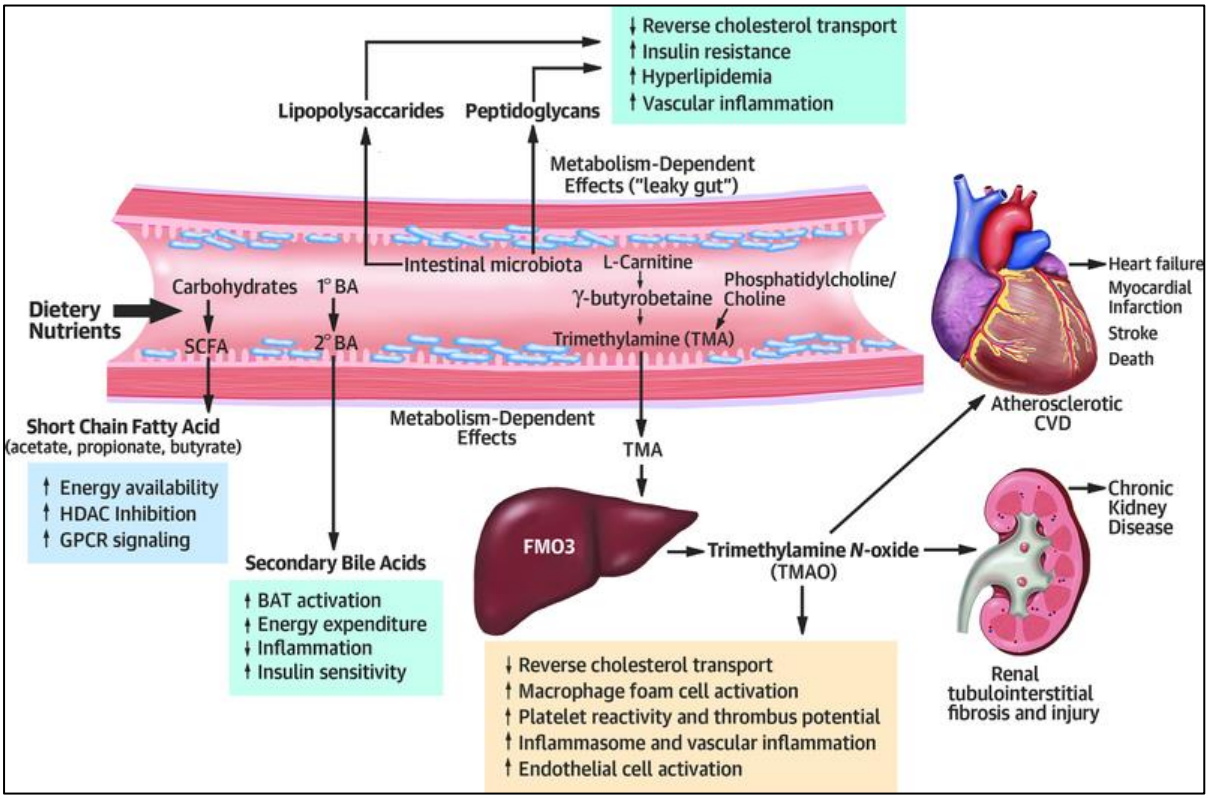
Figure 4- Microbiome gut-brain axis structure (from Carabotti 2015). [13]

Because numerous intestinal microbiota-generated metabolites are biologically active and affect host phenotypes, the intestinal microbiome also functions as a major endocrine organ that is responsive to dietary intake. It communicates with distal organs in the host via intestinal microbiota-generated metabolites. For example, bacterial metabolites as SCFA can directly activate G-coupled-receptors, inhibit histone deacetylases, and serve as energy substrates.[14] In the similar manner, bile acids are an important class of microbially produced metabolites that activate receptors in the gut, the liver, and the periphery, which can regulate several host processes, including metabolic processes.[15] Thus, host metabolism can be affected by microbial modifications of bile acids, leading to altered signaling via bile acid receptors and also by altered microbiota.

1.3 Gut microbiota and its metabolic contributions to cardiovascular health and disease

Several metabolism-dependent and independent effects fo gut microbiota can influence the cardiovascular system (figure 3). Metabolism-dependent effects include the microbial fermentation of dietary carbohydrates to generate short-chain fatty acids (SCFAs), which signal to the host to increase energy expenditure, inhibit histone deacetylase (HDAC) activity, and enhance G protein-coupled receptor (GPCR)

signaling. Another metabolism-dependent effect is the microbial conversion of primary bile acids (BA) to secondary bile acids signals to increase host brown adipose tissue (BAT) activation, energy expenditure, and insulin sensitivity while dampening inflammation. Additionally, the gut microbiota metabolism can affect the cardiovascular system by the microbial conversion of choline and L-carnitine to trimethylamine (TMA). TMA is afterward converted by the host flavin monooxygenase (FMO) enzyme family to trimethylamine-N-oxide (TMAO) in the liver. TMAO accelerates atherosclerosis by altering sterol and bile acid metabolism, increases in macrophage activation, and likely other mechanisms. Furthermore, the gut microbiota can influence host health through metabolism-independent effects, including gut hyperpermeability (leaky gut), allowing bacterial cell wall products such as lipopolysaccharide (LPS), and peptidoglycans to enter the bloodstream. Low circulating levels of these bacterial components can activate macrophages, reducing reverse cholesterol transport and increasing insulin resistance, hyperlipidemia, and vascular inflammation. The metabolic-dependent and independent effects of the gut microbial endocrine organ converge to modulate the risk of developing atherosclerotic CVD, MI, stroke, and death.[7]



Dietary nutrients are filtered by intestinal microbiota by both metabolism-dependent effects (generation of microbial metabolites such as short-chain fatty acids and trimethylamine from dietary carbohydrates and choline/carnitine, respectively) and metabolism-independent effects (lipopolysaccharides and peptidoglycans) leading to downstream metabolic alterations that affect cardiovascular and end-organ functions..BA: bile acids; BAT : brown adipose tissue; CVD: cardiovascular disease; FMO3:flavin monooxygenase 3; GPCR :G-protein-coupled receptors; HDAC: histone deacetylase.

Figure 5- Gut microbiota and its metabolic contributions to cardiovascular and disease (from Tang, 2019). [7]

1.4 TMAO association with CV events

One of the gut microbiota-dependent metabolites that is extensively reported to correlate with CVD and major cardiovascular events (MACE) is trimethylamine *N*-oxide (TMAO)[16]. In this manner, TMAO is associated with atherosclerotic burden[17] and is an independent predictor of myocardial infarction, stroke, and cardiovascular death,[16,18,19] as well as all-cause mortality.[20]

1.5 TMAO regulatory pathways

TMAO is a metabolite of dietary quaternary ammonium compounds, largely choline[21] and carnitine[22]. Different taxa of gut microbiota[23] metabolize these dietary precursors to trimethylamine (TMA), converted in the liver to TMAO by flavin monooxygenase (FMO) family enzymes, particularly FMO3.[21]

1.6 TMAO Atherogenic Mechanisms

Several mechanisms propose TMAO atherogenic effects. There is evidence that one of these mechanisms is by inhibiting reverse cholesterol transport (RCT). Koeth *et al*. showed that TMAO, and its dietary precursors choline and carnitine, suppress reverse cholesterol transport.[22] TMAO can also promote macrophage cholesterol accumulation. Diet supplementation with TMAO or its precursors enhanced macrophage levels of cluster of differentiation 36 (CD36) and scavenger receptor A (SR-A1), two scavengers receptors implicated in atherosclerosis.[21] Additionally, TMAO affected cholesterol metabolism through inhibiting hepatic bile acid (BA) synthesis, partially mediated through the enterohepatic farnesoid X receptor (FXR)-fibroblast growth factor 15 (FGF15) axis.[24] Another mechanism implicated in TMAO contribution to CVD events is enhancing human platelet activation and potential augmentation in thrombosis.[25] TMAO also induced the NLRP3 inflammasome formation and activation, [26] and endothelial disfunction[27]. Gut microbiota suppression through broad-spectrum antibiotic administration decreases or even extinguishes plasma TMAO in mice and humans[16,21,24,25], and its atherogenic effects, corroborating the role of gut microbiota on TMAO generating and pathogenesis.

1.7 Gut microbiota modulation – Red wine and gut microbiota

In the three first years of life, the gut microbiota will acquire the main profile for the rest of one's life. Gut microbiota is profoundly and initially shaped by birth mode, breast feeding, use of antibiotics and environmental factors in this initial "seeding" process.[8,28] During adult life, one of the main determinants of gut microbiota changes is nutrition. Diet has a fundamental role in tailoring gut microbiota composition and function, as well as its interaction with the human host.[8]

Red wine (RW) and its polyphenol components, especially resveratrol, have been proposed as health promoters and correlate with fewer CV events[29], cancer[30], and overall mortality[29,31]. Previous studies from our group studied some aspects of RW consumption related to CV prevention. It was shown that RW consumption can prevent plaque formation in hypercholesterolemic rabbits[32] and preserve vascular function, in rats [33] . In 16 hypercholesterolemic patients, RW improved flow-mediated dilation and enhanced endothelium-independent vasodilation.[34]

Polyphenols' chemical structure, the food matrix, and the enterohepatic circulation can influence polyphenols' bioavailability and absorption, and a large percentage is not absorbed in the small intestine. [35]Consequently, they reach the colon, intact, where they can exert their regulatory function and probably act as prebiotics, serving as fuel to bacterial fermentation and are accessible to a large proportion of gut bacteria. Therefore, some of RW beneficial effects might occur through modifications in gut microbiota[36], fecal metabolome[37], and plasma metabolites [38].Additionally, RW contains 3,3-dimethyl-1-butanol (DMB), a structural analogous of choline, an important diet precursor of TMAO,[39], which may inhibit TMA formation in the gut and decreased plasma TMAO levels in mice.[40]

2. Rationale and Objectives

Although RW effects on gut microbiota have been reported, and its potential interference on plasma TMAO was shown in mice, simultaneous evaluation of the gut microbiota, TMAO and plasma metabolomics in patients with coronary artery disease remains unsettled.

The primary objective of this study is to evaluate if RW, through gut microbiota modifications, can influence TMAO plasma levels. Secondary objectives are to interrogate RW influences on plasma metabolomics and analyze the correlations of plasma metabolomics and TMAO with gut microbiota profile.

3. Methods

3.1 Trial design

This was a randomized, controlled (1:1) crossover trial composed of two 3-week interventions: one with the consumption of RW, 250 mL a day, five days a week, or alcohol abstention. Each intervention was preceded by a 2-week washout (WO) period, without alcoholic beverages, prebiotics, or probiotics consumption. The trial was registered at www.clinicaltrials.gov (NCT03232099). The trial was conducted from August 2016 to May 2018.

3.2 Participants

Participants were recruited from the Heart Institute, University of Sao Paulo, São Paulo, Brazil, and also from advertisements in local newspapers and local radio stations. Participants had established coronary artery disease (CAD), were male, aged 46–69 years, had BMI < 30, were stable, and non-symptomatic. Only men were selected with the intention to homogenize our sample since the metabolization of alcohol is different between the sexes[41], and there is a possible different metabolism of TMAO in females - the activity of FMO3 seems to be greater in female mice than in males[42]. Established CAD included diagnoses, at least 30 days before the study, myocardial infarction, angiographic evidence of ≥ 50% stenosis of one or more epicardial vessels, coronary angioplasty or coronary artery bypass grafting. Importantly, participants had no evidence of acute coronary syndrome (cardiac troponin T level, <0.1 μg per liter), nor coronary angioplasty or coronary artery bypass grafting, at least 30 days prior to the protocol initiation. Exclusion criteria included antibiotic treatments (< 2 months before study start), heart failure (New York Heart Association functional class ≥ II), renal

failure (creatinine clearance <30 mL/min by the Cockcroft-Gault formula), hepatic failure (thrombocytopenia, reduced serum albumin and prolonged prothrombin time), digestive tract cancers, intestinal inflammatory diseases, obstructive biliary diseases, prior digestive surgeries (cholecystectomy, gastrectomy or colectomy), and diabetes mellitus or use of antidiabetic drugs. Patients were also required to have an AUDIT score of 7 or less (AUDIT[43] score is used to identify hazardous or harmful drinkers; scores up to 7 suggest low risk of alcohol abuse) . The Scientific Committee of the Heart Institute (Instituto do Coração- InCor-HCFMUSP) and the Institutional Ethical Committee (CAPPesq) of the *Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo* (HCFMUSP) approved the study protocol (SDC 4257/15/084 - CAAE 57379216.0.0000.0068). All participants gave written informed consent before participating in the study.

3.3 Sample size

For sample size calculation**,** we assumed that the TMAO would decrease by 1.0 µM after the red wine intervention, with a standard deviation of 1.5, type I error for p <0.05, and 80% test power, and it was estimated that 38 patients would be necessary. Then, considering 10% of losses during the intervention, 42 patients should be included, with calculation performed by the E-prime® Statistics program.

3.4 Randomization

The random allocation sequence of the participants was generated by the Random Integer Generator (random.org). The random allocation sequence was implemented using the participant IDs matched with allocated sequences.

3.5 Interventions

The participants were randomly assigned to start on either a 3-week intervention with RW or an intervention of 3-week of alcohol abstention. RW was provided for all patients at the beginning of the intervention period. Ibravin (Brazilian Wine Institute) produced and supplied the red wine, which was elaborated with the Merlot grape variety. The quality of the wine was assessed by Embrapa (Brazilian Agricultural Research Corporation) and released for consumption after their technical approval. It was a Merlot wine from the 2014 vintage, bottled in August 2016, in 250 mL bottles, customized for the study. The sample has an alcohol content (% v / v) of 12.75, a total acidity (mEq / L) of 95.58, and a volatile acidity (mEq / L) of 7.44. It contained 109 mg / L of total anthocyanins (DescSO2) and 2155 mg / L of total polyphenols in Catechin. Patients received 21 labeled bottles and written consumption guidance to drink 250 mL/ day, five days a week, for three weeks. It was allowed to split the doses into two meals, or periods of the day, as long as the whole bottle was consumed each day. They were also handed a printed dietary recall for the daily register of the wine consumption. After the RW intervention period, they returned the 21-red wine's empty bottles and the filled consumption diary.

3.6 Overview of protocol visits and measures

After written informed consent and randomization, patients returned five times to in-hospital visits for clinical and nutritional evaluation, anthropometric measures, and sample collections. On the first visit, there were no sample collections, and a trained nurse or physician interviewed the patients, recorded vital signs, weight, height and waist circumference, and gave instructions about the fecal sample collection that would be carried out at home one day prior to the subsequent four visits. Additionally, in the first visit, patients underwent nutritional assessment by a trained dietitian nutritionist, who orientated the food frequency diaries' filling and instructed them to maintain their routine diet and physical activity. Patients also received written and verbal instructions regarding the 2-week WO period: when they should avoid any alcoholic beverages, fermented products, prebiotics or probiotics. After the 2 WO periods and after the 2 interventions, four hospital visits were carried out in which blood sample collections, clinical interview and nutritional evaluation were performed. They brought the stool samples, collected at home, one day before the visit. The primary endpoint was modifications on the gut microbiota composition and in plasma TMAO after consumption RW compared with alcohol abstention. Gut microbiota was assessed by 16 S rRNA gene sequences from fecal samples, and plasma levels of TMAO were analyzed by Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy. We also evaluated, at every visit, body weight, waist circumference, fasting concentrations of plasma glucose, triglycerides (TAG), total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), serum albumin, serum bilirubin, serum lactate dehydrogenase, gamma-glutamyl transferase, prothrombin

time, high sensitive C-reactive protein, interleukin-6, complete blood count, creatine, urea, serum lipopolysaccharides (LPS). Further analysis included, in 20 patients, the plasma metabolomic profile, four times, after the two washout periods and after the two interventions. For biochemical analysis, TMAO analysis, microbiota analysis, and metabolomic analysis, the assessors that collected outcome data and data analysts were unaware of the assigned intervention.

3.7 Fasting blood samples' biochemical analyses

Blood samples were collected at all four examination days, after fasting for 12 hours. Blood sample analyses were performed on the same day of collection. These routine laboratory tests' samples were measured on the Dimension EXL analyzer, Siemens Healthcare (Newark, USA), except for testing of troponin I, which was measured using chemiluminescent immunometric assay ADVIA Centaur® - XP; TnI-Ultra ™ (Siemens Healthcareneers). Blood counts of total hemoglobin, leucocytes, neutrophils, lymphocytes, and other immune cells (including monocytes, mast cells, basophils and eosinophils) were obtained using a Sysmec XN-2000™ automated hematology analyzer (Sysmex America Inc., Lincolnshire, Illinois, USA). Creatinine clearance was estimated using the Cockcroft–Gault equation, and the Friedewald equation estimated LDL cholesterol.

3.8 Dietary intake assessment and compliance

A trained dietitian nutritionist conducted an in-person interview at every in-hospital visit, focusing on the filling of the dietary diaries and adherence to the dietary

guidelines: avoidance of other high polyphenolic sources and any alcoholic beverages other than the provided RW, prebiotics and probiotics or fermented products; and mostly to keep a similar diet pattern throughout the study. These orientations aimed to decrease the interference on the gut microbiota of other changes in the habitual diet rather than the RW. Participants completed a 3-day dietary record, filled out on two weekdays and one weekend at the end of both interventions. Daily consumption (amount and type) of dietary products throughout both interventions as well as any deviations from the dietary instructions, were recorded. The diary was used to measure adherence with the dietary recommendations and calculate the absolute consumption of dietary products. For the nutritional calculation, the foods described in homemade measures were converted into grams (g) or milliliters (ml), according to Pinheiro *et al.* [44]. Food labels were analyzed using the data available in the Brazilian Food Composition Table - TACO[45], and in the second Brazilian Food Composition Table – TBCA (http://www.fcf.usp.br/tbca/). Daily intake of total energy, macronutrients and micronutrients were calculated.

3.9 Interruption Criteria

Patients were periodically evaluated regarding their safety profile. In addition to hospital visits, patients were encouraged to contact the study staff by phone or email if they experienced any adverse health-related implications of the interventions. In the case of RW intolerance - limiting headaches, dizziness, gastrointestinal complaints, or other forms of intolerance - patients would be excluded. If there were any major cardiac events such as myocardial infarction, stroke, heart or digestive tract surgery, the use of antibiotics or antidiabetic drugs, the protocol would be interrupted.

3.10 TMAO assessment

During the protocol, after overnight 12-hour fasting, all the 42 patients had blood samples collected in every one of the four examination visits. Shortly subsequent collection, the blood samples were stored in the refrigerator, separated into serum and plasma, placed in Eppendorf® tubes, and immediately stored at −80°C until analyzed. All blood sample analyses were performed in only two batches to ensure low variability.

3.11 TMAO Validation tests

Ten patients were submitted to TMAO validation tests, in 4 further visits (twice a week), after finishing the protocol: after 12-hour fasting, blood samples were collected and analyzed by 3 techniques. Patients were advised not to modify medication, diet, nor exercise. TMAO validation tests were performed with 3 different collection techniques: 1) Blood samples were collected, separated into serum and plasma, placed in Eppendorf® tubes and immediately frozen at -80ºC after collection, 2) Blood samples were collected, separated into serum and plasma, placed in Eppendorf® tubes and refrigerated for 2 hours after collection and then frozen at -80ºC, or 3) Blood samples from fingertips were used for dried blood spots (DBS) analysis.

3.12 TMAO quantification

The methodology used was described by Wang et al. [46] TMAO was extracted from 50 μL of plasma sample using 200 μl of the methanol (containing the internal

standard TMAO-d9 at 10 μM) to denatured the proteins after mechanical agitation (vortex). The plasma samples were centrifuged to precipitate the proteins, and an aliquot of the supernatant was taken for direct injection into the LC-MS/MS system. The analytical curve (from 0.25 to 200 μM of TMAO) and control samples (0.5, 5 and 100 μM of TMAO) were prepared through TMAO (μM) addition in 5% bovine serum albumin (Sigma Aldrich, USA) aqueous solution containing 0,7% sodium chloride (w/w). TMAO and TMAO analytical standards were purchased from Sigma-Aldrich. The DBS samples were collected from 2 drops of whole blood from fingertips punched and displaced at Whatman Paper 903 (Protein Saver Card, GE Healthcare, USA). The DBS cards were dried at room temperature for 1 hour. To extract TMAO from DBS, two of 3.0 mm circular "punches" were sampled from the DBS card direct to 1.5 mL Eppendorf tube, added 150 μL of 75% methanol/25% water (v /v) solution containing 1.0 μM the internal standard TMAO-d9. The tube was mixed in a vortex for 10 seconds and then ultrasound for 3 minutes, vortex for another 10 seconds, and centrifuged at 10.000 g for 3 minutes. An aliquot of the extracted solution was transferred to a 96-well plate (or vials) and injected into de LC-MS/MS system. The analytical curve (from 0.25 to 200 μM of TMAO) and control samples (0.5, 5, and 100 μM of TMAO) were prepared through TMAO (μM) addition in the whole blood (lyophilized form, ControLab, Brazil) without the presence of TMAO and displaced in the card followed extraction procedure and analyzed. To quantify TMAO and its internal standard TMAO-d9 in the extracted plasma and DBS samples, a Liquid Chromatography (Agilent 1260, Agilent Technologies, USA) coupled to a hybrid triple quadrupole linear ion trap mass spectrometer 3200 QTRAP® (Sciex, Canada) were used. The compounds were separated in the LC system using an Atlantis HILIC Silica (100 x 3.0 mm, 3μm) (Waters Corp, USA) column maintained at 35 ºC and the following mobile phases (A)

aqueous solution containing 10 mM of ammonium formate (LC-MS grade, Sigma Aldrich) and (B) solution 90% acetonitrile/10% water (v/v) containing 10 mM of ammonium formate. 10 uL of sample volume was injected, and the compounds were eluted through the column using the following gradient condition at 500 uL/min, flow: from 0 to 1 min. 0% mobile phase (A); from 1.0 to 5.20 min. 90% (A) – 10% (B); from 5.20 to 7.50 maintained 90% (A); 7.50 min. turned to initial condition 0% until 11.5 min., (increased the flow to 600 uL/min.) and let equilibrate for an extra 1.0 min before re-injection. TMAO/TMAO-d9 retention time 6,15 min. After the separation, the compounds were ionized by Electronspray (Turbo V, Sciex, Canada) source in positive mode using 5200 V; nebulizer gas 45 psi; heater gas 50 psi; curtain gas 15 psi and analyzed in the MS/MS system by Multiple Reaction Mode (MRM) optimized for the following m/z transitions: 76 (precursor ion)>58 (product ion) and 85>66, TMAO and TMAO-d9 respectively.

3.13 Plasma Metabolomics

Twenty patients were randomly assigned to have their plasma metabolomic profile analyzed by Random Integer Generator (random.org). From the blood samples collected at the four visits, 20 patients and 80 samples of their four visits were selected for untargeted plasma metabolomics analysis. The samples had been stored at −80°C and were assayed with an untargeted, gas chromatography/mass spectrometry and liquid chromatography/mass spectrometry-based metabolomic protocol by Metabolon, Inc. (Durham, NC).

3.13.1 Plasma Metabolomics Analysis: Metabolon Platform - *Metabolon, Inc. (Durham, NC)*

3.13.1.1 Sample Accessioning

Following receipt, 80 plasma samples were inventoried and immediately stored at -80ºC. Each sample received was accessioned into the Metabolon Laboratory Information Management System (LIMS) and was assigned by the LIMS a unique identifier that was associated with the original source identifier only. This identifier was used to track all sample handling, tasks, results, etc. The samples (and all derived aliquots) were tracked by the LIMS system. All portions of any sample were automatically assigned their own unique identifiers by the LIMS when a new task was created; the relationship of these samples was also tracked. All samples were maintained at -80ºC until processed.

3.13.1.2 Sample Preparation

Samples were prepared using the automated MicroLab STAR® system from Hamilton Company. Several recovery standards were added prior to the first step in the extraction process for quality control (QC) purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI,

one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for analysis.

3.13.1.3 QA/QC

Several types of controls were analyzed in concert with the experimental samples: a pooled matrix sample generated by taking a small volume of each experimental sample (or alternatively, use of a pool of well-characterized human plasma) served as a technical replicate throughout the data set; extracted water samples served as process blanks; and a cocktail of QC standards that were carefully chosen not to interfere with the measurement of endogenous compounds were spiked into every analyzed sample, allowed instrument performance monitoring and aided chromatographic alignment. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the pooled matrix samples.

3.13.1.4 Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS)

All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate

mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid. Another aliquot was also analyzed using acidic positive ion conditions; however it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same afore mentioned C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, however with 6.5mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate, pH 10.8. The MS analysis alternated between MS and data-dependent MS^n scans using dynamic exclusion. The scan range varied slighted between methods but covered 70-1000 m/z. Raw data files are archived and extracted as described below.

3.13.1.5 Bioinformatics

The informatics system consisted of four major components, the Laboratory Information Management System (LIMS), the data extraction and peak-identification software, data processing tools for QC and compound identification, and a collection of information interpretation and visualization tools for use by data analysts. The hardware and software foundations for these informatics components were the LAN backbone, and a database server running Oracle 10.2.0.1 Enterprise Edition.

3.13.1.6 LIMS

The purpose of the Metabolon LIMS system was to enable fully auditable laboratory automation through a secure, easy to use, and highly specialized system. The scope of the Metabolon LIMS system encompasses sample accessioning, sample preparation and instrumental analysis and reporting and advanced data analysis. All of the subsequent software systems are grounded in the LIMS data structures. It has been modified to leverage and interface with the in-house information extraction and data visualization systems, as well as third party instrumentation and data analysis software.

3.13.1.7 Data Extraction and Compound Identification

Raw data was extracted, peak-identified and QC processed using Metabolon's hardware and software. These systems are built on a web-service platform utilizing Microsoft's .NET technologies, which run on high-performance application servers and fiber-channel storage arrays in clusters to provide active failover and load-balancing.

Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Metabolon maintains a library based on authenticated standards that contains the retention time/index (RI), mass to charge ratio (*m/z)*, and chromatographic data (including MS/MS spectral data) on all molecules present in the library. Furthermore, biochemical identifications are based on three criteria: retention index within a narrow RI window of the proposed identification, accurate mass match to the library +/- 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the ions present in the library spectrum. While there may be similarities between these molecules based on one of these factors, the use of all three data points can be utilized to distinguish and differentiate biochemicals. More than 3300 commercially available purified standard compounds have been acquired and registered into LIMS for analysis on all platforms for determination of their analytical characteristics. Additional mass spectral entries have been created for structurally unnamed biochemicals, which have been identified by virtue of their recurrent nature (both chromatographic and mass spectral). These compounds have the potential to be identified by future acquisition of a matching purified standard or by classical structural analysis.

3.13.1.8 Curation

A variety of curation procedures were carried out to ensure that a high-quality data set was made available for statistical analysis and data interpretation. The QC and curation processes were designed to ensure accurate and consistent identification of true chemical entities, and to remove those representing system artifacts, mis-assignments,

and background noise. Metabolon data analysts use proprietary visualization and interpretation software to confirm the consistency of peak identification among the various samples. Library matches for each compound were checked for each sample and corrected if necessary.

3.13.1.9 Statistical Calculations

For many studies, two types of statistical analysis are usually performed: (1) significance tests and (2) classification analysis. Standard statistical analyses are performed in ArrayStudio on log transformed data. For those analyses not standard in ArrayStudio, the programs R (http://cran.r-project.org/) or JMP are used

3.14 Stool Sampling

Patients received verbal and written instructions regarding the collection of stool samples. They were oriented to collect the samples a day prior to the visit to the hospital or on the same day of the visit. They were stored in provided coolers at 4ºC immediately and then transported to the hospital where they were immediately frozen at -20ºC until analysis. Total DNA was extracted from the intestinal microbiota with the PSP® Spin kit Stoll Plus DNA (STRATEC Biomedical AG, Germany) from 200 mg of stool.

3.15 High throughput sequencing targeting the 16S rRNA gene

Twenty milliliters of raw cow's milk were added to 30 ml of physiological solution. First, this solution was centrifuged at 1,000 x g for 5 min at 4°C. The supernatant was centrifuged a second time (5,000 x g for 15 min at 4°C) to collect the bacterial cell. The repetition of this step three times increased the number of fecal pellets. After collecting the fecal pellets, DNA was extracted with the SPIN Kit for Soil (MP Biomedicals, Italy), according to the manufacturer's manual. Extracted DNA was stored at -20°C. The assessment of the DNA concentration was with the fluorimeter Qubit® 2.0 (Invitrogen, Italy). Processing of DNA was at the Research and Testing Laboratory (RTL; Lubbock, TX, United States) (https://rtlgenomics.com). The amplification of the 16S ribosomal RNA (rRNA) gene (V1-V3) used the bacterial primers 5′-GAGTTTGATCNTGGCTCAG-3′ and 5′-GTNTTACNGCGGCKGCTG -3′. The sequencing of the PCR products was done using the Illumina MiSeq platform, following the company's standard protocols.

3.16 Gut microbiota bioinformatic analysis

DADA2 tool was used to analyze fastq sequences[47] in order to recover single-nucleotide resolved Amplicon Sequence Variants (ASVs) from amplicon data. Default parameters were used to improve the overall quality of the sequences. Besides that, reads were filtered and trimmed using the "filterAndTrim" function implemented in DADA2 as described in https://benjjneb.github.io/dada2/tutorial.html. Additionally, low-quality bases in the beginning and at the end of the reads from the forward and reverse fastq files respectively were removed, as according Magro *et al*[48]. The

taxonomic assignment was subsequently performed using the naïve Bayesian classifier method implemented in DADA2 using as reference the SILVA database. Finally, the final phylogenetic tree of the ASVs was carried out using DECIPHER[49] R package (version 2.18), precisely the function AlignSeq to create the multiple sequence alignment and the Fast Tree program[50].

3.17 Gut microbiota and plasma metabolomic data integration analysis

We used the Diablo platform version 6.3.2 (http://mixomics.org/mixdiablo/) to evaluate these two datasets. DIABLO is a mixOmics framework for integrating multiple data sets in a supervised analysis, and it stands for Data Integration Analysis for Biomarker discovery using latent variable approaches for 'Omics studies. This analysis aims to identify correlated (or co-expressed) variables measured on heterogeneous data sets, which also explain the interest-supervised analysis's categorical outcome. First, we fit a DIABLO model without variable selection to assess the global performance and choose the number of components for the final DIABLO model. The function perf is run with 16-fold cross-validation repeated 10 times. From the performance plot, we observe that both overall and balanced error rate (BER) decreased from 1 to 2 components. The standard deviation indicates a slight potential gain in adding more components. The Mahalanobis distance seems to give the best accuracy. The next step was to tune the keepX parameters in the block.splsda function. The model has identified 8 taxa for the first component and 30 for the second component, 30 metabolites for the first component, and 5 for the second.

3.18 Statistical analysis

All statistical analyses were performed using either SPSS v.25 software for Windows © or R (R version 4.0.3). The variables were assessed by descriptive measures, according to the type of variable (qualitative or quantitative). For qualitative variables, frequencies and percentages were calculated. Comparisons between paired groups were performed using the two-sided Student's t-test when the data followed the normal distribution. The comparison between two dependent measures was performed using the Wilcoxon non-parametric test in non-normality. For comparisons between independent groups, we used Student's t-test for independent groups or the Mann-Whitney non-parametric test. The level of significance adopted for all hypothesis tests was 5%. The correlation between TMAO measures and urea, creatinine, glomerular filtration rate (GFR), lipid profile measures, macronutrients, and micronutrients were calculated using Spearman's rank correlation test. The Generalized Estimating Equation (GEE) method was used to evaluate the pattern of TMAO measures within the individual, longitudinally, in the different intervention groups (RW and abstemious) and related to actual function parameters and the use of angiotensin-converting enzyme (ACE) inhibitors, angiotensin receptor blocker (ARB), and diuretics. This effect verified the effect of time with an independent variable of interest for the measure assessed over time. We wanted to observe whether there was a difference between groups, between times, and the interaction between the variables and time. Peer comparisons were performed using the Bonferroni test. We investigate plasma TMAO intraindividual concordance in repeated measures with the Intraclass Coefficient (ICC).

3.19 Data availability

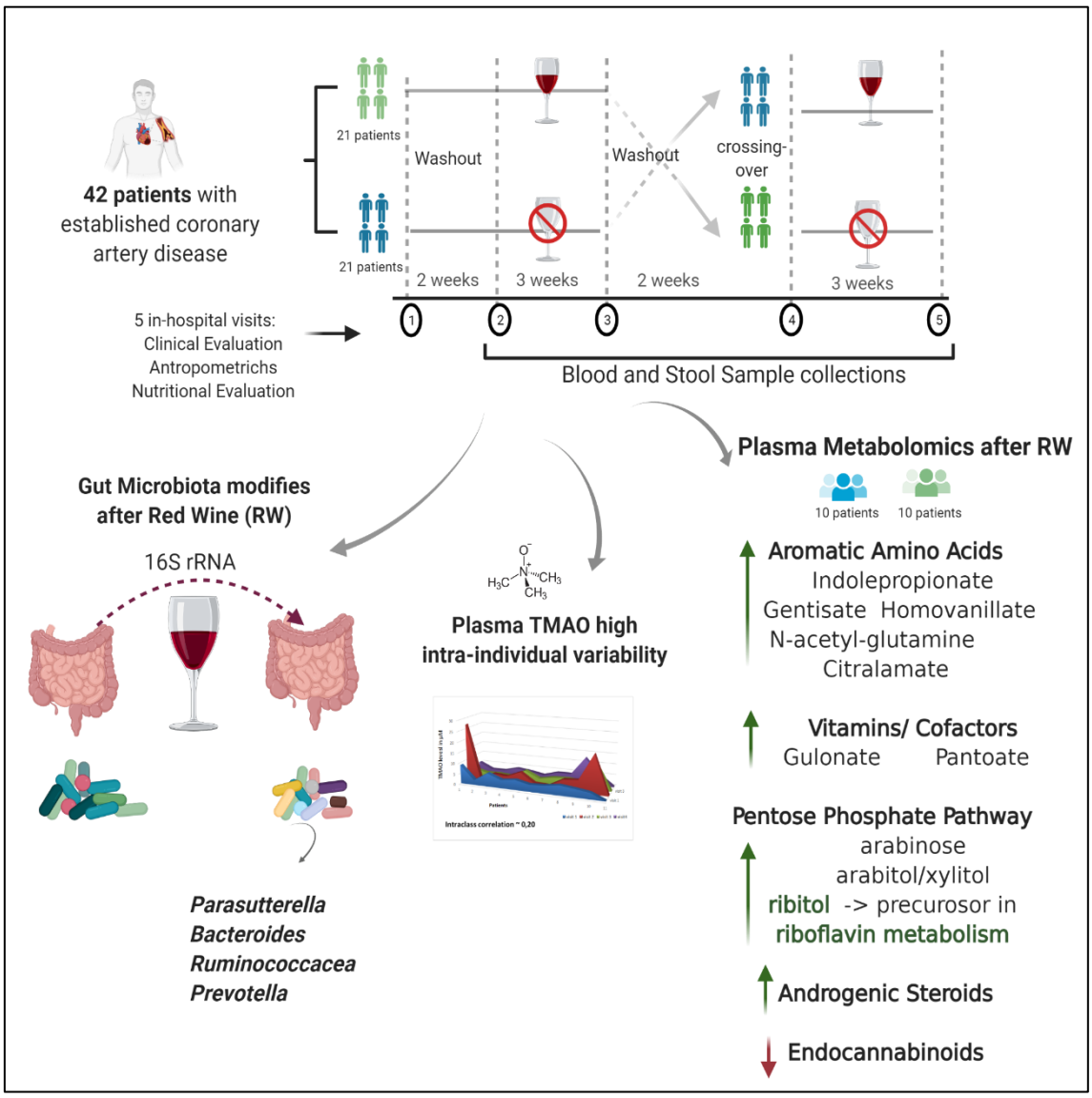
The raw Illumina read data for all stool samples have been deposited in the NCBI SRA repository under the BioProject ID: PRJNA726242. The metabolomic datasets for this study can be found in the repository MetaboLights under the public URL https://www.ebi.ac.uk/metabolights/editor/study/MTBLS310/.

4. Results

4.1 Crossover intervention

This was a randomized, controlled, crossover trial with two 3-week interventions comprising a RW consumption period or a period of alcohol abstention, both preceded by a 2-week washout period. Fifty-seven patients were initially recruited, of which 15 were excluded. Among the excluded patients, eight withdrew from the protocol, two used antibiotics, one had hypertriglyceridemia on the first laboratory exam, and one had a leukemia diagnosis and was referred to the Hematology Clinic. One patient used metformin during the protocol, one patient had gouty arthritis, and one suffered acute myocardial infarction shortly after randomization. Finally, 42 male patients completed the protocol. An overview of the study design and main findings are shown in figure 6. The patient's baseline characteristics are shown in Table 1. They had CAD documented by coronary angiography or clinical event and were clinically stable, and ages ranged between 46 and 69 years old; they were on average overweight and had increased mean waist circumference. Blood pressure, lipid profile, and glycemic profiles were well controlled. After initial clinical evaluation, study participants were randomly assigned to two interventions: (1) 3 weeks of total alcoholic abstention followed by 3 weeks RW consumption, or (2) 3 a week RW consumption period followed by alcohol abstention, both preceded by a 2-week washout period. All the volunteers tolerated RW ingestion well. In the RW period compared to abstinence, there were no significant changes in blood pressure, heart rate, or body mass index. Also, there were no significant changes in high sensitive C-reactive protein, interleukin-6, lipopolysaccharides, glucose, and glomerular filtration rate. However, after RW, there was an increase in high-density lipoprotein (HDL), low-density lipoprotein (LDL)

cholesterol, and total cholesterol. Additionally, liver enzymes did not change, except for minor increases in gamma-glutamyl transferase and prothrombin time (Tables 2 and 3).



In 42 patients with established atherosclerotic disease, RW modified gut microbiota and, in a subset of 20 studied, also altered plasma metabolomics. Trimethylamine-*N*-oxide varied highly intra-individually in the control period and in further validation tests. Coronary artery disease, CAD. 16S rRNA refers to high throughput sequencing targeting the 16S rRNA gene analysis of gut microbiota. Green arrows denote an increase and red arrow, a decrease

Figure 6- Visual Abstract. Study design, data overview and summary of the crossover trial comparing RW consumption vs. abstinence from alcohol

Table 1- Baseline participant's demographic and clinical data

Variable		Value N= 42 (SD and %)
Age, y		60.4 (5.4)
SBP, mmHg		119.8 (16.0)
DBP, mmHg		72.3 (9.3)
Heart Rate,		68.1 (9.3)
BMI, kg/ m^2		27.1 (25.2-28.3)
Waist circumference, cm		98.0 (8.1)
Current smoker		3(7.1%)
Former smoker		23 (54.7%)
Hypertension		21 (50.0%)
Dyslipidemia		18 (42.9%)
Physical activity		23 (53.8%)
5-10 METs.h/wk	7 (30.4%)	
10-40 METs.h/wk	10 (43.5%)	
>40 METs.h/wk	6 (26.1%)	
Physical inactivity		19 (45.2%)
History of myocardial infarction		28 (66.7%)
> 50% stenosis epicardial artery		25 (59.5%)
CABG		13 (31.0%)
PCI		19 (45.2%)
Medications		
Beta-blockers		30 (71.4%)
ACE inhibitors		21 (50.0%)
ARB		10 (23.8%)
CCB		3 (7.1%)
Aspirin		37 (88.1%)
Clopidogrel		10 (23.8%)

(continues)

Table 1- Baseline participant's demographic and clinical data (continued)

Variable	Value N= 42 (SD and %)
Statins	38 (90.5%)
PBI	8 (19.0%)
Diuretics	
Total Cholesterol, mg/dL	143 (127-170)
High-density lipoprotein, mg/dL	46 (39-54)
Low-density lipoprotein, mg/dL	77 (66-97)
Triglycerides, mg/dL	85 (66-111)
Apolipoprotein A, g/L	1.37 (1.22-1.51)
Apolipoprotein B, g/L	0.7 (0.62-0.86)
Lipoprotein a, mg/dL	60.5 (4.3-91.3)
Fasting Glucose, mg/dL	90 (86-100)
GFR, mL/min/1,73 m^2	85.5 (72-99)
hsCRP, mg/L	1.01 (0.58-2.77)
IL-6, pg/mL	0.75 (0-4.8)
LPS, EU/ mL	0.76 (0.63-0.81)

Data are presented as mean (standard deviation); medians (interquartile range); n= numbers and percentages. SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; CABG, previous coronary artery bypass grafting; PCI, previous percutaneous coronary intervention; METs, metabolic equivalents; METs.h/wk, METS-h/week score = (MET level × hours × times/week); ACE, angiotensin-converting enzyme; ARB angiotensin-receptor blocker; CCB, calcium channel blockers, PBI, proton bomb inhibitors; GFR, glomerular filtration rate; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin 6; LPS, lipopolysaccharides.

Table 2- Changes in laboratory measures in the beginning and at the end of both intervention periods: abstention (Abst) and red wine (RW) periods. Values are presented in mean (SD) and median. SD: standard deviation. Wilcoxon test.

	Washout		RW			Washout		Abstemious		
	Mean (SD)	Median	Mean (SD)	Median	p	Mean (SD)	Mean	Median (SD)	Mean	p
Hematocrit %	42,98 (3,23)	43	42,93 (3,19)	43	0,930	43,19 (3,26)	43,00	42,95 (3,46)	43,00	0,502
Hemoglobin g/dL	14,59 (1,21)	14,65	14,57 (1,23)	14,4	0,940	14,60 (1,14)	14,60	14,55 (1,31)	14,60	0,618
Leukocytes in mm³	5986,19 (1605,70)	5660	6091,90 (1490,92)	5865	0,399	6171,43 (2063,13)	5895	6191,19 (1887,99)	5960	0,938
Neutrophils in mm³	3595,45 (1174,60)	3423,5	3642,17 (1106,33)	3533,5	0,468	3712,14 (1725,50)	3496,50	3699,48 (1376,55)	3462,50	0,442
Neutrophils%	58,62 (8,98)	60	59,31 (7,83)	60,5	0,659	57,14 (12,50)	59	58,88 (8,21)	60	0,070
Eosinophils in mm³	193,64 (173,04)	150	177,43 (134,82)	144	0,653	179,74 (165,98)	137,50	189,71 (164,42)	155,00	0,545
Eosinophils %	3,14 (2,37)	2	2,69 (1,44)	2	0,373	3,05 (2,46)	2	3,14 (2,55)	2,50	0,735
Basophils in mm³	39,45 (38,80)	46,5	37,12 (31,81)	48	0,969	42,57 (34,58)	54	44,31 (32,98)	48	0,911
Basophils %	0,64 (0,53)	1	0,62 (0,49)	1	0,763	0,69 (0,47)	1	0,71 (0,46)	1	0,763
Lymphocytes in mm³	1711,60 (540,79)	1749	1709,17 (478,39)	1628	0,955	1709,88 (541,16)	1676	1723,79 (576,57)	1654,5	0,985
Lymphocytes %	28,26 (8,82)	27,5	28,55 (6,40)	28	0,943	28,98 (9,60)	29	28,43 (7,16)	29	0,209
Monocytes in mm³	513,98 (135,29)	507,5	526,43 (125,24)	509	0,361	523,21 (176,08)	483	526,95 (180,82)	488	0,406
Monocytes %	8,79 (2,05)	8,5	8,83 (1,92)	8,5	0,971	9,26 (3,91)	8	8,79 (2,09)	8	0,727
Platelets in mm³	203047 (45933)	194500	193262 (45595)	182000	0,020	195690 (49069)	187500	202167 (54550)	192500	0,109
Total Cholesterol mg/dL	151,26 (38,06)	148	153,76 (29,93)	156	0,108	151,40 (32,63)	143,50	147,44 (36,69)	139	0,089
High-density lipoprotein Cholesterol mg/dL	46 (12,05)	46,5	48,59 (12,77)	48	0,004	46,90 (12,35)	46	46,15 (11,48)	46	0,159
LDL Cholesterol mg/dL	81,10 (23,89)	77	84,15 (24,71)	79	0,357	83,31 (26,85)	77	76,90 (25,36)	73	0,036
Non-HDL cholesterol mg/dL	105,26 (40,04)	96,5	105,17 (30,67)	97	0,288	104,50 (32,57)	97	101,10 (37,49)	93	0,121
Triglycerides mg/dL	116,48 (95,33)	84	110,15 (67,60)	88	0,793	107,17 (67,94)	85	112,29 (81,46)	85	0,444
Apolipoprotein (A1) g/L	1,35 (0,22)	1,31	1,38 (0,23)	1,37	0,202	1,36 (0,23)	1,37	1,37 (0,22)	1,34	0,258
Apolipoprotein B g/L	0,78 (0,28)	0,72	0,79 (0,20)	0,73	0,581	0,76 (0,19)	0,70	0,76 (0,25)	0,70	0,693
Lipoprotein (a) mg/dL	45,71 (42,09)	34,9	52,76 (47,02)	54,4	0,394	50,58 (42,84)	60,5	46,52 (41,67)	48,3	0,056
Troponin I ng/mL	0,009 (0,0103)	0,006	0,0070 (0,0034)	0,006	0,309	0,0088 (0,0108)	0,0060	0,0074 (0,0046)	0,0060	0,593
Glucose mg/dL	93,41 (9,24)	92	93,43 (9,54)	93	0,684	92,45 (11,12)	90,5	94,52 (8,46)	93	0,168
Urea mg/dL	37,88 (10,23)	36	37,39 (9,23)	38	0,690	39,60 (9,170	38	38,24 (8,61)	39	0,194
Creatinine mg/dL	1,01 (0,20)	0,99	1,01 (0,19)	0,97	0,695	1,01 (0,19)	1	1,02 (0,17)	1,01	0,840
Sodium mEq/L	140,33 (1,82)	140	140,59 (2,07)	140	0,322	140,57 (2,09)	141	140,79 (1,59)	141	0,520
Potassium mEq/L	4,25 (0,30)	4,25	4,18 (0,24)	4,20	0,140	4,24 (0,32)	4,25	4,28 (0,30)	4,25	0,371
Alanine transaminase U/L	28,43 (11,00)	24,5	27,50 (9,55)	24,5	0,645	28,62 (11,95)	25	27,31 (9,96)	24,5	0,428

(continues)

Table 2- Changes in laboratory measures in the beginning and at the end of both intervention periods: abstention (Abst) and red wine (RW) periods. Values are presented in mean (SD) and median. SD: standard deviation. Wilcoxon test. (continued)

	Washout		RW			Washout		Abstemious		
	Mean (SD)	Median	Mean (SD)	Median	p	Mean (SD)	Mean	Median (SD)	Mean	p
Aspartate transaminase U/L	41,93 (26,30)	35	37,33 (15,21)	34	0,303	39,5 (18,54)	35,5	38,93 (20,25)	34	0,800
Gamma-glutamyl transpeptidase U/L	44,03 (22,94)	37,5	44,76 (18,39)	40	0,058	44,10 (19,72)	39	42,88 (18,14)	38,5	0,221
Alkaline phosphatase U/L	78,62 (20,81)	74	76,60 (20,14)	73	0,153	77,44 (20,34)	74	77,88 (21,17)	75,5	0,576
Albumin g/dL	4,05 (0,29)	4,1	4,00 (0,26)	4	0,108	4,05 (0,25)	4	4,01 (0,26)	4	0,041
Total bilirubin mg/dL	0,77 (0,33)	0,69	0,77 (0,30)	0,75	0,821	0,80 (0,34)	0,76	0,78 (0,32)	0,77	0,702
High-sensitivity C-reactive protein mg/L	2,37 (3,24)	1,07	2,71 (3,23)	1,49	0,193	3,86 (9,71)	1,01	2,90 (3,82)	1,32	0,361
Prothrombin Time seconds	11,69 (1,00)	11,6	11,43 (0,69)	11,3	0,004	11,58(0,77)	11,5	11,59 (0,67)	11,5	0,555
International normalized ratio (INR)	0,99 (0,09)	1	0,97 (0,07)	1	0,090	0,98 (0,07)	1	0,99 (0,07)	1	0,386
Glomerular filtration rate mL/min/1,73 m2	86,5 (18,94)	84,5	85,9 (17,11)	88	0,572	86,21 (19,69)	85,5	85 (16,97)	84	0,851

Table 3- Comparison using the Wilcoxon test of the differences in clinical and laboratorial measures in the beginning and at the end of the two interventions periods between the groups: abstention and red wine (RW) periods. Values are presented in values mean (SD) and median. SD: standard deviation.

	Abstention		RW		p
	Mean (SD)	Median	Mean (SD)	Median	
Systolic Blood Pressure in mmHg	4,13 (14,21)	0	-0,37 (13,09)	0	0,065
Diastolic Blood Pressure in mmhg	3,71 (19,24)	0	2,31 (12,80)	0	0,907
Heart Rate beats per minute	3,32 (20,42)	0	1,98 (13,82)	0	0,924
Weight in kg	0,43 (2,41)	0	2,16 (11,65)	0,27	0,173
Body Mass Index	0,82 (2,56)	0	0,16 (1,97)	0,18	0,813
Waist circumference in cm	-0,26 (2,18)	0	0,32 (2,95)	0	0,226
Hematocrit in mm^3	-0,45 (5,31)	0	-0,01 (4,01)	0	0,489
Hemoglobin in g/dL	-0,31 (4,83)	-0,73	-0,10 (3,25)	0	0,613
Leukocytes in mm^3	1,21 (14,54)	-1,21	3,92 (19,05)	3,78	0,463
Neutrophils in mm^3	3,69 (20,37)	1,51	4,50 (22,26)	6,85	0,809
Neutrophils %	10,74 (54,83)	1,63	2,14 (10,69)	0,71	0,354
Eosinophils in mm^3	9,74 (45,51)	0	6,64 (41,76)	0,17	0,963
Eosinophils %	8,35 (46,24)	0	4,09 (44,04)	0	0,737
Basophils in mm^3	-17,25 (40,65)	-6,90	-18,36 (45,71)	-6,66	0,679
Basophils in mm^3 %	-17,24 (38,44)	0	-21,15 (40,43)	0	0,636
Lymphocytes in mm^3	19,96 (145,30)	-1,64	2,30 (17,77)	-1,51	0,591
Lymphocytes %	3,62 (30,32)	0	8,99 (50,81)	0	0,355
Monocytes in mm^3	1,01 (26,11)	4,17	5,38 (23,10)	3,15	0,837
Monocytes %	3,62 (30,32)	0	2,49 (20,67)	0	0,895
Platelets in mm^3	1,01 (26,11)	3,11	-3,67 (16,79)	-4,36	0,007
Total cholesterol mg/dL	-2,38 (10,15)	-2,75	3,41 (14,25)	4,74	0,013
High-density lipoprotein (HDL) cholesterol mg/dL	-0,79 (10,92)	-2,17	6,22 (13,39)	6,38	0,002
Low-density (LDL) lipoprotein cholesterol mg/dL	-4,63 (14,91)	-7,72	5,27 (19,03)	2,47	0,027
Non-HDL cholesterol mg/dL	-2,96 (13,79)	-3,65	2,99 (17,74)	2,47	0,050
Triglycerides mg/dL	6,67 (28,50)	7,84	5,13 (39,33)	5,13	0,587
Apolipoprotein (A1) g/L	1,99 (10,94)	2,09	3,03 (13,37)	1,74	0,613
Apolipoprotein B g/L	-0,48 (13,81)	-0,23	4,48 (23,11)	1,11	0,361
Lipoprotein (a) mg/dL	-7,97 (13,08)	-5,19	-3,14 (32,31)	0	0,279
Troponin I ng/mL	29,54 (166,55)	0	3,57 (56,04)	0	0,640
Glucose mg/dL	3,01 (9,67)	1,62	-0,20 (8,86)	-1,19	0,188
Urea mg/dL	-1,92 (18,47)	-2,39	1,29 (26,49)	-1,04	0,603
Creatinine mg/dL	1,19 (9,98)	-0,53	0,73 (8,36)	0	0,771
Sodium mEq/L	0,17 (1,49)	0	0,27 (1,60)	0	0,836
Potassium mEq/L	1,14 (6,50)	1,14	-1,51 (7,20)	-2,27	0,091
Alanine transaminase U/L	-1,14 (18,03)	-4,00	-0,59 (17,22)	0	0,785
Aspartate transaminase U/L	0,73 (19,31)	-2,86	-2,24 (23,07)	0	0,514
Gamma-glutamyl transpeptidase U/L	-2,11 (13,62)	0	4,35 (15,88)	3,87	0,025
Alkaline phosphatase U/L	0,74 (8,79)	0	-2,14 (9,51)	-2,54	0,196
Albumin g/dL	-1,21 (3,76)	-2,33	-1,33 (6,21)	-2,33	0,638
Total bilirubin mg/dL	2,56 (29,44)	-0,89	3,78 (29,40)	-1,22	0,956
High-sensitivity C-reactive protein mg/L	105,42 (355,64)	16,27	107,38 (272,08)	16,31	0,474
Prothrombin Time seconds	0,35 (2,91)	0,84	-1,98 (6,01)	-2,40	0,003
International normalized ratio (INR)	1,16 (6,12)	0	-1,84 (7,10)	0	0,055
Glomerular filtration rate mL/min/1,73 m^2	-0,38 (9,83)	-0,52	0,06 (9,50)	0	0,826

4.2 Diet was constant during the protocol

Patients underwent nutritional evaluation at five in-hospital visits and filled a three-day food frequency questionnaire at the end of each intervention period (Abst or RW). Patients were compliant with nutritional guidance and kept the same food ingestion pattern documented in both food diaries. No significant differences in macronutrient and micronutrient consumption between the two intervention periods were noted (Table 4).

Table 4- Food frequency questionnaires (FFQ) data quantification

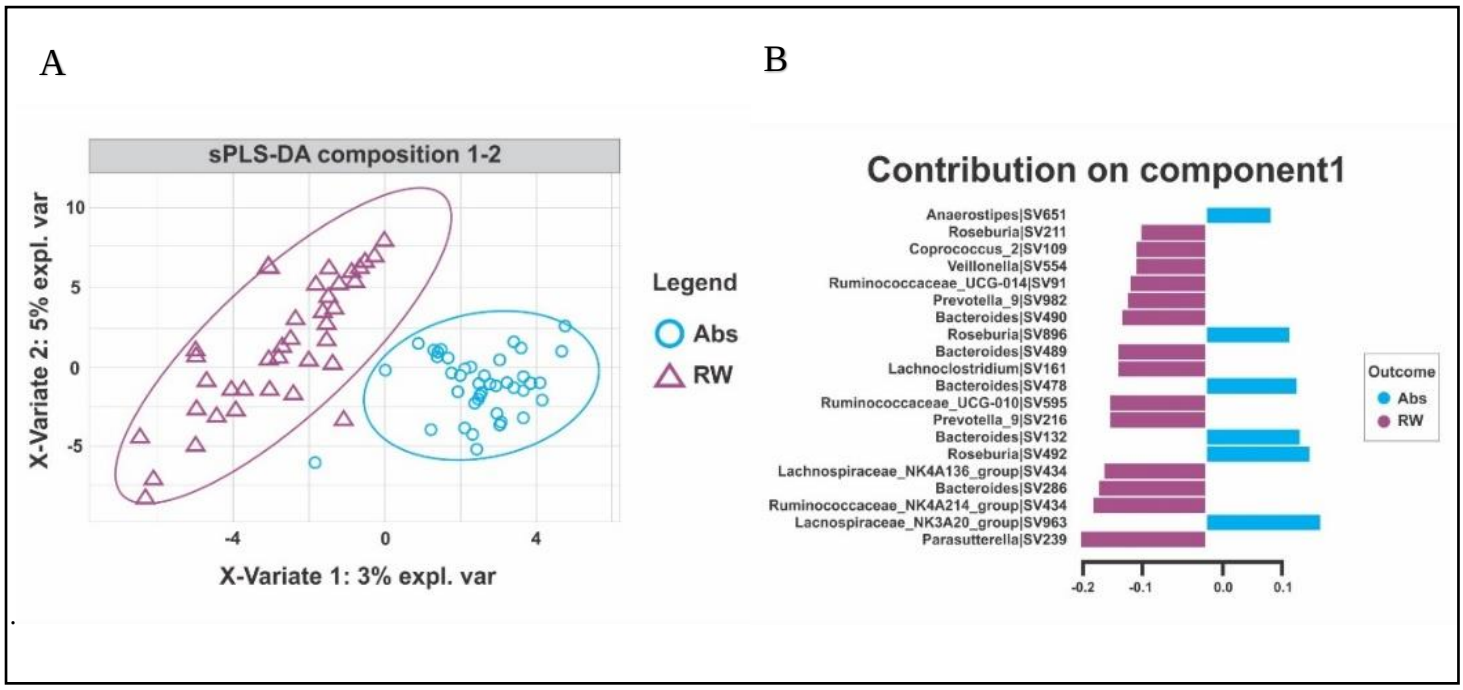
		Abstention	Red Wine	p-value*
Macronutrients				
	Total energy intake, Kcal	1995.5(±608.8)	2082.5(±513.3)	0.485
	Protein, g	97.2(±34.3)	99.5 (±28)	0.735
	Lipids, g	62.7(±24)	63.6 (±22.9)	0.861
	Carbohydrates, g	258.4(±92.6)	250.8(±83.2)	0.699
	Fiber,g	23.7 (±12.8)	22.9 (±11.2)	0.753
Micronutrients				
	Cholesterol, g	326.1 (±156.7)	342.4 (±140.8)	0.624
	Calcium,g	611.6 (±428.8)	506.4 (±308.5)	0.207
	Iron,g	10.6 (±9.1)	16.9 (±53)	0.466
	Sodium,g	1603.7 (±784.1)	1589.4(±768.3)	0.934
	Potassium,g	2509.1 (±904.4)	2278.3 (±777)	0.221
	SFA,g	22.5 (±9.8)	22 (±8.4)	0.808
	MUFA,g	18.5 (±7.8)	18.3 (±9.2)	0.934
	PUFA,g	9 (±4.8)	9.3(±5.2)	0.813

Three-day food frequency questionnaires were applied at the end of each intervention period. Values are presented in mean (± SD) per day. *T-test. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids. No differences were observed in macronutrient or micronutrient consumption between periods.

4.3 Modifications in Gut Microbiota identified the RW consumption

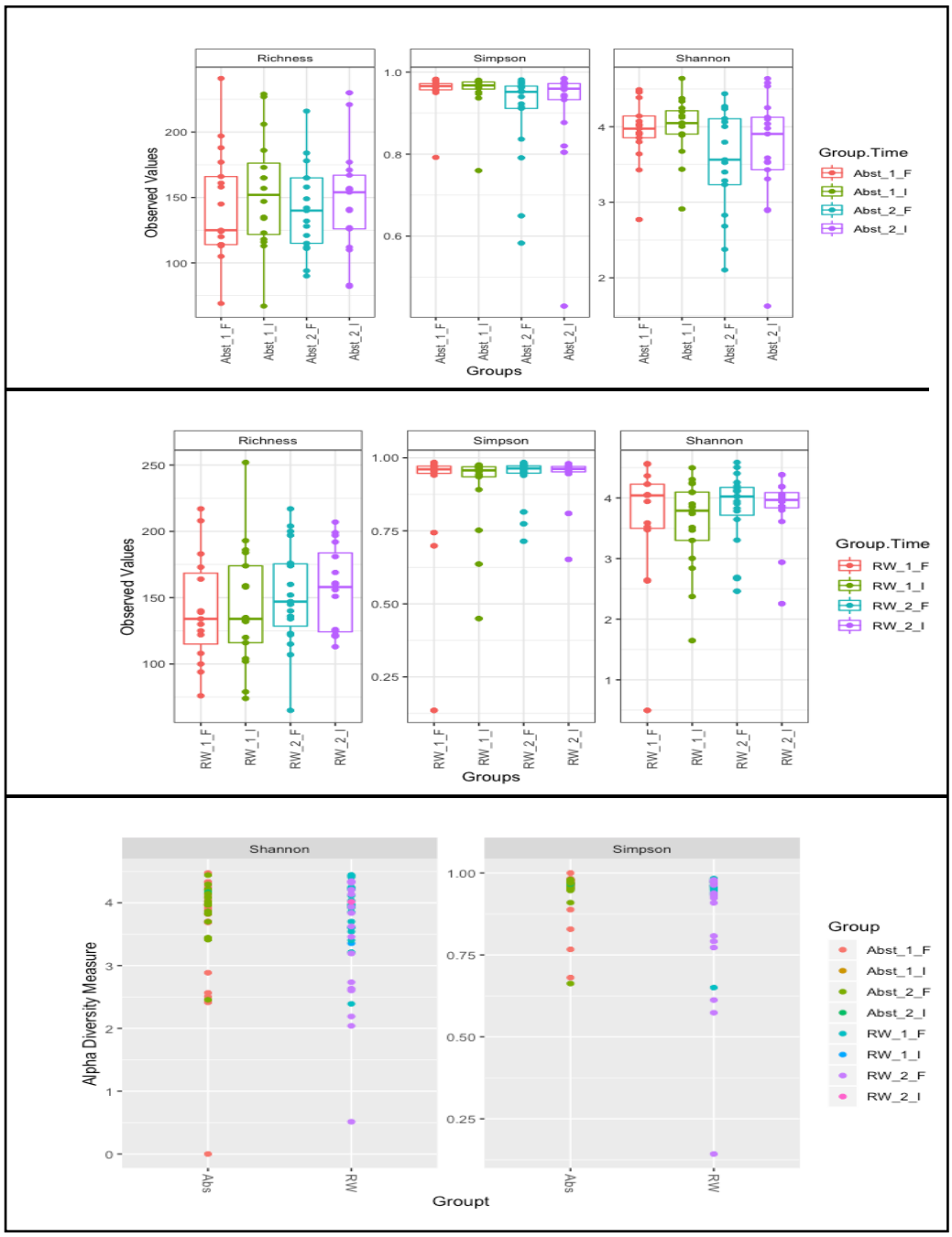
After a two-week washout period, RW modified gut microbiota was analyzed by 16S amplicons and compared with the abstention period. Beta diversity, as highlighted in the sparse partial least squares regression - discriminant analysis (sPLS-DA), shows a clear separation between groups (figure 7 A), and a gut microbiota profile could pin-

point RW consumption. On the other hand, alpha diversity did not change after RW consumption (figure 8). All gut microbiota evaluations were compared pairwise. Figure 7B shows the contribution to the composition of the main bacterial genera that predominate in each period's composition, RW and abstention periods. *Parasutterella* genus was prominent in gut microbiota composition in the RW period and is associated with succinate - a key intermediate precursor of short-chain fatty acids (SCFA), aromatic amino acid, purine and bile acids (BA) derivatives.[51] *Ruminococcaceae* and several *Bacteroides* were also relevant in the RW period. *Prevotella*, a genus related to fiber digestion and fiber consumption[52], was also prominent in the RW period. Three fecal samples from 3 different patients were removed from analysis due to low count read. In the end, 39 fecal samples in the RW period were compared to 39 fecal samples of the abstention period. These three losses did not impact statistical analysis since 38 patients were required to power the crossover intervention.



(A) The plot of two-component (comp 1 and 2) sPLS-DA model showing fecal samples clustering according to red wine consumption (RW) or not (abst) with the percentage of variance captured for each principal component for each period of the study (RW vs. abstention). First, a prediction of the classification performance (error rate) was carried out concerning the number of selected variables in the model with the function tune.splsda. The tuning is performed one component at a time, and we set a maximum of ncomp = 3, as suggested from the PLS-DA performance assessment. To reach optimal performance, we estimate that 2 components were sufficient for our final sPLS-DA model. We chose 5-fold cross-validation (folds = 5) repeated 10 times. The sPLS-DA plot based on the relative abundance of bacterial taxa of gut microbiota from the Abstention (Abs) group (blue circle) or Red Wine (RW) group (orange triangle) and their 95% confidence ellipses. **(B)** Contribution plot indicating genera contributing to component 1 of sPLS-DA analysis blue color (ABS) and orange color (RW).

Figure 7- Sparse partial least squares - discriminant analysis (sPLS-DA)



(a). The different numbers of asterisks above each box plot indicated significant differences (paired-wise Anova, P<0.05 * and P<0.01 **). Box Plots represent mean and standard deviation. **a** Control group Abs 1_I (group 1 and initial timepoint), Abs 1_F, (group 1 and final timepoint) Abs 2_I (group 2 and initial timepoint), and Abs 2_F (group 2 and final timepoint). N=20. B) Patients group with consuming of wine RW 1_I (group 1 and initial timepoint), RW 1_F (group 1 and final timepoint), RW 2_I (group 2 and initial timepoint), and RW 2_F (group 2 and final timepoint). N=19. **b** All in the RW period vs. all abstained. No significant differences in alpha diversity between periods.

Figure 8- Alpha diversity: Timepoint patients' differences in alpha diversities (Richness, and Simpson and Shannon indexes) of stool samples

4.4 Plasma metabolomic analysis shows that RW remodels several metabolic pathways

Untargeted plasma metabolomic analysis of 20 patients in the four visits revealed microbiome-related changes associated with RW consumption. The main changes occurred in pathways of amino acids, lipids, carbohydrates, and vitamins and cofactors (figure 9A). Metabolites that RW consumption modified significantly compared to the abstention period were identified by paired Wilcoxon signed-rank test for non-parametric data and paired t-test for parametric data. All metabolites affected during the protocol that achieved statistical significance ($p < 0.05$) with their fold change in percentage are listed in Supplementary material – Table S1. In total, 39 metabolites were significantly different after RW ingestion (figure 9B), compared to the abstention period: 24 of putative beneficial pathways, 5 potentially harmful, and 10 without clear descriptions in the medical literature. Although some pathways might suggest risk, the net results indicate a beneficial direction, as the majority of metabolites changed suggest CV protection.

4.5 RW altered pentose phosphate, ascorbate and aromatic amino acid pathways

Pentose phosphate pathway (PPP), glucuronate/ascorbate pathway, and aromatic amino acid metabolism exhibit several interconversions amongst them (figure 9C). Several metabolites in these pathways rose. Pentose phosphate and glucuronate pathways support redox homeostasis systems, contributing – although not exclusively – to antioxidant / reductive pathways[53,54] and both comprise precursors to aromatic amino acid metabolism[55]. After RW, there was a significant increase in sedoheptulose, arabinose, ribitol, arabitol, and xylitol, components of PPP's non-oxidative branch. The

increase in ribitol, an important metabolite in the pentose phosphate pathway and an integral part of riboflavin (vitamin B2),[56] might indicate a modulation of the riboflavin metabolism pathway. Riboflavin can act as an antioxidant against oxidative stress, especially lipid peroxidation and reperfusion oxidative injury.[57] The mechanisms by which riboflavin protects the body against oxidative stress may be attributed to its role in the glutathione redox cycle, directly scavenging free radicals or reinforcing the effect of other antioxidants such as vitamin C.[57] Riboflavin acts as a coenzyme for redox enzymes bearing flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) cofactors or prosthetic groups. FAD, among many other functions, is required as a coenzyme for glutathione reductase activity. Additionally, FAD transports hydrogen from the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) to oxidized glutathione to convert it into its reduced form. Reduced glutathione, the most abundant non-protein reducing thiol in the cells, plays a key role in protective and signaling events in cellular processes linked to reactive oxygen species.[58]

In the glucuronate/ ascorbate pathway, there was a significant increase in gulonate, which is an obligatory intermediate in ascorbic acid generation.[53] Gulonate is also involved in the pathway leading to xylulose, a key metabolite in PPP.[59]

Additionally, there were modifications in microbiome-associated products of aromatic amino acids, i.e., tryptophan, tyrosine and phenylalanine. After RW, indole propionate (IPA) significantly increased. IPA, a putatively beneficial microbial-derived metabolite of tryptophan, associates with a reduced risk of type 2 diabetes and, in rats, oral administration of IPA improved glucose metabolism.[60] The tyrosine metabolites, gentisate and homovanillate, also increased, but thyroxine decreased. RW consumption also augmented 1-carboxyethylphenylalanine, derived from phenylalanine metabolism.

Gentisate and homovanillate are phenolic catabolites - with antioxidant activities[61], may exert beneficial effects in the metabolic syndrome[62] and may be related to RW's influence on gut microbiota[38,63,64]. Reduction in thyroxine may reflect a gut microbial and bile acid modulation of the G-protein-coupled bile acid receptor 1 (also known as TGR5). TGR5 regulates energy metabolism promoting the conversion of inactive thyroxine into active thyroid hormone, leading to thermogenesis.[15,65]

4.6 Amino acid metabolites changed after RW ingestion

RW consumption elevated the glutamate metabolites, citralamate and N-acetylglutamine. These microbial-derived metabolites may influence weight control by reducing appetite.[66] *N*-acetylglutamine is a marker of adhesion to the Mediterranean diet[67] and its derivatives also associate with leanness[68]. Nonetheless, some amino acid changes did not relate to putative beneficial effects. Branch-chain amino acid (BCAA) metabolites 3-methyl-2-oxovalerate and 2,3-dihydroxyisovalerate increased. BCAA are a risk factor for CAD and may participate in insulin resistance[69]. There was also an increase in the polyamine metabolite, acisoga, related to atrial fibrillation[70]. Lanthionine, a metabolite in cysteine metabolism, also augmented after RW and is a uremic toxin[71].

4.7 RW consumption altered lipid metabolites, including androgens, endocannabinoids, and fatty acids

RW, when compared to abstention, induced increases of androgens 5alpha-androstan-3beta,17beta-diol monosulfate (2), androsteroid monosulfate C19H28O6S (1)* and androstenediol (3beta,17beta) monosulfate (1). Increased androgenic steroids plasma levels were previously associated with alcohol consumption,[72,73]. Low levels of androgen steroids are also markers of metabolic syndrome,[74] insulin resistance[75] and cardiovascular disease[76]. Furthermore, we detected a reduction of the endocannabinoid metabolites, linoleoyl ethanolamide and its intermediate linoleoyl-linolenoyl-glycerol (18:2/18:3) [2]* with RW. Endocannabinoid signaling is a potent mediator of food intake, influences energy metabolism,[77] and adipogenesis[78]. Gut microbiota is implicated in these interactions[78], and its metabolites are increased in obesity and insulin resistance and involved in hedonic eating.[79] In addition, RW consumption modified fatty acid metabolites, as shown by a decrease in 3-decenoylcarnitine and increase on 2R,3R-dihydroxybutyrate and 3-hydroxystearate. Long-chain acylcarnitines, which are intermediates of fatty acid oxidation, such as 3-decenoylcarnitine, are associated with insulin resistance[80] and also with recurrent CV events.[81] The increase 2R,3R-dihydroxybutyrate and 3-hydroxystearate could represent increased ketone body oxidation, a potentially beneficial and adaptive mechanism in other clinical conditions as heart failure.[82]

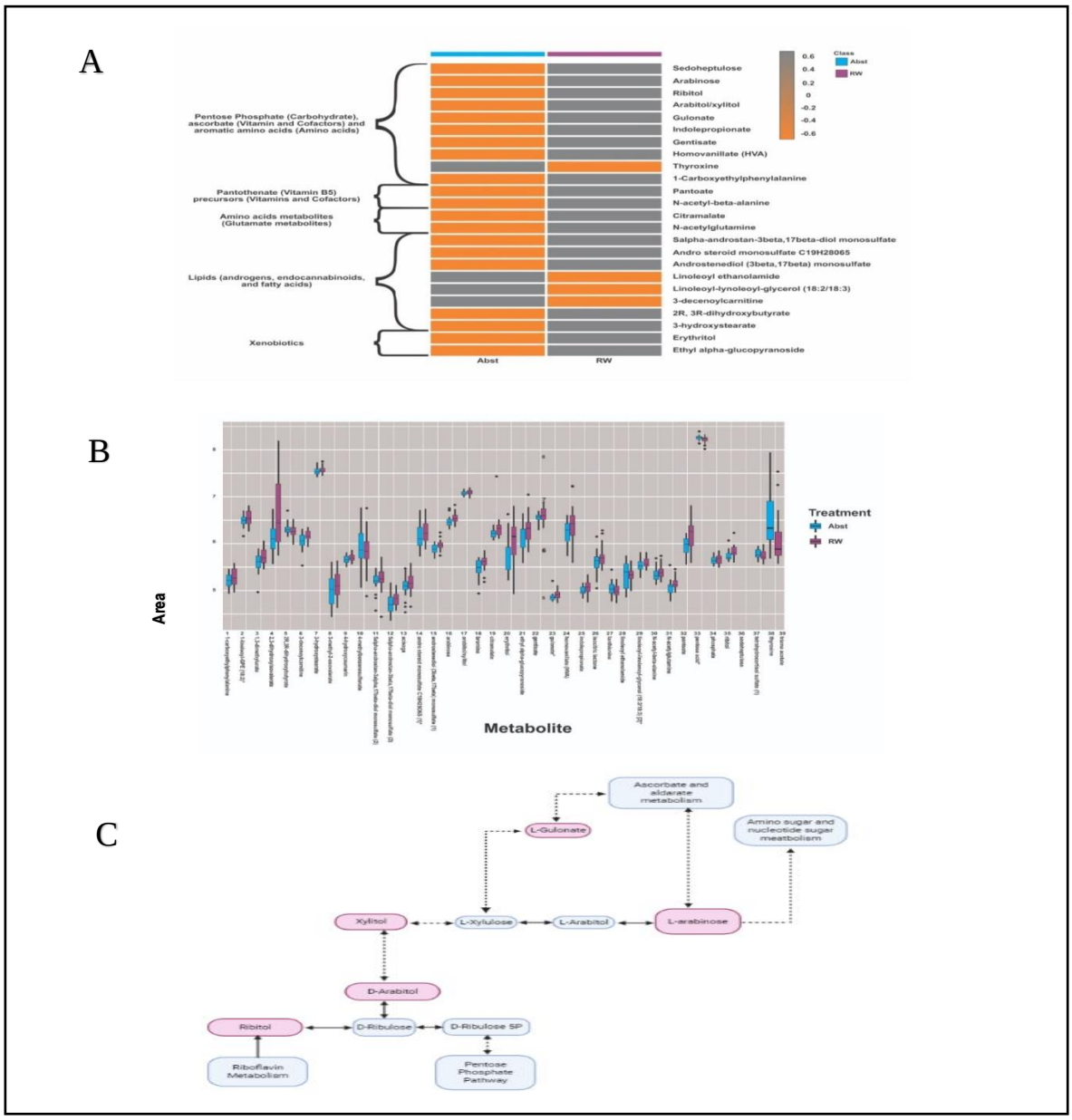
4.8 Xenobiotics metabolites corroborate the RW ingestion

RW consumption significantly increased erythritol and ethyl alpha-glucopyranoside compared to abstention. Erythritol is a natural product of RW fermentation[83]. Glucopyranosides are described as grape metabolites of fermentation[84],

and may inhibit alpha glucosidases, which are therapeutic targets in diabetes[85]. Nevertheless, there was also an increase in 1.3.dimethylurate, a metabolite of theophylline, in the xanthine pathway, implicated in xanthine oxidase activation and pro-oxidant activity[86]. Hence, these findings indicate that subjects adhered to RW intervention.

4.9 Pantothenate (Vitamin B5) precursors increased post RW

RW consumption significantly increased pantoate and N-acetyl-beta-alanine, precursors of pantothenate,[87,88] compared to abstention. B complex vitamins are vital for several human metabolic processes, are directly absorbed on the upper digestive tract, but also derive from gut microbiota metabolism. Pantothenate is involved in Acetyl Coenzyme A biosynthesis and several *Bacteroides* seem to contribute to its gut metabolism[87]. Several B vitamins also are a marker of gut microbiota stability[89] and are associated with butyrate producers bacteria[90].



(**A)** Heatmap showing metabolites significantly different between RW and abstention periods. To analyze the differences in metabolite intensity between RW and abstemious conditions, paired Wilcoxon signed-rank test was performed for non-parametric data, and paired t-test for parametric data. According to its metabolic pathways and biochemical functions, the graph highlights the most clinically relevant metabolites significantly different in the two periods of the study (p-value <0.05, without adjustments for multiple comparisons). The heatmap was created using Euclidean distance measurement and Ward clustering algorithm to show the average difference between the groups. (**B**)Box-whisker plot of the distribution of the discriminating metabolites that significantly modified after RW consumption compared to the abstention period. Data were log-transformed**.** (**C)** Pentose and glucoronate interconversions adapted from Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.[91] In red are the metabolites that were significantly increased after RW, and in blue, the putative pathways these metabolites are involved with. Arrows indicate the direction of the reaction, reversible and irreversible reactions, which are indicated by bi-directional and uni-directional arrows, respectively. Bold lines indicate activation or interaction. Dashed lines indicate an indirect link or unknown reaction.

Figure 9- Untargeted plasma metabolomics modifications of 20 subjects

4.10 Plasma TMAO analysis

TMAO has been correlated with atherosclerotic burden and MACE.[16,17] However, its predictive value regarding future cardiovascular events as well as its significance as a marker of atherosclerosis is still controversial[92]. Therefore, we analyzed TMAO to gain insights into its role in the context of RW consumption.

Plasma levels of TMAO did not differ significantly after the consumption of RW, compared with the abstention period (Table 5). TMAO levels varied substantially in the samples collected both at the beginning and the end of the abstention: the Intraclass correlation (ICC) coefficient between these two samples was 0.049 (CI 95% -0.255,0.345; p - 0.377).

To address whether technical issues related to blood collection could affect our measurements and test whether TMAO levels assessed sequentially from the same individual would show consistent similarity, 10 patients had 4 fasting blood samples collected twice a week for 2 weeks, without any intervention. Three collection techniques were used: 1. Collection, centrifugation and immediate freezing at -80º; 2. Collection, refrigeration for 2 hours, then centrifugation and freezing at -80ºC; 3. Dried-bloodspot (DBS) analysis (Table 6). High concordance was observed amongst the three different sample collection techniques, with an ICC of 0.915 (CI 95%0.861;0.951) and a higher concordance between immediate freezing and refrigeration (ICC of 0.993) (Table 7). On the contrary, there was a low concordance between repeated measures of TMAO (Table 8), indicating a significant inter-measurement variability for the same individual over time.

Table 5- Fasting TMAO plasma levels in the beginning, after the 2-week washout period, and at the end of each 3-week intervention period

Intervention	**TMAO, µM**		
	After Washout	After Intervention	p[1]value
Abstemious	3.95 (2.70-6.76)	4.99 (3.14-9.28)	0.385
Red Wine	4.58 (2.83-8.39)	3.37 (2.66-7.64)	0.703
p[2] value	0.531	0.217	

Values are presented median (interquartile range). p[1]: Wilcoxon test; p[2]: Mann-Whitney test

Table 6- Validation tests for TMAO plasma levels, in µM - absolute TMAO values. Ten patients had fasting blood samples collected twice a week for two weeks. Three samples from each patient were collected at the 4 visits. The samples were divided and analyzed by 3 different sampling techniques: immediate freezing at -80oC; refrigeration at 4ºC for 2 hours and freezing at -80ºC; and dry blood spot (DBS) in protein saver card.

Sample Collection Time		**Immediate Freezing**	**Refrigeration**	**DBS**
		TMAO, µM	TMAO, µM	TMAO, µM
Visit 1	Mean (± SD)	4.20 (± 1.99)	4.49 (± 1.90)	4.52 (± 2.12)
	Median (min-max)	3.85 (1.42-8.39)	3.94 (2.36-8.89)	3.87 (2.99-10.00)
Visit 2	Mean ± (SD)	7.43 (± 8.07)	7.63 (± 8.33)	5.92 (± 4.93)
	Median (min-max)	4.74 (1.41-26.50)	4.87 (1.49-26.90)	4.86 (1.42-16.90)
Visit 3	Mean (± SD)	4.28 (± 3.16)	4.14 (± 2.94)	3.61 (± 2.08)
	Median (min-max)	3.65 (2.10-12.80)	3.32 (2.07-12.00)	3.20 (1.87-9.18)
Visit 4	Mean (± SD)	4.75 (± 3.01)	4.54 (± 3.04)	4.93 (± 4.26)
	Median (min-max)	4.12 (2.38-12.60)	4.05 (2.11-12.60)	3.95 (2.19-16.60)

Values are presented as mean ±SD, and median (minimum and maximum values)

Table 7. Agreement of the TMAO levels by collections techniques assessed by Intraclass Correlation Coefficient (ICC). Values close to 1 indicate greater agreement between the methods. ICC values between all 3 methods (immediate freezing, refrigeration and dry blood spot -DBS), paired by techniques for all visits and stratified by visit. The ICC values indicated a strong agreement between the techniques.

		ICC	IC95%	p value §
All visits	Immediate freezing, Refrigeration and DBS	0.915	0.861-0.951	<0.001
	Immediate freezing and Refrigeration	0.993	0.988-0.997	<0.001
	Immediate freezing and DBS	0.865	0.759-0.926	<0.001
	Refrigeration and DBS	0.864	0.757-0.925	<0.001
Visit 1	Immediate freezing, Refrigeration and DBS	0.904	0.749-0.973	<0.001
	Immediate freezing and Refrigeration	0.911	0.685-0.977	<0.001
	Immediate freezing and DBS	0.882	0.597-0.969	<0.001
	Refrigeration and DBS	0.920	0.712-0.979	<0.001
Visit 2	Immediate freezing, Refrigeration and DBS	0.913	0.771-0.975	<0.001
	Immediate freezing and Refrigeration	0.999	0.995-1.000	<0.001
	Immediate freezing and DBS	0.855	0.522-0.962	<0.001
	Refrigeration and DBS	0.845	0.495-0.959	0.001
Visit 3	Immediate freezing, Refrigeration and DBS	0.949	0.859-0.986	<0.001
	Immediate freezing and Refrigeration	0.992	0.968-0.998	<0.001
	Immediate freezing and DBS	0.908	0.675-0.976	<0.001
	Refrigeration and DBS	0.931	0.749-0.982	<0.001
Visit 4	Immediate freezing, Refrigeration and DBS	0.933	0.820-0.981	<0.001
	Immediate freezing and Refrigeration	0.995	0.981-0.999	<0.001
	Immediate freezing and DBS	0.903	0.659-0.975	<0.001
	Refrigeration and DBS	0.922	0.719-0.980	<0.001

ICC: Intraclass Correlation Coefficient; CI95%: 95% confidence interval. § Under the null hypothesis that the intraclass correlation coefficient is equal to zero.

Table 8- TMAO validation tests. The intraclass correlation coefficient for each TMAO sample collection technique, between 4-time sample collections showed a similar low concordance of TMAO values.

Technique	ICC	**CI95%**	p[1]value
Freezing	0.224	-0.047 – 0.627	0.060
Refrigeration	0.208	-0.058 – 0.613	0.072
DBS	0.219	-0.051 – 0.623	0.063

ICC: Intraclass correlation coefficient; CI 95%: confidence intervals 95%. 1.Under the null hypothesis that the intraclass correlation coefficient is equal to zero.

4.11 Clinical correlations of TMAO plasma levels

We sought clinical variables that could explain the high variability observed in our samples for TMAO levels. First, there was an inverse, but weak correlation of TMAO with renal function variables. Spearman correlation identified positive correlation of TMAO and urea (rho 0.272, p<0.001), creatinine (rho 0.254, p 0.001) and negative with GFR (rho -0.124 p 0.109). This pattern was similar in all the samples. This finding was expected, as the kidney clears TMAO,[93] and it rises in subjects with impaired renal function[20].

A second important factor is the relation of nutrient consumption patterns and TMAO levels(Supplementary material – Table s2), since proteins and fats are the dietary sources of choline and carnitine, and subsequently of TMA, TMAO's precursors[94]. Macronutrients and micronutrients consumption were constant during all the protocol. There was a weak and positive correlation for the final measurement of TMAO and protein (rho 0.235, p 0.046) and cholesterol (rho = 0.303, p 0.009). However, the initial measure of TMAO and carbohydrates correlated negatively (rho = 0.230, p 0.05). Analyzing the two distinct study periods revealed no correlations of

TMAO levels and nutrients in the abstention period. In the RW period, fiber consumption and TMAO levels correlated negatively only in the samples collected at the beginning of the period. The samples collected at the end of the RW period showed a positive correlation of TMAO levels and protein (rho 0.478, p 0.025) and cholesterol (rho 0.336, p 0.045). Likely, the unsteady correlations in different time points occurred due to high intraindividual variations of TMAO levels.

Concerning patient medication, there was no difference in TMAO levels between the subjects using ACE inhibitors or diuretics. ACE inhibitors may enhance TMAO elimination[95], while diuretics may increase TMAO[96]. We investigated whether these medications influence TMAO levels and its variations with time. Application of the Generalized Estimating Equation (GEE) found no interaction of TMAO levels with the use of these drugs nor within time (Supplementary material (Supplementary Table 04).

4.12 TMAO and gut microbiota

Regarding TMAO and gut microbiota relationships, during the abstention period, TMAO correlated weakly with *Roseburia* and negatively with *Lachnospiracea*. After RW consumption, TMAO plasma levels correlated positively with the genus *Bacteroides* (Figure 10, C and D). These results contrast with the literature, as members of *Bacteroidetes, as Roseburia* and *Bacteroides,* should not be capable of converting choline to TMA in the gut, as these taxa lack the gene cluster necessary for this conversion.[97] Furthermore, TMAO correlated negatively with *Blautia* after RW consumption. Taken together, these data did not show a consistent correlation between nutrients and TMAO, nor to gut bacterial taxa implicated in TMAO formation.

4.13 Microbiome remodeling during RW intake: correlation with clinical, biochemical and nutritional variables (Figure 10)

Spearman correlations of gut microbiota taxa and clinical, biochemical and nutritional variables appear on Supplementary material: Table S3(A) represent clinical variables during abstention period and table S3 (B), during RW period; table S3 (C) represent biochemical variables during abstention period and S3(D) during RW period table S3 (E) represent nutritional variables during abstention period and tableS3(F), during RW period .

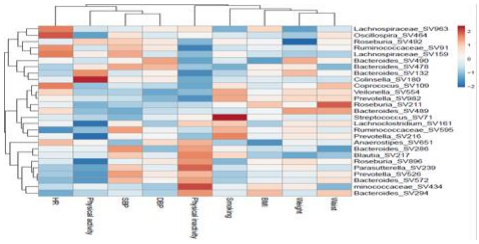
In the abstention period, several positive and negative correlations were found among physical activity, biochemical features, and nutritional components. In the abstention period, there was a positive correlation between physical activity and *Collinsella,* and negative with *Prevotella* and *Lachnoclostridium*. Also, there was a

negative correlation between weight with *Roseburia, Blautia* and *Lachnospiraceae*, and a negative correlation of *Anaerostipes* with BMI. Analyzing the correlations of biochemical features and taxa, we found a positive correlation of renal function (GFR) with *Roseburia, Parasutterella* and *Ruminococcaceae*, and negative with *Collinsella*. In addition, a negative correlation of triglycerides levels and *Oscilospira* occurred, as well as negative correlation of several *Bacteroides* with Total Cholesterol and LDL; LPS too, correlated negatively with *Bacteroides* and *Ruminococcaceae*. Regarding the nutritional components of the patients' FFQ, there was, in the abstention period, a positive association of fiber consumption and *Roseburia* and *Oscillospira*, but a negative correlation with *Bacteroides* and *Collinsella*. There was also a positive association between sodium intake and *Roseburia* and *Lachnospiraceae*; as well as a negative correlation between lipid intake and *Anaerostipes* and *Lachnoapiracea*.

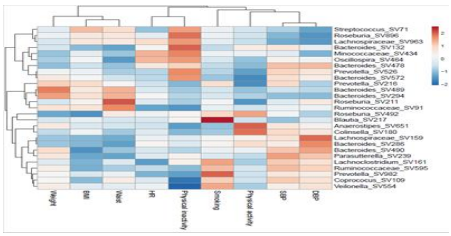
During RW, a negative correlation of waist and BMI with *Parasutterella* was observed; also negative was the correlation between glucose, *Roseburia* and *Streptococcus*. Notably, there was a positive correlation of fiber intake and *Roseburia* and *Oscillospira*, which also was present in the abstention period. In accordance with these data, *Roseburia* is well described as a plant component degrader[98]. *Oscillospira* is a genus associated with SCFA production[99], which links to fiber consumption, concordant with our findings. Hence, RW and abstention were associated with different gut microbiota profiles, suggesting that RW indeed remodels the microbiota in potentially beneficial ways.

Correlations of **clinical features** and microbial taxa in the **abstention (a)** and in the **RW (b) periods.**

A

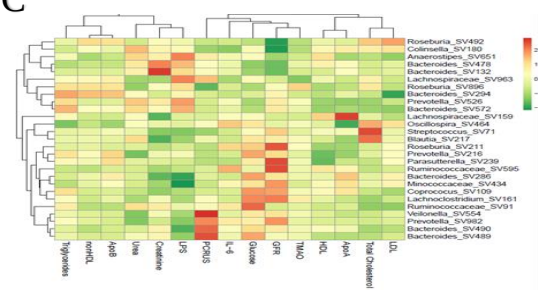


B

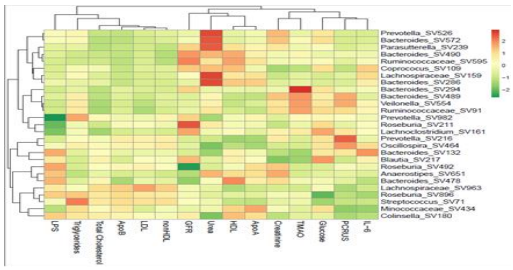


Correlations of **biochemical features** and microbial taxa in the **abstention (c)** and in the **RW (d) periods.**

C

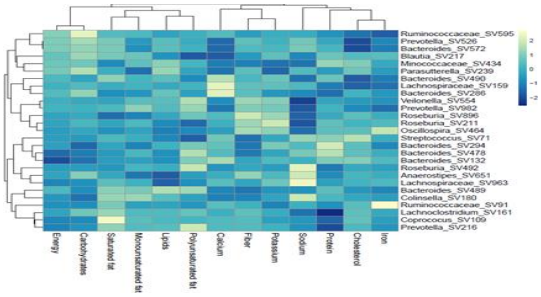


D

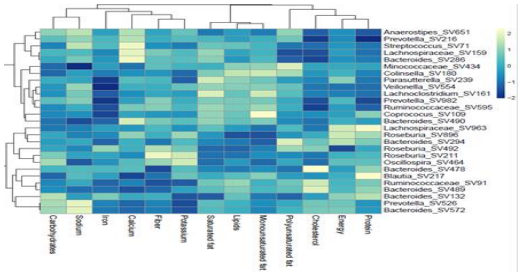


Correlations of nutritional **components** and microbial in the **abstention (e)** and in the **RW (f) periods.**

E



F

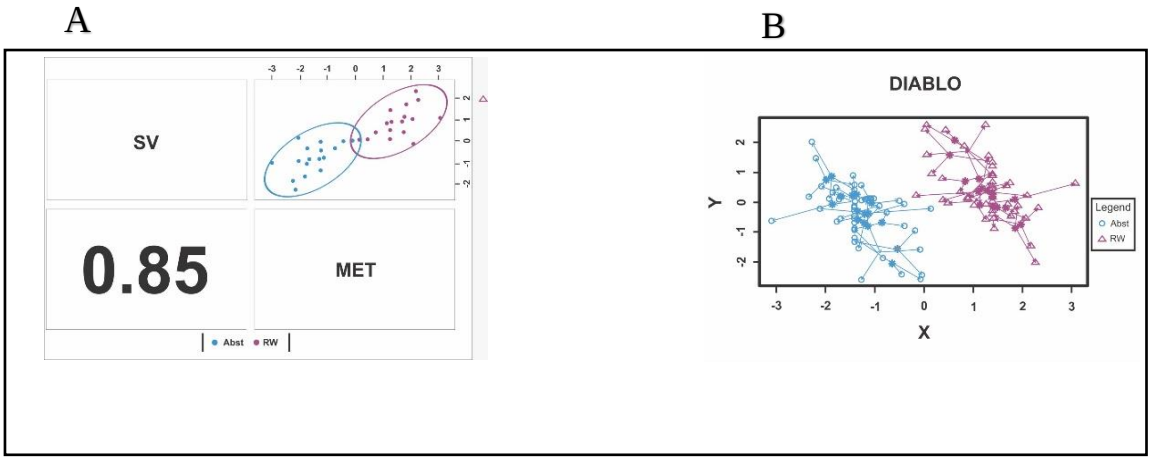


(A) and RW period **(B)**. Rows correspond to bacterial taxa quantified using 16S rRNA; columns correspond to clinical parameters measured in the in-hospital visits. Red and blue denote positive and negative associations, respectively. Correlation of laboratory tests and gut microbial taxa identified in the abstention period **(C)** and RW period **(D)**. Rows correspond to bacterial taxa quantified using 16S rRNA; columns correspond to blood exams collected in the in-hospital visits. Red and green denote positive and negative associations, respectively. Correlation of macronutrients and micronutrients, and gut microbial taxa identified in the abstention period **(E)** and RW period **(F)**. Rows correspond to bacterial taxa quantified using 16S rRNA; columns correspond to nutrients quantified in the FFQ. Light green and dark blue denote positive and negative associations, respectively. The intensity of the colors represents an overview of the numeric differences and correlations between the taxa abundances and clinical features. Columns and rows are clustered by Euclidean distance.

Figure 10- Associations of gut microbial genera with clinical features, laboratory blood tests and nutrients. Correlation of clinical features and gut microbial taxa identified in the abstention period

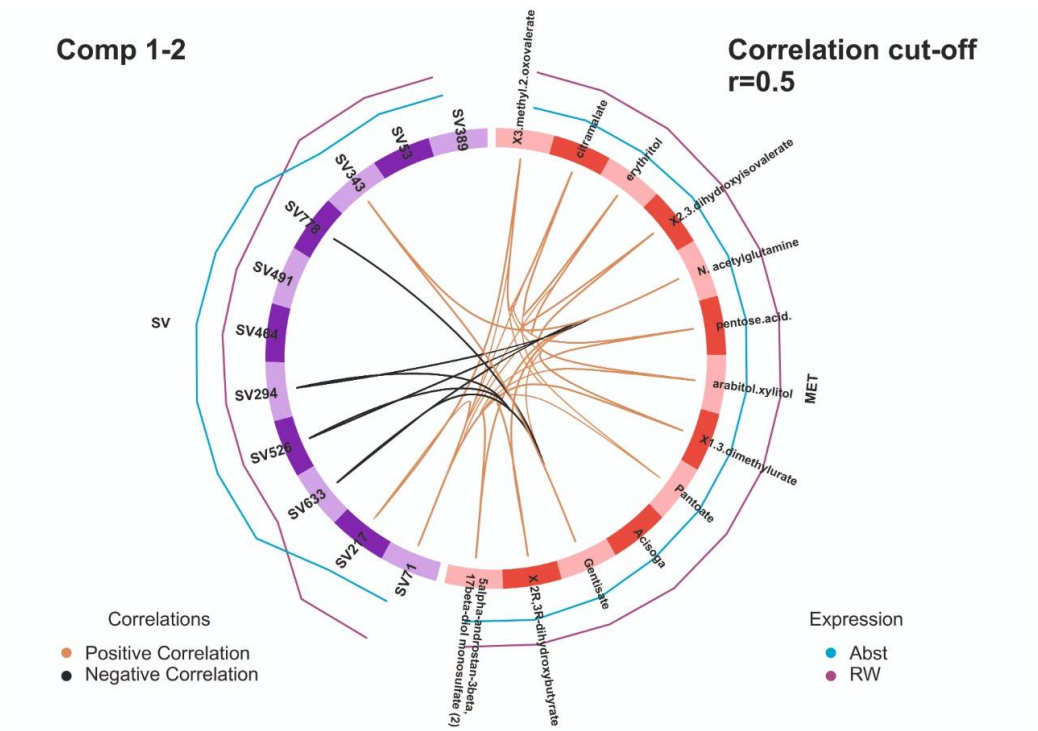
4.14 Integration of plasma metabolomics data and gut microbiota analysis separated well the RW period and the abstention period

In 20 patients selected randomly, using the DIABLO platform for multi-omics integration data, the first components from each data set are highly correlated to each other, indicating a high discriminative power of each component to separate the different groups: RW and abstention (figure 11A). Metabolites and taxa of the first component are displayed in figure 7. The optimally selected key predictors included several taxa of gut microbiota, i.e., *Streptococcus, Blautia, Ruminococcaceae, Bacteroides, Prevotella* and metabolites in amino acid pathways, lipid pathways and cofactors, such as pantoate. The agreement between all data sets is shown in the arrow plot (figure 11 B), which indicates that the two omics (microbiota and metabolomics) can separate well the two conditions. A correlation matrix (figure 12) integrated metabolomics and taxa in the two intervention periods. The taxa and metabolites vary during the different interventions and also the high correlations among key predictors (figure 13).



(A) integration of microbiota analysis (SV) and plasma metabolomics analysis (MET): the colors and ellipses indicate the discriminative power of each component to separate the different groups (Abst vs. RW). Correlation index of 0.85. **(B)** In the arrow plot, the arrow origin indicates the centroid between all data sets for a given sample, the tips of the arrows indicate the location of that sample in each block. These graphs highlight the agreement between all datasets at the sample level when modeled with DIABLO. The two omics (microbiota taxa and metabolomics) performed well in separating the two interventions (RW and abst).

Figure 11- Multi-Omics data integration analysis



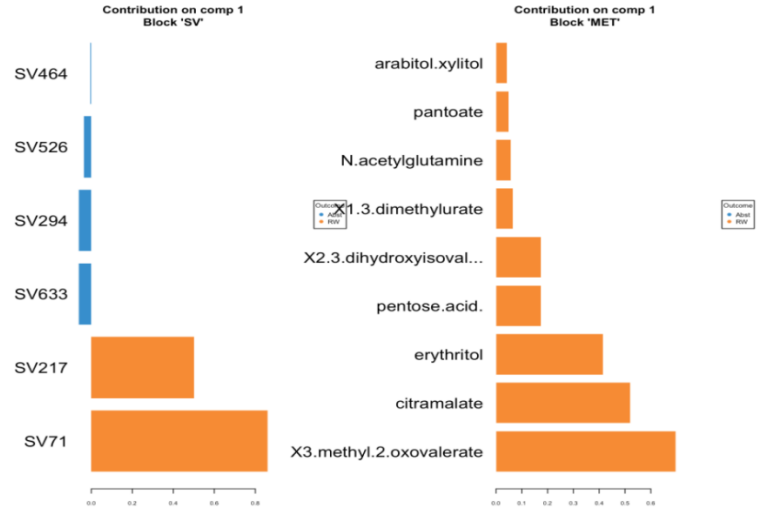
List of Samples

SV	Kingdom	Phylum	Class	Order	Family	Genus	Species	Species.1
SV71	k__Bacteria	p__Firmicutes	c__Bacilli	o__Lactobacillales	f__Streptococcaceae	g__Streptococcus	NA	NA
SV217	k__Bacteria	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Lachnospiraceae	g__Blautia	NA	obeum
SV633	k__Bacteria	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Ruminococcaceae	g__	NA	NA
SV526	k__Bacteria	p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__Prevotellaceae	g__Prevotella	NA	NA
SV294	k__Bacteria	p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__Bacteroidaceae	g__Bacteroides	NA	NA
SV464	k__Bacteria	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Ruminococcaceae	g__Oscillospira	NA	NA
SV491	k__Bacteria	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Christensenellaceae	g__	NA	NA
SV778	k__Bacteria	p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__[Odoribacteraceae]	g__Odoribacter	NA	NA
SV343	k__Bacteria	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Lachnospiraceae	g__Coprococcus	NA	hadrus
SV53	k__Bacteria	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Lachnospiraceae	g__Lachnospira	NA	NA
SV389	k__Bacteria	p__Proteobacteria	c__Betaproteobacteria	o__Burkholderiales	f__Alcaligenaceae	g__Sutterella	NA	secunda

The line outside the circle indicates to which group the feature is associated (taxa- SV or metabolites-MET). The orange line represents biomarkers associated with the RW period, while the blue lines represent those associated with the abstention period. The higher the line, the higher the discrimination power of the feature. The line inside the circle represents the correlation between the taxa and the metabolites (orange line indicates positive correlation, black line a negative correlation). The correlation cutoff was set to 0.5.

Figure 12- Correlation Matrix

A



B

Here below simple tables of Taxa and metabolites that have been identified as biomarker to separate RW and Abst treatment. See the loading contribution to see the treatment each variable is associated.

List of selected taxa feature on the first component

List of samples

	Kingdom	Phylum	Class	Order	Family	Genus	Species	Species.1
SV71	k__Bacteria	p__Firmicutes	c__Bacilli	o__Lactobacillales	f__Streptococcaceae	g__Streptococcus	NA	NA
SV217	k__Bacteria	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Lachnospiraceae	g__Blautia	NA	obeum
SV633	k__Bacteria	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Ruminococcaceae	g__	NA	NA
SV294	k__Bacteria	p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__Bacteroidaceae	g__Bacteroides	NA	NA
SV526	k__Bacteria	p__Bacteroidetes	c__Bacteroidia	o__Bacteroidales	f__Prevotellaceae	g__Prevotella	NA	NA
SV464	k__Bacteria	p__Firmicutes	c__Clostridia	o__Clostridiales	f__Ruminococcaceae	g__Oscillospira	NA	NA

List of selected Metabolites feature on the first component:

- X3.methyl.2.oxovalerate
- citramalate
- erythritol
- pentose.acid.
- X2.3.dihydroxyisovalerate
- X1.3.dimethylurate
- N.acetylglutamine
- pantoate
- arabitol.xylitol

(A) Shows the loading weights of each selected variables on each component and each data set. The color indicates the class in which the variable has the maximum level of expression. (**B)** The list of selected metabolites and taxa on the first component.

Figure 13- Metabolites and taxa of the first component on DIABLO analysis – multi omics integration analysis

5. Discussion and Conclusions

This study showed that in patients with stable CAD, short-term, moderate RW consumption modified gut microbiota as well as plasma metabolomics, particularly in pathways affecting amino acids, vitamins and cofactors, lipids, and carbohydrates. These effects were not reflected in plasma TMAO which displayed a high intraindividual variability.

After the RW intervention, analysis of the gut microbiota revealed a preponderance of *Ruminococcaceae*, several *Bacteroides*, and *Prevotella;* based on discriminant analysis, *Parasutterella* was the main genus responsible for modifications on the composition of the gut microbiota. *Parasutterella* is a genus considered beneficial for its relationship to fiber digestion, succinate production, salutary aromatic amino acid metabolism, and BA metabolism[51]. In line with this, the increase in *Parasutterella* is associated with some of the features found in plasma metabolomic analysis after RW consumption. In particular, there were modifications in tryptophan and tyrosine metabolism, since increases in IPA, gentisate and homovanillate were observed. *Parasutterella* is also a member of the *Proteobacteria* phylum, which harbours predicted ascorbate-producing genera, that could modify the cellular redox state.[100] In accordance with this, an increase in gulonate, a key cofactor in the glucoronate/ascorbate pathway, was noted. Also, the group formed by *Ruminococcaceae*, *Bacteroides* and *Prevotella* are part of the core microbiota in humans and were previously described as one enterotype identifiers, and are associated with high taxa abundance.[101] Taken together, these modifications on gut microbiota taxa suggest beneficial modifications after RW ingestion, which could too explain some of the modifications observed in plasma metabolomics.

RW has long been proposed to reduce oxidative stress. Previous analysis has shown that RW could reduce oxidative stress on rodent's colonic mucosa[30], and in healthy men, RW increased plasma and LDL polyphenols and enhanced antioxidant activity[102]. An important component of RW is resveratrol, which stimulates Sirtuin2, a complex of proteins with anti-aging properties[103]. Our findings are in line with these RW proposed redox effects. We found plasma modifications that indicate modulations in redox signaling, such as the increase in gulonate,[53] in metabolites on PPP[54] and in ribitol, an integral part of riboflavin, recently studied as important redox pathway[57]. Another putative beneficial redox influence was the augmentation of tyrosine metabolites gentisate and homovanillate, derived from polyphenols with redox capability.[61] In parallel, we noted metabolite modifications that could be beneficial towards insulin resistance and DM2, as the increase in the tryptophan metabolite IPA,[60] increase in the g*lucopyranosides,*[84] erythritol and ethyl alpha-glucopyranoside, and the decrease in long-chain acylcarnitines[80]. Furthermore, RW consumption might also have beneficially influenced energy expenditure and metabolism, as shown by the decrease in endocannabinoids[77,78] and the augmentation of pantothenate precursors.[87] Moreover, Bolte et al., observed in a large human cohort that long-term dietary patterns, including RW, are associated with anti-inflammatory features of gut microbiome.[104]

Somewhat unexpected findings relate to TMAO. TMAO has been considered a pro-atherosclerotic metabolite as well as a target to reduce cardiovascular risk. Previous studies proposed that dietary modifications could alter plasma TMAO in humans, with mixed results. Early studies investigating TMAO proposed that the higher levels of TMAO in omnivores vs. vegetarians due to exposure to dietary L-carnitine promoting its production.[22,105] To assess this possibility, dietary patterns were investigated related

to TMAO levels. Introduction of a diet rich in animal protein, for 4 weeks,[106] the putative precursors of TMA in the gut, did not increase TMAO plasma levels. Also, a six-week intervention with a Mediterranean diet did not reduce TMAO concentration.[107] Nevertheless, in a randomized trial with older healthy males, there was an increase in TMAO levels with higher dietary protein intake [108]. In another analysis, meat, egg, or fish consumption was not associated with TMAO, but there is positive association of dairy consumption and TMAO levels.[109] Also, dietary prebiotics and probiotics supplements might modulate gut microbiota and were investigated related to TMAO. For instance, inulin (a prebiotic fiber) supplementation did not reduce TMAO in individuals at risk for T2DM,[110] and in a clinical trial in subjects with reduced insulin sensitivity, resistant starch did not alter TMAO plasma levels[111]. Another dietary modification that could modulate TMAO is RW. RW contains DMB, a possible choline competitor for the gut microbiota,[39] and resveratrol decreased plasma TMAO in mice.[40] Notwithstanding, this study did not show a similar response in humans. Although gut microbiota was modified, plasma TMAO did not differ significantly after RW consumption.

TMAO concentrations fluctuated considerably and varied within individuals over time. We found the ICC of TMAO of 0.049 in the control arm and around 0.21 in four further validation tests. Admitting that a test is considered reliable when the ICC is above 0.80, [112], the high intra-individual variability might hamper the interpretation of the effect of interventions upon TMAO. Such intra-individual variability may explain why some studies have correlated positively with TMAO and CV events and risk factors[20,113], while others have not[114, 115]. Additionally, most of the studies that correlated TMAO with clinical outcomes longitudinally had an initial single TMAO measurement

followed by clinical follow-up, without further measures[16,20]. Few studies have compared TMAO values intra-individually over time. TMAO's high intraindividual variability was already described, but with only 2 measures,[116] here 4 measures were made within two weeks.

It is important to note that TMAO has relevant physiological functions. Firstly described in deep-sea marine animals, TMAO acts as a piezolyte and osmolyte. Marine animals use TMAO to halt the protein-destabilizing outcomes of osmotic and hydrostatic pressures.[117,118] TMAO serves as a chemical chaperone inhibiting endoplasmatic reticulum stress and attenuating the unfolded protein response.[119] TMAO was shown to counteract the effects of various protein denaturants, such as urea,[120] heat,[121] and pressure, [122]corroborating these features. In accordance with these, TMAO reduced cardiac fibrosis and improved hemodynamic parameters in spontaneously hypertensive rats model.[123] In a HF experimental model, TMAO supplementation prevented impaired mitochondrial energy metabolism by preserving fatty acid oxidation and subsequently decreasing pyruvate metabolism. This reaction led to preserved cardiac energy metabolism and resulted in a tendency to restore ventricular function.[124] Additionally, TMAO supplementation in spontaneously-hypertensive-heart-failure rats reduced mortality and was associated with diuretic, natriuretic and hypotensive effects.[125] In two different studies in mice model, TMAO supplementation did not increase aortic atherosclerotic lesion size,[126] and even a significant reduction of aortic lesion occurred.[127]

The discrepancy between studies investigating the effects of TMAO on the CV system might occur not only for different methodologies applied in the investigations, but mainly because of different doses of TMAO's precursors used and the large

difference in TMAO fold change in different studies. For instance, in the study that associated TMAO with an increase in aortic root size and decrease in RCT, the mice received 2000 mg/kg/day of L-carnitine, generating an increase of plasma TMAO of 10-fold.[22] Meanwhile, the finding of TMAO protecting aortic atherosclerotic root enlargement was found in mice that received 352mg/kg/day of L-carnitine, showing plasma TMAO arising of 2 -fold.[127] The supplementation with choline in mice that led to 14-fold higher levels of TMAO showed enhanced expression of inflammatory genes. [128] On the other hand, TMAO beneficial effects on hypertensive heart failure rats occurred after TMAO supplementation in tap water and a 3–4-fold increase plasma TMAO levels.[125]

Taking all together, it could be suggested that TMAO acts in a hermetic dose-response manner [129], exhibiting a dual effect, both beneficial in low levels and harmful in higher levels.

Additionally, several confounders should be considered when evaluating TMAO adverse effects. TMAO could be considered a microbial deviation of choline and carnitine metabolism that the host would otherwise absorb. Choline is an essential nutrient, a precursor of acetylcholine, a crucial neurotransmitter[130]; and carnitine is fundamental as an energy source as it is involved in the transport of fatty acids for β-oxidation process in the mitochondria[131]. Thus, TMAO could be a surrogate marker for disruptions of gut microbiota with a negative impact on the host. TMAO is also described at higher levels in patients with renal failure, perhaps not only because of its renal clearance but also because it could have a protective effect on the pressure and osmotic overload features of renal failure, such as hypertension and uremia[132]. In this manner, as renal failure increases cardiovascular risk, TMAO could be confounded by

renal impairment and poor metabolic control[133]. It is also possible that TMAO is a proxy for the detrimental effects of FMO3 activation, which can have deleterious effects on diabetes, regulating obesity and the beiging of white adipose tissue, regulating cholesterol balance, and acting in both platelet responsiveness and thrombosis potential in vivo.

Our TMAO analysis was very rigorous, with strict methodology, several measures and coherent validation techniques that corroborated the analysis. Thus, it is very unlikely that the TMAO variation observed was due to methodological issues. In addition, TMAO's high intraindividual variability was unlikely explained by medication use, renal function, diet, or gut microbiota profile. Hence, the main described possible interferents on TMAO levels were investigated and did not explain its varying levels.

This study has some limitations. RW intake was not identified by direct sample tests but through diary registers and the return of empty bottles. Additionally, because we investigated RW's effects only on males in a short-term period, our results may not reflect the long-term effects of RW consumption and thus should not, in principle, be generalizable to the overall population. On the other hand, the crossover design is a positive strength because it precludes interferences of confounding factors since each individual is his own control.

In summary, our findings indicate potential beneficial modifications of the gut microbiota and plasma metabolomics associated with RW consumption, especially regarding energy and redox metabolism, amino acid and vitamins. Also, the instability of serial TMAO measurements over time posed challenges for its role as a biomarker, and possibly other unknown interferences might affect its behavior over time. The study findings add new knowledge to the pathophysiological role of gut microbiota in

cardiovascular diseases and demonstrate that it can be modified by a non-lethal intervention such as RW. Beneficial modulation of gut microbiota and plasma metabolomics adds further support to the importance of diet in cardiovascular prevention.

6. Supplementary material

Supplementary Table 1 : Metabolites that significantly modified after RW consumption compared to the abstemious period

Supplementary Table 1 Metabolites that significantly modified after RW consumption compared to the abstention period. Univariate analysis showed metabolites changes related to RW intake during the study. We performed paired Wilcoxon signed-rank test (for non-parametric data) and paired t-test (for parametric data) to analyse metabolites changes in the samples collected at the end of the intervention with RW versus the samples collected at the end of the Abstemious period (A), RW period versus pre-wine washout (B) and RW period versus pre-abstained (C) conditions. For each scenario, significantly different compounds (p-value<0.05, no adjustment for multiple comparisons) are listed, and also the fold change in percentage.

			Wine vs. Abstained		Wine vs. Pre-wine Washout		Wine vs. Pre-Abstained Washout	
Super pathway	Sub Pathway	Metabolite	p-value	% of change	p-value	% of change	p-value	% of change
Amino Acid	Creatine Metabolism	guanidinoacetate					0,04	-26,0
	Glutamate Metabolism	citramalate	0,001	30,3				
		gamma-carboxyglutamate			0,04	4,0		
		glutamate, gamma-methyl ester					0,03	-18,5
		N-acetylglutamine	0,008	14,8				
	Glutathione Metabolism	cysteinylglycine disulfide*			0,04	7,6		
	Leucine, Isoleucine and Valine Metabolism (BCCA)	2-ketocaprylate			0,02	-11,4		
		3-methyl-2-oxovalerate	0,001	8,1				
	Methionine, Cysteine, SAM and Taurine Metabolism	lanthionine	0,02	34,6			0,02	50,4
		S-methylcysteine sulfoxide			0,05	-16,7		
	Phenylalanine Metabolism	1-carboxyethylphenylalanine	0,01	26,3				
		2-hydroxyphenylacetate					0,04	-12,8
	Polyamine Metabolism	acisoga	0,04	21,3	0,03	31,5		
	Tryptophan Metabolism	C-glycosyltryptophan			0,02	-5,6		

		indolepropionate	0,03	67,3			0,04	56,1
		N-formylanthranilic acid					0,05	-15,5
	Tyrosine Metabolism	serotonin			0,02	-48,6		
		gentisate	0,02	57,7				
		homovanillate (HVA)	0,03	13,4				
		thyroxine	0,03	-5,8	0,001	-6,4		
		vanillactate			0,04	-13,6		
Carbohydrate	Aminosugar Metabolism	N-acetylneuraminate			0,03	-12,1	0,04	-16,9
	Pentose Metabolism	arabinose	0,02	20,8			0,04	20,8
		arabitol/xylitol	0,004	17,9	0,0003	23,5		
		arabonate/xylonate					0,01	32,1
		ribitol	0,03	7,5				
		sedoheptulose	0,05	23,4	0,004	33,9	0,02	30,0
		xylose			0,02	28,1	0,03	12,3
Cofactors and Vitamins	Ascorbate and Aldarate Metabolism	gulonate*	0,04	8,1				
	Pantothenate and CoA Metabolism	pantoate	0,007	26,8	0,02	26,0		
Energy	Oxidative phosphorylation	phosphate	0,01	-9,2				
	TCA Cycle	isocitric lactone	0,01	25,0				
		succinylcarnitine (C4)					0,004	15,7
Lipid	Androgenic Steroids	5alpha-androstan-3alpha,17beta-diol monosulfate (1)			0,04	26,9		
		5alpha-androstan-3alpha,17beta-diol monosulfate (2)	0,04	-11,1			0,02	-12,4
		5alpha-androstan-3beta,17beta-diol monosulfate (2)	0,03	23,7				
		andro steroid monosulfate C19H28O6S (1)*	0,04	18,1				
		androstenediol (3beta,17beta) monosulfate	0,02	26,5				

		(1)						
	Corticosteroids	tetrahydrocortisol sulfate (1)	0,003	20,5				
	Diacylglycerol	linoleoyl-linolenoyl-glycerol (18:2/18:3) [2]*	0,02	-22,9	0,03	-33,5	0,02	-27,1
		linoleoyl-linoleoyl-glycerol (18:2/18:2) [1]*			0,01	-30,1		
		linoleoyl-linoleoyl-glycerol (18:2/18:2) [2]*			0,02	-29,8		
	Endocannabinoid	linoleoyl ethanolamide	0,03	-14,9				
	Fatty Acid Metabolism (Acyl Carnitine, Dicarboxylate)	adipoylcarnitine (C6-DC)			0,02	-26,7		
		octadecanedioylcarnitine (C18-DC)*			0,0003	-19,6		
		octadecenedioylcarnitine (C18:1-DC)*			0,02	-16,7		
	Fatty Acid Metabolism (Acyl Carnitine, Hydroxy)	3-hydroxyhexanoylcarnitine (1)					0,04	-36,3
	Fatty Acid Metabolism (Acyl Carnitine, Long Chain Saturated)	lignoceroylcarnitine (C24)*			0,02	-20,0		
	Fatty Acid Metabolism (Acyl Carnitine, Monounsaturated)	3-decenoylcarnitine	0,04	-15,8	0,02	-19,4		
		5-dodecenoylcarnitine (C12:1)			0,05	-15,0	0,04	-26,5
		cis-4-decenoylcarnitine (C10:1)			0,00	-15,6	0,04	-20,9
	Fatty Acid Metabolism (Acyl Carnitine, Short Chain)	acetylcarnitine (C2)			0,02	-14,3		
	Fatty Acid, Dicarboxylate	dodecadienoate (12:2)*			0,05	-18,1		
		hexadecenedioate (C16:1-DC)*					0,03	-44,0
		octadecenedioate (C18:1-DC)*			0,03	-21,4		
	Fatty Acid, Dihydroxy	2R,3R-dihydroxybutyrate	0,009	17,7				
	Fatty Acid, Monohydroxy	13-HODE + 9-HODE			0,004	-24,9		
		2-hydroxyoctanoate			0,03	-22,0		
		3-hydroxystearate	0,04	22,1				
	Hexosylceramides (HCER)	glycosyl-N-behenoyl-sphingadienine (d18:2/22:0)*			0,03	-14,6		

	Lysophospholipid	1-linoleoyl-GPE (18:2)*	0,02	15,3				
	Lysoplasmalogen	1-(1-enyl-stearoyl)-GPE (P-18:0)*			0,03	l-9,9		
	Phosphatidylcholine (PC)	1-linoleoyl-2-linolenoyl-GPC (18:2/18:3)*			0,05	-24,0		
		1-oleoyl-2-docosahexaenoyl-GPC (18:1/22:6)*			0,008	-13,4		
		1-myristoyl-2-palmitoyl-GPC (14:0/16:0)					0,02	22,6
		1-stearoyl-2-arachidonoyl-GPC (18:0/20:4)			0,02	-6,4		
		glycerophosphorylcholine (GPC)			0,02	43,2		
		1-(1-enyl-palmitoyl)-2-palmitoyl-GPC (P-16:0/16:0)*			0,04	-7,0		
	Secondary Bile Acid Metabolism	deoxycholic acid glucuronide			0,04	-33,2		
		glycodeoxycholate 3-sulfate					0,04	-25,3
		isoursodeoxycholate					0,02	-29,8
	Sphingomyelins	sphingomyelin (d18:1/19:0, d19:1/18:0)*			0,05	-11,2		
		sphingomyelin (d18:1/20:1, d18:2/20:0)*			0,001	-8,2	0,05	-5,8
		sphingomyelin (d18:1/22:1, d18:2/22:0, d16:1/24:1)*			0,03	-7,1		
		sphingomyelin (d18:2/18:1)*			0,02	-13,7	0,04	-10,7
		sphingomyelin (d18:2/21:0, d16:2/23:0)*			0,01	-11,0		
Nucleotide	Pyrimidine Metabolism, Uracil containing	2'-deoxyuridine					0,04	14,1
		N-acetyl-beta-alanine	0,01	18,8				
Partially Characterised Molecules	Partially Characterised Molecules	branched-chain, straight-chain, or cyclopropyl 10:1 fatty acid (1)*			0,03	-22,9		
		branched-chain, straight-chain, or cyclopropyl 10:1 fatty acid (2)*			0,04	-17,7		
		glycine conjugate of C10H14O2 (1)*			0,01	-23,3		
		pentose acid*	0,008	71,1	0,003	74,3	0,04	77,8
Xenobiotics	Benzoate Metabolism	3-methoxycatechol sulfate (1)			0,04	175,6		

	Chemical	4-methylbenzenesulfonate	0,006	10,6			0,03	11,0
		bromine	0,009	7,1				
		perfluorooctanesulfonate (PFOS)					0,03	8,3
		trizma acetate	0,04	-75,6				
	Drug - Cardiovascular	4-hydroxycoumarin	0,04	31,3				
	Food Component/Plant	2,3-dihydroxyisovalerate	0,01	1152,9	0,0002	1463,8		
		alliin			0,03	-43,9		
		erythritol	0,002	20,2	0,001	23,6	0,02	14,0
		ethyl alpha-glucopyranoside	0,007	124,9	0,001	240,6	0,003	186,5
	Xanthine Metabolism	1,3-dimethylurate	0,04	34,4				
		3-methylxanthine			0,02	-22,9		

Supplementary Table 2- Spearman correlation for plasma TMAO values, macronutrients and micronutrients consumption measured based on Food Frequency Questionnaires at the end of each study period: Abstention (Abst) or Red Wine (RW). The measures and values were analysed as continuous variables.

Group Period			TMAO final	Energy	Protein	Lipids	Carbohydrates	Fiber	Cholesterol	Calcium	Iron	Sodium	Potassium	Saturated fat	Monounsaturated fat	Polyunsaturated fat
Abst	**TMAO initial**	r	0,217	-0,092	0,057	-0,084	-0,188	-0,033	0,123	-0,089	-0,040	-0,250	0,085	-0,123	-0,081	0,008
		p-value	*0,185*	*0,586*	*0,740*	*0,623*	*0,266*	*0,845*	*0,468*	*0,602*	*0,813*	*0,135*	*0,615*	*0,468*	*0,632*	*0,963*
		n	39	37	37	37	37	37	37	37	37	37	37	37	37	37
	TMAO final	r	1	-0,029	0,068	0,000	-0,114	-0,039	0,286	-0,190	-0,097	-0,224	-0,145	-0,123	-0,080	-0,088
		p-value		*0,863*	*0,691*	*0,999*	*0,503*	*0,818*	*0,086*	*0,259*	*0,569*	*0,183*	*0,392*	*0,467*	*0,637*	*0,606*
		n		37	37	37	37	37	37	37	37	37	37	37	37	37
RW	**TMAO initial**	r	0,238	-0,197	-0,042	-0,125	-0,283	-0,374*	0,037	0,131	-0,256	0,308	-0,258	0,055	0,007	-0,248
		p-value	*0,144*	*0,250*	*0,806*	*0,468*	*0,094*	*0,025*	*0,831*	*0,446*	*0,133*	*0,068*	*0,129*	*0,749*	*0,966*	*0,144*
		n	39	36	36	36	36	36	36	36	36	36	36	36	36	36
	TMAO final	r	1	0,032	0,478**	0,047	-0,020	-0,063	0,336*	0,254	0,107	-0,008	0,278	0,095	0,106	0,002
		p-value		*0,853*	*0,003*	*0,787*	*0,909*	*0,714*	*0,045*	*0,135*	*0,534*	*0,965*	*0,101*	*0,582*	*0,538*	*0,992*
		n		36	36	36	36	36	36	36	36	36	36	36	36	36

Spearman Correlation Coefficient for all the measures – RW and abstemious TMAO values combined.

		TMAO	Energy	Protein	Lipids	Carbohydrates	Fiber	Cholesterol	Calcium	Iron	Sodium	Potassium	Saturated fat	Monounsaturated fat	Polyunsaturated fat
TMAO initial	r	0,224*	-0,142	0,008	-0,105	-0,230	-0,215	0,085	0,020	-0,168	0,024	-0,094	-0,020	-0,055	-0,115
	p-value	*0,049*	*0,230*	*0,945*	*0,375*	*0,050*	*0,068*	*0,473*	*0,865*	*0,155*	*0,840*	*0,431*	*0,870*	*0,644*	*0,333*
	n	78	73	73	73	73	73	73	73	73	73	73	73	73	73
TMAO final	r	1	-0,004	0,235*	0,022	-0,067	-0,061	0,303**	0,016	-0,011	-0,121	0,061	0,008	0,012	-0,073
	p-value		*0,973*	*0,046*	*0,856*	*0,576*	*0,605*	*0,009*	*0,894*	*0,929*	*0,309*	*0,611*	*0,945*	*0,918*	*0,541*
	n		73	73	73	73	73	73	73	73	73	73	73	73	73

*. Significant correlation p <0.05 (two-tailed

Supplementary Table 3- Spearman correlation for gut bacteria taxa and clinical (A and B), biochemical (C and D) and nutritional (E and F) variables.

Supplementary Table 3 (A) Spearman correlation, the columns correspond to gut bacteria taxa and the rows correspond to clinical parameters during the abstention period. SBP, systolic blood pressure in mmHg; DBP, diastolic blood pressure in mmHg; HR, heart rate in bpm; weight in kg; BMI, body mass index; waist in cm.

Taxa	SBP	DBP	HR	Smoking	Physical inactivity	Physical activity	Weight	BMI	Waist
Anaerostipes_SV651	-0,143582	-0,0022895	0,01771037	-0,1279364	0,02576971	-0,051571727	-0,0641608	-0,1838069	-0,0819079
Roseburia_SV211	-0,0994144	-0,0892468	-0,0348399	-0,0126096	-0,08275823	-0,122009356	-0,0093829	0,06117769	0,13229607
Coprococus_SV109	-0,0079202	-0,0446982	0,22874644	0,10080442	-0,11127459	-0,077339898	0,0571069	-0,0122109	0,15869094
Veilonella_SV554	-0,0399015	-0,1246344	0,27370861	0,33975271	-0,08688534	-0,047949949	0,25025013	0,11864731	0,22877038
Ruminococcaceae_SV91	0,17384992	0,09494142	0,23027367	-0,0433051	-0,18994004	-0,032767195	-0,04532	0,0294154	0,05440705
Prevotella_SV982	-0,0217643	-0,1218834	0,16189218	0,37397889	-0,2114276	-0,00118398	0,27664203	0,17900473	0,27039439
Bacteroides_SV490	0,02786693	0,16135138	0,00778696	-0,0557738	-0,1834464	-0,086613073	0,15220364	-0,18664	0,02897798
Roseburia_SV896	0,07927281	0,15630691	0,04953025	-0,0605923	0,29647663	-0,161101393	0,17947382	0,13135108	0,12331954
Bacteroides_SV489	-0,157769	-0,0770236	0,12371249	-0,053108	-0,02805265	-0,123791834	0,22020839	0,03233274	0,21429153
Lachnoclostridium_SV161	0,12589641	-0,0345113	0,08737712	0,20968655	0,20231688	-0,160306436	0,13271804	-0,0248548	0,15695892
Bacteroides_SV478	0,21795057	0,20650658	-0,0218588	-0,0685038	-0,03879526	0,127101728	0,12333785	0,10096698	-0,0577226
Ruminococcaceae_SV595	0,21129023	0,0065392	-0,2235491	0,18484996	-0,08730364	-0,140666048	0,08160992	0,01655753	-0,0365725
Prevotella_SV216	0,15127474	0,1339234	0,11626837	0,34695577	-0,02632287	-0,166168724	0,17760553	0,10942082	0,16180735
Bacteroides_SV132	-0,0025287	0,11591906	-0,0488466	-0,0504836	-0,18876651	0,313715881	0,25362787	0,11699025	0,09029331

Roseburia_SV492	-0,0081277	-0,0404117	-0,087268	-0,0549853	-0,14973254	-0,006769612	-0,2165099	-0,0683754	-0,0549967
Lachnospiraceae_SV159	0,09885615	-0,1619423	0,15546754	-0,0576133	-0,24673342	-0,043601829	-0,2136099	-0,1760101	-0,209587
Bacteroides_SV286	0,12453602	-0,2641121	-0,1072875	-0,0677008	0,10984346	-0,122586698	-0,1482856	-0,2032157	-0,3293779
Minococcaceae_SV434	-0,1293892	-0,1545551	-0,1325262	-0,0794977	0,13285944	-0,077899977	-0,0297474	0,01455768	-0,177622
Lachnospiraceae_SV963	-0,0084522	0,04646928	0,16944056	-0,0897125	0,07632414	-0,033315226	-0,1105442	0,09485105	-0,0242953
Parasutterella_SV239	0,1573265	-0,017777	-0,1230621	-0,0737779	0,2805055	-0,185546532	0,0679986	0,0253454	-0,0793332
Colinsella_SV180	0,10743763	0,11290955	-0,0149028	-0,1067635	-0,30389697	0,610038544	-0,1206303	-0,0373484	-0,1304766
Streptococcus_SV71	-0,254934	0,0566693	0,03019484	0,95357882	0,24241513	-0,12836584	0,03285807	0,17407403	0,0243832
Blautia_SV217	0,20573294	-0,1913361	-0,1806922	0,15775585	0,40624667	-0,24997058	-0,5590642	-0,4170841	-0,4483554
Bacteroides_SV294	-0,0789603	-0,1680435	-0,2548398	-0,0578396	0,30877915	-0,16255594	-0,0916582	0,23752545	0,21144616
Oscillospira_SV464	0,01768852	-0,0889139	0,16411164	-0,090732	-0,03007564	-0,20247066	-0,1488508	-0,1128677	0,01457457
Prevotella_SV526	0,3122898	0,16400078	-0,0545514	-0,0526316	0,28097574	-0,14791891	0,07604112	0,02842235	0,15942552
Bacteroides_SV572	0,28486245	0,12232335	-0,1076431	-0,0633393	0,33813955	-0,17801264	0,05375568	0,07865464	0,19967002

Supplementary Table 3 (B) Spearman correlation, the columns corespond to gut bacteria taxa and the rows correspond to clinical parameters during the consumption of Red Wine (RW). SBP, systolic blood pressure in mmHg; DBP, diastolic blood pressure in mmHg; HR, heart rate in bpm; weight in kg; BMI, body mass index; waist in cm.

Taxa	SBP	DBP	HR	Smoking	Physical inactivity	Physical activity	Weight	BMI	Waist
Anaerostipes_SV651	0,17620879	0,12922096	-0,0359659	-0,1162652	-0,2048481	0,4412295	0,16150438	0,00261927	-0,0144717
Roseburia_SV211	-0,03786233	0,0153494	-0,0343327	-0,0117808	-0,0757147	-0,1203714	-0,0476751	-0,0005298	0,11350497
Coprococcus_SV109	0,15508232	0,10594362	-0,0056733	0,14819112	-0,1950884	-0,0397817	0,05087396	-0,0984827	0,07693233
Veilonella_SV554	0,07208987	0,10757979	0,02519587	0,2012729	-0,2479332	-0,0885559	0,07776305	-0,0066456	0,00228234
Ruminococcaceae_SV91	0,01655619	0,01737274	-0,1249281	0,00447817	-0,1436721	-0,03873	0,08409316	0,0775803	0,18670406
Prevotella_SV982	0,22762795	0,18706243	-0,1870876	0,55396648	-0,2391751	-0,0448288	0,1300291	0,00299903	0,08594297
Bacteroides_SV490	0,10891393	0,15148418	-0,0943952	-0,0429457	-0,1432591	-0,0463179	0,06456198	-0,2564136	-0,1703409
Roseburia_SV896	-0,29707077	-0,3767068	-0,0920048	-0,0675683	0,32382576	-0,1698714	-0,0677371	0,04770013	0,05749891
Bacteroides_SV489	-0,05902751	-0,0752017	-0,0393045	-0,0499281	-0,0956284	-0,1053273	0,17934076	0,05013226	0,1298978
Lachnoclostridium_SV161	0,28420127	0,20701409	-0,2467138	0,3585891	0,12395275	-0,1199674	-0,0015188	-0,1880149	-0,0076303
Bacteroides_SV478	0,36173893	0,36338976	0,20483479	-0,0870132	0,30722773	-0,1922758	-0,183774	-0,0746309	-0,1691162
Ruminococcaceae_SV595	0,25905786	0,21635558	-0,105299	0,26154699	-0,1931493	-0,0610158	0,10093648	-0,2170027	-0,1475476
Prevotella_SV216	0,13398067	-0,0458903	-0,0231004	0,11847047	-0,0774845	-0,1316116	0,14603462	0,1285219	0,05630791
Bacteroides_SV132	0,05508393	-0,0103994	0,01967423	-0,0630144	0,17049877	0,03564206	-0,0404135	-0,098265	-0,1229496

Roseburia_SV492	-0,12177116	-0,1990297	-0,1288752	-0,0541369	-0,1169244	-0,0127108	-0,2393232	-0,2615917	-0,0920165
Lachnospiraceae_SV159	0,03456036	0,26652111	-0,1083053	-0,0726805	-0,149826	-0,0365542	-0,281639	-0,3212193	-0,345285
Bacteroides_SV286	-0,02296785	0,2061998	-0,0648452	-0,0646632	-0,16618	0,04299382	-0,2916464	-0,3265619	-0,3795172
Minococcaceae_SV434	-0,09624985	-0,2258081	0,10973603	-0,0625143	0,16831958	-0,0709076	-0,1997982	-0,083061	-0,1925322
Lachnospiraceae_SV963	-0,26401204	-0,303295	0,13309891	-0,0557668	0,2672664	-0,1402017	-0,1042434	0,08016555	0,06715193
Parasutterella_SV239	0,16296238	0,12023647	-0,0635923	-0,0651449	0,01236019	-0,1110959	0,0472819	-0,1925464	-0,1976708
Colinsella_SV180	0,23058935	0,17118186	0,01863219	-0,1077798	-0,1490521	0,42002644	-0,0681351	0,07460843	-0,1106516
Streptococcus_SV71	-0,265951306	-0,4048346	-0,3113029	-0,0742722	0,30085814	-0,1813841	-0,1146831	0,23564452	0,13006941
Blautia_SV217	-0,111712648	0,07285508	0,05185198	0,86771858	0,22446003	0,00319354	0,0560308	0,1438204	0,04281335
Bacteroides_SV294	-0,221722516	-0,2328075	0,08340115	-0,0660933	-0,1557052	-0,1663371	0,40930679	0,21320509	0,37091619
Oscillospira_SV464	0,043289545	-0,0025255	0,24294202	-0,1069865	0,30239282	-0,2279297	-0,2003629	0,00230841	-0,3389476
Prevotella_SV526	0,198642079	0,14857486	-0,0552502	-0,0588235	0,3040345	-0,1480412	0,06637302	0,0500096	-0,0690225
Bacteroides_SV572	0,161283993	0,11060766	-0,0416063	-0,0674835	0,27434813	-0,1698358	0,12625252	0,08087661	-0,0120497

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Supplementary Table 3 (C) Spearman correlation, the columns corespond to gut bacteria taxa and the rows correspond to nutritional parameters, macronutrients and micronutrients , during abstention period.

Taxa	Energy	Protein	Lipids	Carbohydrates	Fiber	Cholesterol	Calcium	Iron	Sodium	Potassium	Saturated fat	Monounsaturated fat	Polyunsaturated fat
Anaerostipes_SV651	-0,1404981	-0,1968668	-0,281324	-0,001189633	0,01187706	-0,0918897	-0,1135132	-0,114364	0,08880425	-0,0811421	-0,131289828	-0,235308343	-0,1516888
Roseburia_SV211	-0,0892756	0,00551155	-0,1531086	-0,02988932	0,09650242	-0,0426589	-0,0562104	-0,0174771	-0,2608367	0,16215989	-0,086870518	-0,024270905	-0,21495714
Coprococus_SV109	-0,1714951	-0,217074	-0,0795669	-0,166205241	-0,0708161	-0,0553688	-0,0766368	-0,1310188	-0,1448522	-0,1300951	0,092990623	-0,065230173	-0,08961901
Veilonella_SV554	-0,0006499	-0,0204799	0,03934427	-0,011742428	0,1192819	-0,0461461	-0,0695237	-0,0608612	-0,1927871	0,09397039	-0,024866794	0,038212903	0,14222284
Ruminococcaceae_SV91	-0,1833486	-0,1799195	-0,1406634	-0,187731874	-0,100862	-0,0646147	-0,0640666	0,17158616	-0,1062522	-0,206448	-0,013164476	-0,145182698	-0,16758858
Prevotella_SV982	0,01241131	0,01218959	0,03599908	0,006989392	0,0898029	-0,0668502	-0,098672	-0,109424	-0,1414408	0,07551561	0,025355716	0,100287805	0,11039844
Bacteroides_SV490	0,09452718	0,12335772	0,10674632	0,053556863	0,09016949	-0,0382431	0,18529948	0,00152484	-0,0139164	0,12145753	0,153522991	0,088092307	0,01967442
Roseburia_SV896	-0,106726	-0,0920775	-0,0995314	-0,095197803	0,06888759	-0,0438306	-0,0581108	-0,0849569	-0,2046856	0,02294931	-0,191478709	-0,161046804	-0,02189973
Bacteroides_SV489	0,08338881	0,11142683	0,25820417	-0,060688676	-0,1236976	-0,0336643	-0,081549	-0,0865232	-0,0456497	-0,0422435	0,198605389	0,165701308	0,23565405
Lachnoclostridium_SV161	-0,1633647	-0,4021123	-0,0911916	-0,041240768	-0,0710016	-0,0696142	-0,1290445	-0,1424712	-0,2706382	-0,1388533	0,008342175	-0,092974759	-0,06208279
Bacteroides_SV478	-0,2028647	0,04665765	-0,1476596	-0,175990364	-0,1782408	-0,0453056	-0,0557744	-0,0912997	-0,0177314	-0,106692	-0,127361378	-0,069985666	0,03888607
Ruminococcaceae_SV595	0,07978908	-0,0471876	-0,0202926	0,169060617	-0,0262522	-0,0869496	0,03043329	-0,1168117	-0,0063211	-0,001767	0,028385229	0,020829646	0,00445064
Prevotella_SV216	-0,1827123	-0,2704039	-0,0418666	-0,151700004	-0,0469217	-0,0613599	-0,0538987	-0,1431157	-0,1506816	-0,0816037	0,028634878	-0,048393756	0,09859057
Bacteroides_SV132	-0,3327325	-0,036824	-0,2107874	-0,266359171	-0,1590751	-0,0278056	0,01928959	-0,1029751	-0,1321533	-0,0985194	-0,183093246	-0,168655613	-0,03823621
Roseburia_SV492	-0,078482	-0,1690478	-0,0219566	-0,078361441	-0,0452236	-0,0371552	-0,0982402	-0,0642432	0,29365644	-0,0778742	-0,097752575	0,003873711	0,23005354
Lachnospiraceae_SV159	0,20838038	0,02390077	0,17484479	0,252882144	0,28635084	-0,0483771	0,57114254	-0,0103318	0,24564268	0,28294574	0,186686587	0,220011091	0,2175508
Bacteroides_SV286	-0,0198288	-0,2053497	0,00617559	0,067342153	-0,0126772	-0,0503529	0,17955951	-0,1259569	-0,1497274	-0,1239324	-0,148283562	-0,126809996	-0,10312719
Minococcaceae_SV434	-0,0774545	-0,0389047	-0,0497317	-0,073989235	-0,2095126	-0,0571979	-0,2128432	-0,1452474	-0,2263583	-0,2309957	-0,196829269	-0,094413753	-0,11990293
Lachnospiraceae_SV963	-0,1446509	-0,1006454	-0,1945003	-0,129228047	-0,0907815	-0,0652467	-0,0393219	-0,0517644	0,11792486	-0,0151231	-0,037125285	-0,06990695	-0,12744048
Parasutterella_SV239	-0,0473007	-0,1433396	-0,0192619	0,003378469	-0,07313	-0,0553143	-0,1975103	-0,1416193	-0,0839771	-0,2151941	-0,111245559	-0,206742133	-0,12949767

Colinsella_SV180	-0,128146	-0,0050562	0,03364703	-0,249450316	-0,1959599	-0,0746027	-0,2078886	-0,1053671	0,2692141	-0,0919929	0,166660461	0,175913527	-0,10206059
Streptococcus_SV71	-0,1946765	-0,0335122	-0,2394118	-0,20241008	-0,2875441	0,01919976	-0,0673195	-0,1588327	-0,0160846	-0,1714499	-0,121429271	-0,15357796	-0,3171053
Blautia_SV217	-0,0390917	-0,0273489	-0,258877	0,07467645	-0,2270988	-0,0866565	-0,4201271	-0,0742371	-0,2942539	-0,0528924	-0,006258315	-0,03195906	-0,4341533
Bacteroides_SV294	-0,0218992	0,31426388	0,03743148	-0,17743733	-0,1361793	0,10293622	0,11330291	-0,0925747	0,07734137	0,15910977	-0,007974709	-0,10617641	0,1487251
Oscillospira_SV464	-0,0779134	0,09627738	-0,186703	-0,03158535	0,27476025	0,10024141	-0,0711616	0,33993457	-0,2429968	0,22235848	-0,118678285	-0,04994077	-0,1467057
Prevotella_SV526	0,41944729	0,21358377	0,33017658	0,50018376	0,3399568	-0,0942051	-0,00185	0,09776032	0,38906025	0,25824257	0,407061333	0,16075471	0,2377929
Bacteroides_SV572	0,40074172	0,27417663	0,32723192	0,44527184	0,29927775	-0,0688839	0,02262531	0,07455314	0,39275151	0,28391488	0,391769115	0,13251614	0,2619095

Supplementary Table 3 (D) Spearman correlation, the columns corespond to gut bacteria taxa and the rows correspond to nutritional parameters,macronutrients and micronutrients, during the consumption of RW.

Taxa	Energy	Protein	Lipids	Carbohydrates	Fiber	Cholesterol	Calcium	Iron	Sodium	Potassium	Saturated fat	Monounsaturated fat	Polyunsaturated fat
Anaerostipes_SV651	-0,1271697	-0,2280965	-0,0986361	0,06672656	-0,0849157	-0,2167106	0,11011981	-0,0896754	0,11750499	0,01238007	-0,169544966	-0,16507314	0,071770533
Roseburia_SV211	-0,00121	0,02183246	-0,149494	0,0926934	0,25587524	-0,1375019	-0,0967245	-0,0358082	-0,0381431	0,20549912	-0,145413403	-0,08989799	-0,09557615
Coprococcus_SV109	0,07936406	0,2009489	0,16517793	0,03735368	0,06307073	0,04575998	0,09037706	-0,0570986	0,05053864	0,05333167	0,209747457	0,32709925	0,064199668
Veilonella_SV554	0,0374325	0,0317428	0,19231738	0,0808845	0,11874336	0,04263417	0,1235349	-0,0447418	0,20462732	0,16306178	0,199679141	0,19083986	0,163780578
Ruminococcaceae_SV91	0,11444794	0,1977131	0,23486475	-0,0058115	-0,1136854	0,31494385	-0,0162349	-0,0802751	0,07632467	-0,0978334	0,182443024	0,09783453	0,17909053
Prevotella_SV982	0,32250389	-0,002049	0,41424981	0,35415964	0,43523996	0,01347812	0,23064807	-0,0463029	0,44102105	0,27903613	0,426513685	0,41051363	0,263665092
Bacteroides_SV490	0,13770466	0,10295209	0,19574678	0,02731534	0,22631684	0,0247921	0,33301842	0,00382959	-0,0356583	0,18010012	0,256162291	0,2287502	0,067562268
Roseburia_SV896	0,22962938	0,18031341	-0,1435856	0,01748838	0,03709136	0,02645079	0,15448455	-0,04454	-0,0587989	0,17215872	-0,017055865	-0,14453768	-0,022802363
Bacteroides_SV489	0,13555988	0,24321174	0,22226262	0,01867995	-0,0589337	0,3572529	-0,0970342	-0,0391137	-0,0447287	0,06597255	0,164346417	0,13455461	0,172603536
Lachnoclostridium_SV161	-0,0077927	-0,0345767	0,15751763	0,02155872	0,07480589	-0,009796	0,0154243	-0,0697851	0,17637062	0,05552164	0,306497619	0,28257814	0,133968739
Bacteroides_SV478	-0,1567022	0,03780471	-0,2337402	-0,0831522	-0,3339931	0,2071961	-0,0547788	-0,0735093	-0,0657215	-0,0549619	-0,213051933	-0,19381206	-0,247441127
Ruminococcaceae_SV595	0,14411833	0,01785902	0,29384934	0,1010885	0,31412372	-0,0493982	0,29760037	-0,0176957	0,19029215	0,22122684	0,342798198	0,34600213	0,190139042
Prevotella_SV216	-0,1540435	-0,2831887	-0,1213438	-0,0514724	-0,0937854	-0,2621676	0,0374889	-0,0723034	0,00232254	-0,0610344	-0,071253622	-0,09043442	-0,027000409
Bacteroides_SV132	-0,0460393	-0,0035442	-0,0968178	-0,0020564	-0,1440035	-0,180994	-0,1595023	-0,0638943	-0,1799047	-0,3003358	-0,290642121	-0,24359604	-0,277829587
Roseburia_SV492	-0,2701595	-0,142789	-0,2370701	-0,1476693	-0,0117888	-0,0492516	-0,1268339	-0,0370464	-0,1903213	-0,0697254	-0,230241202	-0,20184908	-0,029051867
Lachnospiraceae_SV159	0,01908156	-0,0769304	0,03606184	0,08867414	0,16002385	-0,1397168	0,31912376	-0,0345484	0,10143348	0,09862091	0,200055309	0,07523782	-0,141637307
Bacteroides_SV286	-0,0484337	-0,0914134	-0,0338693	0,01787201	0,09419445	-0,1958901	0,31179376	-0,0320787	0,13015272	0,08181712	0,158060953	0,06565833	-0,152168715
Minococcaceae_SV434	-0,0030603	-0,0158947	0,12863745	-0,1418729	0,06546633	-0,017266	-0,1226105	-0,0344726	-0,2548071	0,02544683	0,008785016	0,14033932	0,240215502
Lachnospiraceae_SV963	0,1010827	0,13370958	-0,0534941	-0,0961905	-0,0062946	-0,093477	-0,0564279	-0,0476279	-0,083976	-0,063607	0,011209017	-0,09646537	-0,003336896

Parasutterella_SV239	0,19559729	0,04922814	0,19758577	0,11816291	0,13935907	0,0635506	0,15303941	-0,0199325	0,10980824	0,02223543	0,253834866	0,17189271	0,093558404
Colinsella_SV180	0,12404885	-0,0620251	0,4389952	-0,145473	-0,0001679	0,01541076	-0,0160998	-0,0853294	0,03794856	-0,1380575	0,288147088	0,44987451	0,381001256
Streptococcus_SV71	-0,1877478	-0,3098411	0,05744257	-0,2636124	-0,2206061	-0,3429493	0,40979855	-0,0121657	0,26688168	-0,2859175	0,064177826	-0,04081584	-0,30707759
Blautia_SV217	0,08976999	0,55289634	0,16262926	-0,1612208	-0,1338671	-0,0332657	-0,2608561	0,01998624	0,1470729	0,20081615	0,065417673	0,26649464	0,01017925
Bacteroides_SV294	0,33913045	0,2258473	-0,087655	0,01216183	-0,0950571	0,18988108	0,08767335	-0,1405315	0,06143867	0,13085925	-0,053967333	-0,16319515	0,41140685
Oscillospira_SV464	-0,0676969	-0,0710414	-0,2447543	0,07421517	0,04781277	-0,3825325	-0,096428	-0,2702289	-0,4476839	0,16833799	-0,384222061	-0,39086751	-0,19501222
Prevotella_SV526	0,21084128	-0,0533307	0,08386146	0,35225195	-0,1286795	0,22126376	-0,2161673	-0,1282516	0,51936521	-0,3037675	0,165929892	-0,08583858	0,0345994
Bacteroides_SV572	0,25718506	-0,0184034	0,06898062	0,34668323	-0,1402098	0,24504678	-0,1985083	-0,1465983	0,51766747	-0,2778061	0,154370398	-0,10846767	0,09545979

Supplementary Table 3 (E) Spearman correlation, the column correspond to gut bacteria taxa and the rows correspond to laboratory mesaures, during abstention period. Total Cholesterol, in mg/dL; HDL, High-density lipoprotein, in mg/dL; LDL, Low-density lipoprotein, in mg/dL; non-HDL, non- High-density lipoprotein, in mg/dL; Triglycerides,in mg/dL; ApoA, Apolipoprotein A, in g/L; Apo B, Apolipoprotein B, in g/L; Glucose, Fasting Glucose, in mg/dL; ; GFR, glomerular filtration rate, in mL/min/1,73m2; hsCRP, high-sensitivity C-reactive protein, in mg/L; IL-6, interleukin 6, in pg/mL; LPS, lipopolysaccharides, in EU/mL.

Taxa	Total Cholesterol	HDL	LDL	nonHDL	Triglycerides	ApoA	ApoB	Glucose	Urea	Creatinine	GFR	hsPCR	TMAO	LPS	IL-6
Anaerostipes_SV651	-0,1297811	0,11303633	-0,1868877	-0,1582496	-0,0039209	-0,1619069	-0,111922	0,01725914	0,13287064	0,0605967	-0,1705487	0,07562883	-0,0952827	0,24848972	-0,0135434
Roseburia_SV211	-0,0938074	-0,1524059	-0,0321546	-0,0701852	-0,0778758	-0,1482255	-0,0860948	0,04479193	-0,0818564	-0,1246792	0,13767807	-0,1256031	-0,0319607	-0,0945677	0,035029
Coprococcus_SV109	-0,1545277	-0,0313569	-0,1967944	-0,1381327	0,0303983	0,01838681	-0,1193658	0,12187294	-0,1601465	-0,2191407	0,10894286	-0,1371972	-0,0667543	-0,1813289	-0,1247153
Veilonella_SV554	-0,21011	-0,1537805	-0,1411211	-0,1545326	-0,0947071	-0,2370728	-0,1709872	0,07259787	-0,3708689	-0,0634023	0,09366957	0,45719135	-0,0810101	-0,2995662	-0,1338981
Ruminococcaceae_SV91	-0,1653915	-0,1033619	-0,1629321	-0,1269065	0,00837401	-0,0875408	-0,1246077	0,14116107	0,03765661	0,01353503	-0,0934593	-0,0346183	-0,069355	-0,0592343	0,01805813
Prevotella_SV982	-0,1822816	-0,1513859	-0,1412005	-0,1274711	-0,0168892	-0,1558483	-0,1678207	0,06291941	-0,3439118	-0,1826954	0,28683375	0,3039161	-0,1014825	-0,3336736	0,08230868
Bacteroides_SV490	-0,2204887	-0,0571263	-0,1941423	-0,1948829	-0,1160233	-0,0793041	-0,1520343	0,0542555	-0,136957	-0,0165238	0,01399564	0,19701476	-0,0757634	-0,3070422	-0,0565819
Roseburia_SV896	0,19924991	-0,0620138	-0,0254531	0,21406887	0,2162453	-0,0291815	0,21027902	-0,063991	-0,095209	0,12229195	0,1152306	-0,1494968	0,25177758	0,2207952	0,01325338
Bacteroides_SV489	-0,2164748	-0,2121802	-0,1512448	-0,1431367	-0,0628697	-0,2252858	-0,1443915	0,27704232	-0,0240823	0,05585238	-0,0263924	0,43202429	-0,046196	-0,2918076	-0,0430515
Lachnoclostridium_SV161	-0,335272	-0,1985262	-0,2590482	-0,2613314	-0,1409633	-0,266349	-0,1945246	0,09430253	-0,3130447	-0,1564766	0,10854105	-0,1444868	-0,0799158	-0,3108135	-0,2440792
Bacteroides_SV478	-0,0903663	0,09789662	-0,040898	-0,1182369	-0,1611543	0,06517101	-0,1235297	-0,2237244	0,13582392	0,34363528	-0,2706514	0,0566108	-0,024287	0,24456413	-0,0078918
Ruminococcaceae_SV595	-0,2726384	0,05194712	-0,2078009	-0,2787802	-0,2469286	-0,0094007	-0,2013482	-0,1393938	-0,2147874	-0,3287104	0,37261042	-0,1857449	-0,1260869	-0,2558622	0,18657107
Prevotella_SV216	-0,0875887	-0,2433883	-0,018342	-0,0086762	0,05798026	-0,2033447	0,08791747	0,14745125	-0,2425951	-0,0540746	0,07908191	-0,1570637	-0,0840944	-0,0425256	-0,120433
Bacteroides_SV132	-0,0444717	-0,043345	-0,0010263	-0,0284867	-0,0319948	0,09029498	-0,0193751	-0,1550526	0,13865181	0,46498468	-0,2222078	0,07353238	-0,0396418	0,21529773	0,02544071
Roseburia_SV492	0,38616947	0,10749191	0,4660392	0,34286679	0,09414644	0,05631645	0,31990568	-0,0495409	0,00035295	0,19294268	-0,2980296	-0,023332	-0,0622825	0,12169167	0,0048063
Lachnospiraceae_SV159	-0,0466462	0,10492643	-0,1137597	-0,0807265	0,02472402	0,25215143	-0,0855451	0,00023154	0,01585145	-0,2059696	-0,0727934	-0,0617785	-0,0770109	-0,0548132	0,00684004
Bacteroides_SV286	-0,0816503	-0,071735	0,00795384	-0,0585713	-0,1074617	0,09155383	0,01997813	0,13190687	-0,0970324	-0,243364	0,0440186	-0,095764	-0,0608624	-0,2742757	-0,120867
Minococcaceae_SV434	-0,0245172	0,04477267	0,05662006	-0,040752	-0,1360519	0,12057808	0,03121451	0,1150682	-0,0889119	-0,1765226	0,23911886	-0,1120766	-0,0825792	-0,3226285	0,05106626
Lachnospiraceae_SV963	0,1555148	0,07812797	-0,0067917	0,12911439	0,10763515	-0,0112057	0,15054586	0,12683803	-0,0744649	-0,0645525	0,0095435	0,25894285	-0,113323	0,33718031	-0,0678323
Parasutterella_SV239	-0,1432749	-0,2238408	-0,055107	-0,0740085	-0,0450579	-0,1394934	0,01294543	-0,0151103	-0,1038022	-0,1105592	0,13957334	-0,1577803	-0,0751578	-0,1265032	-0,1532027

Colinsella_SV180	0,17188143	0,14577051	0,21319007	0,11785669	-0,0054069	0,18401157	0,06221537	0,09883098	0,28989656	0,15473879	-0,2323173	-0,0950231	-0,0600177	0,13802774	-0,1260077
Streptococcus_SV71	0,64406062	0,07967036	0,02099639	0,00235634	-0,0094227	0,06000824	0,05884189	0,11878647	0,01933778	-0,2968508	0,3421271	-0,2437676	-0,0195663	-0,3061367	-0,1019943
Blautia_SV217	0,19929041	-0,232543	-0,1592448	-0,1593175	-0,1604985	-0,3399815	-0,1845686	-0,012277	0,03240468	-0,440672	-0,0352888	-0,1569596	-0,1242491	-0,3432548	-0,25464
Bacteroides_SV294	-0,1203732	-0,1812975	-0,6030995	0,68066772	0,77264535	0,08260177	0,66939359	-0,0040389	0,25022893	-0,0540691	0,3388174	-0,0460746	-0,0849995	0,34080638	-0,1426404
Oscillospira_SV464	0,11265749	-0,0938687	0,04381557	-0,144137	-0,2301972	-0,2717325	-0,1966203	0,03020156	-0,0337128	-0,0787303	-0,0850779	-0,0418286	-0,0472969	-0,0961308	0,08316865
Prevotella_SV526	-0,2905339	-0,2898791	-0,2140182	-0,1684048	0,04613011	-0,3115496	-0,1625502	-0,2719292	0,0867268	-0,0013152	-0,0046279	-0,1890298	-0,0778873	0,15374915	-0,0866809
Bacteroides_SV572	-0,3067828	-0,3192773	-0,3368325	-0,0161244	0,21107524	-0,2833623	-0,0128942	-0,2637317	0,13775212	-0,0129217	0,06853202	-0,1926541	-0,093605	0,22205643	-0,1145254

Supplementary Table 3 (F) Spearman correlation, the column correspond to gut bacteria taxa and the rows correspond to laboratory mesaures, during RW period. Total Cholesterol, in mg/dL; HDL, High-density lipoprotein, in mg/dL; LDL, Low-density lipoprotein, in mg/dL; non-HDL, non- High-density lipoprotein, in mg/dL; Triglycerides,in mg/dL; ApoA, Apolipoprotein A, in g/L; Apo B, Apolipoprotein B, in g/L; Glucose, Fasting Glucose, in mg/dL; ; GFR, glomerular filtration rate, in mL/min/1,73m2; hsCRP, high-sensitivity C-reactive protein, in mg/L; IL-6, interleukin 6, in pg/mL; LPS, lipopolysaccharides, in EU/mL.

Taxa	Total	HDL	LDL	nonHDL	Triglyceride	ApoA	ApoB	Glucose	Urea	Creatinine	GFR	PCRUS	TMAO	LPS	IL-6
Anaerostipes_SV651	-0,0221158	0,21314291	-0,0603721	-0,1186109	-0,1498573	0,20882452	-0,0942313	-0,0482897	0,3078049	0,32525546	-0,1878124	-0,033368	-0,1013298	0,27112173	-0,0597001
Roseburia_SV211	-0,16636	-0,0882418	-0,0763676	-0,0953618	-0,09013	-0,0908216	-0,0791936	0,02175942	-0,0157098	-0,1763925	0,16081173	-0,1172672	-0,1034765	-0,274197	-0,0686167
Coprococcus_SV109	-0,041929	0,06112409	-0,069843	-0,069469	-0,0359846	-0,0428349	-0,1023161	0,03060684	0,06108365	-0,128231	0,05359465	-0,1047125	-0,1084225	-0,083464	0,05128822
Veilonella_SV554	-0,2330278	-0,1902301	-0,2036498	-0,1418066	0,01839599	-0,1530482	-0,1674216	0,0307769	-0,0079577	0,0398476	-0,1221417	0,13818287	0,15869595	-0,1814907	-0,0186233
Ruminococcaceae_SV91	-0,4852682	-0,3203778	-0,4583288	-0,328514	0,06653669	-0,3853206	-0,2905816	0,1744481	0,1020437	0,15374757	-0,1980968	0,173207	0,35458771	-0,3320502	-0,022774
Prevotella_SV982	0,00643518	-0,0326954	-0,0463184	0,01934579	0,16892186	0,01950862	-0,0479989	0,00905544	-0,1913229	-0,0895054	0,10829299	-0,0166014	0,0058911	-0,3355948	-0,0428058
Bacteroides_SV490	-0,1877489	0,07286941	-0,169863	-0,2148382	-0,1818098	-0,0418425	-0,195885	-0,1176946	0,06026562	-0,1028	0,09253347	-0,1074238	-0,0597652	-0,147891	-0,1093988
Roseburia_SV896	0,18854668	-0,0036363	0,19871428	0,18264541	0,28405932	-0,0117446	0,24537072	-0,2760409	-0,0125355	0,05802481	0,05466709	-0,1157687	0,0508213	0,26807652	-0,1623871
Bacteroides_SV489	-0,2667892	-0,2429938	-0,2280797	-0,150588	0,05101596	-0,2531626	-0,1633371	0,05830663	0,00843937	0,19967202	-0,1635513	0,25081635	0,29454683	-0,1660575	-0,1525405
Lachnoclostridium_SV161	-0,333761	-0,1443381	-0,242629	-0,2602922	-0,1794637	-0,2225736	-0,2583707	0,01510627	-0,1830465	-0,1204544	0,01556978	-0,0816462	-0,0935325	-0,3784931	-0,1554705
Bacteroides_SV478	-0,0041134	0,15658817	-0,0601517	-0,0754366	-0,0872978	0,01861797	-0,1610458	-0,0442935	-0,0291906	0,06196357	-0,1879825	-0,0783167	-0,0475421	0,11005696	-0,1425715
Ruminococcaceae_SV595	-0,2029151	0,07711484	-0,1850068	-0,2319034	-0,2020767	-0,021145	-0,2243259	-0,0801744	-0,0204738	-0,1197526	0,11133172	-0,1643086	-0,1060213	-0,1901724	-0,1093633
Prevotella_SV216	0,01504922	-0,3045286	0,13819626	0,14883308	0,04855442	-0,284422	0,16496826	0,21500347	-0,1689814	0,17924169	-0,1154264	0,57198181	-0,1218497	-0,1284821	-0,027584
Bacteroides_SV132	0,06855327	0,10666951	0,01734517	0,01794104	-0,0135935	0,03806444	0,00564447	0,14341707	-0,1431931	-0,0904839	-0,0365667	0,14948526	-0,1349141	0,26159749	0,29661585
Roseburia_SV492	0,04828647	0,01099928	0,08875655	0,0407565	-0,0853303	0,16941334	0,07380997	-0,0750605	-0,0803098	0,21315886	-0,349049	0,01828675	-0,0994172	0,26777975	-0,01124
Lachnospiraceae_SV159	-0,1640919	0,0114933	-0,1741264	-0,1648095	-0,0556303	0,02052479	-0,2051183	-0,1864898	0,24302946	-0,2080183	-0,0731845	-0,1465776	-0,1406367	-0,1651331	0,04376129
Bacteroides_SV286	-0,1263082	0,12528053	-0,1688161	-0,1792073	-0,1205128	0,12324665	-0,2154243	-0,1616516	0,32451263	-0,148833	-0,1231363	-0,161935	-0,118617	-0,0995999	0,02117442
Minococcaceae_SV434	-0,0135357	0,05876498	-0,0854965	-0,0377392	0,04878356	0,10076749	0,02360978	0,04196576	-0,0448952	-0,0319265	-0,0560336	-0,1642396	-0,0720551	-0,1238295	-0,1570695
Lachnospiraceae_SV963	0,36462091	-0,1189004	0,5021889	0,40450213	0,15995086	-0,0362804	0,40372146	0,150815	0,06503499	-0,0146996	0,12235993	-0,1365388	-0,0277164	0,30086896	-0,0432634
Parasutterella_SV239	-0,2466869	-0,0126542	-0,211144	-0,2330446	-0,1547956	-0,0682689	-0,2257678	-0,1036592	0,16804926	-0,0207106	0,02513856	-0,1973519	-0,1292218	-0,1700391	-0,1651101

Colinsella_SV180	0,03463197	0,13100607	-0,0178928	-0,0269636	0,0621161	0,08521635	0,01551823	-0,0696293	-0,2284798	-0,1484093	0,03892157	-0,1721602	-0,0792943	0,11018385	-0,1528489
Streptococcus_SV71	0,3890832	-0,1426016	0,44209666	0,45158301	0,81803084	-0,1554761	0,4713905	-0,2729211	0,11750443	0,04726063	0,2000071	-0,1340999	0,094127	0,14414877	-0,1251406
Blautia_SV217	0,1495296	0,15821009	0,08593968	0,06728852	-0,0684981	0,09685135	-0,0563407	0,39255487	-0,0175679	-0,2439418	0,32105345	-0,0872936	-0,1919224	0,19459731	0,14300848
Bacteroides_SV294	-0,346528	-0,1674878	-0,2536743	-0,2613121	-0,1897079	-0,1844987	-0,2000723	0,07330496	-0,17204	-0,1448608	0,14475599	-0,1621418	0,52428541	-0,2895176	-0,1122652
Oscillospira_SV464	0,2156556	-0,2884089	0,27882775	0,351056	0,29420603	-0,3902428	0,2764153	0,41352933	-0,5502754	-0,1661968	-0,0401227	0,77483563	-0,1618199	0,09275211	0,47055362
Prevotella_SV526	-0,2413211	-0,1490653	-0,2015094	-0,1664345	-0,033745	-0,1051326	-0,2526419	0,03623165	0,32722094	0,13789005	-0,1315662	-0,2041804	-0,1421531	-0,0488911	-0,1976547
Bacteroides_SV572	-0,2881329	-0,1710107	-0,2352564	-0,2020607	-0,0614357	-0,130546	-0,2772923	0,0464451	0,29460292	0,11331231	-0,1071368	-0,2241693	-0,0606874	-0,0912053	-0,2103142

Supplementary Table 4 - Generalized estimating equation (GEE) approach verified time effects with the medications for the measures for TMAO, longitudinally

Variable	TMAO				
	Wald chi-square test	p value[1]	Pairwise Comparisons		p value[2]
Group	0,014	0,904	RW and Final	RW and Initial	1
Period	0,471	0,493		Abstemious and Final	1
Group * Period	1,807	0,179		Abstemious and Initial	1
			RW and Initial	Abstemious and Final	1
				Abstemious and Initial	1
			Abstemious and Final	Abstemious and Initial	1
ACE inhibitor	2,523	0,112	Yes and Final	Yes and Initial	1
Period	0,461	0,497		No and Final	1
ACE inhibitor * Period	0,043	0,836		No and Initial	1
			Yes and Initial	No and Final	1
				No and Initial	0,814
			No and Final	No and Initial	1
				No and Final	1
ARB	0,791	0,374	Yes and Final	Yes and Initial	1
Period	0,126	0,722		No and Final	1
ARB * Period	1,185	0,276		No and Initial	1
			Yes and Initial	No and Final	1
				No and Initial	1
			No and Final	No and Initial	1
Diuretics	2,745	0,098	Yes and Final	Yes and Initial	1
Period	0,216	0,642		No and Final	0,746
Diuretics * Period	0,689	0,406		No and Initial	1
			Yes and Initial	No and Final	1
				No and Initial	1
			No and Final	No and Initial	1
Spironolactone	3,649	0,056	Yes and Final	Yes and Initial	1
Period	0,826	0,363		No and Final	1
Spironolactone * Period	0,006	0,939		No and Initial	1
			Yes and Initial	No and Final	0,472
				No and Initial	0,436
			No and Final	No and Initial	1

1 p value for Wald's chi-square test; 2 p-value for the Bonferroni's test for pairwise comparisons

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Appendix

Appendix 1- Curriculum Summary

Education

Degree in Medicine, 2005, Universidade Federal de Santa Catarina (UFSC), SC, BR

Medical Training

Internal Medicine, 2008, Universidade Federal de Santa Catarina (UFSC), SC, BR

Cardiology, 2010, Instituto Dante Pazzane de Cardiologia, São Paulo, SP, BR

Echocardiography, 2012, Instituto de Radiologia HCFMUSP, São Paulo, SP,BR

Certification

Título de Especialista em Cardiologia, 2010, Sociedade Brasileira de Cardiologia.

Título de Especialista em Ecocardiografia 2012, Sociedade Brasileira de Cardiologia.

Relevant Publications and Presentations

Book Chapter

Clinical Endothelial Dysfunction: Prognosis and Therapeutic Target Haas, E.A., Nishiyama, M., da Luz, P.L. Endothelium and Cardiovascular Diseases: Vascular Biology and Clinical Syndromes, 2018, pp. 683–697

Journal article

Intestinal Microbiota and Cardiovascular Diseases. International Journal of Cardiovascular Sciences, 2020. DOI: 10.36660/ijcs.20200043

Poster Presentation

ESC Congress/ World Congress of Cardiology, 2019, Paris: “Beneficial effects of red wine intake upon gut microbiota and parallel effects upon plasma metabolomics”.

Next Gen Immunology 2020, Weizzmann Institute, Israel: “Red wine effects upon gut microbiota, plasma levels of trimethylamine-n oxide (tmao) and plasma metabolomics - the Wineflora study”.

Appendix 2- Paper: "Intestinal Microbiota and Cardiovascular Diseases"

International Journal of Cardiovascular Sciences. 2020; 33(5):462-471

ORIGINAL ARTICLE

Intestinal Microbiota and Cardiovascular Diseases

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Abstract

Background: Gut microbiota is essential to metabolize proteins, carbohidrates, aminoacids, fibers and essentially everything we eat. On the other hand, foods that we ingest powerfully influences gut miocrobiota.

Objetive: to analyze the contribution of human gut microbiota on cardiovascular disease.

Methods/Results: We summarized current knowledge regarding microbiota on cardiovascular diseases, emphazysing clinical relevance. The intestine harbors a great variety of bascteria and at the same time produces substances that can act locally as well as at a distance. Thus, the intestine is now considered an endocrine organ. The appropriate balance between the external environment and the composition of the gut microbiota is pivotal for human health. Alterations in gut microbiota are known broadly as disbiosis. A number of human diseases have been associated with disbiosis, including psychiatric, metabolic and chronic degenerative entities. In the cardiovascular system alterations of gut microbiota have been associated with atherosclerosis, hypertension, heart failure, obesity and diabetes. These influences occur through profound effect of gut microbiota upon the metabolism of proteins, carbohidrates, biliary acids, aminoacids and intestinal barrier among others.

Conclusion: Given the profund influences of gut microbiota upon mechanisms underlying cardiovascular diseases, microbiota is a potential therapeutic target.

Keywords: Cardiovascular Diseases; Gastrointestinal Microbiome/physiology; Risk Factors; Hypertension; Diabetes Mellitus; Obesity; Neoplasms; Alzheimer Disease; Metabolism; Atherosclerosis; Diet, Mediterranean.

Abstract

Recently, gut microbiota has emerged as an important mediator of several diseases such as diabetes, atherosclerosis, arterial hypertension, obesity, cancers and neuropsychiatric diseases including Alzheimer, autism and depression. Intestinal microbiota is formed by bacteria, fungi and viruses and its main function is to facilitate the absorption and metabolism of foods (protein, fat and carbohydrate). One example of the multiple actions of the gut microbiota is the bidirectional relationship between the intestine and the brain, the so-called "gut/brain axis". Furthermore, metabolites produced by gut microbiota can induce effects locally or at distance, which suggests that the intestine is an endocrine organ.

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Given the participation of the gut microbiota in several diseases, there is great interest in strategies that may positively affect the gut flora and prevent or even treat diseases. Among these strategies, lifestyle change, but specially diet modulation has gained importance. In this article, we review the mechanisms through which intestinal microbiota participates in cardiovascular diseases and possible therapeutic interventions.

Introduction

For long time traditional risk factors such as hypertension, diabetes, smoking and hypercholesterolemia have been considered the main promoters of atherosclerosis, and their control has been the cornerstone treatment for cardiovascular

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diseases. More recently, a new independent risk factor has emerged: the gut microbiota.[1,2,3]

Intestinal microbiota is made up of trillions of cells – about 10 times more than all the cells of the human organism – consisting of bacteria, viruses, fungi and archea. The phyla Firmicutes (mainly Clostridia species) and Bacteroidetes represent about 90% of gut microbiota, which is also composed of Actinobacteria, Proteobacteria and Verrucomicrobia.[2]

Until recently, studies on intestinal microbiota relied on culture of bacteria, providing limited information regarding a small fraction of the gut microbiota. Lately, culture-independent techniques, such as the marker gene analysis (16 S rRNA gene sequences), metagenome and metatranscriptone enabled the identification of previously unculturable bacteria.[1]

Gut flora remains relatively constant during an individual´s lifetime. However, it changes considerably from childhood to adult life and then during aging (Figure 1).[4] Thus, total gut microbiota is estimated to be small during childhood, increases considerably during adult life and decreases in old age. Infants have unstable, distinct and heterogeneous microbiota, characterized by low levels of total bacteria. On the other hand, elderly subjects have high levels of E-coli and Bacteroidetes. In the study by Maritat et al.[4] the measured ratio of Firmicutes to Bacteroides was 0.4, 10.9 and 0.6 for children, adults and elderly, respectively. It is tempting to speculate that these two extremes may be related to vulnerability of children and old people.

More recently, clusters or enterotypes in intestinal microbiota have been identified (Figure 2). Arumugan et al.[5] studied fecal metagenomes of 39 individuals from France, Italy, Spain and Denmark by 16S ribosomal RNA-encoding gene. They identified three clusters that are not nation or continent specific. They also found that 12 genes correlated significantly with age and three functional modules with body mass index. There were three main enterotypes –Bacteroidetes/Roseburia, Akkermansia/Alistipes/Ruminococcus and Prevotella. The authors concluded that intestinal microbiota variation is generally stratified, not continuous. Wu et al.[6] also described the link between dietary habits and gut microbial enterotypes (see ahead).

Gut microbiota varies individually and in populations as well, mainly due to different cultures and diets. Diet is a major element; for instance, vegans and vegetarians have higher counts of certain Bacteroidetes compared to omnivores.[7-9] Ayenic et al.[9] compared gut microbiome in rural Bassa with urban individuals from Nigeria. In rural Bassa they documented a predominance of bacteria with high capacity for fiber degradation and almost absence of common members of urban/industrial microbiomes. They also observed an adaptation of intestinal microbiota to urbanization.

Intestinal microbiota also varies in different intestinal regions as in the upper and lower small intestine and the colon.[10] This distribution explains the preferential absorption and metabolization of proteins, lipids and carbohydrates throughout the gut. The question regarding the "normal

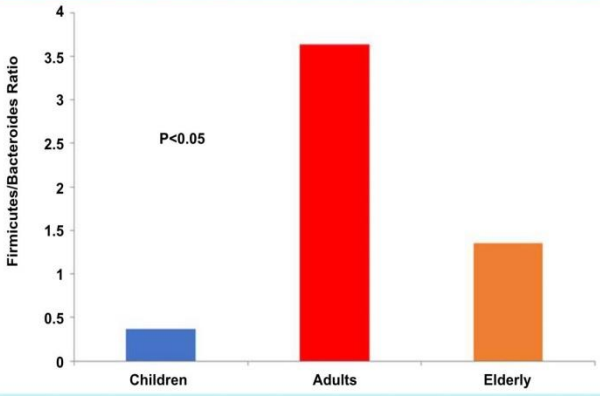

Figure 1 – Firmicutes/Bacteroides ratio in different phases of life. Modified from Mariat, et al.[4]

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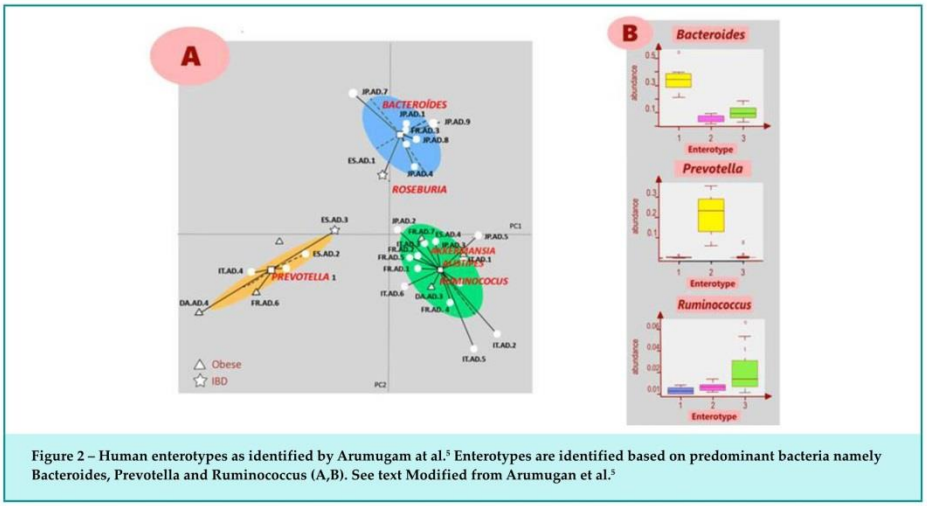

Figure 2 – Human enterotypes as identified by Arumugam at al.[5] Enterotypes are identified based on predominant bacteria namely Bacteroides, Prevotella and Ruminococcus (A,B). See text Modified from Arumugan et al.[5]

flora" is still open. Probably there are different enterotypes depending on diet, geographic location and genetic background.[2] On the other hand, the term "dysbiosis" describes a primary imbalance of gut microbiota. Some gut microbiota metabolites detected in plasma correlate directly with plasma trimethylamine-N-oxide (TMAO)[11] indicating the influence of gut microbiota on the pathogenesis of atherosclerotic disease.

Expansion of the knowledge in this area, both in mechanisms and identification of bacteria is expected in the near future. Understanding the functional role of bacteria and their relationship with plasma metabolome is pivotal issues for new research. However, our present understanding in this area is still superficial.

Main roles of the gut microbiota

The primary role of gut flora is the promotion and regulation of the absorption and metabolism of what we eat, *i.e.*, proteins, carbohydrates, fibers, nucleic acids, macro and micronutrients. Figure 3 summarizes the main functions of human gut microbiota. For instance, fermentation of non-digestible fibers and starch by microbiota in the colon leads to the production of short chain fatty acids (SCFAs), especially acetate, butyrate and propionate. Fatty acids are essential energetic sources of various organs including the heart, acting in the metabolism of proteins and carbohydrates.[12,13] Although only 5-10% of the energy consumed is from SCFAs, they play fundamental roles as in the signaling of molecules.[14]

The wide range of modulatory effects of SCFAs embrace the nervous system, blood pressure, histone deacetylases, inflammation, production of reactive oxygen species (ROS), inhibition of chemostasis, phagocytosis modulation, maintenance of intestinal barrier integrity and modulation of immune system responses.[2,14] SCFAs act through G-protein receptors, specifically the GRP41 and the olfactory receptor-78 (Olfr78). Olfr78, highly expressed in renal just-glomerular apparatus, mediates renin secretion induced by SCFAs. GPR41 and Olfr78 are also expressed in smooth muscle cells (SMC) of resistance vessels, and studies with KO mice indicate their influence on blood pressure. Thus, while GRP41 KO-mice are hypertensive, Olfr78 KO-mice are hypotensive.[1] Animal studies also indicate that SCFAs are essential in cardiac repair after myocardial infarction and immune response.[13,14]

Few interventions have focused on SCFAs; diet modulation represents the major tool to alter the gut microbiota. David et al.[15] examined, in 10 normal individuals, the effect of a shift from a plant-based diet to an animal-based diet. The animal-based diet increased bile–tolerant microorganisms, like Bacteroides, and decreased the levels of Firmicutes that metabolize polysaccharides, such as Roseburia. Consequently, there was a reduction in fecal acetate and butyrate when subjects were switched from plant to animal-based diets. This occurred within just a few

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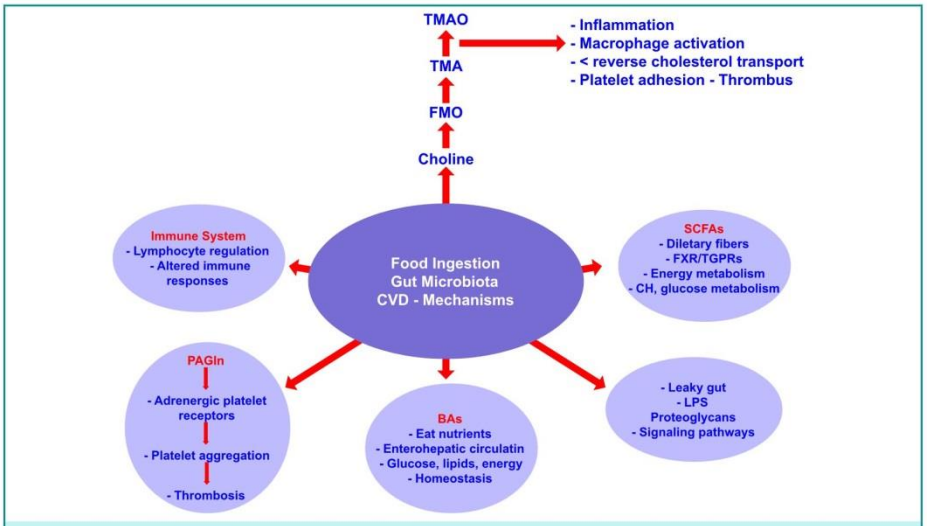

Figure 3 – Schematic summarized representantion of the main mechanisms through which gut microbiota may induce cardiovascular diseases (CVD). PAGIn: phenylacethyl glutamine; BAs: bile acids; SCFAs: short-chain fatty acids; FMO: flavin monooxygenases; TMA: trimethylamine; TMAO: trimethylamine-N-oxide; LPS: lipopolyssacharides.

days, indicating that human intestinal microbiota can be manipulated very rapidly.

In insulin–resistant patients with metabolic syndrome, fecal transplantation from lean donors led to improved insulin sensitivity and abundance of Roseburia, which is a butyrate–producing bacterium.[2]

Effects of bile acids upon intestinal microbiota

Bile acids (BAs) are synthesized from cholesterol in the liver. This is an important way to eliminate cholesterol from the body. The rate-limiting enzyme is hepatic cholesterol 7 α-hydroxylase ($CYP7A_1$). BAs are conjugated to taurine and glycine, which enhances their water solubility and their secretion into the bile; they facilitate fat digestion.[16] The main conjugated BA are chenodeoxycholic acid and cholic acid (primary BAs); the secondary or deconjugated BAs are lithocholic acid, ursodeoxycholate and deoxycholic acid. About 95% of the bile acids are reabsorbed in the terminal ileum and colon. These molecules are then recirculated to the liver through the portal vein; this process is known as the *enterohepatic circulation*.

BAs regulate energy metabolism through activation of the membrane Takeda G protein-coupled bile acid receptor 1 (TGR5) and the nuclear Farnesoid X receptor (FXR). Both TGR5 and FXR are highly expressed in the intestine and the liver. Humans produce a large conjugate BA pool which is maintained by a feedback mechanism of the FXR in the liver and intestine. BAS act as direct antimicrobial agents because of their detergent properties and hydrophobicity.[2]

BAs exert important effects as hormones dependent on activation of TGR5 and FXR by gut microbiota.[17] These receptors are implicated in lipid and glucose metabolism. In the ileum, FXR activation by BAs induces fibroblast growth factor 19, which circulates to the liver and reduces CYP7A1; such reduction then inhibits the synthesis of BAs, specifically lithocholic acid and deoxycholic acid.

One important observation is that reduced BAs levels in the gut are associated with inflammation and bacterial growth.[17] In this sense, obeticholic acid, a BA analogue and FXR agonist, was approved in the USA for treatment of bacterial translocation and inflammation in steatohepatitis. Also, FXR activation in mice decreased cholesterol absorption by 50%. FXR activation increases apoptosis and reduces inflammation and cell migration; FXR is expressed in endothelial cells, where it increases endothelial nitric oxide synthase (eNOS) expression and reduces endothelin-1 (ET_1). Glucose stimulates FXR and CYP7A1, but insulin inhibits them.[17]

On the other hand, TGR5 is involved in energy metabolism, and its activation has an anti-atherogenic effect. Given these multiple physiological functions, FXR and TGR5 are potential therapeutic targets. Both synthetic agonists and inhibitors have been tested, with conflicting results in animal models and humans. More research is still necessary to establish the role of the intervention on these receptors before clinical application.

Microbiota and Immunity

The immune system, either innate or adaptive, is clearly linked to gut microbiota, which plays a role in modulating the relation regulatory-to-effector T cells.[18,19]

To reach distant organs, microbial signals need to cross the intestinal epithelium. Structural components of the microbiota such as lipopolysaccharides (LPS) and peptidoglycans interact with mucosal surface cells through pattern recognition receptors (PRR). PRR recognize pathogen-associated molecular patterns (PAMPs), which modulate immune responses. LPS and peptidoglycans can trigger a cascade of downstream signaling pathways.[2]

Gut commensal microbiota maintains a balance of immune effectors, to protect the gut against dangerous invaders, and at the same time tolerate innocuous microbial antigens. A thick mucus layer in the intestinal mucosa, together with the epithelial wall, is essential to maintain homeostasis. The contribution of intestinal mononuclear phagocytes (MNPs) has being recognized as a potential targetable pathway in inflammatory disease.[18] The normal intestinal microbiota can inhibit innate lymphoid cells, which are major producers of interleukin-22 (IL-22), a cytokine that acts in epithelial cells to promote healing during infection. IL-22 also induces antimicrobial peptide production.[18]

In addition, commensals can affect the adaptive immune system by inducing T cell differentiation. Also, Clostridium clusters can induce colonic regulatory T cells (Tregs) that produce anti-inflammatory interleukin-10 (IL-10); to do this, Clostridium provides a transforming growth factor β (TGFβ) and high luminal concentrations of SCFAs, especially butyrate. Thus, SCFAs participate actively in the process called "homeostatic induction", in which bacteria exert immune effects through the differentiation of lymphocytes.[19]

Segmental filamentous bacteria (SFB) induce CD4+T helper cells in the ileal epithelial surface. CD4T helper cells produce IL-17, IL-17f and stimulate Th17 cells. All these cytokines are involved in inflammatory diseases such as inflammatory arthritis, psoriasis and inflammatory bowel disease.[18] These findings suggest that the inflammatory environment of the intestine modulate the differentiation of effector lymphocytes, highlighting the intimate interplay of gut microbiome and immunity.

Not only bacteria, but viruses can influence immunity; enteric viruses are frequent causes of human gastrointestinal diseases. Recent studies have also suggested interactions between viruses and bacteria – the so called "transkingdom interaction"; an example is the presence of virus-like particles correlated with significant changes in gut microbiome in intestinal bowel disease patients. Also, helminths such as Schistosoma mansoni and Trichinella have been found to modulate immunity.

These inflammatory cytokines can profoundly alter intestinal motility and permeability. One major effect of this phenomenon is the translocation of intestinal bacteria to plasma which can cause bacteremia and sepsis.

Taken together, these data indicate a significant modulatory role of gut microbiota - bacteria, viruses and helminths – in the immune system. Mechanistic studies are needed to further our knowledge in this emerging field.[18,19]

The real impact of gut microbiota in cardiovascular diseases

It has been recognized that gut microbiota is involved in the development of atherosclerosis, diabetes, hypertension, obesity, stroke, heart failure and neuropsychiatric diseases such as depression, autism and Alzheimer.[2] Even birth circumstances affect gut microbiota; in normal deliveries the child is exposed to the vaginal flora of the mother, which is beneficial to the health of the child. On the contrary, cesarean section deprives the baby of such exposure, and asthma and allergies have been encountered more frequently among these children. Furthermore, another gut microbiome metabolite, phenylacetyl glutamine (PAGIn), has been recently associated with cardiovascular disease in humans. PAGIn acts through adrenergic platelet receptors facilitating platelet aggregation and thrombus formation.[20]

A characteristic of the intestine and its microbiota is that they produce substances that act locally and others that act at distance, such as cytokines and noradrenergic products. These features led to the concept that the intestine is an endocrine organ.

Atherosclerosis

The metabolism of phosphatidylcholine, carnitine and choline found in abundance in red meat, milk and eggs has as its final compound trimethylamine-N-oxide (TMAO).

These substances enter the intestine and suffer a series of metabolic reactions under the influence of microbiomes. The fundamental reaction is the conversion of choline into trimethylamine (TMA), which is then metabolized by flavin monooxygenases (FMOs), especially FMO3, of the liver into TMAO.[1] TMAO production is entirely dependent on the gut microbiota. In experimental animals fed a choline enriched diet, TMAO production is abolished when animals received broad spectrum antibiotics.[3] The authors also showed that TMAO induced foam cell formation and atherosclerotic plaques in aortic root of rabbits. Seldin et al.[21] observed elevated inflammatory gene expression compared to controls in the aortas of $LDLR^{-/-}$ mice fed a choline diet. Chronic choline feeding led to inflammatory gene expression of cyclooxygenase 2 (COX^{-2}), E-selectin, monocyte chemoatractic 1 (MCP-1); macrophage inflammatory protein2 (MIP-2), TMAO and tumor necrosis factor α (TNF-α). In addition, acute injection of TMAO at physiological levels induced the same inflammatory markers and mitogen activated protein kinase (MAPK), extra-cellular signal related kinase (ECSRK) and nuclear factor kappa beta (NFK-β). To further explore the effects of TMAO, the authors[21] studied human aortic endothelial (HAEC) and vascular smooth muscle cells (VSMC) in culture. Treatment of these two cell lines with TMAO also induced gene expression of inflammatory markers: NFK-β; COX-2, interleukin 6, E-selectin and intercellular adhesion molecule (ICAM). In addition, TMAO enhanced endothelial recruitment of leukocytes.[21] These data indicate that TMAO activates inflammatory pathways in the vasculature, causing recruitment of endothelial cells and leukocytes, and atherosclerosis; these actions are mediated by NFK-β pathway.

Human studies documented participation of TMAO in atherosclerotic disease. Tang et al.[22] examined the effects of a phosphatidylcholine challenge (two hard-boiled eggs and deuterium-labeled phosphatidylcholine) in 40 normal individuals; they documented a significant increase in plasma and urine TMAO; in six of them, broad-spectrum antibiotics were administered, which completely suppressed TMAO increases. In a second study, 4,007 patients with documented coronary artery disease (CAD) were followed for three years and a graded increase in event risk in relation to TMAO plasma levels was documented, specifically death, non-fatal myocardial infarction or stroke (Figure 4).

In addition, TMAO levels correlate with atherosclerotic burden, as measured by the Syntax score, as well as to early atherosclerosis.[23,24] Furthermore, in a group of patients similar to those of the Courage trial,[25] i.e., with documented CAD and treated medically, higher TMAO levels were associated with worse prognosis due to cardiovascular events.

Emoto et al.[26] compared 39 CAD patients with 30 patients with risk factors and 50 normal controls. They

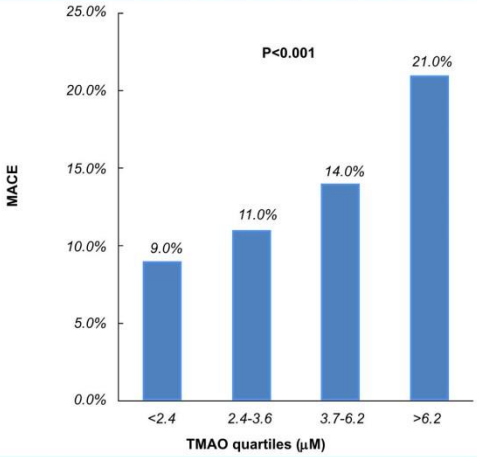

Figure 4 – Incidance of MACE (myocardial infarction, death and stroke) in 4007 CAD patients over 3 years period. Modified from Tang et al.[22]

observed that in CAD patients, order Lactobacillales was significantly increased and the phylum Bacteroidetes/ Prevotella was decreased when compared to controls.

On the other hand TMAO is an inductor of atherosclerosis or simply a marker of it. TMAO is clearly dependent on renal function and increases with age. Thus, individuals with even moderately decreased glomerular filtration rates have higher TMAO plasma concentrations.[2] Elderly individuals also have higher TMAO levels compared to middle age persons. One finding that supports the active role of TMAO as an atherogenic molecule is that it induces platelet hyperreactivity and thrombotic risk, both experimentally and in humans.[27,28] Furthermore, the ingestion of deep-sea fish increases urinary TMAO levels.[29]

The mechanisms underlying the atherogenic effects of TMAO include: a. induction of inflammation by expression of inflammatory genes in both vascular SMC and endothelial cells; b. induction of ROS production; c. impairment of bile acids synthesis through interference in the FXR/TGPR5 axis; d. increase in platelet adhesiveness and thrombus formation; e. impairment of reverse cholesterol transport; f. promotion of oxLDL receptors expression in macrophages facilitating foam cells formation[3]

Taken together these experimental and clinical studies indicate that dietary derived TMAO is closely associated with atherosclerosis, is entirely dependent on gut microbiota and is a marker of clinical outcomes; however, it is not yet entirely clear whether it is a marker or a true causative factor of atherosclerosis. It should also be mentioned the physiological functions of TMAO, specifically cell protection against hydrostatic and osmotic stress cells in deep sea fish and humans.[30,31]

Gut microbiota in diabetes and obesity

Patients with type 2 diabetes (DM_2) have typical intestinal flora compared with non-diabetic individuals; lower concentrations of butyrate-producing bacteria, such as Roseburia intestinalis and Faecalisbacterium, and higher concentrations of Lactobacillus gasseri and Streptococus mutans are found in DM2 patients. Also, insulin-resistant patients have increased concentrations of branched-chain amino acids,[32] which are associated with Prevotella copri and Bacteroids vulgatus.[33] In addition, in DM_2 individuals, postprandial glucose in response to diet can be modulated by intestinal microbiota.[34] Also, imidazole propionate, a metabolite produced by microbiota is elevated in DM2 and impairs glucose tolerance.[35]

Hypertension

It is well known that elevated salt intake is implicated in hypertension. In mice, high salt intake induced significant changes in gut microbiota associated with a reduction in Lactobacilus murinus. When this species was added to the diet, hypertension was no longer induced,[36] partially due to modulation of TH17 cells. Another mechanism involves G-protein coupled receptors (GRCRs) that are regulated by SCFAs. SCFAs can stimulate GPCRs, affect renin secretion and hence blood pressure;[37] in this line of evidence, KO mice for $GPCR_{41}$ showed systolic hypertension and SCFAs lowered blood pressure through regulation of $GPR_{41.}$[37]

Furthermore, Olfr78 and GPR41 are expressed in vascular SMC of resistance vessels; interestingly, propionate can cause vasodilation in mice through modulation of Olfr78 and GPR41. Also, high levels of oxLDL contribute to hypertension through inhibition of NO, which is a classic endothelial vasodilator. In summary, the link between diet, microbiota and hypertension includes two branches: a) production of SCFAs that are the final substances of fiber fermentation in the gut and their effects upon GRPs and Olf78 that are present in SMC and control blood pressure; b) increases in oxLDL from diet which inhibits NO and increases endothelin-1, which acts on SMC.

Cheema et al.[38] investigated metabolites associated with infused Ang II in mice. They found four up-regulated and eight down-regulated plasma metabolites; in feces there were 25 unregulated and 71 down regulated. These effects did not occur in germ-free mice. Thus, the relationship between AngII and hypertension is differentially regulated by microbiota-dependent metabolites, by complex mechanisms. Karbach et al.[39] also observed that gut microbiota facilitates AngII- induced vascular dysfunction and hypertension. Clinical observations have indicated that butyrate-producing bacteria is associated with lower blood pressure in pregnant women.[36]

Despite these strong mechanistic studies that support the interaction of diet, gut microbiota and hypertension, the role of human microbiota in hypertension needs further studies.

Heart failure

The participation of intestinal microbiota in heart failure (HF) has been suggested in many studies.[1] For instance a depletion of gut microbiota and its diversity has been observed.[40] Tang et al.[41] also showed that elevated TMAO in HF patients indicates higher long-term mortality risk, independent of traditional risk factors

and cardiorenal function.[41] Although mechanisms are not clear, one hypothesis is that bacterial translocation, inflammation and oxidative stress make these patients more vulnerable; that is an explanation well-suited to the "gut/brain axis hypothesis". In support of this hypothesis is the observation that HF patients are more prone to Clostridium difficile infection.[42]

Intervention on gut microbiota

Diet is the main tool to modulate intestinal microbiota. De Fillipis et al.[7] analyzed gut microbiota in 153 individuals who were omnivorous, vegetarians and vegans. There were significant associations between the consumption of vegetable-based diets and increased levels of fecal SCFAs, Prevotella and some fiber-degrading bacteria. On the contrary, higher urinary TMAO levels were observed among those who did not follow a Mediterranean diet. These data indicate that a Mediterranean type of diet influences gut microbiota and protect against atherosclerosis.[45]

Resveratrol, a polyphenol encountered in grapes, vegetables, berries and red wine may influence gut microbiota. Ingested resveratrol has low bioavailability due to its metabolization in the liver and intestine. Bifidobacteria infantis and Lactobacillus acidophilus are bacteria involved in the metabolism of resveratrol. Chaplin et al.[43] also showed, in animals, potential beneficial effects of resveratrol in fat accumulation, adipose deport extension, hepatic fat accumulation, glucose intolerance and insulin resistance, high blood pressure and lipids; in view of these effects, the authors concluded that resveratrol might be useful in metabolic syndrome.

Chen et al.[44] investigated the effects of resveratrol on TMAO and BA synthesis by gut flora in Apo $E^{-/-}$ mice. Resveratrol attenuated TMAO- induced atherosclerosis in these mice. Resveratrol also increased Lactobacillus and Bifidobacterium levels, which increased bile salt hydrolase activity, thus enhancing BA deconjugation and fecal excretion. In addition, resveratrol suppressed the FXR-TGR_5 axis and increased CYP7A1 and hepatic BAs neosynthesis. In antibiotic- treated mice none of these effects were noted. The authors concluded that resveratrol attenuated TMAO-induced atherosclerosis by decreasing TMAO levels and augmenting hepatic BA neosynthesis via gut microbiota remodeling. As indicated before, BA synthesis is an important pathway to eliminate cholesterol from the body.

Enterotypes have been linked to dietary patterns. Thus, the first enterotype described by Arumugan et al.[5] which is high in Bacteroides and low in Prevotella, is found in long-term Western diets, rich in animal proteins, choline and saturated fats; the second enterotype is high in Prevotella, low in Bacteroides and is associated with plant-based diets rich in fibers and simple sugars; the third enterotype has a slightly higher population of the genus Ruminococcus of the phylum Firmicutes.[45] Wu et al.[6] confirmed, in 98 individuals, that enterotypes are strongly associated with long-term diets, especially protein and animal fats with Bacteroides, in contrast to Prevotella which is preferentially linked to carbohydrate metabolism. Taken together, these data suggest that diet modulation, especially the Mediterranean diet, may beneficially influence the gut microbiota. Personalized diets according to the intestinal microbiota is a promising approach for glycemic control, as suggested by Zeevi et al.[34] In our group, we tested the effects of red wine on gut microbiota and plasma metabolomics in CAD patients (Wineflora Study). Preliminary results suggest a potential beneficial effect on gut microbiota by induction of anti-atherosclerotic bacteria.

Another possibility is *enzymatic blockade* of TMA formation by suppressing FMO3. However, this approach leads to TMA accumulation in plasma and consequent fish odor syndrome, which hampers its clinical application.[46]

Also, bacterial enzyme inhibitors, such as choline TMA lyase and carnitine TMA lyase, represent another approach to reduce TMA production.[47] However no human data is yet available. Another approach would be the use of long-term broad-spectrum antibiotics to suppress TMAO formation, as mentioned before. Unfortunately, this is not possible in clinical practice. Further, the use of antibiotics in patients produced no effects in preventing coronary events.[48]

Prebiotics and *probiotics* are potential ways to interfere with gut microbiota. Probiotics are substances that contain live bacteria such as Lactobacillus.[45] Tannock et al.[49] gave a milk compound containing Lactobacillus rhamnosus to 10 normal individuals; they observed transient changes in fecal microbiota, specifically lactobacillus and enterococcus, but no concomitant modifications in biochemical parameters. Experimental clinical studies have offered promising results related to BA metabolism. Prebiotics are foods such as fibers whose metabolism provide the growth of "protective bacteria"; for instance, ingestion of nondigestible fibers may induce the growth of commensals and alter intestinal motility.[47] Prebiotics and probiotics are in early phases of development but will likely constitute valuable alternatives for gut microbiota modulation.

Another intervention that impacts on intestinal microbiota is *bariatric surgery*, in which increased circulating levels of primary and secondary BAs were observed.[2]

Finally, *fecal transplantation* can be employed in especial circumstances.[50] Few experiments have been conducted on humans, showing inconsistent results. A series of technical and ethical problems, such as the definition of healthy donors, still need clarification. However, in special circumstances such as IBD resistant to conventional treatment, fecal transplantation may be a valuable alternative.

Conclusions

Gut microbiota plays a pivotal role in atherosclerosis, heart failure, diabetes, and obesity, acting as an independent risk factor. Gut microbiota is essential for metabolism of nutrients like proteins, carbohydrates, and plant derivates. It interferes directly in the metabolism of SCFA, BAs, inflammation and immune system. It also induces the formation of TMAO, an atherogenic molecule. The intestine is considered today an endocrine organ since it produces substances that act locally or at distance. The intestine and the brain maintains constant and bidirectional influences through the 'gut-brains axis'. Human intestinal microbiota is profoundly influenced by diet, and for this reason, diet modulation, especially by adopting a Mediterranean type diet, is the most promising approach to beneficially influence gut microbiota. However, there are no clinic studies analyzing the long-term effectiveness of dietary interventions on gut microbiota. Further research is needed to clarify the roles of intestinal microbiota in health and human diseases.

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Author contributions

Conception and design of the research: Luz PL. Statistical analysis: Favarato D. Critical revision of the manuscript for intellectual content: Haas EA, Favarato D.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

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