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Sara Raquel Ramalho Pereira Nunes

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Tese no âmbito do Doutoramento em Ciências Farmacêuticas, ramo de Farmacologia e Farmacoterapia, orientada pelo Doutor Flávio Nelson Fernandes Reis, pela Professora Doutora Maria Manuela Estevez Pintado e pela Professora Doutora Cláudia Margarida Gonçalves Cavadas e apresentada à Faculdade de Farmácia da Universidade de Coimbra.

Dezembro de 2021

Faculdade de Farmácia da Universidade de Coimbra

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Dissertation presented to the Faculty of Pharmacy of the University of Coimbra in fulfillment of the requirements for a Doctoral degree in Pharmaceutical Sciences, branch of Pharmacology and Pharmacotherapy.

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UNIVERSIDADE DE COIMBRA

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"Success is the sum of small efforts repeated day in and day out"

Robert Collier

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Abbreviations

Δp	Proton motive force
ΔpH	Proton gradient
ΔΨm	Mitochondrial membrane potential
8-OHdG	8-hydroxy-2'-deoxyguanosine
3-HB	3-hydroxybutyrate
ABTS$^{\bullet+}$	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation
Ac-CoA	Acetyl-coenzyme A
ACACA	Acetyl-CoA carboxylase
ACAD	Acyl-CoA dehydrogenase
ACADL	Acyl-CoA dehydrogenase long-chain
ACOX	Acyl-CoA oxidase
ADA	American Diabetes Association
ADP	Adenosine diphosphate
AFLD	Alcoholic fatty liver disease
Akt	Protein kinase B
ALT	Alanine aminotransferase
AMPK	5' adenosine monophosphate-activated protein kinase
ANT	Adenine nucleotide translocase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AUC	Area under the curve
BAE	BB anthocyanins extract
BB	Blueberry
BCAA	Branched-chain amino acids
BJ	Blueberry juice
BMI	Body mass index
BSA	Bovine serum albumin
BW	Body weight
C3G	Cyanidin-3-O-beta-glucoside
Ca^{2+}	Calcium
CD	Cafeteria diets
CGI	Combined glucose intolerance
ChREBP	Carbohydrate-responsive element-binding protein
CoA	Coenzyme A
COX	Cytochrome c oxidase
CPT	Carnitine palmitoyl transferase
CPT1	Carnitine palmitoyl transferase 1
CRP	C-reactive protein
CsA	Cyclosporine A
Cu/ZnSOD	Copper/zinc superoxide dismutase
Cyt c	Cytochrome c

DAG	Diacylglycerol
DF	Dilution factor
DNL	*De novo* lipogenesis
DNP	2,4-dinitrophenol
DPP-4	Dipeptidyl peptidase-4
Drp1	Dynamin-related protein 1
EGCG	(-)-Epigallocatehin-3-gallate
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	Endoplasmic reticulum
ETC	Electron transport chain
FABP	Fatty acid binding protein
FAD	Flavin adenine dinucleotide, oxidized form
$FADH_2$	Flavin adenine dinucleotide, reduced form
FASN	Fatty acid synthase
FAT/CD36	Fatty acid translocase/cluster of differentiation 36
FATP	Fatty acid transporter protein
FCCP	Carbonyl cyanide p-(tri-fluromethoxy)phenyl-hydrazone
FFAs	Free fatty acids
FMN	Flavin mononucleotide
FMT	Fecal microbiota transplantation
FOXO1	Forkhead box protein-O1
FPG	Fasting plasma glucose
FRAP	Ferric reducing antioxidant potential
FXR	Farnesoid X receptor
G6Pase	Glucose-6-phosphatase
GAE	Gallic acid equivalents
GCK	Glucokinase
GIMM	Gastrointestinal microbiome modulator
GIP	Gastric inhibitory polypeptide
GIT	Gastrointestinal tract
GK	Goto-Kakizaki
GLP-1	Glucagon-like peptide-1
GLUT	Glucose transporter
GM	Gut microbiota
GP	Glycogen phosphorylase
GPRs	G-protein-coupled receptors
GPx	Glutathione peroxidase
GR	Glutathione reductase
GS	Glycogen synthase
GSH	Reduced glutathione
GSK3	Glycogen synthase kinase
GSSG	Oxidized glutathione
GST	Glutathione-S-transferase
GTT	Glucose Tolerance Test

H_2O	Water
H_2O_2	Hydrogen peroxide
H&E	Hematoxylin-eosin
HbA1c	Glycated hemoglobin
HDL	High-density lipoprotein
HDL-c	High-density lipoprotein cholesterol
HFD	High-fat diet
HFHSD	High-fat/high-sugar diet
HGP	Hepatic glucose production
HMDM	Human Metabolome Database
HMG-CoA	Hydroxymethyl glutaryl-CoA
HNE	Hydroxynonenal
HO1	Heme oxygenase
HOMA-IR	Homeostatic model assessment of insulin resistance
HPLC	High-performance liquid chromatography
HRCECs	Human retinal capillary endothelial cells
hs-CRP	High-sensitivity C-reactive protein
HSD	High-sugar diet
HSuHF	High-sucrose/high-fat
i.p.	Intraperitoneally
ICAM-1	Intercellular adhesion molecule-1
IDF	International Diabetes Federation
IEC	International Expert Committee
IFG	Impaired fasting glucose
IGF-1	Insulin-like growth factor-1
IGT	Impaired glucose tolerance
IKK-β	Inhibitor of nuclear factor kappa B kinase subunit beta
IL-6	Interleukin-6
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
InsR	Insulin receptor
IRS	Insulin receptor substrate
JAK-STAT	Janus kinase/signal transducers and activators of transcription
JNK	c-Jun N-terminal kinase
K_{ITT}	Rate constant for glucose clearance
LDL	Low-density lipoprotein
LDL-c	Low-density lipoprotein cholesterol
LPS	Lipopolysaccharide
LXR	Liver X receptor
MAM	Mitochondria-associated ER membrane
MAO	Monoamine oxidases
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde

Mfn	Mitofusins
mGPDH	Mitochondrial glycerol-3-phosphate dehydrogenase
MMP	Mitochondrial membrane permeability
MnSOD	Manganese superoxide dismutase
MPT	Mitochondrial permeability transition
mPTP	Mitochondrial permeability transition pore
mRyR	Mitochondrial ryanodine receptor
MSI	Metabolomics Standards Initiative
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
Muc-2	Mucin-2
NAD^+	Nicotinamide adenine dinucleotide, oxidized form
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide-adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEFAs	Non-esterified fatty acids
NF-κB	Nuclear factor-kappa B
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NO_3^-	Peroxynitrite
NQO1	NAD(P)H quinone oxidoreductase 1
NRF	Nuclear respiratory factor
$^1O_2$	Singlet oxygen
$O_2^{-\bullet}$	Superoxide anion radical
OD	Optical density
OGTT	Oral glucose tolerance test
$OH^\bullet$	Hydroxyl free radical
OLEFT	Otsuka Long-Evans Tokushima fatty
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate-buffered saline
PC	Pyruvate carboxylase
PCA	Principal component analysis
PCR	Polymerase chain reaction
PEPCK	Phoshoenolpyruvate carboxykinase
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator-1α
PI3K	Phosphatidylinositol 3-kinase
PK	Pyruvate kinase
PKC	Proteins kinase C
PLS-DA	Partial least square discriminant analysis
PP	Polyphenols
PPARs	Peroxisome proliferator-activated receptors

PPAR-γ	Peroxisome proliferator-activated receptor gamma
Prxs	Peroxiredoxins
PTP1B	Protein tyrosine phosphatase 1B
PYY	Peptide YY
QH_2	Ubiquinol
QUICKI	Quantitative insulin sensitivity index
RaM	Rapid calcium mode
RCR	Respiratory control ratio
RCT	Randomized controlled trial
ROS	Reactive oxygen species
SCD1	Stearoyl-CoA desaturase-1
SCFAs	Short-chain fatty acids
SIRT	Sirtuins
SOD	Superoxide dismutase
SREBP-1c	Sterol regulatory element-binding protein-1c
STZ	Streptozotocin
T2DM	Type 2 diabetes mellitus
TAS	Total antioxidant status
TBARS	Thiobarbituric acid reactive substances
TCA	Tricarboxylic acid
TEM	Transmission electron microscopy
TFAM	Mitochondrial transcription factor A
TGs	Triglycerides
TJ	Tight junctions
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-α
Total-c	Total-cholesterol
TOCSY	Total correlation spectroscopy
TPC	Total phenolic content
TPP^+	Tetraphenylphosphonium
Trxs	Thioredoxins
TTFA	Thenoyltrifluoroacetone
UCPs	Uncoupling proteins
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor
VIP	Variable importance in the projection
VLDL	Very low-density lipoproteins
WD	Western diets
WHO	World Health Organization
ZO-1	Zonula occludens-1
ZDF	Zucker Diabetic Fatty

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Publications

Part of the work presented in this Thesis have been published in international peer-reviewed scientific journal as follows:

Nunes S, Vieira P, Gomes P, Viana SD, Reis F. Blueberry as an attractive functional fruit to prevent (pre)diabetes progression. *Antioxidants* 2021; 10(8): 1162. doi: 10.3390/antiox10081162. – **Chapter 1**

Preguiça I, Alves A, **Nunes S**, Fernandes R, Gomes P, Viana SD, Reis F. Diet-induced rodent models of obesity-related metabolic disorders - A guide to a translational perspective. *Obes Rev*. 2020; 21(12): e13081. doi: 10.1111/obr.13081. – **Chapter 1**

Nunes S, Viana SD, Preguiça I, Alves A, Fernandes R, Teodoro JS, Figueirinha A, Salgueiro L, Silva S, Jarak I, Carvalho RA, Cavadas C, Rolo AP, Palmeira CM, Pintado MM, Reis F. Blueberry consumption challenges hepatic mitochondrial bioenergetics and elicits transcriptomics reprogramming in healthy wistar rats. *Pharmaceutics*. 2020; 12(11): 1094. doi: 10.3390/pharmaceutics12111094. – **Chapter 2**

Nunes S, Viana SD, Preguiça I, Alves A, Fernandes R, Matos P, Teodoro JS, Figueirinha A, Salgueiro L, André A, Silva S, Jarak I, Carvalho RA, Cavadas C, Rolo AP, Palmeira CM, Pintado MM, Reis F. Blueberry counteracts prediabetes in a hypercaloric diet-induced rat model and rescues hepatic mitochondrial bioenergetics. *Nutrients*. 2021; 13(12): 4192. doi: 10.3390/nu13124192. – **Chapter 3**

The following publications were performed during the course of this thesis:

Nunes S, Alves A, Preguiça I, Barbosa A, Vieira P, Mendes F, Martins D, Viana SD, Reis F. Crescent-like lesions as an early signature of nephropathy in a rat model of prediabetes induced by a hypercaloric diet. *Nutrients*. 2020; 12(4): 881. doi: 10.3390/nu12040881.

Preguiça I, Alves A, **Nunes S**, Gomes P, Fernandes R, Viana SD, Reis F. Diet-induced rodent models of diabetic peripheral neuropathy, retinopathy and nephropathy. *Nutrients*. 2020; 12(1): 250. doi: 10.3390/nu12010250.

Fernandes R, Viana SD, **Nunes S**, Reis F. Diabetic gut microbiota dysbiosis as an inflammaging and immunosenescence condition that fosters progression of retinopathy and nephropathy. *Biochim Biophys Acta Mol Basis Dis*. 2019; 1865(7): 1876-1897. doi: 10.1016/j.bbadis.2018.09.032.

Teodoro JS*, **Nunes S***, Rolo AP, Reis F, Palmeira CM. Therapeutic options targeting oxidative stress, mitochondrial dysfunction and inflammation to hinder the progression of vascular complications of diabetes. *Front Physiol*. 2019; 9: 1857. doi: 10.3389/fphys.2018.01857. *Equal authorship.

Abstract

Prediabetes, recognized as a state of high risk for the development of type 2 diabetes mellitus (T2DM) and related complications, is certainly a privileged phase to implement strategies to prevent or delay the disease progression to more advanced stages. Unhealthy lifestyles, marked by sedentarism and unbalanced dietary habits, are important contributors to the growing incidence of various metabolic disorders, namely prediabetes. Several recent evidence point towards unbalanced diets, including the hypercaloric ones, as major drivers of gut microbiota (GM) dysbiosis, which may be involved in the onset of metabolic deregulation. Although the putative engagement of dysbiosis as early as prediabetes remains to be elucidated, the adoption of healthier dietary regimens as a first-line therapy is now well established.

During the recent years, we have witnessed a change in consumers´ attitude towards the adoption of healthier dietary patterns to avoid the onset of certain diseases while ensuring a better life quality. Thus, the demand and consumption of functional foods and natural supplements rich in phytochemicals, namely polyphenols, are a contemporary trend. Still, high doses of phenolic compounds have already been associated with adverse effects, namely at the hepatic level, a controversial issue within the scientific community that warrants further studies.

Blueberry (BB) fruit has become popular, both to be consumed as a food or as a supplement, by virtue of their claimed health benefits (e.g. antioxidant, prebiotic), as they are a valuable source of bioactive compounds (i.e. polyphenols and fiber). Even so, the effective consequences of its excessive intake in a healthy condition are yet to be defined. Likewise, a lack of consistent evidence subsists regarding the expected positive impact of a BB-derived nutraceutical intervention in a prediabetic condition, presumed on the basis of its panoply of health-related properties in several metabolic diseases, including in T2DM.

Considering these rationales, the main aims of this thesis were the evaluation of the effects arising from a long-term BB supplementation, in healthy and prediabetic conditions, with particular focus on GM and liver tissue. BB was administered in the juice form, in a dose of 25 g/Kg of body weight for 14 weeks in two experimental conditions: a) in healthy Wistar rats and b) in a hypercaloric diet- induced prediabetic rat model.

When a healthy condition was foreseen, our observations corroborated the expected increase in antioxidant activity BB-driven, accompanied by an unchanged metabolic profile. Somewhat surprisingly, no profound changes were observed in the composition and function of the microbial community. In the liver, BB supplementation elicited a scenario of mitochondrial adaptation without functional and structural changes in the hepatic tissue. Indeed, we found a marked remodeling of mitochondrial bioenergetics, accompanied by a reprogramming of metabolic transcriptomics and an expressive repression of inflammation-related genes. Collectively, these observations suggest the activation of an adaptive cellular response to an overload of the antioxidant cargo triggered by a prolonged consumption of BB.

Regarding the impact of BB on prediabetes, we observed a prevention of hypercaloric diet-evoked metabolic changes. Apparently, this metabolic protection could not be explained by the impact on GM. Conversely, and paralleling an antioxidant effect, we observed a reduction in hepatic steatosis along with a clear improvement in hepatocyte mitochondrial bioenergetics, suggesting that mitochondria may be a key player in early disease progression. Moreover, BB consumption has positively modulated the hepatic gene expression of key targets involved in fatty acid oxidation, insulin signaling, inflammation, as well as the mitochondrial respiratory chain, all of which were found compromised in the liver of prediabetic rats. Collectively, these results advocate that the improvement of hepatic mitochondrial function may be a crucial mechanism by which BB exerts its protective effect in prediabetes.

In conclusion, the results of this thesis suggest that the long-term supplementation of BB may be an effective nutraceutical intervention to avoid the progression of prediabetes; still, the marked impact on hepatic mitochondrial bioenergetics in a healthy condition raises the need of further studies for full clarification.

Keywords: Blueberry; long-term supplementation; healthy condition; prediabetes; hypercaloric diet; gut microbiota; hepatic mitochondria bioenergetics.

Resumo

A pré-diabetes, reconhecida como um estado de risco elevado para o desenvolvimento de diabetes mellitus tipo 2 (DMT2) e de complicações a ela relacionadas, é certamente uma fase privilegiada para implementar estratégias para prevenir ou retardar a progressão da doença para fases mais avançadas. Os estilos de vida pouco saudáveis, marcados pelo sedentarismo e hábitos alimentares desequilibrados, têm contribuído para a crescente incidência de várias doenças metabólicas, nomeadamente a pré-diabetes. Diversas evidências recentes apontam no sentido de que dietas desequilibradas, incluindo as hipercalóricas, contribuem para a disbiose da microbiota intestinal, que poderá estar envolvida na origem da desregulação metabólica. Embora uma ligação entre a disbiose e a pré-diabetes permaneça por provar, é hoje perfeitamente aceite que a adoção de regimes alimentares mais saudáveis é a primeira linha de intervenção terapêutica.

Nos últimos anos, temos assistido a uma mudança de atitude dos consumidores no sentido de privilegiar padrões alimentares mais saudáveis, de forma a evitar o aparecimento de certas doenças e garantir uma melhor qualidade de vida. Assim, o aumento da procura e do consumo de alimentos funcionais e suplementos naturais ricos em fitoquímicos, nomeadamente polifenóis, é uma tendência contemporânea. Contudo, doses elevadas de compostos fenólicos foram já associadas a efeitos adversos, nomeadamente a nível hepático, um assunto controverso dentro da comunidade científica que reforça a importância de estudos adicionais.

O mirtilo (BB) tornou-se popular, tanto para ser consumido sob a forma de alimento como de suplemento, em virtude dos seus alegados efeitos benéficos para a saúde (p.ex., antioxidante e pré-biótico) uma vez que são uma valiosa fonte de compostos bioativos (i.e., polifenóis e fibras). No entanto, as consequências precisas do seu consumo excessivo numa condição saudável ainda não estão definidas. Da mesma forma, persistem dúvidas no que diz respeito à presumível proteção do BB como intervenção nutracêutica eficaz na pré-diabetes, apesar de já terem sido descritas propriedades benéficas em várias doenças metabólicas, incluindo na DMT2.

Tendo em consideração estes fundamentos, os principais objetivos desta tese foram a avaliação do impacto de uma suplementação prolongada de BB numa condição saudável e na pré-diabetes, com particular destaque para a microbiota intestinal e o tecido hepático. O BB foi administrado sob a forma de sumo, numa dose de 25 g/Kg de peso corporal, durante 14 semanas, em duas condições experimentais: a) em ratos Wistar saudáveis e b) num modelo de pré-diabetes induzido com recurso a uma dieta hipercalórica.

Na condição saudável, as nossas observações corroboraram o esperado aumento da atividade antioxidante nos animais suplementados com BB, sem alterações do perfil metabólico. De forma algo surpreendente, não foram observadas modificações na composição e função da comunidade microbiana intestinal. A nível hepático, o BB desencadeou um cenário de adaptação mitocondrial sem alterações tecidulares, tanto estruturais como funcionais. De facto, constatámos uma acentuada remodelação da bioenergética mitocondrial, acompanhada de uma reprogramação da transcriptómica metabólica e uma repressão expressiva de genes relacionados com a inflamação. Coletivamente, estes resultados sugerem a ativação de uma resposta celular adaptativa a uma sobrecarga antioxidante desencadeada por um consumo prolongado de BB.

Relativamente ao impacto do BB na pré-diabetes, observámos uma prevenção das alterações metabólicas induzidas pela dieta hipercalórica. Aparentemente, esta proteção metabólica não pode ser explicada pelo impacto na microbiota intestinal. Em contrapartida, e em paralelo ao efeito antioxidante, observámos uma redução da esteatose hepática acompanhada por uma melhoria significativa da bioenergética mitocondrial do hepatócito, sugerindo que a mitocôndria possa ser uma peça-chave na progressão inicial da doença. Além disso, o consumo de BB modulou positivamente a expressão génica a nível hepático de alvos-chave da oxidação de ácidos gordos, sinalização de insulina, inflamação e cadeia respiratória mitocondrial, todos eles comprometidos nos animais pré-diabéticos. Coletivamente, estes resultados defendem que a melhoria da função mitocondrial hepática pode ser um mecanismo crucial através do qual o BB exerce o seu efeito protetor na pré-diabetes.

Em conclusão, os resultados desta tese sugerem que a suplementação prolongada de BB pode ser uma intervenção nutracêutica eficaz para prevenir a progressão da pré-diabetes, embora o impacto significativo na bioenergética mitocondrial hepática, em condição saudável, recomende estudos adicionais para cabal clarificação.

Palavras-chave: Mirtilo; suplementação prolongada; condição saudável; pré-diabetes; dieta hipercalórica; microbiota intestinal; bioenergética mitocondrial hepática.

CHAPTER 1

General introduction

(Part of this chapter was published in *Antioxidants 2021; 10(8): 1162; doi: 10.3390/antiox1008116;* and in *Obesity Reviews 2020; 21(12): e13081; doi: 10.1111/obr.13081*)

1. Prediabetes: a wake up call

1.1 Definition and diagnostic criteria

Prediabetes represents a stage of a mild hyperglycemia, broadly defined by blood glucose levels higher than normal but lower than diabetes thresholds, implying a high-risk for the overt type 2 diabetes mellitus (T2DM) and diabetes-related complications development [1, 2].

The term prediabetes has been largely controversial because slightly increased glucose levels was not reason for alarm since many prediabetic subjects do not necessarily progress to diabetic state, and this discussion still persists nowadays [2]. In this sense, alternative terminologies have been concomitantly used for this condition: the World Health Organization (WHO) use the term intermediate hyperglycemia [3] and an International Expert Committee (IEC) convened by the American Diabetes Association (ADA) prefers the "high-risk state of developing diabetes" rather than "prediabetes" [4, 5]. Moreover, diagnostic criteria for prediabetes are not also yet consensual and vary depending on the international organizations, as shown in Table 1.1. Prediabetes might be identified based on fasting plasma glucose (FPG), blood glucose levels 2 h after a 75 g oral glucose tolerance test (OGTT), or a glycated hemoglobin (HbA1c) test. This condition encompasses the clinical states defined as impaired fasting glucose (IFG), impaired glucose tolerance (IGT) or both. According to the WHO, the intermediate hyperglycemia can be diagnosed by two distinct states: IFG, defined as a FPG from 110 to 125 mg/dL (from 6.1 to 6.9 mmol/L), and a normal response to glucose load (<140 mg/dL or < 7.8 mmol/L); and IGT, defined as 2 h post-load plasma glucose concentration from 140 to 199 mg/dL (from 7.8 to 11.0 mmol/L), measured after a 75 g OGTT or a combination of both [3]. While WHO guidelines do not approved the use of increased HbA1c for defining prediabetes, the IEC recommended that an individual with HbA1c levels ranged between 6.0 to 6.4 % (from 42 to 46 mmol/mol) were at particularly high risk for diabetes and might be considered for diabetes prevention interventions [4, 6]. The ADA still use the term prediabetes and although applying the same thresholds established by WHO for IGT, uses a lower cutoff value for IFG, defined as FPG levels from 100 to 125 mg/dL (from 5.6 to 6.9 mmol/L) and/or recommend the HbA1c values from 5.7 to 6.4% (from 39 to 47 mmol/mol) as an additional diagnostic screening tool for prediabetes [5], which leads to higher prevalence rates compared with those defined by other guidelines [1, 7].

It is worth mentioning that many people with IGT are normoglycemic in their daily lives, and individuals with IFG or IGT may have normal or near normal HbA1c levels. Notwithstanding, considering any of these criteria to define prediabetes, individuals are at high risk for progression to T2DM and at risk for adverse outcomes [2, 8-10]. However, depending on the definition used for prediabetes, the magnitude of the major clinical outcomes differs [9].

Table 1.1 | Current prediabetes´ diagnostic criteria and terminology according to ADA, WHO and IEC.

Criteria	WHO	ADA	IEC
Terminology	Intermediate hyperglycemia	Prediabetes	High risk for T2DM progression
IFG (assessed using FPG)	110 – 125 mg/dL (6.1 – 6.9 mmol/L)	100 – 125 mg/dL (5.6 – 6.9 mmol/L)	NA
IGT (assessed 2 h after an OGTT)	140 – 199 mg/dL (7.8 – 11.0 mmol/L)	140 – 199 mg/dL (7.8 – 11.0 mmol/L)	NA
HbA1c	NA	5.7 – 6.4% (39 – 47 mmol/mol)	6.0 – 6.4% (42 – 46 mmol/mol)

ADA, American Diabetes Association; IEC, International Expert Committee; HbA1c, glycated hemoglobin; IGT, impaired glucose tolerance; OGTT, oral glucose tolerance test; NA, not applicable; WHO, Word Health Organization.

Other non-traditional serum biomarkers of hyperglycemia such as glycated albumin and fructosamine have a potential for identifying prediabetes. These markers strongly correlate with HbA1c and FPG [11, 12], predict macrovascular [13] and microvascular complications [14], and provide similar prognostic value to HbA1c concerning the risk of cardiovascular disease, end-stage renal disease, and diabetic retinopathy [15]. There is also compelling evidence for a role of glycated albumin and fructosamine as useful complementary or alternative tests when HbA1c testing is problematic or when there is divergence in glucose and HbA1c test results [14, 16]. However, these biomarkers have not been integrated into guidelines, and currently, there is no consensus on the use of glycated albumin or fructosamine in clinical practice for defining the glycemic status [16].

The lack of consensus regarding a single best definition of prediabetes continues to be a major challenge for the field and for clinical practice. It is important to recognize that due to an asymptomatic phenotype, prediabetes tend to remain undiagnosed for many years or people are only diagnosed opportunistically when tested for other conditions

[17]. IGT and IFG are associated with metabolic syndrome, which includes obesity (especially abdominal or visceral obesity), dyslipidemia and hypertension [18].

Prediabetes screening is critical given that the diabetes-related complications and morbidities are already present at the time of the clinical T2DM diagnosis. Indeed, long standing glucose imbalances is a cause of serious complications: the microvascular, such as peripheral neuropathy, retinopathy and nephropathy, which are generally classified into small vessel diseases [19-22], and the macrovascular complications, which encompass the large vessel diseases like peripheral vascular disease, coronary artery disease, stroke and other cardiovascular diseases [23-25]. These usually begin slightly, but in the long-term, it results in prolonged organ damage leading to overt disruption of organs function. Thus, timely detection of prediabetes and the implementation of early measures aiming to prevent or to mitigate the incidence of diabetes-related complications have been a priority focus of research during the last decades [18, 26].

1.2 Prevalence and risk factors

Prediabetes is a major public health challenge globally. As with diabetes and obesity, the prediabetes prevalence is increasing rapidly worldwide and continues to rise in parallel with increased cases of obesity and sedentary lifestyle [1, 27].

Prediabetes prevalence´ estimates vary greatly among the diagnostic criteria selected for evaluation (ADA or WHO), whether definitions are examined individually or combined, and also due to the population in study. While International Diabetes Federations (IDF) has no data about the prevalence of IFG, 373 million of adults worldwide are estimated to have IGT, a number that is predicted to increase to 548 million (representing about 8.6 % of the global adult population) by 2045 [28, 29]. In Europe, it is estimated that the number of people with IGT will rise from around 36.6 million (corresponding to 5.5% of global adult population) in the year of 2019 to 40.3 million people in 2045 (Figure 1.1) [28, 29].

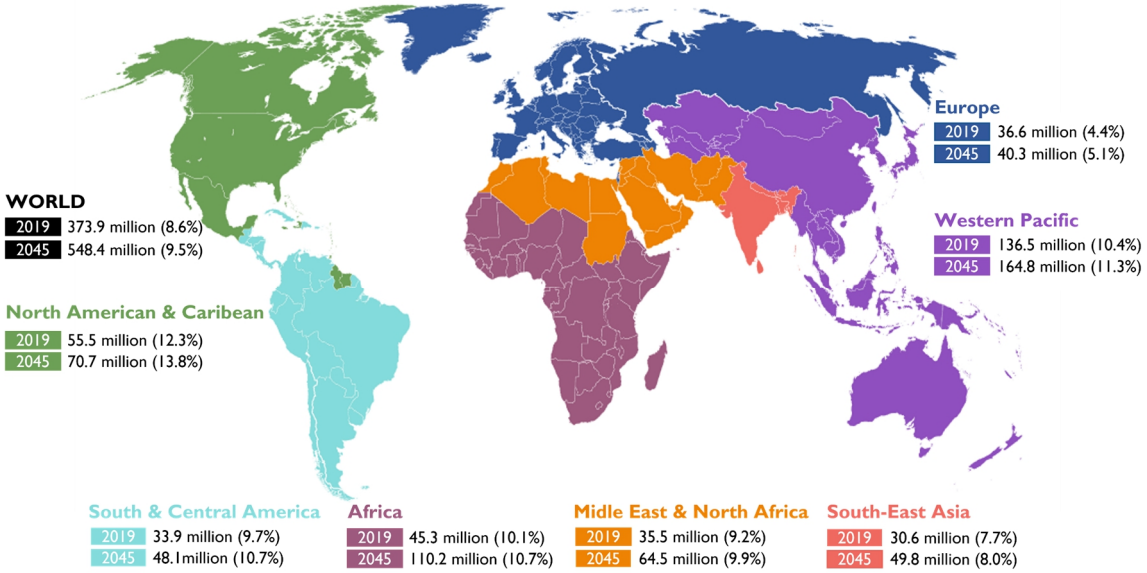

Figure 1.1 | Global prevalence estimates of IGT in adults (20-79 years) by IDF region in 2019 and 2045. Data source from International Diabetes Federation (IDF) Diabetes Atlas – 9th Edition [28]. Numbers represent the estimated number of people with impaired glucose tolerance (IGT) in each region. Percentages in parenthesis represent age-adjusted comparative regional prevalence estimates. (Adapted from Hostalek, 2019 [1]).

The recent statistics report by the United States Center for Disease Control and Prevention estimated that in 2018, 88 million of American adults, aged 18 years or older, were prediabetics, based on fasting glucose values of 100 to 125 mg/dL or HbA1c levels (5.7 to 6.4%) [30].

Regarding Portugal, the estimated prevalence of prediabetes in 2018 was 28.0%, corresponding to 2.1 million of the Portuguese adults, where: 10.6% (0.8 million) displayed IFG, 14.6% (1.1 million) presented IGT and 2.8% (0.2 million) of this population share both conditions (Figure 1.2) [1]. Furthermore, about 41.6% of the Portuguese adult population were estimated as prediabetic or diabetic, corresponding to 3.2 million of Portuguese adults [1].

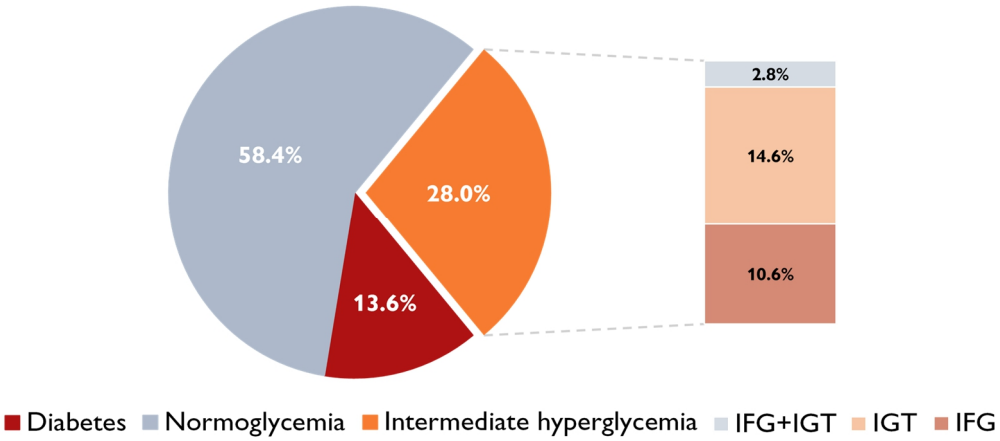

Figure 1.2 | Prevalence estimates of diabetes and intermediate hyperglycemia in Portugal in 2018. IFG, impaired fasting glucose; IGT, impaired glucose tolerance. (Adapted from Annual Report of the Portuguese National Diabetes Observatory, Edition 2019 [31]).

IFG and IGT prevalence vary between ethnic groups and in majority populations studied. Additionally, IFG is more prevalent in men, except in people older than 70 years, whereas IGT is slightly more common in women. The prevalence of IGT, more common than IFG, rises with age; however, in some populations there is a plateau in middle age. In contrast, the IFG prevalence reach a plateau in middle age (40-50 years), with the exception of European women where it rises until 70 years [26].

According to ADA expert panel, 70% of prediabetic individuals will develop overt diabetes within their lifetime and annually around 5–10% of prediabetic patients progress to T2DM and have increased risks for early development of diabetes-related complications. IFG and IGT have additive roles in predicting the risk of developing T2DM. According to the IDF, the cumulative incidence of T2DM progression is estimated to be between 26% and 50%, five years after diagnosis of IGT or IFG, respectively [28]. These values are alarming, and it means that the prevalence of T2DM is greatly increasing. Concurrently, a strong predisposition to development of chronic T2DM-related complications ensure that the disease has a huge economic impact on healthcare system, mainly due to hospitalizations, early disability, morbidity and reduced the overall quality of life, becoming a serious public health problem [2]. Nonetheless, it is important to note that some prediabetic subjects can also experience reversal to normal glucose tolerance [32].

Due to the etiology of the disease, the same multiple genetic and environmental factors that increase the risk of developing T2DM also contribute to the prediabetes

development. There are non-modifiable risk factors such as age, sex and genetic background (e.g. race and ethnicity); however, they are not the sole determinant for (pre)diabetes development [24, 33]. Indeed, the modifiable factors linked to the increased body weight (BW), dietary factors, and physical activity are more likely to impact glycemic traits [34, 35]. Obesity and sedentary lifestyle driven by unhealthy dietary patterns are etiological factors behind the dramatic rise in (pre)diabetes prevalence [27, 35, 36]. Moreover, in addition to the modern lives, based on high energy intakes and physical inactivity, other risk factors such as the high levels of psychosocial stress, depression and smoking, also contribute to the T2DM development [37, 38]. As the T2DM development is highly dependent on lifestyles modification, approaches towards implementing healthy lifestyle choices can have lasting impacts in early stage of diseases and can be effective in delaying or even preventing progression to T2DM.

1.3 Main pathophysiological features of prediabetes - emphasis on gut and liver paths

1.3.1 Insulin resistance, glucose intolerance, glucotoxicity and lipotoxicity

The transition from a normal state to prediabetes, and then to T2DM, is the consequence of a series of pathophysiological changes. Each of these changes makes the individual gradually more susceptible to the subsequent disruption of glucose homeostasis, which is mainly governed by insulin sensitivity of peripheral tissues and pancreatic β-cell function. In most subjects, insulin resistance - the inability of target cells to adequately respond to normal concentrations of insulin - is often the primary metabolic defect leading to the development of T2DM. In fact, insulin resistance can be observed several years before the appearance of any impairment in glucose homeostasis [2, 39]. As long as the pancreatic β-cell is able to increase insulin secretion to compensate for the progressive resistance to insulin action (compensatory hyperinsulinemia), glucose tolerance remains normal [40]. However, the increased workload associated with different levels of genetic susceptibility ends up leading to β-cell dysfunction, with the subsequent insufficient and delayed profile of insulin secretion, which in turn is unable to maintain glucose homeostasis, prompting the glucose intolerance. Consequently, insulin-resistant

individuals first develop postprandial hyperglycemia and subsequently develop fasting hyperglycemia. A state of chronic hyperglycemia contributes to a further suppression of insulin secretion from pancreatic β-cells and to worsen insulin resistance, leading to T2DM progression (Figure 1.3). Although there has been a significant debate in the past on whether insulin resistance or β-cell dysfunction is the primary driver in the pathogenesis of prediabetes as well as on their relative contributions to disease development [41], the current view is that both conditions dynamically influence each other and presumably synergistically exacerbate diabetes [40].

Increased peripheral insulin resistance and altered β-cell function lead to major biochemical alterations in metabolic tissues, including the skeletal muscle, liver, and adipose tissue, among other organs.

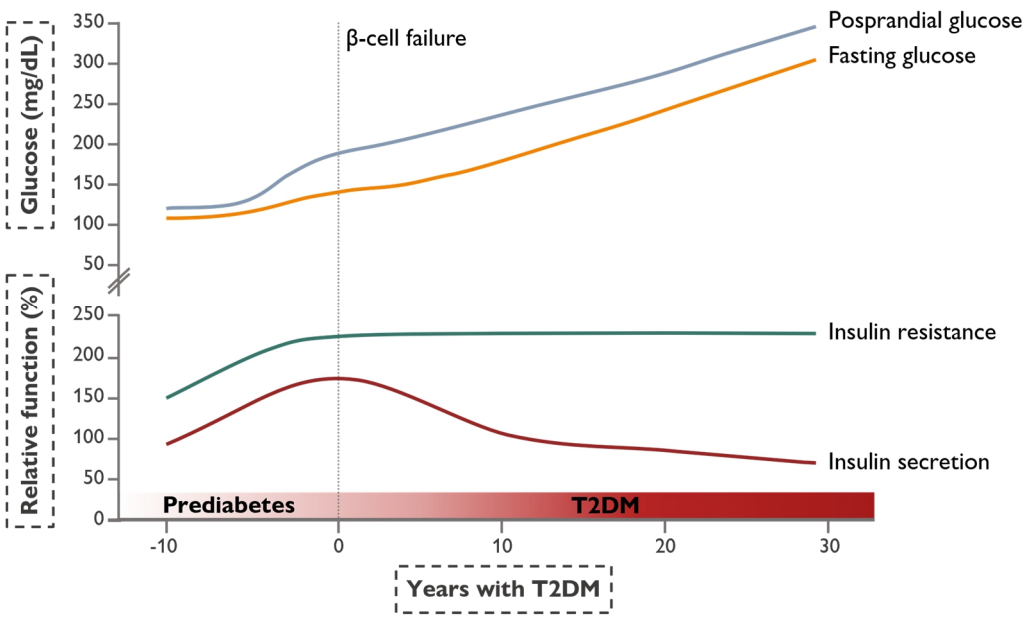

Figure 1.3 | Schematic representation of the natural history of T2DM. (Adapted from Perreault, 2019 [42]).

On a simplified view, the long-term exposure to high levels of glucose and free fatty acids (FFAs) promotes insulin resistance as well as impair β-cell function and survival through diverse, complex and interrelated mechanisms, which have contributed to the concepts of glucotoxicity, lipotoxicity and glucolipotoxicity [43-45]. The combination of lipotoxicity and glucotoxicity creates an environment for the downward spiral leading to β-cell failure [44]. These molecular mechanisms involved in the progression of

(pre)diabetes include oxidative stress and low-grade chronic inflammation [46-48], which can be both a cause and a consequence of hyperglycemia and hyperinsulinemia.

The chronic exposure to hyperglycemia leads to oxidative stress through various biochemical pathways, such as increased activation of the polyol and hexosamine pathways, increased formation of advanced glycation end-products and activation of classical isoforms of proteins kinase C (PKCs), which induce increased production of mitochondrial reactive oxygen species (ROS), nonenzymatic glycation of proteins and glucose auto-oxidation. Activation of these pathways promotes glucotoxicity, which may cause cellular injury and results in an irreversible deleterious effect on β-cell function and survival, such as reduced insulin secretion and action [43]. On the other hand, hyperglycemia impairs the endogenous antioxidant defense systems both in prediabetes and diabetes [49, 50]. A large body of experimental and clinical studies has established a close link between oxidative stress and prediabetes [51-53]. In fact, prediabetic states have been associated with an increase in the levels of oxidative stress-induced DNA damage marker 8-hydroxy-2'-deoxyguanosine (8-OHdG), lipid peroxidation products measured as thiobarbituric acid reactive substances (TBARS) and malondialdehyde (MDA), protein oxidation products such as nitrotyrosine and carbonyl levels, and also lowered activity of antioxidant enzymes and endogenous antioxidants, such as myeloperoxidase and reduced glutathione (GSH) [51, 53-55].

Paralleling with hyperglycemia, the prolonged exposure of FFAs, resulting from chronic, excessive calorie intake, also have negative cellular consequences for β-cell function and lead to the accumulation of toxic lipid intermediates within the pancreatic islet cell, thus fostering lipotoxicity [45]. In addition to insulin resistance, high levels of FFAs also contribute to β-cell injury through endoplasmic reticulum (ER) stress and inflammation, intracellular accumulation of toxic fatty acids intermediates (e.g. ceramide and diacylglycerol (DAG)), as well as the ROS formation [44, 45]. Some of the mechanisms by which glucotoxicity and lipotoxicity contributes to insulin resistance at the subcellular level will be expanded latter in this thesis.

As mentioned above, another important player in the pathophysiology of prediabetes is inflammation. During the transition from normal glucose tolerance to T2DM, having prediabetes (or intermediate hyperglycemia) as a middle ground, there is a gradual accumulation of pro-inflammatory factors, ultimately leading to a state of chronic low-grade inflammation, which contributes to the progression of diabetes and its vascular

complications. Various studies have reported higher concentrations of cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and other inflammatory markers, such as C-reactive protein (CRP) and plasminogen activator inhibitor-1 (PAI-1), in the blood of prediabetic patients relative to healthy subjects [47, 48, 51, 56]. Under these conditions, three major inflammation-associated signaling pathways are activated, namely nuclear factor-kappa B (NF-κB), mitogen-activated protein kinase (MAPK), and the Janus kinase/signal transducers and activators of transcription (JAK-STAT) [57]; each pathway can promote tissue inflammation, particularly in the adipose tissue, liver, muscle and pancreas [58]. As a result, pro-inflammatory macrophages are recruited predominantly to the adipose tissue, leading to cytokine and chemokine secretion into the circulation, promoting further inflammation in other tissues [59]. Additionally, the activation of pro-inflammatory mediators (e.g. inhibitor of nuclear factor kappa B kinase-subunit beta (IKK-β), c-Jun N-terminal kinase (JNK) and PKCs) can negatively interfere with insulin signaling [60, 61]. Moreover, the accumulation of FFAs may directly activate toll-like receptors (TLRs), mediating inflammatory signals via IKK-β/ NF-κB, and JNK activation, thus downregulating insulin signaling [62].

Interestingly, the pro-inflammatory state in prediabetes appears to result preferentially from insulin resistance rather than from impaired β-cell function, as increased levels of high-sensitivity CRP (hs-CRP) and PAI-1, common markers of inflammation, were found elevated in prediabetic individuals who are predominantly insulin resistant but not in those with a primary defect in β-cell function [63]. In addition, adiponectin is an anti-inflammatory adipokine known to be inversely linked with insulin resistance, lipid accumulation, inflammation, and non-alcoholic fatty liver disease (NAFLD) [64]. Adiponectin may reduce ceramide levels in the liver by activation of ceramidase activity to promote insulin sensitivity [65]. The two adiponectin receptors, adiponectin receptor 1 (ADIPOR1) and adiponectin receptor type 2 (ADIPOR2) are involved in the glucose metabolism that links adiponectin to the amelioration of insulin resistance. Moreover, low hepatic mRNA expression of adiponectin and ADIPOR2 (mainly expressed in the liver) were associated with increased steatosis [64].

Thus, a better understanding of the exact sequence of pathophysiological events underlying progression of prediabetes to T2DM might allow the development of improved therapeutic and preventive strategies.

1.3.2 The gut path

1.3.2.1 Main metabolic functions of the gut

The intestine is a crucial organ for the digestion and energy extraction from nutrients, namely from lipids, carbohydrates and proteins, using a diverse enzymatic machinery that includes lipases, glucosidases and peptidases, among others. However, the contribution of gut for metabolism regulation goes beyond this primary role, extending to the level of the organism [66]. In fact, meal ingestion and digestion/absorption phases determine the two main functional states that regulate energy homoeostasis: the catabolic (interdigestive/postabsorptive) and the anabolic (digestive/absorptive). In addition, since the gastrointestinal tract (GIT) is the port of entry for water and salts, the gut is pivotal for fluid and electrolyte homoeostasis, which is another key area of metabolism because governs cells' ability to access nutrients and energy. In addition, the gut plays major endocrine roles which have been increasingly explored in the last decades, particularly those related with regulation of glucose homoeostasis and of appetite/satiety [66].

The gut endocrine system was firstly explored regarding the role of gut hormones in the peripheral regulation of GIT function, such as secretin on pancreatic secretion. The term "secretin" used to describe potential mediators regulating pancreas secretion was later replaced by the term "incretin", when became evident that factors released from the gut are able to stimulate insulin secretion [67]. This incretin effect, which is responsible for 50-70% of the total insulin secreted by the pancreas after glucose ingestion, is carried out by two peptides secreted in response to food ingestion: glucagon-like peptide-1 (GLP-1), mainly expressed in mucosal enteroendocrine L-cells located predominantly in the distal intestine (ileum and colon), and glucose-dependent insulin releasing polypeptide (GIP, formerly called gastric inhibitory polypeptide), released from the duodenum and jejunum. The GLP-1-mediated effects have been translated into antidiabetic pharmacological approaches using inhibitors of GLP-1-degrading enzyme dipeptidyl peptidase-4 (DPP-4) and analogues of GLP-1 [68]. The metabolic roles of GLP-1 go far beyond glycemic control since influences BW and regulates gastric emptying and satiety, via crosstalk communication between the gut and other organs and tissues, clearly illustrating the complexity of gut-dependent regulation, not only within the digestive organs, but also with regard to distant organs [69]. Many other peptides, such as peptide YY (PYY) and ghrelin, are also produced and secreted by the gut, influencing key metabolic

functions by interaction with various body organs (i.e. pancreas, liver, adipose tissue and brain), including the regulation of glucose metabolism, energy storage, lipolysis, appetite and satiety, thus showing major implications of the gut in metabolic diseases [70, 71]. In addition, it is nowadays clearly recognized that the intestinal microbial community (also referred as gut microbiota: GM) has important metabolic, structural and protective functions that will be addressed more prominently considering the marked prebiotic effect of blueberry (BB), later discussed.

Hundreds of trillions of microorganisms inhabit the human body, the overwhelming majority of which are bacteria from two main phyla (Bacteroidetes and Firmicutes). This complex bacterial community is present in many body fluids and surfaces but has a huge implantation in the GIT, in particular in the large intestine. GM can be seen, in a way, as a supra-organism that performs crucial functions for host homeostasis (co-metabolism), which include metabolic and energetic effects, protection against pathogens, maintenance of intestinal barrier integrity and shaping/education of immune system [72, 73]. In metabolic terms, the intestinal microbiota contains an enzymatic machinery that extracts energy from dietary components (namely cellulose digestion, functions that the host cannot do by itself) and deposit it in fat stores [74].

The composition and function of the intestinal flora are dynamic and strongly affected by the properties of the diet, including the amount and composition of lipids, carbohydrates, and proteins, which can influence the host's physiology through interaction with the GM [75, 76]. Conversely, the intestinal bacteria affects the metabolism of dietary constituents [77]. In this framework of strong interaction between the diet and the gut microbial community, it is not surprising that metabolic diseases (such as obesity and diabetes, highly influenced by eating habits and the amount of energy consumed), could be associated with changes in microbiota profile.

1.3.2.2 (Pre)diabetic gut microbiota dysbiosis

Disturbances in the composition and/or functions of GM cause a situation referred as dysbiosis which has been associated not only with disorders of the GIT, but also with extra-intestinal diseases, including metabolic disorders and in particular obesity and diabetes [78, 79]. Evidences from animal and human studies suggests a strong association between diabetes phenotypes and changes in the relative abundance and richness/diversity

of the intestinal bacterial community [80]. Although there is no consensus regarding which species are altered in diabetic patients, some studies reported a decline in butyrate-producing bacteria (eg. *Clostridiales sp*. SS3/4, *Eubacterium rectale* and *Faecalibacterium prausnitzii*) and an increase in several opportunistic pathogens (eg. *Bacteroides caccae*, *Clostridium hathewayi*, *Clostridium ramosum*, *Clostridium symbiosum* and others) [81].

The influence of this community of microorganisms on the host's metabolism and immune response can be mediated by metabolites produced by it, such as fatty acids, bile acids and other intermediary products of metabolism (such as trimethylamine), and by pro-inflammatory factors derived from bacteria, such as lipopolysaccharide (LPS) [82-85]. Several mechanisms have been proposed to explain the possible association between the intestinal microbiota and T2DM, including energy metabolism, barrier permeability, inflammation, and the immune system response [78, 79, 86].

The intestinal microbiota converts dietary polysaccharides by hydrolysis and fermentation into monosaccharides and short-chain fatty acids (SCFAs), which are used as a source of energy for the host, including by colonocytes, thus protecting intestinal barrier structure [87]. Between 5% and 10% of total human body energy resources come from the large intestine [88], in particular from SCFAs (namely from acetate, propionate and butyrate), which participate in several important processes for the host, including lipid (e.g. lipogenesis and cholesterol synthesis) and glucose (e.g. gluconeogenesis) metabolism [83]. In addition to being metabolic substrates, SCFAs act as signaling molecules, through G-protein-coupled receptors (GPRs), namely GPR41 and GPR43, whose energy-regulating effects are dependent on the microbiota [89, 90]. Activation of GPR43 protects against diet-induced obesity in animal models, with an increase in GLP-1 due to activation in L-cells, as well as increased anti-lipolytic activity and improved lipid and glucose metabolism by activation in white adipose tissue [89, 91-95]. GRP41 has also been shown to regulate metabolism and increase the production of the PYY through interaction with GM [90]. Butyrate and propionate also showed the ability to activate peroxisome proliferator-activated receptor gamma (PPAR-γ) and thus modulate lipid metabolism by increasing energy expenditure, reducing BW and decreasing hepatic triglycerides (TGs) [96-98]. Since SCFAs have a positive impact on metabolic health, with benefits in terms of obesity and diabetes [83], GM dysbiosis associated with the reduction of SCFAs-producing bacteria can contribute to T2DM progression.

The intestinal barrier, made up of several layers (including the physical barrier composed of GM, mucus, epithelial cells with tight junctions that regulate permeability, and innate and adaptive immune cells), plays an important role in health and disease. Excess body mass index (BMI), consumption of high-fat diets (HFD) and increased exposure to FFAs, among other situations, can cause disturbances in the structure of the intestinal wall, particularly in tight junctions, which seems to interfere with the release of peptides that play an important role in regulating appetite and satiety, including PYY and GLP-1/2 [99, 100]. However, the most relevant aspect seems to be the impact on intestinal permeability (Figure 1.4).

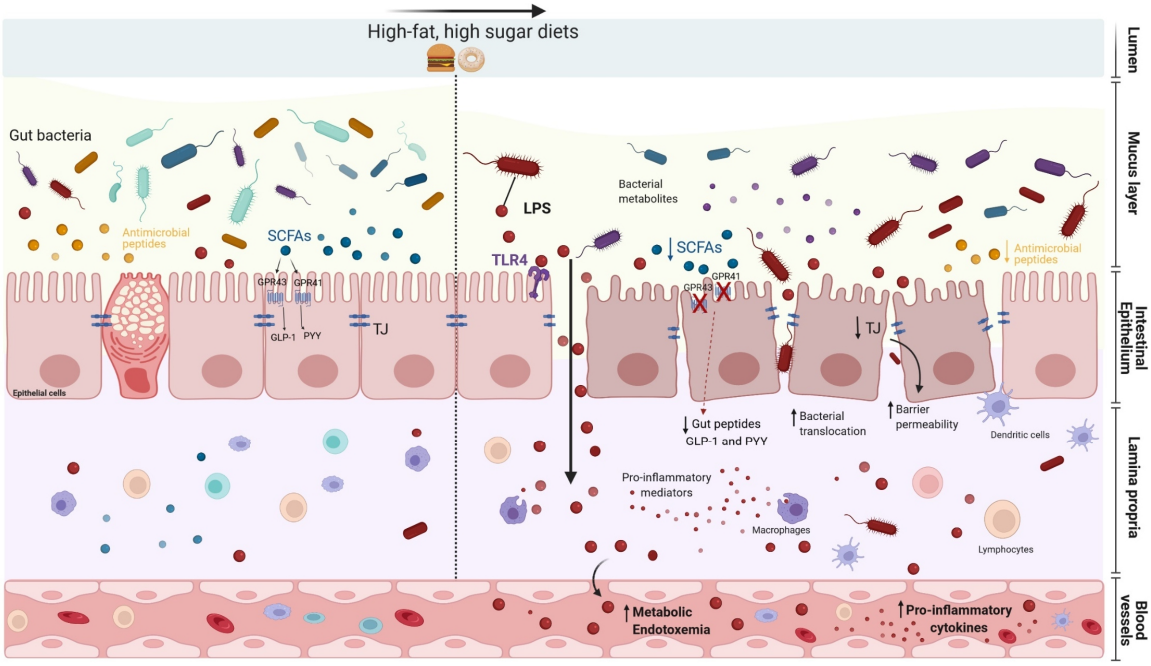

Figure 1.4 | Schematic overview of the influence of high-fat diets feeding on gut microbiota. High-fat diets (HFD) are associated with changes on gut microbiota (GM) composition towards dysbiosis. This leads to alterations in intestinal barrier, namely impaired distribution of tight junctions (TJ), favoring the translocation to circulation of bacteria and bacterial components, such as lipopolysaccharide (LPS), which results in metabolic endotoxemia and systemic low-grade inflammation. In addition, a decrease in short-chain fatty acids (SCFAs) may result in a reduction of the gut hormones secretion, such as glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), which could affect glucose homeostasis and insulin sensitivity. GPR, G-protein-coupled receptors; TLR4, toll-like receptor 4. (Adapted from Yang et al. 2021 [100], and created with *BioRender.com*).

When the intestinal barrier is damaged, an increase in permeability can occur, which allows the translocation of bacterial products to the host's circulation, which can result in a pro-inflammatory state, also called metabolic endotoxemia because it results from the effect of endotoxins [101]. The most prevalent of all is LPS, a structural component of the

outer membrane of gram-negative bacteria, which can enter the host's circulation, where it is recognized by TLRs on the surface of immune cells (including in macrophages) and other cells (namely hepatocytes and adipocytes) [102].

Overall, accumulating evidence indicates that GM is involved in host metabolism by increased energy extraction, immune system modulation, and altered lipid and glucose metabolism, all which have been demonstrated to contribute to progression of prediabetes to T2DM. Taking this into account, over the past few years, many studies have attempted to answer the following questions about GM and the onset and progression of T2DM: i) When does dysbiosis begin? ii) Is there a state of prediabetic (or early) dysbiosis? And, most important of all: iii) Is dysbiosis a cause of the metabolic imbalance leading to prediabetes, which can then progress to diabetes, or is another consequence of it?

A recent study from Wu and colleagues analysed and compared GM of individuals with isolated IFG, isolated IGT, combined glucose intolerance (CGI), and treatment-naïve T2DM patients [86]. They found that GM changes are already present in prediabetic states, being more pronounced in subjects with CGI and strongly linked to insulin resistance. These evidences corroborate earlier studies [81, 103, 104] but extends the previous knowledge. In fact, apart from confirming differences between the IFG and IGT groups, with more prominent bacterial alterations in IGT subjects (notwithstanding comparable BMI between IFG and IGT groups) [86], they showed that this profile was independent of age, sex, and BMI, as well as of metformin treatment, which is a major modulator of microbiota and, thus, an important confounding factor [105-107].

Recent longitudinal studies reported a robust association between gut microbiome and insulin resistance and identified distinct interactions microbe-host in insulin-sensitive versus insulin-resistant subjects [108, 109]. In CGI subjects, circulating levels of the microbial metabolite imidazole propionate were augmented and the microbial ability to produce branched-chain amino acids (BCAA) was also increased [86]. Since both type of metabolites can affect insulin signaling via mammalian target of rapamycin (mTOR) complex 1 (mTORC1) [110, 111], these effects might be suggested as causal links between the microbiota and insulin resistance.

It has been also shown that an individual's glucose response after a meal is influenced by a combination of their GM composition and host physiology [112, 113]. Several studies have reported decline of butyrate-producing bacteria in prediabetes cohorts of distinct

ethnicities [81, 103-105]. The study of Wu and colleagues suggests that the loss of butyrate producers and genes for butyrate synthesis from both carbohydrates and proteins occurs already in prediabetes and to a greater extent in people with IGT and CGI [86]. These findings suggest another causal link between GM (butyrate producers and/or butyrate itself) and glucose intolerance/progression to T2DM.

Overall, the connection of GM and prediabetes not only provides a clue to monitor the natural course of diabetes, but also raises the possibility of target intestinal microflora as an early strategy to prevent or delay the development of T2DM. There are several strategies capable of modifying the GM, including prebiotics, probiotics, synbiotics and post-biotics, in addition to antibiotics and other drugs, including the first-line oral antidiabetic agent – metformin [114-118]. Indeed, renewal of GM community able to produce SCFAs could improve glucose tolerance and insulin sensitivity as will be further discussed for BB.

1.3.3 The liver path

1.3.3.1 Main metabolic functions of the liver

In addition to being the main detoxifying organ of the body, the liver also plays a fundamental role in maintaining energy and metabolic homeostasis. The liver is a major regulator of glucose and lipid metabolism, which are both metabolic processes tightly coupled to fluctuations in nutrient availability (e.g. fed vs fasted state) and strongly regulated by the pancreatic hormones insulin and glucagon as well as by various intracellular signaling pathways and transcription factors [119-121]. In addition, the liver expresses several nutrient- and energy-sensing pathways, including the sirtuins (SIRT1-7) and AMP-activated protein kinase (AMPK), which are activated in the fasted state, and insulin/insulin-like growth factor-1 (IGF-1) and the mTOR, which are activated in the fed state [122-124]. These pathways play an essential role in the maintenance of hepatic energy homeostasis, by regulating downstream metabolic processes.

Because glucose is a readily available energy source for cells, the maintenance of blood glucose levels within a relatively narrow range during periods of nutrient shortage or excess is critical for survival. The liver plays a major role in maintaining whole body glucose homeostasis by regulating several metabolic processes involved in glucose transport, oxidation, storage and production. In the fed (anabolic) state, glucose is taken up by

hepatocytes via glucose transporter-2 (GLUT-2) and used to produce energy through glycolysis in the cytoplasm, and pyruvate is subsequently oxidized in the mitochondria to generate adenosine triphosphate (ATP) through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS). Excess glucose is stored within the liver in the form of glycogen, and this process – glycogenesis - is stimulated by insulin. During this period, insulin binds to its receptor (InsR), which promote the phosphorylation of intracellular substrate such as insulin receptor substrates (e.g. IRS-1, IRS-2) leading to activation of phosphatidylinositol 3-kinase (PI3K), and protein kinase B (also known as Akt). The Akt activation inactivates glycogen synthase kinase (GSK3), allowing dephosphorylation and activation of glycogen synthase, which stimulates glycogen synthesis. In addition, insulin suppresses gluconeogenesis in the liver by decreasing the expression and activity of several key enzymes controlling the gluconeogenic pathway such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) through Akt and forkhead box protein-O1 (FOXO1)-dependent mechanisms [120, 121, 125]. In the fasted (catabolic) state, the liver increases hepatic glucose production (HGP) to provide cells with glucose and to restore normoglycemia. This is achieved initially through the hydrolysis of glycogen stores with the release of glucose, via glycogenolysis. Upon glycogen depletion, the liver switches to gluconeogenesis, a process of glucose synthesis from non-carbohydrate precursors, such as lactate, pyruvate or glycerol, and this process is stimulated by glucagon and inhibited by insulin [125].

Hepatic lipid metabolism encompasses fatty acid uptake, synthesis and oxidation, storage, and secretion, which requires a well-orchestrated balance between these processes, dietary intake and energy expenditure [126]. Insulin plays a crucial role in long-term energy storage in the form of fat. Upon food ingestion, insulin promotes the conversion of excess carbohydrates into fatty acids (*de novo* lipogenesis, DNL), through upregulation and activation of sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP), that are then esterified into TGs for storage as lipid droplets; suppresses mitochondrial fatty acid β-oxidation; and inhibits TGs secretion in the form of very low-density lipoproteins (VLDL) into the blood [127, 128]. These two transcriptional regulators, ChREBP and SREBP-1, have important roles in the nutritional regulation of lipogenesis. These factors are involved in the regulation of the expression of genes of several key enzymes involved in lipid and glucose metabolism such as pyruvate kinase (PK), acetyl-CoA carboxylase (ACACA), fatty acid synthase (FASN) and stearoyl-CoA desaturase 1 (SCD1) [120].

Excessive dietary fat will also allow for increased fatty acid uptake from plasma into hepatocytes via specialized transporters, including fatty acid transporter proteins (e.g. FATP2, FATP5), fatty acid binding protein (FABP), and fatty acid translocase/cluster of differentiation 36 (FAT/CD36). Under fasting conditions, fatty acids released by adipose tissue through lipolysis are transported to the liver and then translocated into the mitochondrial matrix by carnitine palmitoyl transferase 1 (CPT1), where they are oxidized for ATP production or alternatively condensed into ketone bodies, namely into acetoacetate and 3-hydroxybutyrate (3-HB). CPT1 which mediates the transfer of activated long-chain fatty acid into the mitochondrial matrix towards oxidation, is the rate-limiting enzyme in mitochondrial fatty acid β-oxidation. The ketone bodies synthetized by liver from fatty acid β-oxidation-derived acetyl-coenzyme A (Ac-CoA) readily diffuse into the circulation and are used peripherally as an energy by extrahepatic tissues such as the brains, heart, kidney and skeletal muscle [129, 130]. These alternative fuels are not considered a part of the true triglycerides pool of energy, however become necessary when there is a limited availability of glucose. Moreover, ketones can also serve as anabolic substrates in the liver [129].

Transcription factors such as PPARs (i.e. PPARα, PPARγ, PPARδ), liver X receptor (LXR) and farnesoid X receptor (FXR) are also important regulators of gluconeogenesis and lipid metabolism, controlled by different fatty acids [120]. PPARα is activated by fatty acids released in the fasting state, and plays an essential role in the metabolic adaptation of liver to fasting situations by inducing the genes for mitochondrial and peroxisomal fatty acids oxidation as well as those for ketogenesis in mitochondria, such as the acyl-CoA dehydrogenase (ACAD) family, mitochondrial hydroxymethyl glutaryl-CoA (HMG-CoA) synthase and acyl-CoA oxidase (ACOX) [120, 130].

Indeed, disturbances in hepatic glucose and lipid metabolism similarly contribute to the development of metabolic diseases, including T2DM and NAFLD [131]. Furthermore, as described above, mitochondria are central organelles in hepatic metabolism, regulating the flow of metabolites in the cell in response to changes in energy demand and supply. Alterations in mitochondrial function also play a critical role in the pathogenesis of insulin resistance and hepatic steatosis, which will be explained in more detail below.

1.3.3.2 (Pre)diabetic hepatic steatosis

Hepatic steatosis – the hallmark and earliest feature of NAFLD – is defined as an excessive accumulation of TGs in the cytoplasm of hepatocytes due to imbalances in the processes that maintain the normal hepatic fatty acid metabolism, and often occurs in the setting of overnutrition and obesity [132, 133]. Conceptually, the onset of hepatic steatosis results from lipid acquisition pathways (uptake of fatty acids and DNL) exceeding or not being compensated by possible increases in lipid disposal pathways (mitochondrial fatty acid β-oxidation and VLDL secretion) [132, 133].

Previous studies have investigated the potential sources of increased storage of hepatic TGs in NAFLD patients and suggested that it results predominantly from increased adipose tissue lipolysis, DNL, and possibly VLDL export [134, 135], with scarce *in vivo* data to address the role of mitochondrial metabolism in the pathogenesis of hepatic steatosis and NAFLD. However, a growing body of evidence suggests that mitochondrial dysfunction may play a role in these disorders of abnormal lipid storage [136]. Possible mechanisms that explain the mitochondrial dysfunction in NAFLD include inefficient fatty acid β-oxidation, excessive production of ROS and pro-inflammatory factors, and altered expression of the peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), a master regulator of mitochondrial biogenesis and function [137]. These mechanisms connecting mitochondrial dysfunction with insulin resistance will be further better discussed in subchapter 1.3.4.

NAFLD is currently the most common liver disease worldwide, with a global prevalence estimated at 25% in the general population and greater than 70% in the high-risk groups of obese subjects and T2DM patients [138]. However, the pathophysiological relationship between hepatic steatosis and T2DM is complex and most likely bidirectional. While steatosis increases the risk of developing T2DM, the latter has also the potential to promote the progression of simple steatosis to more aggressive forms of NAFLD, such as non-alcoholic steatohepatitis (NASH) and cirrhosis [139, 140]. To date, only a few studies have examined the prevalence of fatty liver in prediabetes, but the available information indicates that it can range from 40% up to 60%, depending on the characteristics of the population studied and the diagnostic method and surrogate marker used [141-144].

Collectively, the data presented above support the notion that hepatic steatosis is a favourable biological milieu for the development and progression of (pre)diabetes [145].

Several potential mechanisms could explain the association between hepatic steatosis and the onset of prediabetes, including increased levels of hepatokines and the presence of hepatic insulin resistance [146, 147].

Hepatic insulin resistance is largely characterized by defects in the pathway of insulin signaling (Figure 1.5).

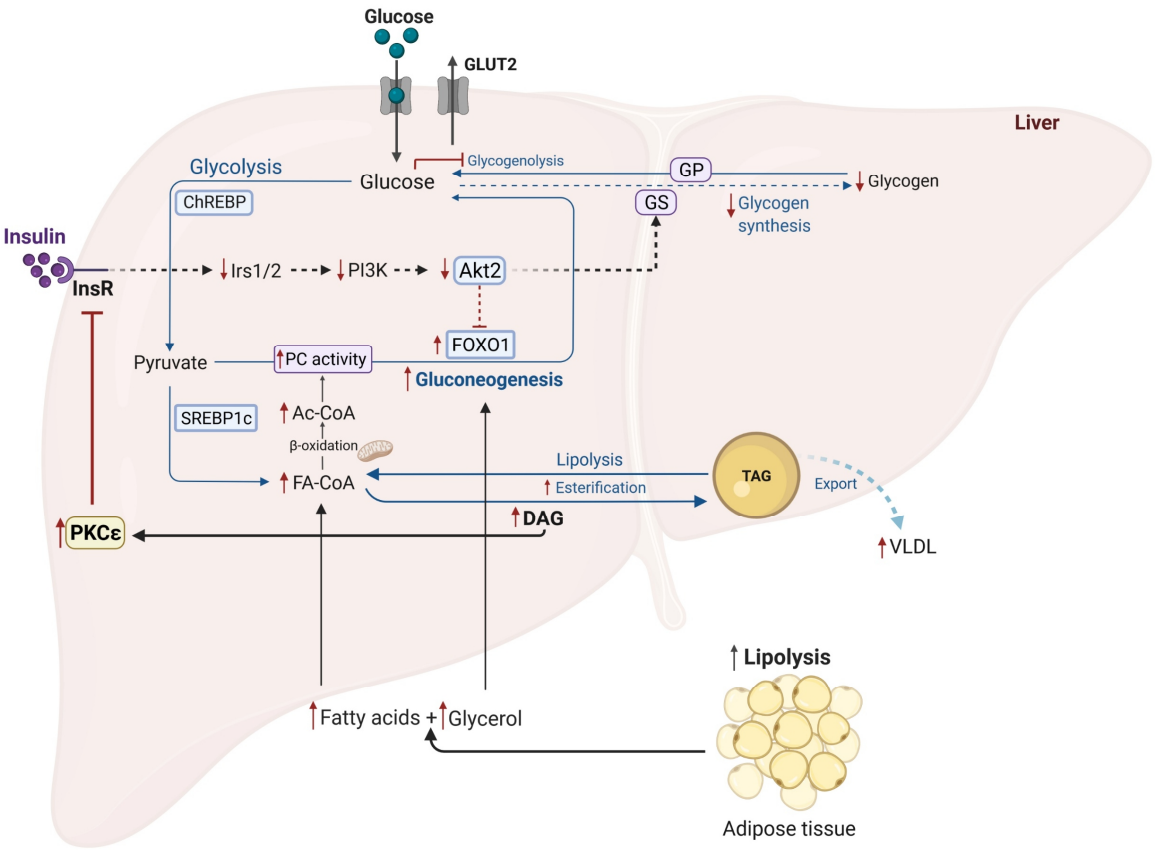

Figure 1.5 | Molecular events involved in hepatic insulin resistance. Adipose tissue inflammation and adipose insulin resistance results in increased rates of lipolysis and increased rates of fatty acids and glycerol delivery to the liver despite high plasma concentrations of insulin. Lipid-induced hepatic insulin resistance increases hepatic levels of diacylglycerol (DAG), which results in the activation of protein kinase Cε (PKCε), thereby direct impairing hepatic insulin signaling through PKCε-dependent phosphorylation of insulin receptor (InsR) at Thr1160. This inhibits the InsR-dependent stimulation of hepatic glycogen synthesis in response to insulin. Continued delivery of non-esterified fatty acids (NEFAs) and glycerol to the liver promotes hepatic lipid accumulation and gluconeogenesis by increasing hepatic acetyl-CoA (Ac-CoA) contents, leading to allosteric activation of pyruvate carboxylase (PC) activity and flux that increases hepatic gluconeogenesis. Impaired net hepatic glycogen synthesis and unrestrained hepatic gluconeogenesis together lead to increases in hepatic glucose production (HGP). Akt2, protein kinase B isoform 2; ChREBP, carbohydrate-responsive element-binding protein; FoxO1, forkhead box protein-O1; IRS1/2, insulin receptor substrate 1/2; GLUT-2, glucose transporter-2; GP; glycogen phosphorylase; GS, glycogen synthase; PI3K, phosphatidylinositol 3-kinase; SREBP1c, sterol regulatory element-binding protein-1c; TAG, triacylglycerol; VLDL, very low-density lipoprotein. (Adapted from Samuel et al. 2016 [148], and created with *BioRender.com*).

A blunted InsR phosphorylation, accompanied by the suppression of IRS transcription and a loss of IRS associated PI3K activity, results in impaired insulin signal transduction [121, 127]. Thus, the capacity of insulin to suppress gluconeogenesis fails, which promotes increased HGP resulting in fast hyperinsulinemia that is accompanied by a decrease in glycogen synthesis by the liver in the postprandial state [125]. Moreover, insulin resistance is often accompanied by hepatic steatosis and NAFLD, reflecting at least in part, increased hepatic lipogenesis yielding by deregulation of lipid metabolism. In turn, this ectopic lipid accumulation may also impair insulin signaling, via activation of PKCε and JNK1-dependent pathways [146].

Importantly, it remains to be clarified whether it is the insulin resistance that cause the accumulation of excess lipid droplets in the liver or if it is the accumulation of TGs (or their intermediates) that causes the hepatic or systemic insulin resistance [149]. Nonetheless, there is now compelling evidence suggesting that increased liver fat may precede and even act as an independent risk factor for the development of impaired glucose metabolism as well as T2DM. In longitudinal studies, the prevalence of newly diagnosed impaired fasting glucose and T2DM was significantly higher in the participants with, versus without, fatty liver [150-152]. Therefore, it may be more beneficial to predict IFG, a prediabetic status, rather than T2DM itself. Future studies need to address whether reducing hepatic steatosis in prediabetic subjects delays the onset and progression of T2DM.

1.3.4 Mitochondria as a central target in (pre)diabetes

1.3.4.1 Mitochondrial structure and function

Mitochondria, the main energy-producing organelles of the cells, are a double membrane structure composed of an outer mitochondrial membrane (OMM), an intermembrane space (IMS), an inner mitochondrial membrane (IMM) and a protein-rich matrix responsible for distinct metabolic pathways [153-155].

The double membrane provides strict transport regulation for metabolites and ions both into and out of the mitochondria. The OMM separates the mitochondria from the cytosol and contains large aqueous protein channels formed by porins such as voltage-dependent anion channels which makes it permeable to ions and molecules of up to

around 5 kDa, allowing its free diffusion across the OMM [156]. The OMM can associate with the ER membrane in a structure called MAM (mitochondria-associated ER membrane). This is important in ER-mitochondria calcium (Ca^{2+}) signaling and is also involved in the transfer of lipids between the ER and mitochondria.

The highly convoluted IMM is highly impermeable to ions and hydrophilic molecules creating a barrier between the matrix and IMM, but contains specific transport proteins (e.g. for respiratory substrates, inorganic phosphate, adenosine diphosphate (ADP) and ATP), providing the stringency of mitochondria transport in and out of the matrix, essential for preventing uncoupled proton translocation and for maintaining the electrochemical gradient required for mitochondrial respiration [155]. The IMM forms multiple invaginations into the matrix compartments, the so-called cristae, responsible for an increase membrane surface area, where is housed the main enzymatic machinery that form the OXPHOS system [155]. IMS contents are similar to cytosol because the porins present in OMM allow free movement of ions and small molecules. The most abundant protein found is cytochrome c (Cyt c), responsible for shuttle electrons from complex III to complex IV and release into the cytoplasm during apoptosis [156]. In addition, the IMS also contains an important antioxidant defense system including the glutathione redox buffer, crucial for redox homeostasis, among others [157].

The mitochondrial matrix is the space enclosed by the IMM and its composition is different from that of cytosol and IMS, due to the IMM impermeability. The matrix contains the metabolites and enzymes involved in central biochemical processes, such as fatty acid β-oxidation, the Kreb´s cycle or TCA cycle, OXPHOS, amino acid and ROS metabolism [158, 159]. These metabolic pathways are closely associated with the electron transport chain (ETC) and the ATP production through ATP synthase contained in the IMM. Moreover, mitochondrial genetic system is also found in the matrix [159, 160].

Mitochondria possess their own circular DNA (mtDNA) and its genome includes 37 genes: 13 polypeptides, two ribosomal RNA and 22 transfer RNAs [153, 160, 161]. All 13 polypeptides are essential components of the core subunits of respiratory chain complexes (I, III and IV) or the F1F0-ATP synthase, whereas the remaining subunits are encoded by nuclear DNA. Complex II is entirely encoded by nuclear DNA. The other mitochondrial proteins required for OXPHOS but also to other vital mitochondrial functions are encoded from the nucleus, translated in cytosol, and imported to mitochondria by chaperones [153].

Mitochondrial integrity, organization and quality are crucial to maintain mitochondrial homeostasis. The mitochondrial content depends on the balance between mitochondrial biogenesis and mitophagy [162]. Mitochondria are highly dynamic organelles that frequently undergo fusion (joining) and fission (division), processes that result in the formation and breakdown of complex mitochondrial tubular networks, critical for a constant elimination and regeneration cycles [155, 161, 163]. These two dynamic processes are crucial for the maintenance of cellular homeostasis, as perturbation in this balance are linked to apoptosis and metabolic diseases [162, 164, 165]. Moreover, the shape and dynamics of mitochondria are tightly linked to their bioenergetic status [153, 166]. Mitochondrial autophagy (also called mitophagy), is a complex self-degradative process by which old or damaged mitochondria are engulfed by autophagosomes and trafficked to lysosomes, thus preventing the accumulation of damaged organelles, while maintaining a healthy and functioning mitochondrial pool [167]. Moreover, mitophagy is also important to protect the cell against oxidative stress, mitochondrial dysfunction and pro-apoptotic factors [168].

Several genes and transcription factors are responsible for the tight regulation of mitochondrial biogenesis, function and energy metabolism, namely PGC-1α [169]. PGC-1α is a co-transcriptional regulation factor that activates different transcription factors, including the nuclear respiratory factors (NRFs) which, in turn, stimulate the transcription of nuclear mitochondrial genes and hence induce mitochondrial biogenesis [161]. In addition, the activation of PGC-1α induces the expression of genes involved in fatty acid β-oxidation, antioxidant enzymes, lipid transport, and gluconeogenesis [169]. Thus, the impairment of mitochondrial life cycle (biogenesis and mitophagy) can lead to detrimental consequence on cellular bioenergetics which contribute to pathogenesis of several diseases [164].

1.3.4.1.1 Electron transport chain and oxidative phosphorylation system

The central role of mitochondria is to generate energy, in the form of ATP, through the OXPHOS system. This energy production process in mitochondria uses a complex system that interplay fatty acid β-oxidation, glycolysis, TCA cycle and OXPHOS, being the latter responsible for more than 95 % of the ATP requirement within the cell [170, 171]. Indeed, the energy from carbohydrate and fatty acid metabolism, namely from glycolysis

and fatty acid β-oxidation, leads to a production of acetyl-CoA via a series of catabolic reactions, that converge at the TCA cycle within the mitochondria. The main role of the TCA cycle is to use various substrates to generate the intermediates, nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) required for OXPHOS system [153, 172]. Moreover, the TCA cycle also leads to the succinate production, which also enter in the ETC displayed along the IMM.

The OXPHOS, taking place at the IMM, consists of two functionally independent processes, namely the oxidation of reduced substrates by ETC and the phosphorylation of ADP by inorganic phosphate. The OXPHOS systems consist of five complexes where Complex I to IV constitute the ETC whereas Complex V is the F1F0-ATP synthase. Electrons released from reducing pyridine nucleotide equivalents (NADH and $FADH_2$) are faithfully transferred via the complexes (Complex I to IV), to molecular oxygen (O_2) with the help of two mobile electron carrier molecules, the coenzyme Q (also known as ubiquinone) and Cyt c. During electron transfer, the protons are transfer from the matrix to the IMS, creating an electrochemical gradient which drives the activation of ATP synthase, the final enzyme of OXPHOS, and the production of ATP [173].

More specifically, mitochondrial respiration involves the transfer of electrons from the intermediates NADH and $FADH_2$ to the ETC (Figure 1.6). The electrons carried by NADH enter in ETC through NADH: ubiquinone oxidoreductase (Complex I), thus catalyzing the NADH oxidation through a chain of co-factors including a flavin mononucleotide (FMN) subunit which accept electron from NADH to generate $FMNH_2$. The electrons are subsequently transferred through a chain of iron-sulfur (FE-S) clusters to reduce ubiquinone (oxidized form of coenzyme Q) to ubiquinol (QH_2) [153, 172, 174]. During this electron transfer process, complex I pumps 4 protons (H^+) from the matrix to the IMS [174]. Alternatively, electrons from $FADH_2$ arising from fatty acid β-oxidation and TCA cycle, can enter to respiratory chain through the succinate: ubiquinone oxidoreductase (Complex II), also called succinate dehydrogenase, which is also a component of TCA cycle, serving as a link between metabolism and OXPHOS [158]. This complex also donates electrons to ubiquinone by succinate oxidation to fumarate in a reaction that is coupled to the reduction of FAD to generate $FADH_2$. This is re-oxidized by passing its electrons along a series of Fe-S clusters within this complex to ubiquinone, in a similar manner as at Complex I [158]. Unlike the other respiratory chain complexes, electron transport at Complex II is not accompanied by proton translocation across the

IMM [153, 158]. Furthermore, there are other substrates involved in the electrons delivery from ubiquinone to O_2 including: ACAD that provides electrons through electron-transferring flavoprotein-ubiquinone oxidoreductase system; mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH); dihydroorotate dehydrogenase; and choline dehydrogenase, which is involved in the oxidation of choline to betaine aldehyde. Although these enzymes are characterized by the lack of energy-conserving proton-translocation mechanisms, all of which are also connected to the respiratory chain, resulting in the reduction of ubiquinone to QH_2 in the ETC, in similar way as the complex II [175, 176].

QH_2, the reduced coenzyme Q, is a highly mobile and hydrophobic molecule that carries the electrons for ubiquinone: cytochrome c oxidoreductase (Complex III), where is catalyzed the reduction of Cyt c, another mobile electron carrier, and ubiquinol oxidation. During this process, for each electron that is transferred to Cyt c, 2 H^+ are taken from the matrix and 4 H^+ are translocated to the IMS [174]. Cyt c then transfers the electrons to cytochrome c oxidase (COX, complex IV), in which molecular O_2 is reduced to water (H_2O) [153]. The reduction of O_2 to H_2O results in the pumping of 4 protons to the IMS, however, 2 protons are consumed in the process, resulting in a total of 2 H^+ pumped into the IMS at Complex IV [158, 174]. Thus, the O_2 consumption (respiration) is final electron transfer step, and it is used as metric of mitochondrial function [177]. According to the chemiosmosis, during this process of electron transfer, the protons movement from the mitochondrial matrix to the IMS induced by complex I, III, and IV, creates an electrochemical proton gradient across the IMM, called as proton motive force (Δp). The Δp displays two components: the chemical potential energy derived from the difference in concentration of H^+ (proton gradient, ΔpH) inside and outside the IMM, and the electrical potential energy known as the mitochondrial membrane potential (ΔΨm) generated from the separation of charge associated to proton migration between the two compartments, corresponding to the voltage difference across the membrane. The Δp provides a favorable passive force for protons to flow back into the mitochondrial matrix through the F1F0-ATP synthase (Complex V). At this complex, the energy from this gradient drives the phosphorylation of ADP to generate ATP [153, 172].

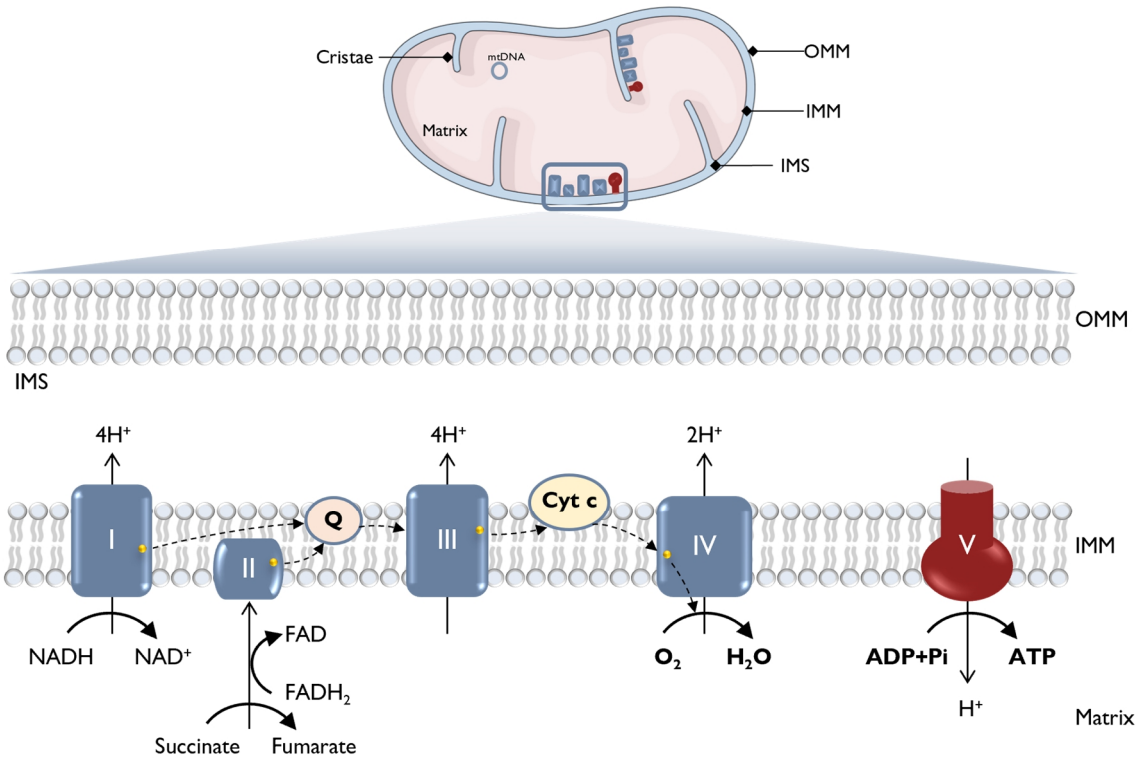

Figure 1.6 | Schematic representation of mitochondrial structure and the ETC. In the first step of oxidative phosphorylation (OXPHOS), a proton gradient is generated across the inner mitochondrial membrane (IMM), which drives the adenosine triphosphate (ATP) synthesis. Reduced electron carriers like nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) donate electrons (e^-) to the complex I and complex II of the ETC respectively. These electrons are subsequently transferred to ubiquinone (Q) thereby reducing it to ubiquinol (QH_2). The electrons are then transferred to complex III via cytochrome C (Cyt C) and then to molecular O_2 via complex IV to form H_2O. Following these reactions, the protons (H^+) that were transferred to the intermembrane space are shuttled back to the mitochondrial matrix by complex V (ATP synthase) by making conformational changes to F1F0-ATP synthase that results in the production of ATP from adenosine diphosphate (ADP) and inorganic phosphate (Pi). IMS, intermembrane space; OMM, outer mitochondrial membrane; (Adapted from Nolfi-Donegan et al [158]).

The Δp over the IMM serves as an indicator for the bioenergetic state of mitochondria and might give a hint at a bioenergetic imbalance resulting from mitochondrial malfunction. The mitochondrial respiration can be measured by the O_2 consumption rate in the presence of specific substrates that feed electrons into different sites of ETC [177, 178]. For instances, in the presence of pyruvate plus malate or succinate (plus rotenone), the electrons fed into Complex I or Complex II, are transferred to ubiquinone, Complexes III and IV, where O_2 plus hydrogen are converted into water.

Moreover, the ETC function depends predominantly on the IMM impermeability and the catalytic integrity of the respiratory chain complexes. Putative events that can interfere with OXPHOS encompass an acute inhibition of the ETC or an uncoupling of the ETC. The direct inhibition of the individual complexes of the ETC leads to inhibition of substrate oxidation and O_2 consumption and membrane potential collapse.

As described above, in coupled respiration, O_2 consumption in the presence of ADP is linked to the ATP synthesis. However, physiologically, OXPHOS is not completely coupled, due to re-entry of protons back toward mitochondrial matrix through alternative leak pathways, without coupling to ATP synthesis [179]. This leads to a mild uncoupling process resulting in Δp dissipation, decreased ΔΨ and ATP synthase activity, lowering the ATP efficiency. This event is associated with the generation of heat instead of ATP. This creates a futile cycle of pump and protons leak across the IMM. Thus, proton leak has beneficial physiological functions including regulation of metabolic rate and thermogenesis, and can be protective in conditions favoring ROS production, including obesity and diabetes [180]. Uncouplers, often hydrophobic weak acids, such as phenols or amides, have a "protonophoric" activity, which means that they may carry a proton into the mitochondria matrix due to their permeability through the IMM.

1.3.4.1.2 Calcium homeostasis and mitochondrial permeability transition pore (MPTP)

Ca^{2+} is an important and versatile intracellular signaling molecule involved in the regulation of several cellular processes ranging ATP production to cell death [166, 181, 182]. Within the cell, Ca^{2+} can be particularly accumulated in the ER but also in mitochondria, Golgi complex cisternae and nucleus. Mitochondria holds an important and efficient role in cytosolic Ca^{2+} buffering and homeostasis, being regulated through a complex system of mitochondrial Ca^{2+} influx and efflux mechanisms. Ca^{2+} influx into the mitochondria is determined by the Ca^{2+} concentrations both in cytosol and mitochondrial matrix and by the ΔΨm. In intracellular stores, Ca^{2+} ions reach concentrations in the micromolar range, while the normal Ca^{2+} concentration in cytoplasm is around 100 nM [182]. When the extra mitochondrial Ca^{2+} concentrations are high (intracellular Ca^{2+} stores and plasma membrane Ca^{2+} channels), mitochondria sense and respond to the Ca^{2+} transients by taking up Ca^{2+}.

One of the main functions of mitochondrial Ca^{2+} transport is to increase NADH production. Mitochondrial Ca^{2+} uptake stimulates the activity of Krebs cycle and OXPHOS, and thus stimulates ATP production [182, 183]. Ca^{2+} transport system in mitochondria comprises specific transporters in the IMM and OMM. Ca^{2+} influx is mainly mediated by a uniporter transporter, powered by the electrochemical gradient, without coupling to ATP hydrolysis [184]. Moreover, Ca^{2+} can also enter to the mitochondrial matrix by the rapid Ca^{2+} mode (RaM) uptake, that was early described in hepatic mitochondria [185]. Other proteins including the mitochondrial ryanodine receptor (mRyR), uncoupling proteins (UCP2 and UCP3) and new mitochondrial Ca^{2+} channels voltage-dependent highly selective for Ca^{2+} have been also showed to be fundamental for Ca^{2+} transport [182, 183, 186].

Mitochondrial Ca^{2+} efflux occurs via two different routes: sodium (Na^{+})-dependent and Na^{+}-independent mechanisms [183]. Since the Ca^{2+} efflux occurs against the electrochemical gradient, both systems require energy, despite distinct kinetics. The mitochondrial Ca^{2+} output through Na^{+}-dependent exchanger is more abundant in excitable cells such as in heart, brain and skeletal muscle cells, whereas the Na^{+}-independent mechanism involving the $2H^{+}/Ca^{2+}$ exchanger occurs predominantly in liver, kidney, lung and smooth muscle [181, 183]. However, both exchangers have been found in mitochondria from all tissues, indicating that Ca^{2+} efflux is not mediated by a single mechanism in any given cell type [182]. The balance between Ca^{2+} influx and efflux across IMM establishes mitochondrial Ca^{2+} homeostasis. The mitochondrial Na^{+}/Ca^{2+} exchanger normally serves as a major Ca^{2+} efflux mechanism, but the transient opening of mitochondrial permeability transition pore (mPTP) represents another important mechanism for Ca^{2+} release from mitochondria in physiological conditions. While mitochondria have a large capacity for buffering Ca^{2+} by forming Ca^{2+} phosphate complexes, excess Ca^{2+} influx into mitochondria can be pathological [182]. As consequence of excessive Ca^{2+} uptake, mitochondria may undergo sustained mPTP opening which may result in Cyt *c* release from the IMS into the cytoplasm, with consequences for cell viability [182].

Besides mitochondrial CA^{2+} overload, several other biochemical events such as generation of ROS, depletion of ATP, increased phosphate concentrations, as well as reduced Ψm are also determinant factors for a sudden increase in the inner mitochondrial membrane permeability (MMP), believed to be caused by the opening of a voltage- and

Ca^{2+}-dependent, cyclosporine A (CsA)-sensitive channel at the juxtaposition of the IMM and OMM. The mPTP is a non-specific large conductance pore that is permeable to non-specific solutes with the molecular weight up to about 1500 Da [183] allowing the free protons influx into the mitochondrial matrix. The pore opening leads to an increase in mitochondrial matrix volume, due to water entry into mitochondria, resulting in mitochondrial swelling associated with membrane depolarization and Ca^{2+} release [182]. The transient opening may be physiologic, being involved in functions such as voltage, redox or matrix pH sensor, divalent cation sensor (role in Ca^{2+} homeostasis), or regulation of adenine nucleotide concentrations [182]. In fact, in intact healthy cells, the mPTP likely fluctuates between the open and closed states with a rapid kinetics [187]. Conversely, large and long-lasting openings can lead to irreversible consequences, including dissipation of Ψm, impairment of ATP generation, excessive Ca^{2+} accumulation, osmotic driven influx of water resulting in matrix swelling and the release of proapoptotic molecules located at the IMS (e.g. Cyt c) to the cytosol, and OXPHOS uncoupling, that can culminate in cell death [183]. Classical mPTP is blocked by high Ca^{2+} levels and CsA, which interacts with the matrix protein cyclophilin D and inhibits the mPTP. Experimentally, the permeability transition is characterized by an abrupt swelling and depolarization of the mitochondria, reflecting the loss of ability to maintain ion and solute gradients across the IMM [183].

1.3.4.1.3 Mitochondrial ROS and antioxidant defense system

In addition to its main role of energy production and regulation of the most important metabolic process, mitochondria are a major intracellular source of ROS [158, 188, 189]. ROS generation during several steps in the respiratory path is a functional consequence of mitochondrial respiration [190]. ROS is a collective term that generally encompasses oxygen free radicals, such as superoxide anion radical ($O_2^{-\bullet}$) and hydroxyl free radical ($OH^{\bullet}$), and nonradical oxidants, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) [182, 191]. It is estimated that approximately 90% of cellular ROS is derived from the mitochondrial ETC due to the higher O_2 consumption [192]; and under physiological respiration, 1-2% of the total O_2 consumed in mitochondria leads to $O_2^{-\bullet}$ production [188]. The $O_2^{-\bullet}$ is the primary and most abundant ROS formed through the interaction of one-electron donors with O_2 in the mitochondria as a functional consequence of electron leak during OXPHOS [188, 192]. Hence, both complex I and III produce the highest

amount of ROS in mitochondria [192]. ROS generated from complex I mainly enter the mitochondrial matrix whereas ROS produced by complex III enter either the matrix or IMS [188, 193]. In addition, ROS can also be produced by TCA cycle enzymes such as α-ketoglutarate dehydrogenase complex, pyruvate dehydrogenase, as well as by other potential electron donors such as α-glycerophosphate dehydrogenase, monoamine oxidases (MAO) and electron transferring flavoprotein quinone oxidoreductase complex but also by nicotinamide-adenine dinucleotide phosphate (NADPH) oxidases [188, 189, 191, 194, 195].

The redox state of the respiratory chain is the primary factor for mitochondrial ROS production [191]. In general, ROS production is increased when an excessive number of electrons are transferred to the ETC as well as when excess protons are loaded into the intermembrane space. The higher rate of ROS formation occurs when there is a high ΔpH and ΔΨ along with a low ATP production. Furthermore, in the presence of ETC inhibitors, such as rotenone or antimycin A, there is an increase of ROS formation and electrons passing through the ETC may leak out to molecular O_2 to form $O_2^{-\bullet}$ [189]. Superoxide dismutase (SOD) in the cell is able to buffer $O_2^{-\bullet}$ by converting it to H_2O_2, which is more stable but still highly toxic. Superoxide is unable to diffuse readily through the IMM and may be subsequently converted either enzymatically or non-enzymatically to highly reactive derivatives, such as $OH^{\bullet}$, H_2O_2 or peroxynitrite (NO_3^-). Most $O_2^{-\bullet}$ is immediately converted to H_2O_2 by superoxide dismutase (SOD) in either the cytosol and mitochondrial intermembrane space by copper/zinc superoxide dismutase (Cu/ZnSOD; SOD1 isoform) or in the mitochondrial matrix by manganese superoxide dismutase (MnSOD or SOD2 isoform) [172].

Apart from SOD, many other antioxidant defense mechanisms are also responsible for limiting the ROS accumulation including enzymatic antioxidants such as glutathione peroxidase (GPx), catalase, thioredoxins (Trxs), and peroxiredoxins (Prxs), together with non-enzymatic antioxidants, such as, α-tocopherol (vitamin E), ubiquinone, β-carotene, ascorbate (vitamin C), and GSH [189, 195]. For instance, mitochondrial H_2O_2 can be completely reduced to water by the activity of catalase, which is mainly expressed in peroxisomes, and by GPx in the mitochondrial matrix as well as in the cytosol [189, 196]. Importantly, GPx, Prxs and Trx systems need an adequate amount of GSH within the mitochondrial matrix to detoxify H_2O_2, so that the depletion of mitochondrial GSH below a crucial level can lead to, or favor, mitochondrial dysfunction and cell death [196].

Physiologically, the greatest amount of mitochondria ROS produced during cellular respiration is detoxified by natural antioxidant defenses and there is a highly controlled balance between ROS generation and scavenging. Lower levels of ROS act as second messengers indispensable for normal cell homeostasis and signaling [197, 198]. However, when ROS are produced in excess and/or antioxidant defense is low or compromised, irreversible damage in membranes, lipids, proteins, and DNA may occur due to a direct interaction with ROS [195]. Indeed, as a consequence of oxidative stress, the overall performance of mitochondria is compromised by impairing function such as ATP production, as well as by disturbing the activity of enzymes involved in a wide range of metabolic functions such as the TCA cycle, fatty acid β-oxidation and amino acid synthesis. Mitochondrial ROS can also facilitate the opening of the mPTP as well as the Cyt c release to cytosol and activated caspases triggering apoptotic and necrotic cell death [182, 188].

Polyunsaturated fatty acid residues of phospholipids contained both in IMM and OMM are prone to peroxidation. The production of cytotoxic lipid peroxides has been suggested to be partly responsible for the changes in mitochondrial membrane fluidity and vital functions [199]. If they are not neutralized by endogenous antioxidants, lipid peroxides will fragment and decompose to form reactive aldehydes such as MDA and hydroxynonenal (HNE), two common markers of lipid peroxidation that can be accumulated in intracellular and extracellular targets, thereby, amplifying the effects of oxidative stress [191].

mtDNA has also a particular susceptibility to ROS attack owing to its proximity to the major site for free radicals' production, the ETC, and due to the lack of protective histones with limited DNA repair mechanisms. The prolonged exposure to non-physiological ROS concentration in mtDNA may lead to DNA strand breaks and the likelihood of mtDNA mutations [188]. Consequently, the accumulation of mtDNA mutations may result in dysfunction of the respiratory chain, leading to increased mitochondria ROS production and subsequent accumulation of more mtDNA mutations, creating a vicious cycle of oxidative damage [190, 200]. Thus, mtDNA represents a critical cellular target for oxidative damage that is implicated in several pathological conditions [201, 202]. Moreover, DNA damage-derived mutations have been associated with development of complications of insulin resistance and T2DM [203].

1.3.4.2 Mitochondrial impairments in (pre)diabetes

Considering the pivotal role of mitochondria in cellular energy metabolism, it is not surprising that disruption in mitochondrial homeostasis is closely linked to insulin resistance and associated metabolic diseases such as T2DM, particularly evident in tissues with higher metabolic demands [159, 164, 204, 205].

Mitochondrial dysfunction elicited by oxidative stress is featured by deletion of mtDNA and reduced mitochondrial mass, reduced oxidative function, reduced expression of genes related with mitochondrial biogenesis and OXPHOS, decreased activity of respiratory chain complexes which collectively may lead to reduced substrate oxidation and lower ATP synthesis. Such changes in mitochondrial network have been reported in early diabetes pathogenesis and may be tightly associated with insulin resistance in multiple tissues [61, 206]. Interestingly, the disruption of mitochondrial function is also implicated in β-cell damage [207], strengthening the key role of mitochondrial dysfunction in insulin resistance and T2DM development. However, whether these events precede, accompany or are a consequence of insulin resistance remains unclear [208]. In this study, we only focused on hepatic tissue, thus the hepatic mitochondrial function will be scrutinized in an insulin resistance scenario.

Glucotoxicity and lipotoxicity along with mitochondrial dysfunction are strictly interconnected in the context of metabolic impairments and deeply impact glucose and lipid homeostasis. In this way, there are consistent evidences for the crucial role of mitochondrial dysfunction in the NAFLD pathophysiology paralleling the insulin resistance [204]. One possible mechanism that have been proposed to link the mitochondrial dysfunction and insulin resistance is via altered fatty acid metabolism [154, 209]. The intracellular lipids accumulation by the decreased activities of carnitine palmitoyl transferase (CPT), which transports long-chain fatty acids into mitochondria, and of acyl-CoA dehydrogenase long-chain (ACADL), an enzyme involved in fatty acid β-oxidation, can yield to insulin resistance in insulin-targeting cells [210]. It has been demonstrated that the reduced oxidation of fuels, particularly fatty acids, facilitate the intracellular accumulation of lipotoxic lipid intermediates (i.e. ceramides and DAG), which might lead to the inhibition of insulin signaling pathway, culminating in the development of insulin resistance and further reduction of the mitochondrial oxidative capacity and ATP synthesis in obese and insulin resistance models [211, 212]. Fatty acids can also change the expression and/or function of key regulatory transcription factors in the liver (e.g.

PGC-1, PPARs, SREBP-1, and ChREBP) (127, 222–224). Additionally, genetic defects in fatty acid β-oxidation (i.e. lack of ACADL) predispose animals to hepatic steatosis and insulin resistance [213]. This suggests that hepatic lipid accumulation may lead to impaired mitochondrial capacity which in turn can trigger hepatic insulin resistance [213, 214].

Another putative mechanism potentially connecting the impaired mitochondrial oxidative function with insulin resistance is the excessive mitochondrial ROS generation – imbalance between oxidative demand and mitochondrial capacity can also impair insulin signaling [215]. In both obese, insulin resistant subjects and insulin resistant rats, hypercaloric diet increases mitochondrial H_2O_2 production, shifts the cellular redox environment to a more oxidized state and decreases the redox-buffering capacity without any changes in respiratory function capacity, which suggest a correlation of mitochondria H_2O_2 generation to the pathogenesis of insulin resistance [216]. Moreover, attenuating mitochondrial H_2O_2 production, by using mitochondrial-targeted antioxidants or via genetically engineering catalase overexpression in mice muscle mitochondria, completely preserved glucose tolerance and insulin sensitivity [216]. Similarly, overexpression of MnSOD ameliorates insulin resistance [217]. Despite aforesaid evidences, the detailed mechanisms linking ROS to insulin resistance remain uncertain. Mitochondrial ROS have been linked to activation of serine/threonine kinases such as IKKβ, PKCs, p38 MAPK, JNK, ERK, and NF-κB, generally ascribed as pro-inflammatory mediators that phosphorylate IRS proteins at serine residues and subsequently disrupts insulin signaling [61]. Increased serine phosphorylation of IRS-1/2 results in a decreased activity of insulin downstream signaling pathways, including PI3K, Akt and PKC, which can culminate in decreased glucose uptake, increased HGP and reduced insulin secretion [61, 121]. Furthermore, oxidative stress not only interferes directly with the insulin signal transduction pathway yielding insulin resistance [216] but can also hamper insulin signaling indirectly by inducing mitochondrial damage and mitophagy. The consequent decrease in mitochondrial function and density compromises overall cellular oxidative capacity, favoring the ectopic accumulation of lipid intermediates of lipid metabolism.

Even though most of studies available focus on skeletal muscle, there are some studies examining the association of mitochondrial dysfunction with insulin resistance and T2DM development in hepatic tissue [218-221]. In general, these studies hypothesize that impaired mitochondrial activity, along with increased ROS production, play a causal role of in the onset of insulin resistance as well as in hepatic steatosis, leading to hepatic

oxidative stress, changes in insulin sensitivity and facilitating the intracellular lipid accumulation [222-224]. Indeed, despite the existence of a plethora of studies in both animals and humans detailing the links between mitochondrial dysfunction and insulin resistance, whether defects in mitochondrial metabolism are primary or secondary culprits in disease pathogenesis remains a matter of debate [214]. Nevertheless, these events clearly occur simultaneously as a result from the chronic high glucose and lipid stress, probably establishing a vicious cycle. Moreover, both lifestyle and therapeutic pharmacologic strategies focused on targeting this vicious cycle to regulate mitochondrial metabolism may be an effective approach to slow down the progression of insulin resistance and NAFLD and thus, further improving the outcomes of the early state of the disease.

1.4 Animal models of prediabetes

Animal models have been used for several decades as essential tools to dissect the impact of various genetic, environmental, behavioral, and pharmacologic factors that play a role in metabolic abnormalities. Furthermore, several animal models may be crucial for studying the pathophysiological mechanisms underlying diabetes progression, to unravel new therapeutic targets, as well as to evaluate the efficacy of new drugs and/or novel therapeutic approaches. The selection of the best animal model, which is dependent of the specific scientific goal, is a challenging process due to the innumerable variables that need to be considered. This is particularly critical with regard to the study of more complex and multifactorial diseases, such as metabolic ones, as our group recently reviewed [225].

The establishment of a prediabetic animal model facilitates a more proper understanding of early pathophysiological features at molecular and cellular levels that may contribute to the progression of overt T2DM. Currently, there is a great diversity of animal models for T2DM sharing many phenotypic features with the human disease, which can be genetically manipulated (spontaneous or transgenic/knockout), surgical treated (partial pancreatectomized animals), chemical induced (e.g. with streptozotocin (STZ) or alloxan) or can be induced by dietary manipulation [226]. Some of these genetically or spontaneously induced diabetic animals, such as: prediabetic spontaneously hypertensive rat - obese strain rats [227], Zucker Diabetic Fatty (ZDF) [228, 229], Goto-Kakizaki (GK)

[230], Otsuka Long Evan Tokushima Fatty [231], diabetes prone BioBreeding [232] rats, nonobese diabetic mouse [233], and spontaneously diabetic Chinese hamster (non-genetic model) [234], are also used as models for prediabetes and insulin resistance in the early stage of their lives; however, these models are relatively expensive, are not widely available compared with experimentally-induced non-genetic models and most of them may not always accurately replicate the natural history of insulin resistance in humans.

The use of hypercaloric diets as a way to induce the disease is an appealing strategy since the excessive consumption of fats and sugars replicates the human unhealthy dietary patterns, which have been claimed as one of the main driver behind insulin resistance, an hallmark of T2DM. Moreover, it is associated with a slow and progressive evolution, mimicking the human disease development. Hence, some diet-induced animal models have been described to mimic the stages of prediabetes and/or insulin resistance (Table 1.2) [235-237].

The most widely used diets for T2DM animal research are high-sugar diets (HSD), high-fat diets (HFD), and the respective combinations of high-fat/high-sugar diets (HFHSD) such as Cafeteria diets (CD) and Western diets (WD). Several experimental studies have shown that dietary macronutrient composition is an important environmental determinant of the efficacy of insulin action and a trigger for the prediabetes stage [238-240]. Indeed, the modern WD and CD, rich in saturated fats and in carbohydrates (such as fructose and sucrose), are able to promote important changes in carbohydrates metabolism, causing prediabetes, although other metabolic features resembling obesity and metabolic syndrome may coexist.

Several experimental studies have shown the use of HSD (mainly, high-sucrose and high-fructose) as an inexpensive nutritional strategy to promote the development of insulin resistance and glucose intolerance in animal models; however, the results were inconsistent, apparently due to variations in the metabolic responses to diets based on species/strain, duration of protocol, percentage of sugar, and route of administration [235, 241-244]. In fact, a study suggested that C57BL/6 mice are more resistant to sucrose treatment than Wistar rats [235]. In that study, male Wistar rats and C57BL/6 mice, both with 7 weeks of age, received 30% sucrose in drinking water for 20 weeks. The mice presented slight obesity and hyperlipidemia without changes in glucose and insulin levels, whereas rats presented hypercholesterolemia, hyperglycemia, hyperinsulinemia, and insulin resistance [235]. Two studies using the same species and strain (male C57BL/6J

mice), but distinct sucrose content and protocol duration, reported absence of weight gain but diverse metabolic responses [241, 245]. In fact, 8 weeks of HSD treatment (10% fat, 32% sucrose) of 3-month-old mice resulted in adipocyte hypertrophy, glucose intolerance, hyperinsulinemia, hyperlipidemia, hepatic steatosis and increased secretion of inflammatory cytokines [241], while 4-week-old mice on 55 weeks of solid food with high sucrose (50%, wt/wt) content did not show changes in BW, but variable degrees of hyperglycemia and hyperinsulinemia [245]. Furthermore, a study using male C57BL/6J mice (8 weeks old) suggested that distinct modes of sucrose delivery differentially affected body regulation and glucose homeostasis [243]. The authors showed that mice exposed to a liquid sucrose solution (50% sucrose in the water plus 30% calories from sucrose in solid food) presented higher adiposity, impaired glucose homeostasis, peripheral insulin sensitivity, and insulin resistance than mice maintained under equivalent levels of sucrose in solid diet (73% calories from sucrose) [243]. Previously, our group was able to reproduce a prediabetic rat model using an oral regimen of 35% sucrose consumption for 9 weeks [236, 237]. The animals were referred to as prediabetic based on fasting normoglycemia, IGT, hyperinsulinemia and insulin resistance without obesity, which is consistent with the results of other study [246]; interestingly, the animals concomitantly displayed some early features of retinal and neuronal impairment [246-248].

The use of fructose-rich diet is also a well-characterized approach for elicit insulin resistance and IGT in rodents [249, 250]. Unlike glucose-fed rodents, fructose-fed rodents displayed disruption of metabolic homeostasis, including IGT and dyslipidemia [212, 251]. In one study, low fructose (7%) content in drinking water was sufficient to cause glucose intolerance with abnormal morphology and function of pancreatic islet cells in Wistar rats, but without changes in FPG, insulin and lipids levels [252]. However, in another study, the same strain of rats fed with fructose-rich diet (10% w/v in drinking water) developed hypertriglyceridemia and a state of insulin resistance (demonstrated by hyperinsulinemia with normoglycemia, high insulin: glucose molar ratio, and HOMA-IR (homeostatic model assessment of insulin resistance) index) after 3 weeks of fructose administration [253].

Long-term HFD intake in several strains of rat and mouse can cause hyperlipidemia, peripheral insulin resistance, moderate hyperglycemia, and IGT, together with increase in the expression of inflammation and oxidative stress markers and hepatic steatosis [254, 255]. In rodents on HFD feeding, insulin resistance is followed by insufficient β-cell compensation, resulting in hyperinsulinemia and altered glucose metabolism [256]. The

effects of HFD depend not only on fat content (% fat; animal/vegetable source) and duration of protocol but also on the animal species and strains used [257-259]. A "humanized" model of prediabetes was suggested for young male Wistar rats under HFD (45% calories from fat) for 6 months [239]. The authors reported a progressive development of the phenotype typical of humans, starting with BW gain, gradual increase of FPG and glucose intolerance, further evolving to hyperinsulinemia and insulin resistance, together with hypertrophy of pancreatic β-cells in the last month [239]. Increased levels of FPG and insulin and augmented HOMA-IR, together with impaired β-cell function and OGTT, were also observed in Sprague-Dawley rats under HFD (46% calories from fat) for 12 weeks [260].

Table 1.2 | Main features of diet-induced rodent models of prediabetes.

Diet type	Composition (%)	Duration (Weeks)	Specie/strain	Main features
HFD	46% kcal from fat, 24% kcal from CHO, 20.3% kcal from protein	12	Male Sprague–Dawley rats (± 8 wo) [260]	- Obesity: data not shown - Increased FPG: ≈115 mg/mL - Hyperinsulinemia - IGT
	45% kcal from fat, 35% kcal from CHO, 20% kcal from protein	17	Male Sprague–Dawley rats (7 wo) [261]	- Obesity: 32.2% Δ weight gain; 21.6 % increase in fat mass - IGT
	45% kcal from fat, 35% kcal from CHO, 20% kcal from protein	17	Male Wistar rats (7 wo) [261]	- Obesity: 66.9% Δ weight gain; 43% increase in fat mass - Fasting hyperinsulinemia (at week 9) - Increased HOMA-IR index (at week 9) - IGT (at weeks 9 and 17) - Hypertriglyceridemia
	59% kcal from fat, 20% kcal from CHO	40	Male Wistar rats (8 wo) [262]	- Obesity: 98 % Δ weight gain; 2-fold increase in total fat mass - Mild elevated FPG: ≈135 mg/mL - Hyperinsulinemia - Mild glucose intolerance
HSD	35 % sucrose in water + standard diet	9	Male Wistar rats (16 wo) [236, 237]	- Obesity: No BW changes - Fasting normoglycemia: 102.90 mg/dL - Increased fed glucose: 162.90 mg/dL - Fasting hyperinsulinemia - Increased HOMA-IR index - Decreased insulin sensitivity - IGT - Hypertriglyceridemia
	7% fructose (w/v) in water	12	Male Wistar rats (6 wo) [252]	- Obesity: No BW changes - Fasting normoglycemia: 70.5 mg/dL - IGT - Loss of β-cells - Increased β-cell mass
	10% fructose (w/v) in drinking water	3	Male Wistar rats (± 8 wo) [253]	- Obesity: No BW changes - Fasting normoglycemia: 90 mg/dL - Fasting hyperinsulinemia - Hypertriglyceridemia
HFHSD	42% kcal from fat %, 37% kcal from CHO, 21% kcal from protein,	22	Male C57BL/6 mice (8 wo) [263]	- Obesity: ≈80% Δ weight gain; increased fat mass - Fasting normoglycemia: 189 mg/dL - Fasting hyperinsulinemia - IGT
		42		- Obesity: ≈60% Δ weight gain; increased fat mass - Fasting normoglycemia: 160 mg/dL - Fasting hyperinsulinemia
	45% kcal from fat, 35% kcal from CHO; 21% kcal from protein	8	Male Wistar rats (4 wo) [264]	- Obesity: 26% Δ weight gain; ≈ 2-fold increase for epididymal and perirenal fat weight - Elevated FPG: 192 mg/dL - Decreased insulin sensitivity - IGT - Hypertriglyceridemia
	30% kcal from fat and 70% kcal from CHO + 30% (w/v) of fructose solution	10	Male Wistar rats (6 wo) [265]	- Obesity: 23% Δ weight gain - Elevated FPG: 105 mg/dL - Impaired glucose tolerance - Hypertriglyceridemia

BW, body weight; CHO carbohydrates; HFD, high-fat diets; HSD, high-sugar diets; HFHSD, high-fat/high-sugar diets; FPG, fasting plasma glucose; IGT, impaired glucose tolerance; wo, weeks old.

As animal strains might present distinct responses to HFD, the metabolic effects of HFD intake for 16 weeks were compared between Wistar and Sprague-Dawley rats. Results showed that most of the metabolic changes (such as BW gain, fasting insulin and leptin levels, HOMA-IR index, and glucose intolerance) were detected earlier and were more pronounced in Wistar rats [261]. A robust mouse model for IGT and early T2DM was established using female C57BL/6J mice under HFD (58% calories from fat) for 12 months [266]. One week after starting the diet protocol, the animals presented elevated baseline glucose and insulin levels and IGT, along with impaired insulin response; over time, progressive worsening of insulin resistance was observed in HFD-fed mice [266]. Interestingly, Kleemann and colleagues showed that HFD-induced insulin resistance primarily in the liver (after 6 weeks of dietary regimen) and then in adipose tissue (after 12 weeks), while skeletal muscle remained sensitive [267].

It is important to highlight that HFD, commonly displaying 45% and 60% fat do not closely recapitulate human dietary Western patterns apart from being able to replicate, in rodents, a huge number of metabolic diseases associated with this human dietary regimen [268]. For instance, humans typically consume ≈ 30% of their calories from fat, which is largely lower than the fat content observed in most of the commercially available standardized rodent HFD [269]. Therefore, it has been suggested that experimental paradigms with 45% fat calories are more relevant for translational purposes. However, important preclinical evidences ensue from diets with 60% fat calories, probably due to an accelerated disease phenotype.

HFHSD is also commonly used to experimentally replicate prediabetes [263, 270]. Different sources and amounts of carbohydrates (mainly fructose or sucrose) and fats have been used [271-273]. In the HFHSD regimens administered to rodents, carbohydrates content typically ranged between 10% and 60% (in the diet, drinking water or both), whereas fat content varied between 20% and 60% [271, 274]. C57BL/6J mice fed HFHSD (45% energy from fat and 20% by weight from sucrose) for 22 weeks showed increased BW, glucose intolerance, fasting hyperinsulinemia and dyslipidemia [263]. Paradoxically, when the same diet was continuously administered for additional 20 weeks, long-term (42 weeks) HFHSD consumption was able to change glucose tolerance and decrease plasma FFAs levels, although the mice displayed elevated FPG levels and dramatic hyperinsulinemia [263]. In Wistar rats, a prediabetic stage was achieved by feeding the animals during 8 weeks with WD (containing 45% kcal from fat and 35% kcal from

carbohydrates) and 20% sucrose solution in drinking water. The animals developed obesity, mild hyperglycemia, hyperinsulinemia, hyperlipidemia, and glucose intolerance [264]. In other recent study from our group, the ingestion of sucrose in drinking solution (35%) for 9 weeks and supplemented by a solid HFD (60%) for additional 14 weeks effectively induced a prediabetic state in Wistar rats, which was confirmed by mild postprandial hyperglycemia, hypertriglyceridemia, IGT and insulin sensitivity, accompanied by increased BW and early renal lesions [275].

Overall, these studies clearly demonstrated that rodent models based on nutritional manipulation can replicate a similar pattern of clinical pathophysiological features found in humans. Notwithstanding, there is a set of conditions and variables that can affect the prediabetic phenotype induced by hypercaloric diets. In fact, factors such as dietary paradigms (e.g. nutrient sources, diet type and specific composition of macronutrients, protocol of exposure), but also the rodent species and strain, as well as the age and sex, of the animal model being used may have a greatly impact on the expected metabolic outcomes.

2. Blueberry as a promising option to protect prediabetes progression

2.1 Phytochemistry, metabolism and bioavailability

Blueberry (BB) is one of the healthy fruits recommended by the Food and Agriculture Organization from United Nations [276]. It belongs to the genus Vaccinium of Ericaceae family which includes approximately 450 species all over the world [277]. Some examples are the well-known *Vaccinium corymbosum*, *Vaccinium ahei*, *Vaccinium poasanum*, *Vaccinium angustifolium* and *Vaccinium myrtillus*, the later commonly denominated as bilberries across Europe [278, 279]. Blueberries are characterized by their enriched nutritive composition, containing high contents of a wide diversity of biologically active components. BB chemical composition greatly varies upon the cultivar, variety, growing location, environmental conditions, plant nutrition, ripeness stage, harvest time as well as the storage conditions, making the content of each individual component highly flexible, with impact on the corresponding antioxidant profiles [280-282]. Nonetheless, BB-derived phytochemicals display a panoply of health-related properties beyond their antioxidant profile able to interfere with chief physiological functions [283-285]. Therefore, public interest in BB as a functional food has increased over the last years [286]. Even though the aerial parts of the shrubs are used as a folk medicine for treating metabolic diseases for years, the fruits have been constantly increasing in popularity due to their pleasant taste and organoleptic acceptance as well as the richness of their bioactive compounds [287].

BB fruits are low in calories (0.046 kcal/g fresh fruit) and high in water, micronutrients (e.g. selenium, zinc, iron), dietary prebiotic fibers (3-3.5% of fruit weight), vitamins (C, B complex, E, and A) and sugar units [e.g. glucose (≈ 26.415 mg/g – 46.495 mg/g fruit), fructose (≈ 22.682 mg/g – 43.074 mg/g/fruit) and other osidic moieties such as ribose, rhamnose, arabinose and maltose] which are often associated with bioactive polyphenols (PP), known as heterosydic forms of PP [277, 288-290]. Briefly, PP are secondary metabolites of the plants that act against ultraviolet radiation as well as in plant-microbe interactions and defense response [291]. Collectively, they encompass an aromatic ring and a minimum of two hydroxyl substituents which impact ROS scavenging ability and subsequent antioxidant effects depending on their number and position [292]. PP comprise a large family of compounds and are classified as flavonoids (including flavonols,

flavanols, flavones, isoflavones, anthocyanidins and flavanones), phenolic acids (including benzoic acids, cinnamic acids and derivatives), chalcones and coumarins (intermediate in the biosynthesis of flavonoids), and lastly, polymers, including tannins and lignin [293]. PP are present in distinct parts of the BB fruit; for instance, while tannins and phenolic acids are mostly predominant in the seeds, anthocyanins and proanthocyanidins are present mainly in the fruit's skin and pulp, respectively [294]. Moreover, PP occur in vegetable cells in soluble forms (mostly localized in the cell vacuoles) as well as covalently bound with cell wall macromolecules such as structural proteins, cellulose, hemicellulose, arabinoxylans and/or pectins, collectively designated as PP insoluble forms [295]. Likewise, insoluble PP naturally present in BB fruits may become tightly linked (e.g. esther; C-C bonds) with pectin and xyloglucan, two main components of BB cell walls with prebiotic activity [296].

The biological properties of PP found in BB fruits greatly depend on effective concentrations reached in internal compartments that need to be maintained for an adequate period of time [297]. Notably, the poor bioavailability of PP present in ingested food, ranging from 1-5% of the total PP intake [298-300], is inversely correlated with their biological effects, a phenomenon stated as the low bioavailability/high bioactivity paradox [301]. Even though PP are extensively metabolized within enterocytes, colonic GM and liver by phase I/II enzymatic reactions (e.g. sulfation, methylation, glucuronidation, PP ring-fission), a wide variety of new chemical structures retaining intrinsic bioactivity along with parent compounds reach systemic circulation and are distributed to different organs and tissues, supplying efficient cellular concentrations that underpin PP efficacy [300-303].

PP physicochemical properties, their release from the food matrix during GIT digestion (bioaccessibility), cellular uptake, metabolism and transport in the circulatory system are key features that determine PP organism availability [298]. To better understand blueberry PP metabolism and pharmacokinetics, Zhong and colleagues carried out a single blind, randomized trial and followed human plasma concentrations of anthocyanins, chlorogenic acid and their metabolites over 24 h after oral ingestion of a wild BB beverage [304]. The total bioavailability of unaltered anthocyanin compounds (displaying their original C6-C3-C6 structure) and chlorogenic acid was 1.1% and 0.2%, respectively. Parent compounds and metabolites (e.g. cyanidin-, delphinidin-, petundin-glucoronide metabolites) peaked in plasma within the first 1-4 h post-ingestion (early phase response) and > 5 h after ingestion (late phase response). This bi-phasic response,

probably reflective of enterohepatic circulation, may imply that different BB-derived PP and metabolites interact with GIT cells and other tissues at different times post-consumption [304]. As a matter of fact, nanostructures comprising PP-BB matrix conjugates may be protected along the transit through the GIT, a phenomenon affecting both PP absorption and cellular uptake. Likewise, *in vitro* gastrointestinal digestion of BB fruits underscored a high stability of total PP and anthocyanins during the gastric digestion step along with decreased contents upon intestinal breakdown (49% and 15%, respectively). This profile paralleled unchanged antioxidant activity following gastric digestion and a significant reduction (< 50%) upon digestion under intestinal conditions [298].

Furthermore, it is generally accepted that the remaining unabsorbed PP glycosides and conjugates cycled through hepatic and ileal metabolism reach the colon and modulate fermentation towards a myriad of health benefits [301]. Since small intestine lacks pectinases and cellulases, PP-dietary fibers conjugates are disrupted by colonic gut microbiota enzymes and liberation of PP metabolites occurs [295, 305]. Such metabolites display chief functions in colon health, modifying gut microbial balance towards beneficial bacteria prevalence and SCFAs production [306, 307]. *In vitro* colonic digestion has also shown BB polyphenols biotransformation to syringic, cinnamic, caffeic and protocatechuic acids [298]. Likewise, microbial metabolism of blueberry PP was found to elicit positive outcomes, namely the inhibition of HT-29 colon cancer cell line proliferation as well as the repression of colonic prostanoid production and anti-inflammatory effects [308, 309]. Overall, *in vivo* bioactivity of BB polyphenols is strictly correlated with their absorption along the GIT as well colonic microbial metabolism [298].

2.2 Blueberry in health and disease

The consumption of fruit and vegetable rich diets throughout the lifespan have been systematically linked with a prevention or reduction of the chronic disease´s risk [310, 311]. Tagged by the media as a "superfruit", BB have drawn remarkable attention during the last decades for their intrinsic health promoting potential [312, 313]. Supported by the information flowing from the scientific community, the awareness of BB health benefits has contributed to the expansion of the BB industry and its products worldwide alongside the rising consumer´s demand for this functional food. The global BB production and trade have seen the most dramatic growth between 2010 and 2019 [314].

As a rich source of PP, BB have been widely studied in several diseases which represent major socioeconomic burdens, such as cardiovascular disease, cancer, and other disorders [315-317], but also as a prophylactic for many conditions [318-320]. Evidences arising from *in vitro*, animal and human clinical studies have disclosed the wide healthful aspects of BB, namely their antioxidant, anticarcinogenic, anti-inflammatory, antimicrobial and antiangiogenic properties; in addition, improvement of markers of vascular function and immune health, as well as also aiding in neurocognitive function, memory and slowing age-related cognitive decline, have been described [315, 317, 321, 322]. For instances, the supplementation of BB into the diet improved cognitive performance and memory function in both children [323] and adults with an increased risk for dementia [324]. Moreover, the decline in functional mobility of older adults may be counteracted after daily BB intake [325, 326]. BB-related improvements in cognitive and motor performance have also been widely reported in rodents [322]. The antioxidants present in BB may contribute to the protection of eyes from aging caused by light-induced damage [327]. Furthermore, health benefits from BB consumption have also been associated with positive modulation of GM, which plays a crucial role in overall health [328-330]. Alongside these promising effects, several *in vitro* and *in vivo* research along with human interventional studies have proposed that BB-derived PP are a viable complementary strategy for various metabolic disorders, including T2DM, which will be detailed in the next subchapter.

The effects of BB and their constituent PP have been examined in many short-term [331-333] and long-term [324, 334] human interventions. The beneficial properties of phenolic compounds, namely anthocyanins, have also been extensively investigated and among them are their high antioxidant and anti-inflammatory potentials that could be, at

least in part, responsible for the most favorable effects observed in BB. Nevertheless, it has also been demonstrated that the combination of BB phytochemicals may exhibit synergistic and multifunctional effects on health [335]. It has been reported that the moderate intake of blueberries (approximately one-third cup) and anthocyanins (<50 mg) daily is associated with disease risk reduction [313, 336]. Higher intakes of BB and total anthocyanins were associated with a 32% lower rate of myocardial infarction, and this association was independent of established risk factors [337]. As a matter of fact, chronic BB consumption, for 1 month, improved endothelial function and lowed systolic blood pressure in healthy adults, and it was associated with anthocyanin intake [338]. However, in 2 prospective cohort studies no association was found between anthocyanin intake and stroke risk [337, 339].

Currently the most of evidences derived from *in vitro* and *in vivo* studies reported BB-derived PP benefits involves their antioxidant activity, however these compounds also have capacity to influence numerous other biological processes that are responsible for the changing the metabolic disease risk. Indeed, direct health benefits may also include the modulation of intercellular signaling and gene expression, the regulation of the endogenous defense mechanisms [337, 340]. In addition, PP can also chelate transition metals, decreasing their ability to promote ROS formation through Fenton reaction [341, 342].

In light of these range of BB-derived phytochemicals benefits, many consumers have sought to increase their PP intake, often turning to dietary supplements to putatively empower overall health status. In particular, the consumption of the PP-rich dietary supplements has risen significantly over the recent years, which contribute to rapidly increased PP consumption. Recent estimates show that 75% of US adults consume at least one dietary supplement and 30% consume PP-rich herbal supplements [42, 343, 344]. Some supplement manufacturers recommend the intake of concentrated doses of purified PP far higher than those commonly encountered in the current typical diet, and this often leads to an indiscriminate use, which has become a controversial issue [340]. The implicated supposition of consumers is based on the misconception that all plant-derived supplements pose no health risk. However, this assumption has not been adequately established, and consuming higher levels of PP in purified forms like dietary supplements presents a variety of challenges that must be investigated to fully understand their impacts. Many studies reported that phenolic compounds may act as pro-oxidants substance under

certain conditions that favor autooxidation, such as high concentrations of PP, pH, the presence of transition metal ions and O_2 molecules [345, 346]. Moreover, while small phenolic compounds (i.e. quercetin and gallic acid) are easily oxidized and possess pro-oxidant properties, phenolic compounds of high molecular weights (i.e. condensed and hydrolysable tannins) have little or no pro-oxidant properties [345, 347]. In addition, phenolic compounds such as caffeic acid, syringic acid, protocatechuic acid, p-coumaric acid, vanillic acid, ellagic acid, and rutin have been reported to possess dual antioxidant and pro-oxidant properties, depending on their concentration, pH and the presence or absence of transition-metal ions [346]. The pro-oxidant activity can be attributed to the production of ROS as a consequence of the possible disturbance of these compounds to the cells; however, their pro-oxidative nature cannot be simply defined as harmful, and the risks and benefits depend on the situation and concentration used [348].

Importantly, the majority of the research regarding the pro-oxidant activity of natural antioxidants are mainly limited to *in vitro* experiments. In fact, there are a few studies showing pro-oxidant activity of PP *in vivo* [349-351]. Wang and colleagues have shown that (-)-epigallocatehin-3-gallate (EGCG) acts as a pro-oxidant in mice at doses higher than 45 mg/kg. Mechanistically, EGCG induces hepatotoxicity via the suppression of nuclear factor erythroid 2-related factor 2 (Nrf2), which is the key protein in the regulation of antioxidant enzymes, including heme oxygenase 1 (HO1), NAD(P)H: quinone oxidoreductase 1 (NQO1), SOD and GSH S-transferase (GST) [349]. Furthermore, high doses of green tea polyphenols in diet (0.5–1%) cause nephrotoxicity and negatively regulate the expression of antioxidant enzymes and molecular chaperones, in addition to aggravating colitis and colon carcinogenesis in mice [348]. Indeed, potential adverse effects observed in connection with consuming high doses of PP have been noted; however, there was little evidence upon which dose threshold for different phenolics and/or food sources of complex mixtures of phenolics [352]. Despite many studies have pointed the potential health benefits of BB-derived phytochemicals, the effectiveness and longevity of the BB intake in both human health and disease has not been established so far.

2.3 Antidiabetic properties of blueberry

2.3.1 Hypoglycemic and insulin sensitizing effects

A number of *in vitro* studies strongly suggests that BB and its bioactive phytochemicals present anti-diabetic properties. The ability of an extract from Lowbush BB fruit to increase pancreatic β-cell proliferation was explored in a cell culture-based bioassay, suggesting a potential capacity to restrain β-cell damage and improve insulin sensitivity [287]. Likewise, whole BB afforded pancreatic β-cell protection and prevented β-cell apoptosis and expansion in HFD-fed C57BL/6J mice [353]. Notably, a tight regulation of pancreatic islet area was observed as BB supplementation induced an increased number of small islets (comprising more β-cells with higher insulin) along with a decreased density of larger islets, probably delaying the overwhelmed burden of β-cells activity that pair obesity and diabetes progression [353, 354]. Similar pro-proliferative effect was observed for a BB leaf extract in pancreatic MIN6 β-cells, accompanied by improved insulin signaling [285]. *In vivo*, this extract was able to decrease BW, plasma glucose, HbA1c, HOMA-IR, TGs and non-esterified fatty acids (NEFAs) levels in C57BL/6J mice fed with HFD [285]. These effects paralleled an increased expression of pancreatic β-cell proliferation-related genes (Ngn3, MafA, Pax4, Ins1, and Ins2) and insulin signaling genes (IRS-1, IRS-2, PIK3ca, PDK1, PKCε, and GLUT-2) while FOXO1, a β-cell apoptosis-related gene, was found downregulated [285]. Similar results were observed in male Wistar rats submitted to a combined STZ+HFD paradigm of experimental T2DM [355].

Furthermore, it has been reported that BB and their bioactive compounds exhibit inhibitory activity toward pancreatic α-amylase and intestinal α-glucosidase, two enzymes involved in the metabolism of starch, which could lead to a delay of carbohydrate digestion and/or decreased glucose absorption [356-358]. Interestingly, *in vitro* studies have also highlighted that PP from BB can act as strong DPP-4 inhibitors, analogous to clinically approved pharmacological options (e.g. gliptins) [359-361].

Protein tyrosine phosphatase 1B (PTP1B) is another promising pharmacological target for the treatment of T2DM as it can reduce blood glucose levels by increasing insulin sensitivity [362]. Notably, Tian and colleagues identified anthocyanins isolated from BB as selective inhibitors of PTP1B able to increase glucose consumption on human hepatoma cells HepG2 in a dose-dependent manner [363]. Moreover, Nachar and colleagues performed an *in vitro* study with phenolic compounds from BB juice fermented with *Serratia vaccinii* and observed a strong antidiabetic potential in the liver and skeletal muscle, as it was able to regulate key enzymes involved in glycogen synthesis, gluconeogenesis and skeletal muscle glucose uptake [364].

In vivo pre-clinical and clinical studies also hint for T2DM improvements upon BB consumption. Regarding animal models, phenolic compounds from a fermented BB – blackberry beverage were able to attenuate FPG and the development of obesity in C57BL/6J mice [284]. Vendrame and colleagues also reported that wild BB consumption significantly decreased plasmatic concentrations of HbA1C, resistin, and RBP4 in the obese Zucker rat model of metabolic syndrome along with the repression of hepatic resistin and RBP4 in the abdominal adipose tissue [365].

Some clinical trials aimed to analyze the potential anti-diabetic effects have also been explored [333, 334]. In T2DM male volunteers, a single oral capsule of 0.47 g standardized bilberry extract (36 % w/w anthocyanins), equivalent to 50 g of fresh bilberries, significantly decreased the area under the curve (AUC) for both glucose and insulin in 18%, with no changes on gut incretin hormones (GIP and GLP-1) nor in glucagon and amylin secreted from the pancreas [333]. Moreover, an intervention study showed that daily consumption of a BB smoothie (45 g of BB bioactive, equivalent to ~2 cups of fresh BB per day) for 6 weeks significantly improved insulin sensitivity in thirty-two obese, insulin resistant adults, despite unchanged fasting serum glucose levels [334].

Nevertheless, recent clinical trials reported inconsistencies on the efficacy of BB in ameliorating glucose profile in both T2DM and insulin resistant subjects [366-368]. A double-blind, randomized controlled trial (RCT) was carried out to evaluate the effect of BB consumption on cardiometabolic parameters in type 2 diabetic men [367]. Twenty-six participants consuming a beverage made from 22g of freeze-dried BB (equivalent to 1 cup of fresh BB) daily for 8 weeks had lower HbA1c and fructosamine levels (two biomarkers of glycemic control) along with lower triglyceridemia when compared to participants consuming the placebo. However, no significant changes in total BW, blood pressure, lipid

profile (low-density lipoprotein (LDL), high-density lipoprotein (HDL) and total cholesterol (total-c)) as well as in serum fasting glucose/insulin concentrations were detected. All of these participants were taking noninsulin diabetes medication and had a good glycemic control [367]. In another study based on a long-term RCT, thirty-seven adults with metabolic syndrome received for 6 months a single-serve sachets with 26 g of freeze-dried BB (1 cup of BB, equivalent to 150 g of fresh BB) daily. Improved markers of vascular function and elements of lipid status (increased high-density lipoprotein-cholesterol (HDL-c) and alipoprotein A-I) were detected following 1 cup of blueberries daily for 6 months. However, no benefits were observed on insulin resistance and sensitivity, measured by HOMA-IR, quantitative insulin sensitivity index (QUICKI) and HbA1c and by the gold standard 2-step, hyperinsulinemic euglyceminc clamp [369]. This ambiguity between human intervention studies may be due to the heterogeneity of study population, the duration of the study and/or with the specific analytical technique used to assess the glycemic control. In addition, variations in sources and doses of BB-derived phytochemicals, distinct food or beverage matrix or processing may be contributing to the inconsistent findings when assessing the effects on metabolic outcomes.

Notably, it is also challenging to identify the optimal dose of polyphenols needed to improve metabolic health since the amount of bioactive compounds present in aforesaid BB paradigms greatly varies between published studies.

2.3.2 Antioxidant and anti-inflammatory properties

BB owe their antioxidant activities mainly to their phenolic compounds including phenolic acids, condensed tannins and principally, anthocyanins [370, 371]. Several studies point to linear correlation between antioxidant activity and the total phenolic concentrations as well as anthocyanins in BB [372-374]. Besides anthocyanins, cinnamic acid derivatives of phenolic acids (e.g. caffeic and chlorogenic acid) were found to be more active antioxidants than benzoic acids derivatives [371].

Chronic hyperglycemia leads to a pro-oxidant and inflammatory scenario. Conversely, increased oxidative stress and inflammation foster insulin resistance and impaired insulin secretion. Hence, treatments aimed to inhibit ROS overproduction and inflammatory markers are welcome to delay the onset and progression of T2DM and related complications [52].

Health beneficial effects of BB phytochemicals are well recognized on the basis of their potent antioxidant and anti-inflammatory properties, particularly in metabolic conditions such as T2DM [358, 375, 376]. In diabetic human aortic endothelial cells, BB phytochemicals were able to restore cell surface glycosaminoglycan, attenuate endothelial inflammation, suppress monocyte binding and reduce endothelial IL-8 and vascular cell adhesion molecule 1 (VCAM-1) [377]. Additionally, BB anthocyanins extract (BAE) was able to decrease ROS generation while increasing catalase and SOD activities in high glucose-induced injury of human retinal capillary endothelial cells (HRCECs); furthermore, BAE was also found to influence angiogenesis by decreasing vascular endothelial growth factor (VEGF), intercellular adhesion molecule-1 (ICAM-1) and Akt pathways inhibition [375].

Several *in vivo* studies highlighted the antioxidant and anti-inflammatory effects of BB and its specific compounds on metabolic impairments. An overall improvement in the inflammatory status was reported in obese Zucker rats supplemented with wild BB powder [378]. The consumption of 8% wild blueberry-enriched diet for 8 weeks significantly decreased plasma levels of TNF-α, IL-6 and CRP and increased adiponectin. In addition, the expression of CRP was reduced in the liver, while TNF-α, IL-6 and NF-κB were down-regulated in both liver and abdominal adipose tissue [378]. In male Wistar rats, BB supplementation in the diet elicited the normalization of TNF-α and IL-1β levels, which were increased by 300% and 500% due to a high-fat feeding paradigm [329]. Other

authors found that BB powder afforded protection against inflammation of adipose tissue by preventing the upregulation of inflammatory genes (i.e. TNF-α and IL-10) and attenuating oxidative stress (with an increased GPx gene expression), collectively ameliorating obesity-induced insulin resistance [379]. In addition, obese Zucker rats treated during 15 weeks with BB showed improved glucose tolerance and renal function, together with amelioration of oxidative balance (upholding SOD/catalase antioxidant levels while attenuating oxygen and nitrogen free radical production) and inflammatory pathways (decreased gene and protein expression of TLR4, phosphorylation of ERK and p38-MAPK, NF-kB activity) [380]. Moreover, other studies showed an antioxidant effect of bilberry leaves extract in serum of male Wistar rats under a double challenge composed of HFD and STZ [355]. Nevertheless, there has been some controversy on BB-derived polyphenols benefits on total antioxidant activity in both *in vivo* and human studies [381-385]. Distinct BB doses, food matrices and/or alternative methodologies to assess total antioxidant status may foster such ambiguity.

In humans, previous studies indicated that BB have immunomodulatory effects and reduce oxidative stress in adults with metabolic syndrome [386]. However, the results so far are relatively inconclusive, possibly due to considerable inter-individual variation. In a 8-week RCT, Basu and colleagues found that circulating levels of oxidized LDL, MDA and HNE decreased in participants with metabolic syndrome that consumed 50 g of freeze-dried BB beverage (approximately 350 g fresh BB) daily, despite no significant changes in serum glucose levels, lipid profiles or inflammation biomarkers [387]. In another RCT designed to evaluate the effects of 6-weeks intake of 45 g of freeze-dried BB per day in subjects with metabolic syndrome, a significant decrease in superoxide and total ROS contents were found in whole blood and monocytes, together with decreased circulatory inflammatory markers and reduced gene expression of TNF-α, TLR4 and IL-6 in monocytes [386]. Similarly, other researchers have found that BB reduced inflammatory markers as well as enhanced antioxidant activity in individuals with metabolic syndrome or diabetes [386, 388].

2.3.3 Prebiotic effects

Given that BB phytochemical composition comprises several bioactive compounds including high molecular weight PP and easily fermentable dietary fibers that are not well absorbed in the small intestine and reach the large intestine, such bioactive components are available to interact with GM [389]. Indeed, prebiotic properties and/or antimicrobial effects against pathogenic intestinal bacteria of BB and their bioactive compounds have been reported in *in vitro* assays [390-392], in animal models [393, 394] and in human intervention studies [328, 395]. An *in vitro* study reported distinct positive correlations between different PP contents and bacterial species; in fact, while PP from BB bagasse powder was correlated with Actinobacteria (*Bifidobacterium* and *Collisella*) and *Akkermansia muciniphila*, the fiber content was associated with *Faecalibacterium* and *Bifidobacterium* [391]. Moreover, the presence of anthocyanins and fibers in BB pomace promoted the growth of *Lactobacillus* and *Ruminococcaceae* genus and was negatively associated with Streptococcus. The addition of a BB extract to mixed human fecal bacterial populations enhanced the population size of *Lactobacilli* and *Bifidobacteria* [393].

Growing evidence hint that the positive effects of BB in improving glucose metabolism and energy balance are closely linked with their ability to modulate the composition and/or function of GM [389, 394, 396, 397]. A recent study reported that upon intestinal absorption BB polyphenols improved metabolic health in diet-induced obese mice via modulation of GM rather than through systemic effects [394]. It was also observed that germ-free mice receiving fecal microbiota transplantation (FMT) from mice fed BB polyphenolic fractions (specifically enriched with proanthocyanins or anthocyanidins) were protected from diet-induced obesity and presented an improved insulin sensitivity and glucose homeostasis when compared to mice receiving GM from HFHSD fed mice. Furthermore, authors postulated that the beneficial effects of FMT could be the result of a decreased proportion of BCAA in the feces of mice fed whole BB or just the PP [394]. Moreover, positive effects on insulin sensitivity and glycemia were found in the mouse model of HFD-induced obesity upon BB juice supplementation during 17 weeks, with or without fermentation [398]; the authors hypothesized that this protection was closely related with BB ability to stimulate SCFAs production and maintain GM homeostasis [398].

Among health commensal bacteria, *Akkermansia muciniphila* has been positively correlated with enhanced mucosal barrier function and improved metabolic phenotype in

obesity ad T2DM [392, 399-401]. Notably, BB interventions enhanced *Akkermansia muciniphila* levels in animal models of metabolic syndrome [397, 402]. Indeed, whilst favoring the growth of healthier bacteria, BB constituents seem to display the ability to restore gastrointestinal integrity by: i) enhancing colonic mucus thickness [397]; ii) increasing the expression of tight junctions proteins [403]; iii) overexpressing mucin-2 (Muc-2) mRNA, the primary glycoprotein of the gastrointestinal mucus layer [329]; iv) reducing endotoxemia and intestinal inflammation [329, 404] and v) fortifying the mucosal immunity [402].

Interestingly, BB polymeric proanthocyanidins have been recently shown capacity to restore the colonic mucus layer, modulate GM and attenuate glucose tolerance in obese mice, in contrast to the anthocyanin fraction from BB [397]. However, other PP subclasses, secondary metabolites and fibers also present in BB may potentially account for these prebiotic effects counteracting the metabolic disorders [296, 392, 405]. Notwithstanding, ambiguous studies and conflicting results still prevail, which could possibly be explained by several factors such as distinct duration and mode of administration of BB interventions, differences in the BB phytochemical composition (i.e. the type and structure of polyphenolic constituents), as well as differences in the dietary regimens. Noteworthy, the inclusion of bilberries in the high-fat setting prompted an increase in SCFA contents accompanied by the normalized α-diversity of GM, enhanced the abundance of Bacteroidetes, *Bifidobacterium* and butyrate-producing bacteria, which collectively contributed to thwart pre-obesity events like hepatic lipid accumulation [406].

The impact of BB on GM has been also evaluated in human interventional studies [395]. Vendrame and colleagues showed that 6 weeks consumption of a BB powder drink rich in anthocyanins significantly enhanced the abundance of *Bifidobacteria* in the cecum from healthy volunteers, whereas no differences were observed for Bacteroides spp., *Prevotella* spp., *Enterococcus* spp., and *Clostridium coccoides* [328]. A pilot RCT was carried out in overweight and obese subjects to evaluate the effects of a gastrointestinal microbiome modulator (GIMM), composed of inulin, β-glucan and anthocyanins and PP from BB, on metabolic parameters, fecal markers of GM, and satiety [407]. Thirty overweight or obese participants consumed the GIMM or placebo, daily, for 4 weeks. Improved glucose tolerance, restored satiety hormones (increased PYY and decreased ghrelin) and fecal SCFAs contents were observed in the GIMM-treated group when compared to the placebo; however, no significant changes were found between groups

regarding insulin sensitivity, fecal markers of GM, plasma satiety hormones or serum lipid concentrations [407]. The prebiotic effects of BB reported in the preclinical ground need further validation in the clinical setting, namely in the (pre)diabetic state, as the literature is particularly scarce in human interventions in this condition. Currently, there are two ongoing clinical trial (NCT03266055 and NCT03934177) exploring the prebiotic effects of BB in obese subjects with metabolic syndrome. The results from this and other trials further conducted will be decisive to achieve more robust conclusions regarding the use of BB to prevent dysbiosis in prediabetes.

2.3.4 Hepatoprotective effects

Apart from high antioxidant and anti-inflammatory properties, BB may exert antidiabetic effects by additional mechanisms. It has been unveiled that BB phytochemicals can provide beneficial effects in T2DM by interfering with energy homeostasis and nutrient metabolism in different tissues, including in the liver [369, 408].

Several phytochemicals abundant in BB, such as anthocyanins, have been shown to display lipid-lowering actions through hepatic metabolism pathways, including the regulation of key hepatic enzymes involved in lipogenesis and fatty acid catabolism [283, 409], as well as in carbohydrate metabolism [356]. A previous *in vitro* study suggested that concentrate anthocyanins and other polyphenols from BB may improve glucose metabolism by repressing glucose production in rat hepatoma cell line H4IIE [410]. Additionally, two BB methanolic extracts showed hypoglycemic effects in human non-tumor hepatic LO2 cells, an effect correlated with the increase of GLUT-2 and PPAR-γ expression as well as with the inhibition of relevant inflammatory pathways [276].

The hepatoprotective effects of the BB phytochemicals have been highlighted in several animal models of chemical-induced liver injury [411-413] but also in obesity and diabetes-associated chronic diseases, including in NAFLD [408, 413-415]. A recent study showed that nonacylated anthocyanins from bilberry reduced plasma glucose, lipids and BCAA level in obese diabetic ZDF rats, suggesting an improvement of insulin sensitivity and reduction of lipogenesis [405]. In another study in diabetic mice, antidiabetic effects of dietary anthocyanin-rich bilberry extract (BBE) were associated with targeting of hepatic AMPK, GLUT-4 and metabolic enzymes; furthermore, BBE downregulated the mRNA expression of two gluconeogenic enzymes, PEPCK and G6Pase, and suppressed

glucose flux into the blood [408]. In addition, an enhanced phosphorylation of ACACA, the key enzyme for fatty acid synthesis that is downstream of AMPK, and the upregulation of PPAR-α, ACOX and CPT1, were found in the liver of diabetic mice supplemented with BBE. Furthermore, supplementation of BB polyphenols extracts also decreased the hepatic mRNA expression of SREBP-1 and FASN, which could contribute to improved hepatic lipid metabolism [378]. Other recent findings suggest that phenolic BB extract improved hepatic lipid metabolism via pathways involving the bile acids receptors FXR and Takeda GPR5 [404].

Moreover, BB phytochemicals elicit mitochondrial-targeted protective properties by scavenging ROS and acting as uncouplers of the OXPHOS, preserving mitochondrial function and protecting cells from apoptosis [416-420]. Likewise, a protective effect of BB anthocyanin-rich extract was found in mice hepatic mitochondria against acrylamide-induced mitochondrial oxidative stress by inhibition of ROS formation [420]. At the cellular level, cyanidin-3-O-beta-glucoside (C3G, one of the main components of BAE) reduced ROS formation, caspase-3 and -9 inactivation, and downregulated the pro-apoptotic Bax protein induced by hyperglycemia, preventing mitochondrial dysfunction through modulation of PI3K/Akt and JNK signaling pathways [417]. The combination of BB juice and probiotics (BJP) reduced NAFLD-induced mitochondrial ultrastructure damage and swelling and hepatic necrosis in parallel with improvements on respiratory function. Moreover, BJP attenuated mitochondrial oxidative stress through elevation of GSH and SOD levels while reducing ROS production in an animal model of NAFLD. The authors demonstrated that the modulation of SIRT1/PGC-1α pathway is a potential target of BJP against hepatic damage induced by NAFLD [418]. More recently, the activation of AMPK/PGC-1α/SIRT3 signaling pathway was associated with beneficial effects of BB leaf polyphenols on hepatic mitochondrial function and oxidative defense [413]. Additionally, improvements on mitochondrial respiratory parameters were found in isolated hepatic mitochondria from non-obese diabetic GK rats after drinking bilberry leaves decoction for 4 weeks [421].

Even though a mechanistic explanation was not scrutinized in this study, quercetin present in bilberry leaves may partially explain the increased mitochondrial oxidative and phosphorylative activities in GK treated rats. In fact, this PP has a positive impact on mitochondrial biogenesis related with an increased mRNA expression of PGC-1α and SIRT1 along with higher contents of mtDNA and Cyt c [422]. Further animal research

also showed that the attenuation of hepatic steatosis and enhancement of lipolysis following exposure of hepatic cells to BB polyphenols can be promoted by modulation of autophagy [413].

In vivo animal studies have showed that BB reduced the aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in both mouse models of liver injury but also in obese and insulin resistant animal models [423, 424]. Conversely, despite changes in lipid peroxidation in the liver, the hepatic TGs accumulation and serum AST and ALT levels were not altered following the administration of whole blueberry powder or polyphenolic-rich extracts in diet-induced obesity and insulin resistant mice [394]. Interestingly, authors found that the improved metabolic outcomes were only exhibited in obese insulin resistant animals supplemented with BB polyphenol-rich fractions and no improvements were found in mice fed high-sucrose/high-fat (HSuHF) diet plus whole BB powder, suggesting that the metabolic benefits are a result of specific classes of PP isolated from the whole fruit.

In humans, some interventional studies have demonstrated the ability of BB to lower hepatic transaminases (such as ALT and AST), which might suggest hepatoprotection [367, 425]. Moreover, a double-blind RCT conducted in patients with NAFLD for 12 weeks demonstrated beneficial effects of purified anthocyanins derived from bilberry and black currant by improving insulin resistance [426].

CHAPTER 2

Rationale and aims

Rationale and aims

In the second half of the last century, we have witnessed a sharper increase in the prevalence of cardiometabolic disorders, such as obesity, diabetes and cerebro/cardio-vascular diseases, in some world regions, namely in the USA and Europe. These effects largely resulted from the lifestyle of these populations in the post-industrialization era, with easier access to food, increasingly processed, and a decline in physical activity. Despite the development of a vast pharmacotherapeutic arsenal, the reality is that its impact on the mortality was much smaller than expected and desirable. Thus, it became clear that a significant impact could only happen with a concomitant change in lifestyles, namely the adoption of a more balanced and healthy diet. Awareness campaigns among populations were intensified and it is likely that they will begin (or have already begun) to display positive results. In fact, the impetus for the improvement of health and quality of life is increasingly present in some fringes of the population, which arouses a growing concern for adopting healthier lifestyle and changing patterns, among which diet plays a preponderant role [427].

In response to the sharp increase in the number of health-conscious consumers, in recent years, there is a strong movement towards the development of nutraceuticals, functional foods and dietary supplements to address the various concerns and constraints [428, 429]. Polyphenols (PP) and fibers are among the most popular and desired compounds by the consumers, due to a set of properties that the scientific community and the food industry have begun to explore and publicize. The consumption of food or dietary supplements enriched in PP and fibers is expected to rise owing to their widespread use [430, 431]. Vegetables and fruits are important food sources of PP and/or fibers and red fruits – namely the blueberry (BB) – are among those that have gained more reputation in recent years.

In addition to other biological effects widely recognized, such as the antioxidant properties, it has been documented that the positive health outcomes associated with phytochemicals-rich foods and supplements (namely those with abundance of PP and fibers) can arise from their beneficial impact on gut microbiota and maintenance of a healthy gut, referred as prebiotic effect [432-434]. However, even though the health benefits of plant-based food products have been widely advertised [434-436], the value of these approaches seems debatable, namely for those enriched in polyphenols. In fact,

unreliable and ambiguous scientific outcomes have been attained in *in vitro* and *in vivo* studies [352, 437]. Importantly, beside dietary PP have been widely touted as antioxidants to explain health benefits, adverse effects probably due to the high load of PP and corresponding pro-oxidant effects have also been claimed [430, 431]. Moreover, this dual behavior of dietary PP in the context of oxidative stress may have a critical role in the modulation of mitochondrial function and consequently impact the cellular redox state [438]; however, the net beneficial or harmful effects of PP supplementation remains to be elucidated, particularly those related with BB-based dietary interventions. Hence, considering the rise of PP intake, not only in food products but particularly as supplements, it is of utmost importance to clarify the safety profile and address the health outcomes associated with this consumption profile. Thus, the first goal of the present thesis was to investigate the impact of long-term supplementation with BB in a healthy condition – **Chapter 3**. To this end, we used young adult healthy Wistar rats.

As pointed out in the introduction of this thesis (**Chapter 1**), several preclinical and clinical studies have revealed a panoply of benefits ascribed from BB-derived PP and fibers (as whole fruits, juices and/or purified extracts forms) in metabolic dysregulation conditions including T2DM and related disorders [283, 287, 290, 388, 439]. Considering this rationale, we hypothesized that BB supplementation may display protective effects against prediabetes progression that goes beyond the well-known antioxidant activity. In particular, we postulated that BB may prevent or delay disease progression by targeting gut and/or hepatic-derived pathophysiological traits which seems to be place as soon as prediabetes. Thus, the second main goal of this thesis was to assess the impact of long-term supplementation with BB in a prediabetic condition – **Chapter 4**. To this end, we used a prediabetic animal model, previously characterized by our team, which were established by the administration of sucrose solution (35%) and further supplemented by high-fat diet (61.6% of energy as fat).

To accomplish these two main purposes, the specific objectives behind this thesis were the following:

- To characterize the metabolic profile through the evaluation of glycemic, insulinemic and lipid profiles;
- To evaluate the impact of BB on the antioxidant status and metabolomic profile;
- To dissect the prebiotic effect of BB on fecal gut microbiota, SCFAs composition, intestinal barrier structure and permeability, as well as on metabolic endotoxemia;
- To assess the effect of BB on hepatic histomorphology and lipid deposition;
- To investigate the impact of BB on hepatic mitochondria bioenergetics;
- To analyze the influence of BB on the modulation of hepatic gene expression involved in chief-metabolic pathways.

CHAPTER 3

Blueberry consumption challenges hepatic mitochondrial bioenergetics and elicits transcriptomics reprogramming in healthy rats

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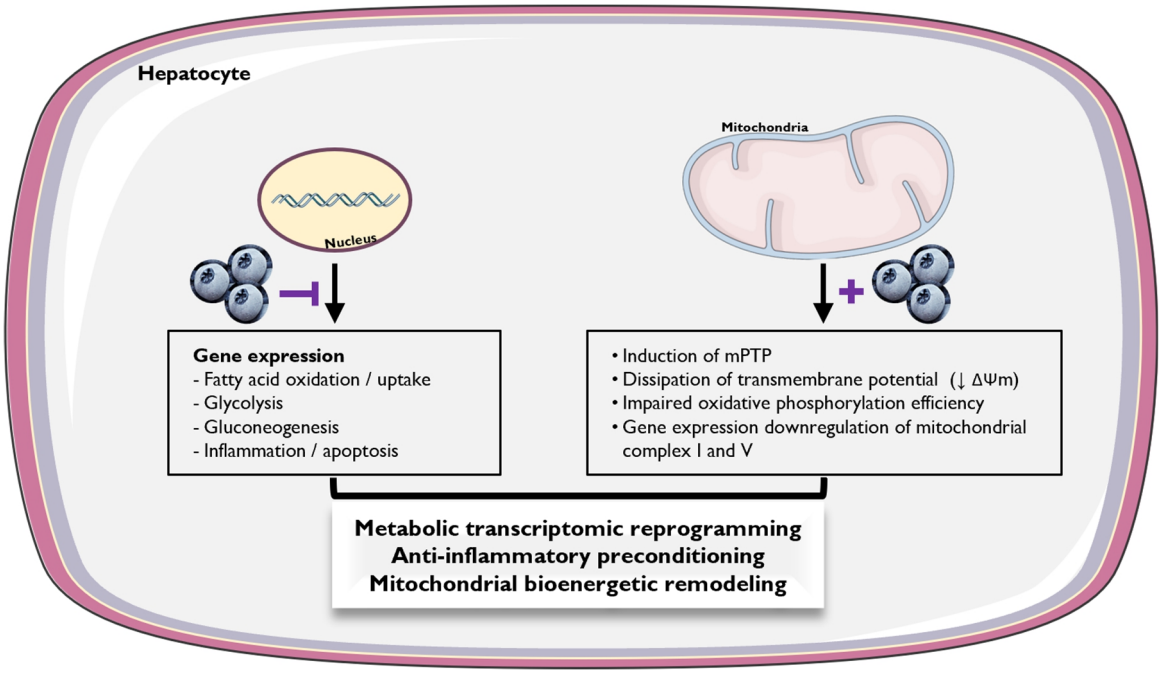
3.1 Abstract

An emergent trend of blueberries' (BB) "prophylactic" consumption, due to their phytochemicals' richness and well-known health-promoting claims, is widely scaled-up. However, the benefits arising from BB indiscriminate intake remains puzzling based on incongruent preclinical and human data. To provide a more in-depth elucidation and support towards a healthier and safer consumption, we conducted a translation-minded experimental study in healthy Wistar rats that consumed BB in a juice form (25 g/kg body weight (BW)/day; 14 weeks' protocol). Particular attention was paid to the physiological adaptations succeeding in the gut and liver tissues regarding the acknowledged BB-induced metabolic benefits. Systemically, BB boosted serum antioxidant activity and repressed the circulating levels of 3-hydroxybutyrate (3-HB) ketone bodies and 3-HB/acetoacetate ratio. Moreover, BB elicited increased fecal succinic acid levels without major changes on gut microbiota (GM) composition and gut ultra-structural organization. Remarkably, an accentuated hepatic mitochondrial bioenergetic challenge, ensuing metabolic transcriptomic reprogramming along with a concerted anti-inflammatory pre-conditioning, was clearly detected upon long-term consumption of BB phytochemicals. Altogether, the results disclosed herein portray a quiescent mitochondrial-related metabolomics and hint for a unified adaptive response to this nutritional challenge. The beneficial or noxious consequences arising from this dietary trend should be carefully interpreted and necessarily claims future research.

Keywords

Blueberries; long-term consumption; bioenergetics remodeling; transcriptomics reprogramming; anti-inflammatory pre-conditioning.

3.2 Graphical abstract



3.3 Introduction

The continuous rise in life expectancy observed in the last decades encloses an ascending trajectory of non-communicable diseases, which are often linked with unhealthy dietary patterns [440, 441]. Society is progressively becoming more aware of healthy eating to prevent diet-related chronic diseases. New trends in food consumption have fostered agro-food and pharmaceutical companies to develop new products, both wellness-focused diets, functional foods, and oral nutraceutical supplements, to meet consumer demands [442]. A good example of this reality is blueberry (BB) market globalization, whose per capita consumption nearly tripled in USA since 2002 and is fast expanding worldwide [443, 444].

The low caloric content of BB (0.046 kcal/g fresh fruit) pair with their enriched nutritional and phytochemical composition [277]. BB is a privileged source of micronutrients (e.g. selenium, zinc, iron), dietary prebiotic fibers (3–3.5% of their fruit weight), and antioxidant polyphenols encompassing anthocyanins, flavonols, phenolic acids, procyanidins, and/or stilbenes derivatives, with an overall content reaching up to 0.3% of fresh fruit weight [277, 289]. These bioactive compounds are extensively metabolized by the colonic microbiota; in addition, regardless of their poor oral bioavailability [445-447], BB-derived phytochemicals can positively modulate chief endogenous functions in distinct organs and tissues that extend beyond their well-documented antioxidant properties [313, 448-450]. In fact, experimental data arising from cell-free systems, standard cell-cultures, and/or isolated organelles highlight BB phytochemicals' ability to modulate non-redox mechanisms through their interactions with functionally diverse cellular targets, such as intercalation with DNA, transcription of several genes associated with key cellular functions, mitochondria dynamics, and even gut microbiota (GM) homeostasis [443, 451-454]. Convergent results are also reported in preclinical animal studies that emphasize BB consumption benefits in a panoply of chronic disorders paralleling obesity-related metabolic diseases such as cardiovascular disease, metabolic syndrome (MS) or type 2 diabetes mellitus (T2DM) [313, 378, 396, 418, 455-458]. Interestingly, the association between human BB consumption and biomarker-based evidence of reduced risk of diseases has been also emphasized [313, 366, 369, 407, 459, 460]. In fact, several clinical trials have progressively emphasized on potential health benefits (e.g. endothelial, gastrointestinal, cardio-metabolic outcomes) arising from long-term BB-enriched human dietary patterns and commercially available BB supplements with

the ultimate goal to empower overall health status [332, 381, 395, 425, 461]. Regardless of this wealth of evidence, incongruent preclinical and human data still remain an open debate and the translation into the clinical practice remains puzzling, mainly due to critical flaws surrounding studies' reproducibility [332, 366, 460, 462, 463]. For instance, *in vitro* assays often employ high-concentrations of BB-derived phytochemicals that are unlikely to reach systemic circulation once orally ingested in living organisms [464]. Besides dose, the compounds' bioactivity is often dependent on (i) how an organism is exposed to, (ii) for how long, and (iii) interindividual variability. The heterogeneity surrounding BB-derived phytochemicals' intake regarding the presentation forms (e.g. fresh/frozen/freeze-dried fruit, distinct cultivars, the range of commercially available supplementation forms) and regimen durations (e.g. short- versus long-term) actually convolutes the interpretation of contemporary preclinical and human data. Moreover, the high interindividual human variability is also well-recognized in terms of BB phytochemicals' bioavailability and bioactivity, which is largely dependent of the individual gut microbiome [332, 366, 369, 442, 465]. Thus, it is currently challenging to judge the benefits and/or hazardous consequences underlying this nutraceutical contemporary trend, which necessarily calls for further research.

To this end, we conducted a translation-minded experimental design in young adult healthy Wistar rats who were daily supplemented with a dose of 25 g/kg of whole fresh BB, mirroring recent clinical trials [332, 465-467] in a long-term regimen (14 weeks) [468]. Particular attention was paid to the physiological adaptations ensuing in the gut and liver tissues regarding their well-known metabolic chief functions.

3.4 Materials and Methods

3.4.1 Preparation of blueberry juice

Blueberries (*Vaccinium corymbosum* L., cultivar "Liberty") were provided from the same variety and in the same maturation stage by COAPE (Farming Cooperative of Mangualde, Mangualde, Portugal) and stored at −80 °C until processing. To ensure that whole parts of BB fruits (peel, pulp, and seeds) were consumed, the BB were weighed, blended with drinking water, and transformed into BB juice (BJ). The amount of drinking water added was adjusted to ensure that 25 g of BB (per kg of rat's BW) were daily consumed. This BB dose was based on previous preclinical and human studies [332, 466, 469], and is equivalent to a daily consumption of 240 g of fresh whole BB (approximately 3/2 daily cups of BB), established taking into account the surface area of a person weighing ≈60 kg [466, 470]. Since whole BB juice loses about 83% of its anthocyanins content and about 40% of its antioxidant activity during storage at 4–8 °C for 10 days, BJ was freshly prepared on a daily basis [471].

3.4.2 Phytochemical analysis of phenolic compounds in BJ

A sample of fresh BJ was concentrated under reduced pressure and freeze-dried for phytochemical analysis. The lyophilized juice was dissolved in water (7 mg/mL) and injected (100 µL) in a high-performance liquid chromatography (HPLC) (Gilson, Middleton, WI, USA) hyphenated with a photodiode array detector PDA (model 170) and a control and processing software (Unipoint® 2.10). The chromatograph was equipped with an auto sampler (234 autoinjector), two pumps (models 305 and 306), a manometric module (model 805), a mixer (Model 811 B), and an C18 analytical column (Spherisorb Waters® S5 ODS2; 250 × 4.6 mm, 5 µm particle size), maintained at 35 °C, preceded by a guard column KS 30/4 Nucleosil 120–5 C-18, Macherey-Nagel (Duren, Germany). A mixture of 5% aqueous formic acid solution (A) and methanol (B) was used as mobile phase, with gradient elution of 0–75 min (0–100% B) at a flow rate of 1 mL/min. The UV-vis spectra were obtained between 200 and 600 nm.

3.4.3 Animals and experimental design

Male Wistar rats (16-weeks-old) were purchased from Charles River Laboratories (Barcelona, Spain) and housed two per cage in ventilated cages, with controlled environmental conditions (22 ± 1 °C, relative humidity of 50–60% and a 12 h light-dark cycle) and ad libitum access to standard rodent chow and tap water. After one week of acclimatization period, rats were randomly assigned into two groups (n = 8 per group): (i) Control group (CTRL), maintained with standard rat chow containing 8.6% kcal from fat (4RF21, Mucedola®, Milan, Italy) and tap water and (ii) Blueberry juice group (BJ), fed the same standard chow and supplemented daily, during the experimental period of 14 weeks, with 25 g/kg body weight (BW)/day of BJ (Figure 3.1). Following the daily dose of BJ intake, drinking water was provided ad libitum.

The experiment was carried out in strict compliance with the National and European Communities Council Directives of Animal Care and with the ARRIVE guidelines for reporting animal research [472]. The animal's protocol was approved by the local (iCBR) Animal Welfare Body (ORBEA, #9/2018, 30 October 2018).

Feed and beverage were provided ad libitum, with exception of the fasting periods. BW was monitored weekly; food and beverage consumption were daily recorded per cage throughout the experimental protocol. Energy intake per week was calculated for each animal by using the former measurements.

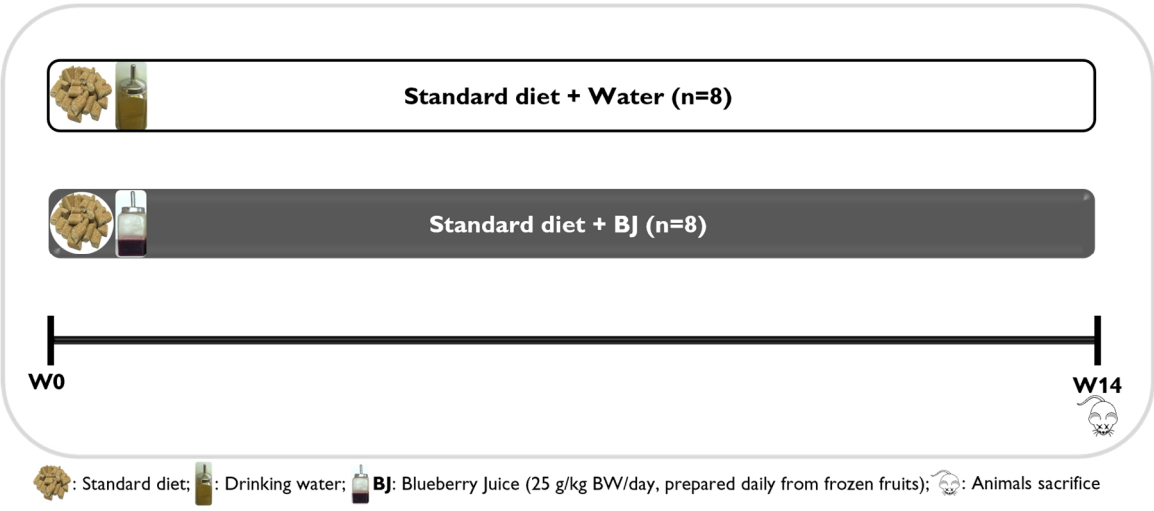


Figure 3.1 | Experimental groups and protocol design.

3.4.4 Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

During the first days of weeks 13 and 14, GTT and ITT were performed to assess the rats' ability to tolerate a glucose load and to evaluate peripheral insulin sensitivity, respectively, as previously described [275]. For the GTT, after 6 h of fasting period (between 8:00 a.m. and 2:00 p.m.), conscious rats were intraperitoneally (i.p.) injected with a glucose solution of 2 g/kg BW and blood glucose levels were measured from the tail blood, recorded immediately before (0 min) and 30, 60, and 120 min after injection, using a portable glucometer (ACCU-CHEK® Aviva, Roche Diagnostics, Mannheim, Germany). Additionally, blood samples (≈30 µL) were collected before glucose challenge to determine the fasting insulin concentration.

For the ITT, animals were fasted during 6 h and injected i.p. with insulin solution (0.75 units/kg BW; Actrapid Novo Nordisk, Bagsvaerd, Denmark). Blood glucose levels were measured in the tail vein blood collected immediately before (0 min) and 15, 30, 45, 60, and 120 min after injection, using the glucometer (ACCU-CHEK® Aviva, Roche Diagnostics, Mannheim, Germany). The rate constant for glucose clearance (K_{ITT}) was calculated using the formula $0.693/t_{1/2}$ where $t_{1/2}$ represents the half-life of plasma glucose decay. The plasma glucose $t_{1/2}$ was calculated from the slope of the least squares analysis of the glycemic concentration during the linear phase of decay [473].

The area under the curve (AUC) of GTT (AUC_{GTT}) and of ITT (AUC_{ITT}) were calculated using the trapezoidal method [474].

3.4.5 Collection of biological samples

At the end of the 14-week protocol, animals were euthanized with isoflurane overdose followed by cervical dislocation. Blood samples were immediately collected by venipuncture from the jugular vein and serum was obtained by centrifugation (3000 *g* for 15 min at 4 °C) and immediately stored at −20 °C until processing for biochemical analysis. The liver and small portions of gastrointestinal tissues (duodenum and colon) were immediately excised, dissected, and stored in conditions according to the assay's technical requirements. The liver was firstly weighed and then divided into four distinct portions: a first piece was immediately used for functional mitochondria assays; the other two parts,

reserved for protein and RNA extraction purposes, were directly frozen in liquid nitrogen and stored at −80 °C until analysis; and a fourth piece was kept in a 10% neutral buffered formalin solution to be used for histological analysis. The relative liver weight was calculated as the ratio of absolute tissue weight (g) to BW (kg). During the last week of experimental protocol, 24-h urine and fecal samples were collected using metabolic cages. During this period, rats had free access to water and food. The volume of urine was recorded; feces were weighed, and samples were stored at −80 °C for later analysis.

3.4.6 Measurement of serum metabolic parameters

Serum samples were used to perform the following measurements, through automatic validated methods and equipment (Hitachi 717 analyzer, Roche Diagnostics GMBH, Mannheim, Germany), as previously described [237]: postprandial glucose, triglycerides (TGs), total-cholesterol (Total-c), low-density lipoprotein cholesterol (LDL-c), and high-density lipoprotein cholesterol (HDL-c) levels, as well as serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations. Hemoglobin A1c (HbA1c) levels were determined using the DCA 2000+ analyzer (Bayer Diagnostics, Barcelona, Spain), according to the manufacturer' instructions. Serum insulin levels were determined by Enzyme-Linked ImmunoSorbent Assay (ELISA) using commercially available kits for rat samples from Mercodia (Uppsala, Sweden). Insulin resistance was evaluated by the homeostatic model assessment of insulin resistance (HOMA-IR) index, which was calculated as previously described [236], using the following formula: HOMA-IR index = [fasting glucose (mmol/L) × fasting insulin (μU/L)]/22.5. High-sensitivity C-reactive protein (hs-CRP) was assayed by using a rat-specific ELISA kit (MBS764381 from Mybiosource, San Diego, CA, USA) according to the manufacturer´ instructions.

3.4.7 Determination of serum Total Antioxidant Status (TAS)

For determination of the serum TAS, ferric reducing antioxidant potential (FRAP) was performed as previously described [475], whereas the scavenging of 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) assay was employed as described by Gião et al. [476].

The $ABTS^{\bullet+}$ stock solution was prepared by reacting equal amount of 7 mM ABTS diammonium salt (Sigma-Aldrich, St. Louis, MO, USA) and 2.45 mM potassium persulphate (Merck, Damstadt, Germany). The reaction was developed for 16 h in the dark. Aliquots of serum samples (10 µL), diluted when needed, were added to 1 mL of $ABTS^{\bullet+}$ solution with an initial optical density (OD) of 0.70 ± 0.02 measured at 734 nm. After allowing the reaction to occur, the OD was recorded using an UV-Vis spectrophotometer (UVmini 1240, Shimadzu, Japan) and the results were calculated as inhibition percentage of $ABTS^{\bullet+}$, according to the equation $ABTS^{\bullet+}$ inhibition (%) = 100 − [(OD sample × DF)/OD ABTS] × 100, where OD sample indicates the sample absorbance following 6 min of reaction, DF is the dilution factor and OD ABTS refers to the initial absorbance of the diluted $ABTS^{\bullet+}$ solution. All measurements were performed in triplicate.

3.4.8 Nuclear Magnetic Resonance (NMR) spectroscopy

Before NMR analysis, 180 µL of the serum samples were mixed with 45 µL of a phosphate buffered (0.2 M) sodium fumarate (10 mM) solution (99.9% ^2D_2O) that was used as internal standard (Sigma-Aldrich, St. Louis, MO, USA) and each sample was loaded into 3 mm NMR grade tubes for high resolution ^1H NMR analysis.

NMR spectra were obtained with a 600 MHz (14.1 T) spectrometer (Agilent, Santa Clara, CA, USA) equipped with a 3 mm indirect detection probe with a z-gradient. 1D-^1H cpmg (Carr-Purcell-Meiboom-Gill spin-echo pulse sequence) experiments with water pre-saturation were acquired (7.2 kHz spectral width, 0.1 s mixing time, 4 s relaxation delay with 3 s of water pre-saturation, 90° pulse angle, 3 s acquisition time and 128 scans at 298 K). Pulse durations and water saturation frequencies were optimized for each sample. Spectra were processed by applying exponential line broadening (0.3 Hz), zero-filling to 64 k, and manual phasing and baseline correction. Chemical shifts were internally referenced to fumarate (singlet at 6.50 ppm).

Spectral assignments were based on matching the recorded spectra to the reference data available in public databases such as Human Metabolome Database (HMDM) [477]. 2D homonuclear total correlation spectroscopy (TOCSY) spectra were recorded for selected samples to help spectral assignment [478]. All metabolites were identified according to Metabolomics Standards Initiative (MSI) guidelines for metabolite

identification [479] and the levels of identification are indicated in supplementary data (Table S3.1).

Processed 1D cpmg spectra were bucketed using one-point bucket (0.6–9.0 ppm, with signal-free, water, and fumarate regions excluded) using Amix Viewer (version 3.9.15, Bruker Biospin GmbH, Rheinstetten, Germany) and aligned using icoshift algorithm [480]. Resulting matrix was normalized by total spectral area included in the analysis. Multivariate statistical analysis was applied on unit variance scaled matrix (SIMCA 14, Umetrics, Sartorius Stedim Biotech, Gottingen, Germany). In order to identify clustering trends or outliers, principal component analysis (PCA) was used to provide the information on global data structure, and partial least square discriminant analysis (PLS-DA) was used to assess class separation and identify the main metabolites that contribute to the class discrimination. A 7-fold internal cross-validation of the PLS-DA model was used to provide the qualitative measure of predictive power (Q2) and to assess the degree of fit to the data (R2). Permutation test (n = 100) was also used to validate the PLS-DA model [481]. The corresponding PLS-DA loadings plot was obtained by multiplying the loading weight factors (w) by the standard deviation of the respective variable and was color-coded according to variable importance in the projection (VIP). Selected signals of chosen metabolites (VIP > 1) were integrated in normalization by ^{1}H-NMR spectra for quantitative assessment of metabolite variations between the groups.

Outliers were excluded based on the quality of the recorded NMR spectra according to the recommendations of MSI [479]. The difference between the means of the two groups was assessed using the *t* test (results reported at a confidence level of 95%).

3.4.9 Evaluation of serum Lipopolysaccharide (LPS) levels

Serum endotoxin LPS concentration was quantified using a Pyrochrome Lisate Mix, a quantitative chromogenic reagent, diluted in glucashield buffer, which inhibits cross-reactivity with (1 → 3)-β-D-glucans (Associate of Cape Cod Incorporated, East Falmouth, MA, USA). Briefly, serum samples were diluted (1:10) in pyrogen-free water (LAL reagent water, W50-100, Lonza, Walkersville, MD, USA) and heated for 10 min at 70 °C. Samples and pyrochrome reagent (1:1) were incubated at 37 °C for 30 min and absorbance was read at 405 nm.

3.4.10 Extraction and quantification of gut microbiota in feces

3.4.10.1 DNA extraction from stool

Genomic DNA was extracted and purified from fecal samples using the NZY Tissue gDNA Isolation Kit (NZYtech, Lisbon, Portugal) according to the manufacturer´s protocol with slight modifications [482]. Briefly, fecal samples (170 to 200 mg) were homogenized in Tris-EDTA buffer solution (10 mM Tris/HCl; 1 mM EDTA, pH 8.0) and centrifuged at 4000 *g* for 15 min. The supernatant was discarded, and the pellet was resuspended in 350 µL of buffer NT1. After an incubation step at 95 °C for 10 min, the samples were centrifuged at 11000× *g* for 1 min. Then, 25 µL of proteinase K was added to 200 µL of the supernatant for incubation at 70 °C for 10 min. The remaining steps followed the manufacturer's instructions. DNA purity and quantification were assessed with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

3.4.10.2 Real-time PCR for microbial analysis of stool

Real-time PCR was performed in sealed 96-well microplates using a LightCycler FastStart DNA Master SYBR Green kit and a LightCycler instrument (Hoffman-La Roche Ltd., Basel, Switzerland) as previously described [482]. The assay was performed in a 50 µL sample containing a reaction mixture of 20 ng of DNA, with 25 µL of SsoAdvanced Universal SYBR Green (Bio-Rad, Hercules, CA, USA), 5 µL of each primer, and 10 µL of water. Primer sequences (Sigma-Aldrich, St. Louis, MO, USA) used to target the 16S rRNA gene of the bacteria and the conditions for PCR amplification reactions are listed in Table 3.1. To verify the specificity of the amplicon, a melting curve analysis was performed via monitoring SYBR Green fluorescence in the temperature ramp from 60 °C to 97 °C. Data were processed and analyzed using the LightCycler software (Hoffman-La Roche Ltd., Basel, Switzerland). Standard curves were constructed using serial tenfold dilutions of bacterial genomic DNA, according to the data provided on the following webpage (http://cels.uri.edu/gsc/cndna.html). Bacterial genomic DNA (DSMZ, Braunschweig, Germany) was used as a standard. Genome size and the copy number of the 16S rRNA gene for each bacterial strain used as a standard were obtained from the NCBI Genome database (www.ncbi.nlm.nih.gov). Data are presented as the mean values of duplicate PCR analysis.

Table 3.1 | Primer sequences and real-time PCR conditions used for gut microbiota analysis.

Bacterial Group	Primer Sequence (5´-3´)	PCR product size (bp)	AT (°C)
Firmicutes	ATG TGG TTT AAT TCG AAG CA	126	45
	AGC TGA CGA CAA CCA TGC AC		
Bacteroidetes	CAT GTG GTT TAA TTC GAT GAT	126	45
	AGC TGA CGA CAA CCA TGC AG		
Clostridium	GCA CAA GCA GTG GAG T	239	45
	CTT CCT CCG TTT TGT CAA		
Universal	AAA CTC AAA KGA ATT GAC GG	180	45
	CTC ACR RCA CGA GCT GAC		
Enterococcus	CCC TTA TTG TTA GTT GCC GCC ATC ATT	144	50
	ACTCGT TGT ACT TCC CT TGT		
Prevotella	CAC RGT AAA CGA TGG ATG CC	513	50
	GGT CGG GTT GCA GAC C		
Bifidobacterium	CGC GTC YGG TGT GAA AG	244	50
	CCC CAC ATC CAG CAT CCA		
Roseburia	TAC TGC ATT GGA AAC TGT CG	230	50
	CGG CAC CGA AGA GCA AT		
Lactobacillus	GAG GCA GCA GTA GGG AAT CTT C	126	55
	GGC CAG TTA CTA CCT CTA TCC TTC TTC		
Akkermansia	CAG CAC GTG AAG GTG GGG AC	327	55
	CCT TGC GGT TGG CTT CAG AT		

AT, annealing temperature; bp, base pairs; PCR, polymerase chain reaction.

3.4.11 Fecal Short-Chain Fatty Acids (SCFAs) and organic acids determination

Short-chain fatty acids (SCFAs) and organic acids (lactic and succinic acid) were measured using an Agilent 1200 series HPLC system with a refractive index—RI detector and with a UV detector. Approximately 200 mg of feces were dissolved in 1 mL of ultrapure water, homogenized in a "mixer" for 15 min, and centrifuged at 10,000× *g* for 10 min; the supernatants were collected and stored at −20 °C until analysis. Briefly, fecal samples were filtered through a 0.22 μm membrane filter (Orange Scientific, Braine-l'Alleud, Belgium) and injected (40 μL) directly into an HPLC System consisting of a

LaChrom L-7100 pump (Merck-Hitachi, Darmstadt, Germany) and an ion exchange Aminex HPX-87H column (300 × 7.8 mm, BioRad Laboratories, Inc., Hercules, CA, USA), operated at 65 °C. The mobile phase used was 0.003 M solution of sulfuric acid at a flow rate of 0.6 mL/min. Data were collected and analyzed with a D7000 Interface (LaChrom, Merck-Hitachi, Fullerton, CA, USA) and using HPLC System Manager® Software 3.1.1 (MerckHitachi, Fullerton, CA, USA). Peak identification was based on the relative retention times determined by injection of standard solutions. Quantification was performed using calibration curves. Fecal SCFAs concentrations were expressed as mean micromoles per gram wet weight.

3.4.12 Colon and duodenum analysis by Transmission Electron Microscopy (TEM)

Duodenum and colon samples were immediately sectioned in small fragments of approximately 1 mm^3 and fixed in 2.5% glutaraldehyde solution in 0.1 M phosphate buffer (pH = 7.2) for 2 h. Sequential post-fixation was performed in 1% osmium tetroxide, for 1.5 h, and 1% aqueous uranyl acetate, for 1 h in the dark. After rinsing with distilled water, samples were dehydrated in a graded acetone series (30–100%) and embedded in an Epoxy resine (Fluka Analytical, Sigma-Aldrich, Darmstadt, Germany). Ultrathin sections obtained with a Leica EM UC6 (Leica Co, Vienna, Austria) ultramicrotome were mounted on copper grids and stained with lead citrate 0.2% for 10 min. Observations were carried out on a TEM Tecnai G2 Spirit Bio Twin at 100 kV (FEI, Hillsboro, OR, USA), and images were processed using AnalySIS 3.2.

3.4.13 Immunohistochemical staining

Cross-sections (10 μm thickness) of rat colon were cut with a cryostat (Leica CM3050S, Nussloch, Germany). Colon cryosection were fixed with an acetone:methanol mixture (1:1) at 20 °C for 2 min and then rehydrated in phosphate-buffered saline (PBS) (3 × 5 min). After washing, sections were permeabilized with 0.5% Triton X-100 in PBS for 15 min and blocked for 40 min with 4% nonfat milk in 20 mM Tris, pH 7.2, and 150 mM NaCl. The sections were incubated with primary antibodies: rabbit polyclonal anti-ZO-1 (ab96587, Abcam, Cambridge, MA, USA) and mouse monoclonal anti-occludin

(OC-3F10, 33-1500, Life Tecnologies, Carlsbad, CA, USA) in PBS containing 1% BSA overnight at 4 °C. After rinsing with PBS (3 × 5 min), the sections were incubated with the secondary fluorescent antibody Alexa Fluor 488-conjugated goat anti-rabbit IgG or Alexa Fluor 568-conjugated donkey anti-mouse IgG (1:200; Molecular Probes, Life Technologies, Paisley, UK) and 4′,6-diamidino-2-phenylindole (DAPI, nuclei dye), for 1 h at room temperature. After incubation, the sections were washed with PBS (3 × 5 min), and the slides were mounted using the Glycergel mouting medium (Dako, Carpinteria, CA, USA). Anti-ZO-1 and anti-occludin immunostaining samples were imaged using a confocal fluorescence microscope (LSM 710, Carl Zeiss, Gottingen, Germany).

3.4.14 Hepatic histological analysis

Hepatic tissue samples were fixed directly in 10% neutral buffered formalin solution and embedded in paraffin wax. Paraffin blocks were cut to sections of 5 μm using a microtome (HM325, Thermo Fisher Scientific, Waltham, MA, USA). Hematoxylin-eosin (H&E) staining was performed according to the manufacturer's guidelines (Merck Millipore, Darmstadt, Germany). Digital images of tissue slices were captured using a Zeiss microscope Mod. Axioplan 2 (Zeiss, Jena, Germany).

Oil Red O staining was performed on frozen liver sections (5 μm) previously fixed in 10% formalin for 5 min as previously described [483]. Briefly, slides were rinsed three times with absolute propylene glycol and then placed in 0.5% Oil Red O stain solution in propylene glycol for 30 min before being rinsed with 85% propylene glycol for 1 min and counterstained with hematoxylin. Thereafter, the slides were washed with distilled water and mounted with aqueous mounting medium (Sigma, St. Louis, MO, USA). Sections were observed with a Zeiss microscope Mod. Axioplan 2 (Zeiss, Jena, Germany).

3.4.15 Quantification of hepatic triglycerides

Hepatic triglycerides levels were measured using a Triglycerides Colorimetric Assay kit (1155010, Cromatest ®, Linear Chemicals, Barcelona, Spain). Briefly, 50 mg of frozen tissue were homogenized in 1 mL of isopropanol using a potter Elvehjem homogenizer (ThermoFisher, Waltham, MA, USA). The homogenate was sonicated and then

centrifuged at 1000× g for 5 min at 4 °C. Triglycerides were detected at 450 nm using an enzymatic-photometric analyzer (BIOTEK ®, Synergy HT, Winooski, VT, USA).

3.4.16 Hepatic mitochondria bioenergetics

Hepatic mitochondria were isolated in homogenization medium containing 250 mM sucrose, 10 mM HEPES (pH 7.4), 0.5 mM EGTA and 0.1% fat-free bovine serum albumin (BSA) [484, 485]. After homogenization of the minced blood-free hepatic tissue, the homogenate was centrifuged at 800× g for 10 min at 4 °C. The supernatant was spun at 10,000× g for 10 min at 4 °C to pellet mitochondria, which were re-suspended in a final washing medium. EGTA and BSA were omitted from the final washing medium, adjusted at pH 7.4. Mitochondrial integrity was evaluated by measuring citrate synthase activity, in the presence and absence of detergent (93 ± 2.5% of intact mitochondria after isolation). Citrate synthase activity serves as a measure for membrane integrity since citrate synthase is located in the inner mitochondrial membrane, and thus should not be present in suspensions of mitochondria with intact membranes. Protein content was determined by the biuret method calibrated with BSA [486].

3.4.16.1 Mitochondrial Permeability Transition (MPT)

Mitochondrial swelling was estimated by changes in light scattering, as monitored spectrophotometrically at 540 nm, as previously described [487]. Reactions were carried out at 25 °C and Ca2+ (20 nmol) was added to the preparation after the start of the experiment. The assays were started by the addition of mitochondria (1 mg) to 2 mL of swelling medium (200 mM sucrose, 10 mM Tris–MOPS, 1 mM KH2PO4 and 10 μM EGTA, pH 7.4) supplemented with 2 μM rotenone and 5 mM succinate. To confirm the relationship between membrane permeability transition (MPT) induction and mitochondrial swelling, cyclosporine A (0.25 μM, a known MPT inhibitor) was added to the mitochondrial preparation before the addition of calcium. All the experiments were performed in triplicate.

3.4.16.2 Mitochondrial respiration (Oxygen consumption)

Oxygen consumption of isolated mitochondria was polarographically monitored with a Clark oxygen electrode (Oxygraph, Hansatech Instruments Ltd., Cambridge, UK) as previously described [484]. Mitochondria (1 mg) were suspended under constant magnetic stirring, at 25 °C, in 1.4 mL of standard respiratory buffer containing 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 50 μM EDTA, and 5 mM HEPES (pH 7.4) and 2 μM rotenone. Mitochondria were energized with succinate (5 mM) and state 3 respiration was induced by the addition of ADP (200 nmol). After the phosphorylation of the ADP to ATP, respiratory rate became slower (state 4). The respiratory control ratio (RCR) was calculated by the ratio between the state 3 and the state 4 respirations and used as a parameter of mitochondrial integrity. The uncoupled respiration was also measured in the presence of carbonyl cyanide-P-trifluoromethoxyphenylhydrazone (FCCP, 1 μM). FCCP is an ionophore that uncouples oxidative phosphorylation by inducing artificial proton permeability in the mitochondria, stimulating the maximum respiration rate. The ADP/O ratio was calculated by the ratio between the amount of ADP added and the O_2 consumed during the state 3 respiration.

3.4.16.3 Mitochondrial membrane potential (ΔΨm)

Mitochondrial membrane potential (ΔΨm) was estimated using an ion-selective electrode to measure the distribution of tetraphenylphosphonium (TPP^+) as previously described [485, 487] using an $Ag/AgCl_2$ electrode as reference. The entrance of TPP^+ in mitochondria was determined by TPP^+ concentration decreasing in the medium, measured by electrode potential. Briefly, mitochondria (1 mg) were suspended by gentle stirring in 1.4 mL of the standard respiratory buffer (as in mitochondrial respiration) supplemented with 3 μM TPP^+ and energized by adding 5 mM succinate. To avoid complex I contribution due to possible endogenous substrates contribution and to prevent retrograde electron flow from the ubiquinone pool back to complex I, 2 μM of rotenone, a complex I inhibitor was added. After the steady-state distribution of TPP^+ occurred, ADP (200 nmol) was added to initiate the phosphorylative cycle. The electrode was calibrated with TPP^+ assuming Nernstian distribution of the ion across the synthetic membrane, and ΔΨ is expressed in -mV. A matrix volume of 1.1 μL/mg protein was assumed. The measured

parameters were membrane potential (-mV), lag phase (seconds), and repolarization (-mV). Respiratory rates and ΔΨm were simultaneously measured.

3.4.17 Gene expression by quantitative Real-Time PCR analysis

Total RNA extraction from flash frozen liver was performed with PureLink RNA Mini Kit (12183018A, Ambion, Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer´s instructions. Total RNA extraction from colon tissue samples was performed using a Trizol protocol (93289, Sigma Aldrich; St. Louis, MO, USA), and stored overnight at −80 °C. RNA concentration from liver was determined by Experion Automated Electrophoresis Station (Bio-Rad, Hercules, CA, USA). The concentration of total RNA from colon samples was measured by Nano Chip® kit in Agilent 2100 bioanalyzer (2100 expert software, Agilent Technologies, Walbronn, Germany). RNA integrity (RIN, RNAIntegrity Number) and purity (A260/A280) of all RNA samples were measured by Nano Chip® kit in Agilent 2100 Bioanalyzer (2100 expert software, Agilent Technologies, Walbronn, Germany) and ND-1000® spectrophotometer (NanoDrop Tecnhologies, Wilmington, DE, USA), respectively.

In the hepatic samples, reverse transcription into cDNA was carried out by using the iScript Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. The colonic cDNA was synthesized from RNA using a Xpert cDNA Synthesis Mastermix (GK81.0100, GRISP, Porto, Portugal) following the manufacturer´s instructions. cDNA samples were then stored at −20 °C until use.

To analyze genes of interest in colon tissue, gene expression quantification was performed as previously described [275]. Real-time PCR were conducted with a SYBR Green real-time PCR kit (Bio-Rad, Hercules, CA, USA), following the manufacturer's recommendations and gene specific-primers for ZO-1 (Tjp1, Unique Assay ID: qRnoCID0001801), occludin (ocln; Unique Assay ID: qRnoCID0005733, Bio-Rad, Hercules, CA, USA), and mucin-2 (muc2; Unique Assay ID: qRnoCID0003629 Bio-Rad), which were normalized with GeNorm algorithm, where gene stability was attained with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine phosphoribosyltransferase (HPRT). The relative expression ratio of each of the target gene was computed on the basis of ΔΔCt ($2^{-\Delta\Delta Cp}$) values.

In liver tissue, a predesigned 96-well Fatty Liver panel (SAB Target List, R96; 10046947, Bio-Rad, Hercules, CA, USA) for SYBR® Green detection (Bio-Rad, Hercules, CA, USA) were used following the manufacturer's instructions. This array includes genes for insulin signaling, adipokines, the inflammatory response, apoptosis, and carbohydrate and lipid metabolism in the liver. Gene expression was performed by SYBR-Green-based real time quantitative PCR using a StepOnePlus PCR system (Applied Biosystems, Foster City, CA, USA).

3.4.18 Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Data was compared and analyzed using Student's unpaired t test for normally distributed data or the Mann Whitney test for non-normally distributed data. One-way or two-way ANOVA followed by Bonferroni post hoc test was used as appropriate. Repeated measures ANOVA, followed by Bonferroni post-hoc test, was used to compare glucose levels throughout the GTT and ITT assays. Values of $p < 0.05$ were considered statistically significant. GraphPad Prism for Windows (Version 6.0, GraphPad Software) was used for all statistical analysis.

3.5 Results

3.5.1 Phenolic composition of BJ

The phenolic composition of BJ was evaluated using the UV-vis spectra obtained on-line from PDA detector, after chromatographic separation. The chromatographic profile obtained for the BJ is represented in Figure 3.2. The main classes of phenolic compounds detected were hydroxycinnamic acids (peaks 1 to 3) and anthocyanins (peaks 4 to 11). Peaks 1 to 3 exhibited spectra profiles characteristic of caffeic or ferulic acid derivatives, with UV maxima near 250 and 324 nm. The other chromatographic peaks (4 to 11) were identified as anthocyanins due to the presence of very characteristic spectra, with a peak between 240 and 280 nm (band II) and a strong visible peak between 450 and 560 nm.

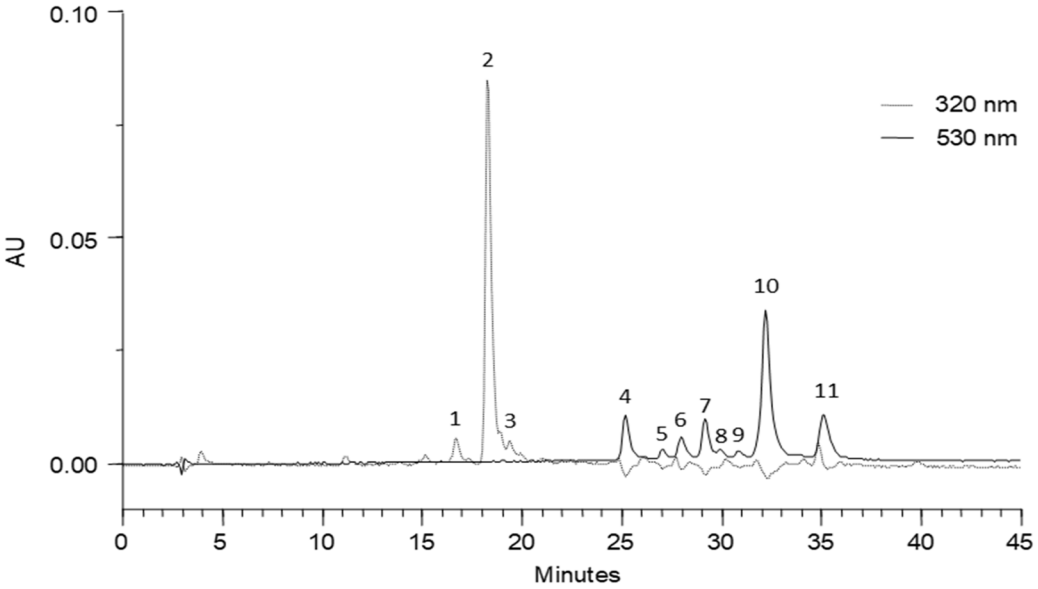


Figure 3.2 | Chromatographic profile of phenolic compounds in BJ, obtained with HPLC-PDA (320 / 530 nm). Peak identification: 1-3, hydroxycinnamic acids; 4-11, anthocyanins.

3.5.2 BW and energy intake

BW remained unchanged following the sustained BJ consumption on despite of the increased consumption of BJ (and consequent increase of carbohydrate load) that paralleled a higher urine output ($p<0.05$). No statistical differences were recorded on solid food ingestion and total energy intake between groups (Table 3.2).

Table 3.2 | BW variation and cumulative energy intakes during the experimental protocol.

	Parameters	CTRL	BJ
Body weight	Initial (g)	461.00 ± 14.00	472.30 ± 14.61
	Final (g)	510.60 ± 20.85	532.40 ± 24.42
	Delta (g)	49.57 ± 8.71	60.13 ± 10.80
Intakes	Food (g/rat/week)	164.90 ± 2.43	162.50 ± 2.62
	Drink (mL/rat/week)	210.20 ± 6.42	323.40 ± 11.20 ***
	Total calories (Kcal/rat/week)	519.30 ± 7.65	542.90 ± 8.51
	Carbohydrates (Kcal/rat/week)	352.70 ± 5.89	382.20 ± 5.77 ***
	Lipids (Kcal/rat/week)	44.52 ± 0.66	43.16 ± 0.67
	Proteins (Kcal/rat/week)	122.00 ± 1.80	118.80 ± 1.95
Urine Output	Urine volume (mL/day)	19.43 ± 2.68	42.25 ± 6.41 **

Data are presented as mean ± SEM (n=8 per group). ** $p < 0.01$ and *** $p < 0.001$ vs. CTRL group.

3.5.3 Glycemic and insulinemic profiles

After BB consumption, serum glucose and insulin levels were not significantly altered in both fasting and postprandial conditions (Table 3.3). In GTT assay, blood glucose levels were transiently higher in BJ group soon after intraperitoneal glucose challenge [at 15 and 30 min ($p<0.01$ and $p<0.05$, respectively)] but quickly recovered in the following time-points, comparable to the control group. Accordingly, no statistical changes were found in AUC values of GTT ($p>0.05$) nor in peripheral insulin sensitivity surrogates (ITT, K_{ITT}, HOMA-IR) (Table 3.3).

Table 3.3 | Glycemic and insulinemic profiles.

Parameters	CTRL	BJ
Fasting glucose (mg/dL)	101.30 ± 2.29	99.50 ± 2.26
Postprandial glucose (mg/dL)	149.30 ± 9.40	159.00 ± 11.15
Glucose levels (mg/dL) during the GTT (min after the glucose load)		
0 min	100.9 ± 1.53	99.71 ± 2.32
15 min	314.4 ± 21.98	412.10 ± 14.19 **
30 min	342.00 ± 37.55	428.40 ± 6.121 *
60 min	248.10 ± 30.30	289.70 ± 16.01
120 min	199.30 ± 18.14	185.60 ± 7.81
AUC_{GTT}	30313.00 ± 2887.00	35174.00 ± 868.70
Fasting insulin (mU/L)	0.65 ± 0.05	0.61 ± 0.11
Postprandial insulin (mU/L)	1.02 ± 0.21	1.24 ± 0.15
Glucose levels (mg/dL) during the ITT (min after insulin injection)		
0 min	100.80 ± 1.82	99.50 ± 2.79
15 min	97.57 ± 4.36	103.10 ± 4.58
30 min	74.57 ± 2.37	80.25 ± 4.97
45 min	60.57 ± 2.06	67.38 ± 3.91
60 min	58.86 ± 2.52	63.25 ± 2.58
120 min	55.43 ± 5.59	64.50 ± 5.26
AUC_{ITT}	8120.00 ± 291.70	8815.00 ± 317.20
K_{ITT} (% min)	0.68 ± 0.06	0.52 ± 0.06
HOMA-IR	3.26 ± 0.60	3.86 ± 0.72

Data are presented as mean ± SEM (n=6/8 per group). AUC, area under curve; GTT, glucose tolerance test; HOMA-IR, homeostatic model assessment of insulin resistance; ITT, insulin tolerance test; K_{ITT}, glucose disappearance rate for ITT; * $p<0.05$ and ** $p<0.01$ vs. CTRL group.

3.5.4 Intestinal microbiota and gut barrier integrity

To assess the impact of a sustained BB consumption on GM composition and function, we next analyzed microbiota and SCFAs/organic acids composition in feces along with colonic ultra-structural morphology, intestinal permeability, and systemic inflammation. While fecal microbiota composition remained unchanged between the two groups (Figure 3.3A), a significant increase in succinic acid ($p<0.05$) was detected in the BJ-treated rats (Figure 3.3B). Moreover, TEM analysis did not reveal any alteration in colonic barrier ultra-organization, namely in the intercellular junctions (Figure 3.3C), which was further corroborated by the expression of ZO-1, occludin and mucin-2 genes, three key players

involved in the maintenance of epithelial integrity (Figure 3.3D). Correspondingly, serum endotoxemia (LPS) and inflammation (hs-CRP) remained unchanged in both experimental groups (Figure 3.3E and 3.3F).

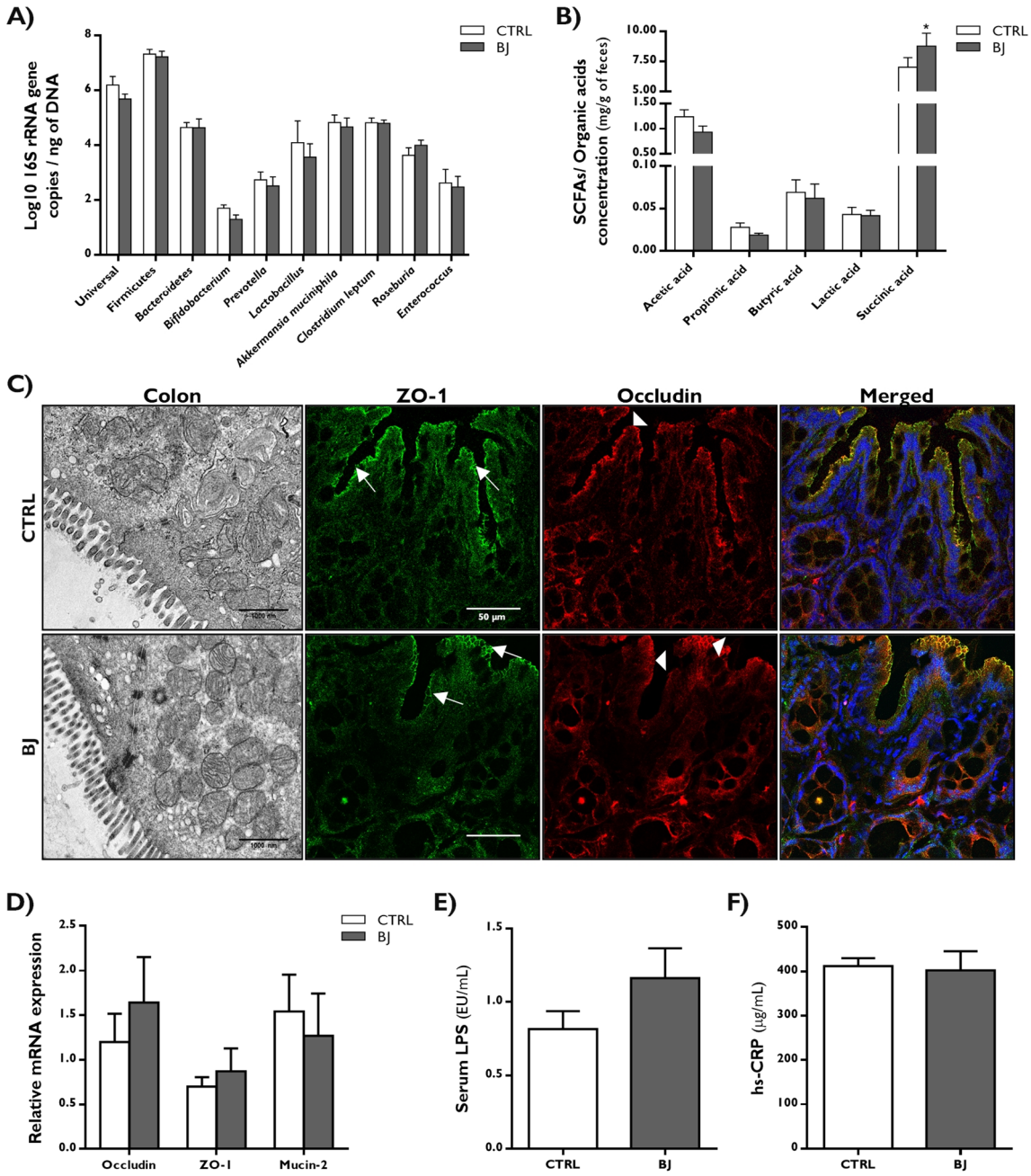


Figure 3.3 | Effects of BJ on gut microbiota composition/function, barrier structure and intestinal permeability. Gut microbiota composition (A), SCFAs and organic acids (B) in feces; ultrastructural distribution of tight junctions and representative confocal images of colon section stained for ZO-1 (arrow) and occludin (arrowhead) (C) and mRNA expression (D) of key players of tight junctions; serum LPS (E) and hs-CRP (F) concentrations. Data are presented as mean ± SEM (n=6-8/group); * $p<0.05$ vs. CTRL group.

3.5.5 Serum antioxidant status and metabolomic profile

The serum antioxidant effect provided by this BJ consumption was evaluated by FRAP and ABTS$^{\bullet+}$ assays. As foreseen, BJ-treated rats presented higher serum TAS ($p<0.01$, Figure 3.4A), along with increased serum ABTS$^{\bullet+}$ inhibition percentage ($p<0.01$, Figure 3.4B).

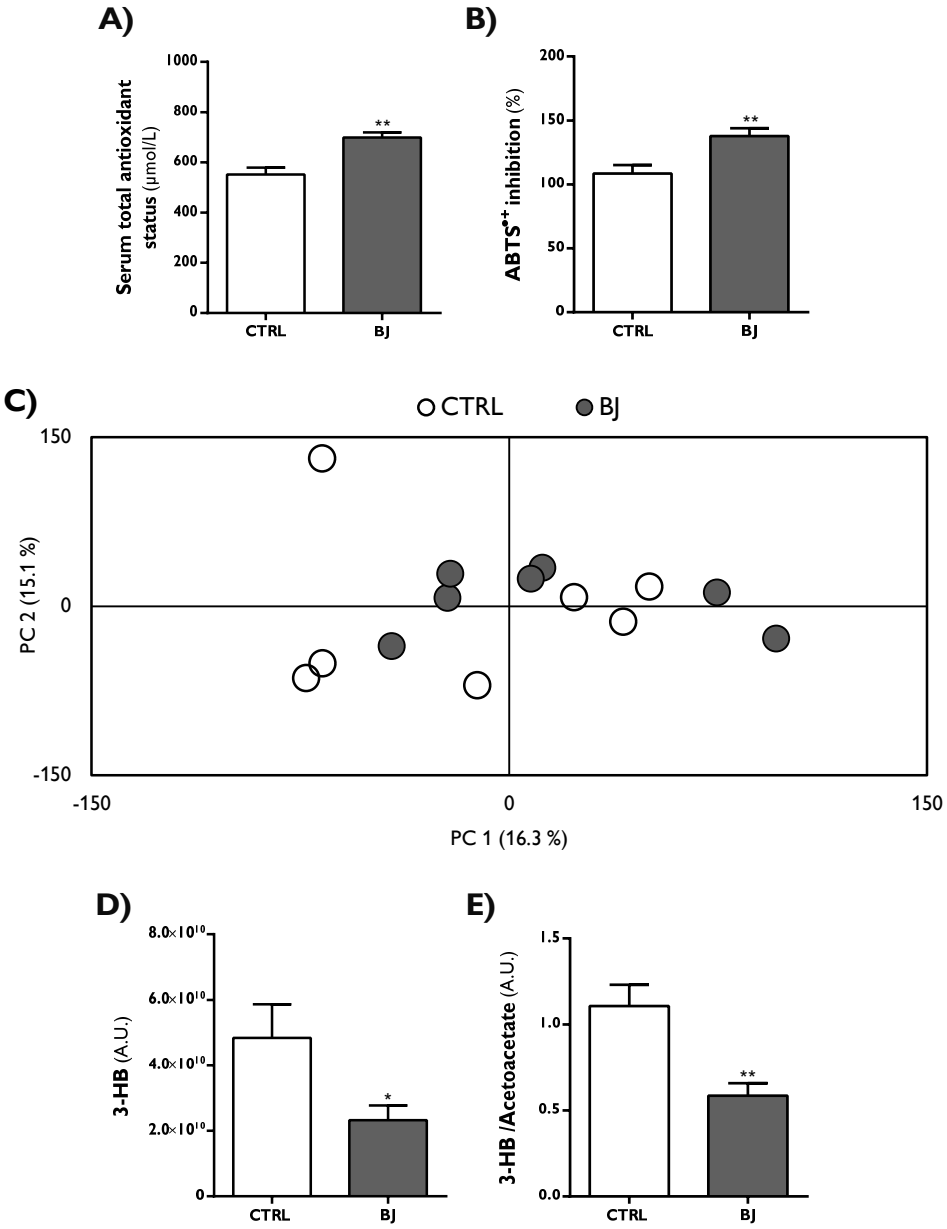


Figure 3.4 | Serum antioxidant profile and metabolomic profile. Serum total antioxidant status (TAS) evaluated by FRAP (A) and ABTS (B) assays; Principal component analysis (PCA) scores plot obtained by multivariate analysis of ^{1}H NMR spectra of serum data (C); Serum levels of 3-hydroxybutyrate (3-HB) (D) and 3-HB/acetoacetate ratio (E). Data are presented as mean ± SEM (n=6-8/group); * $p<0.05$ and ** $p<0.01$ vs. CTRL group.

To scrutinize the metabolomic profile between control and BJ-supplemented animals, a nontargeted ^{1}H NMR-based metabolic analysis was further performed, allowing for the simultaneous assessment of an array of serum metabolites. The resulting spectral profile identified 22 metabolites (supplementary data: Table S3.1) without any global metabolic discrimination between groups (Figure 3.4C), notwithstanding the significant reduction ($p<0.05$) of 3-HB and 3-HB/acetoacetate ratio in serum of BJ-supplemented rats (Figure 3.4D and 3.4E).

3.5.6 Serum and hepatic lipid profile and function

As shown in Table 3.4, comparable values were obtained for both groups regarding serum/hepatic lipid profiles as well as absolute liver weights. These results were substantiated by the regular histologic appearance without signs of lipid deposition observed in hepatic H&E and Oil-red-O-stained sections, respectively (Figure 3.5). Likewise, similar serum ALT and AST activities concurrently hint at a preserved liver function following BJ consumption (Table 3.4).

Table 3.4 | Serum lipid profile and hepatic parameters.

Parameters	CTRL	BJ
Serum lipid profile		
TGs (mg/dL)	130.00 ± 16.34	153.10 ± 16.09
Total-c (mg/dL)	71.25 ± 3.37	72.25 ± 5.28
LDL-c (mg/dL)	20.75 ± 1.51	19.50 ± 1.19
HDL-c (mg/dL)	44.75 ± 1.99	44.63 ± 7.46
TGs / HDL-c ratio	3.04 ± 0.37	3.65 ± 0.54
Hepatic parameters		
Liver weight (g)	13.20 ± 0.44	14.28 ± 0.69
Liver weight/BW (g/kg)	25.94 ± 0.62	26.85 ± 0.55
TGs (mg/g tissues)	12.34 ± 0.88	12.04 ± 0.49
ALT (U/L)	35.50 ± 1.96	36.29 ± 2.39
AST (U/L)	67.13 ± 3.12	68.29 ± 6.30

Data are presented as mean ± SEM (n=6/8 per group). BW, body weight; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; TGs, triglycerides; Total-c, total-cholesterol.

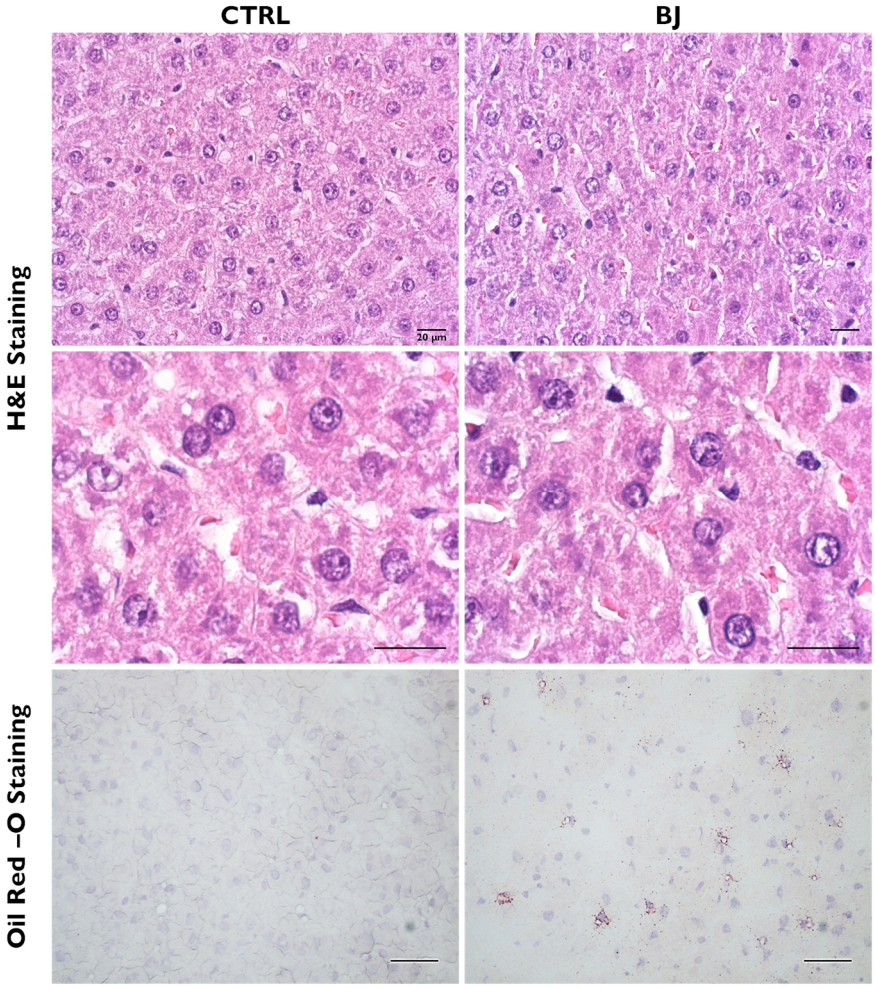


Figure 3.5 | Representative images of hepatic structure and lipid accumulation evaluated by H&E and Oil-Red-O staining, respectively, in the CTRL and BJ-treated rats. Scale bar: 20 µm.

3.5.7 Hepatic mitochondrial bioenergetics

In order to better understand the long-term effects of BB consumption on liver tissue, we assessed several parameters related with mitochondrial bioenergetics, an organelle with a crucial role in energy and metabolic regulation in the liver. Mitochondria have a limited capacity for accumulating calcium before undergoing the calcium-dependent mitochondria permeability transition (MPT), a phenomenon comprising the release of high molecular weight solutes from within the mitochondria, probably through the formation of a multi-channel pore. Mitochondrial swelling, an event that reveals mitochondrial permeability transition pore (mPTP) opening, was evaluated by presenting 20 nmol Ca^{2+} to mitochondrial preparations. In the presence of a relatively high Ca^{2+} dose, on pretreatment of hepatic mitochondria with CsA (MPT specific inhibitor), mitochondria

retained aforesaid Ca^{2+} load and did not undergo Ca^{2+}-dependent mitochondrial swelling, advocating isolated mitochondrial integrity (Figure 3.6A). Surprisingly, BJ induced a more pronounced decline in light scattering, indicating an increased susceptibility to undergo mitochondrial swelling, which will in turn reflect greater susceptibility to pore induction and mitochondrial uncoupling (Figure 3.6A).

Membrane potential (ΔΨm) sustained by mitochondria is central for this organelle function and pinpoints its phosphorylative capacity. As seen in Figure 3.6B, BJ induced a significant reduction in initial ΔΨm (after substrate addition) and in repolarization ΔΨm (mitochondrial capacity to establish ΔΨm after ADP phosphorylation) ($p<0.001$). Moreover, the lag phase (time required for ADP phosphorylation), was significantly longer ($p<0.001$) in hepatic mitochondria of BJ-supplemented animals. Mitochondrial respiration was further determined by evaluating oxygen consumption in energized mitochondria (succinate supply). BJ-isolated mitochondria showed a clear decline of respiratory capacity compared to the CTRL group. A decrease in mitochondrial state 3 (ADP-stimulated respiration) along with a significant increase in state 4 (resting state) respiration were observed ($p<0.001$) in the mitochondria of BJ-treated rats (Figure 3.6C). Additionally, BJ supplementation also caused a significant decrease in uncoupled respiratory rate (V FCCP, a maximal respiratory activity stimulated by FCCP), suggesting that the phosphorylative system of hepatic mitochondria is impaired following a long-term BJ supplementation. Consistently, respiratory control ratio (RCR) was significantly decreased ($p<0.001$) in hepatic mitochondria of BJ-treated animals. This functional parameter was determined by the ratio between state 3 and state 4 respiration and reflects the mitochondrial coupling between respiration and phosphorylation and efficiency. Moreover, the ADP/O ratio (which represents the number of ADP molecules that can be phosphorylated by one atom of oxygen consumed), a surrogate marker of mitochondrial oxidative phosphorylation efficiency, was also significantly decreased in BJ-treated rats (Figure 3.6D).

The expression of key genes encoding mitochondrial respiratory chain complexes was evaluated by RT-PCR. Interestingly, as shown in Figure 3.6E, both *Atp5c1* and *Ndufb6* were downregulated ($p<0.01$) in animals supplemented with BJ when compared to the untreated CTRL animals. These two genes (*Ndufb6* and *Atp5c1*) encode proteins of complex I (subunit NADH:ubiquinone oxidoreductase) and complex V (mitochondrial ATP synthase), respectively.

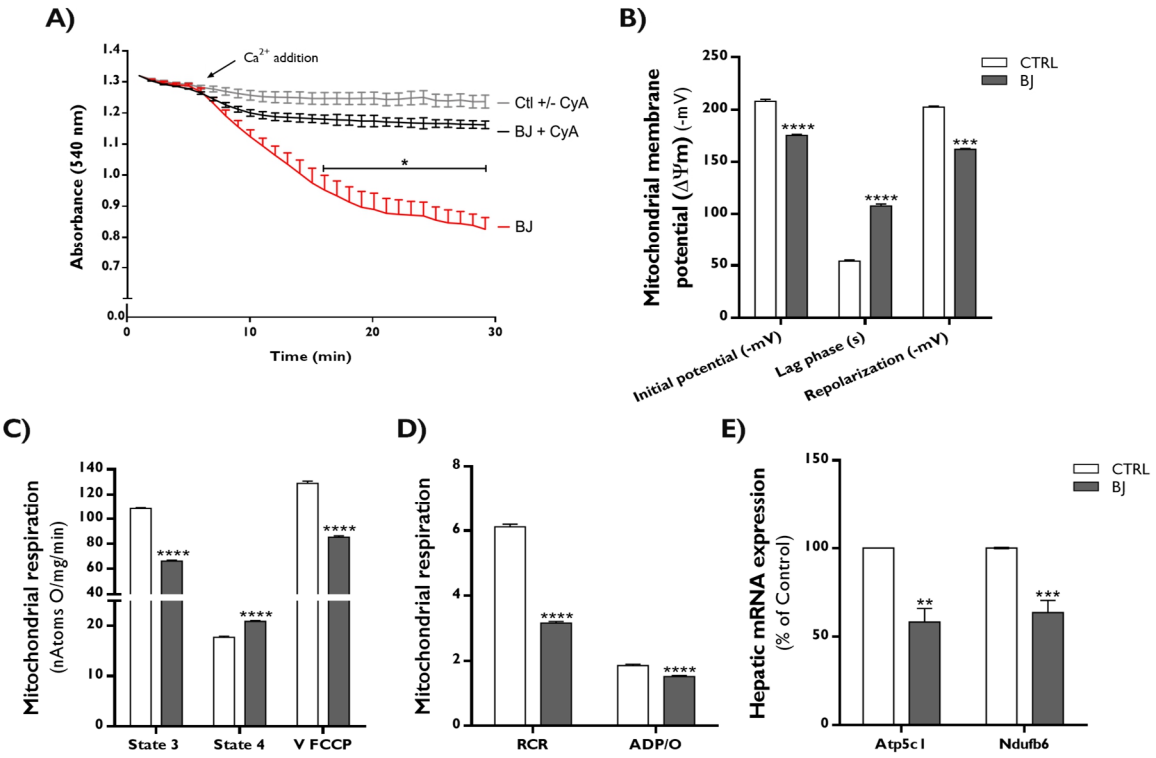


Figure 3.6 | Effects of BJ on hepatic mitochondrial function parameters. Susceptibility to the induction of mitochondrial permeability transition (MPT) (A); mitochondrial membrane potentials and lag phase (B); parameters of mitochondrial respiration (C and D); hepatic mRNA expression of genes involved in mitochondrial respiratory chain (E). Data are presented as mean ± SEM (n=6-8/group); * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001 vs. CTRL group.

3.5.8 Hepatic RNA transcripts encoding functionally diverse cellular targets

A broad analysis of hepatic mRNA expression of BB phytochemical's conceivable cellular targets, from chief-metabolic players to inflammatory components, was performed in both experimental groups.

The hepatic mRNA expression of genes involved in fatty acid uptake and transport, fatty acids oxidation, lipolysis and synthesis (lipogenesis) were analyzed by qRT-PCR. BB consumption significantly decreased the hepatic mRNA expression of genes related with fatty acid transport (*Fabp1* and *Slc27a5*), fatty acid oxidation (*Ppar-α, Acadl*, *Acox1*, *Cpt1a, and Cpt2*) and lipid synthesis (*Dgat-2*) (Figure 3.7). In opposition, an enhanced expression of mRNA levels of *Scd1* (also lipogenic enzyme) (p<0.005) was found in hepatic tissues. Yet, mRNA expression of some genes encoding lipogenic enzymes (*Fasn* and *Lpl*) did not show any significant alteration. Regarding cholesterol metabolism, a significantly increased

mRNA expression of *Apoa1* (the major component of HDL) was found in the BJ group compared with the CTRL one (Figure 3.7). In addition, BJ supplementation significantly decreased mRNA expression of *ApoC3*, which encodes a small protein on the surface of VLDL and LDL. Hepatic alipoprotein (*Apo*) *B* and *Apo E*, *Hmgcr* and *Ldlr* mRNA expression were unchanged between experimental groups. In addition, the impact of BJ on cytochrome P450 2e1 (*Cyp2e1*) was also explored on the basis of its well-known influence in gluconeogenesis as well as in xenobiotic metabolism. Notably, *Cyp2e1* mRNA expression was significantly decreased in animals supplemented with BJ. The expression levels of genes involved in glucose metabolism were also assessed. *Gck*, *Pdk4* and *Ptpn1* mRNA expression were downregulated in livers of BJ-treated rats despite of the normal *G6pd* mRNA expression (Figure 3.7). In addition, BJ treatment enhanced the *Slc2a1* mRNA level (corresponding to the gene encoding glucose transporter, GLUT1), while mRNA expression of *Slc2a2* and *Slc2a4* genes (encoding GLUT2 and GLUT4, respectively) were unchanged (Figure 3.7).

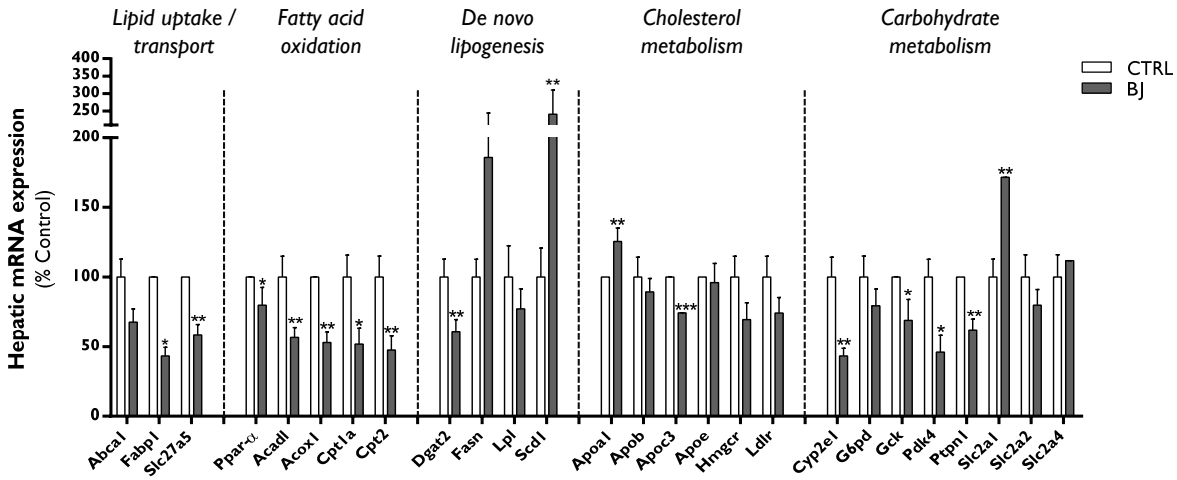


Figure 3.7 | Hepatic mRNA expression of genes involved in fatty acid uptake/transport, fatty acid oxidation and lipogenesis as well as cholesterol and carbohydrate metabolism. Data are presented as mean ± SEM (n=5-6/group); * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs. CTRL group.

Due to the anti-inflammatory properties described for BB in several pathological conditions [379, 488], we further underscored the expression of distinct inflammatory parameters. The mRNA expression of adiponectin receptors (*Adipor1* and *Adipor2*), tumor necrosis factor receptor superfamily member 6 precursor Tnfrsf6 (*Fas*), *Casp3*, *Ifn-γ*, interleukin-1 beta (*Il-1β*), *Nf-kB*, and *Rbp4* (gene that encodes a recently identified

adipokine: retinol binding protein 4) were significantly lower in animals supplemented with BJ when compared to the CTRL group (Figure 3.8). Moreover, the hepatic expression of the heat shock protein (*Hsp90ab1*) was remarkably decreased in animals treated with BJ. No significant changes were found in the expression of the *Stat3* gene, a transcription factor involved in the downstream signaling of several cytokines and growth factors, as well as in the hepatic mRNA expression of tumor necrosis factor-α (*Tnf-α*) (Figure 3.8).

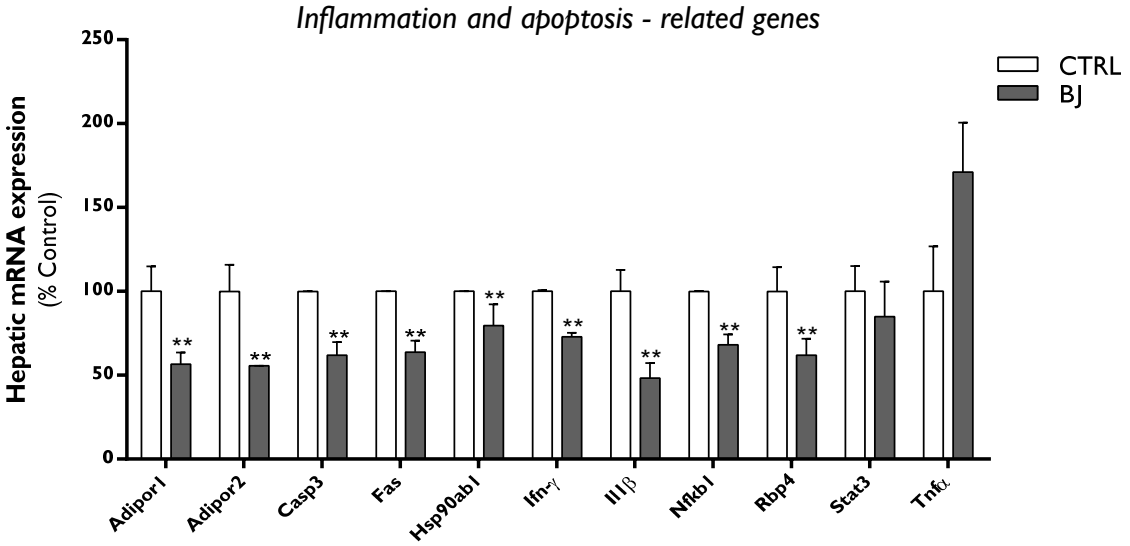


Figure 3.8 | Hepatic mRNA expression of genes involved in inflammatory, stress response and apoptotic processes. Data are presented as mean ± SEM (n=5-6/group); ** p<0.01 vs. CTRL group.

3.6 Discussion

Society globalization, time pressure, and the general concern surrounding a healthier lifestyle are major drivers to the rise of consumers' demand for "superfoods" aimed to reinforce overall health status. Blueberries are classically tagged as "superfruits" on the basis of their richness in a panoply of bioactive phytochemicals throughout their outer and inner layers [277]. An emergent trend of BB "prophylactic" consumption is presently widely scaled-up, not only as a food source but also as commercially available formulations of BB-derived functional foods and supplements whose regulatory oversight varies from country-to-country. In this context, uncertainties regarding their "real-life" efficacy and possible adverse effects subsist [489-491]. Accordingly, reliable and reproducible information from preclinical and human trials is still incongruent and requires a more in-depth elucidation to guide a healthier and safer consumption [332, 366, 460, 462, 463]. Herein, we designed an experimental study in healthy Wistar rats that were exposed to a BB supplementation regimen (BB whole-fruit). Particular attention was given to the dose employed and treatment duration mirroring a long-term human consumption scenario, and gut- and liver-related metabolic parameters were assessed based on BB-health benefit claims within obesity-related metabolic diseases.

We observed a moderate increase in fluid and carbohydrates consumption in BB-supplemented rats, most likely due to the pleasant organoleptic properties of BB fruits. Regardless of the fact that BJ may represent an extra source of carbohydrates, overall BB low caloric content along with the increased urine output observed in BB-supplemented rats favored a final isocaloric intake between groups and the maintenance of comparable BW growth curves. In fact, the lack of BW changes is in line with previous preclinical studies on healthy Sprague-Dawley and Lister hooded rats supplemented with freeze-dried whole BB for short- and long-term periods (3 and 7 weeks), respectively [492, 493].

BB fruits are well-documented in regard to their glucose-lowering and insulin-sensitizing effects in several *in vitro* studies and in diabetic animal models [287, 334, 494-497]. In our study, BB-supplemented rats efficiently managed the higher carbohydrates load arising from BB consumption, without significant interference in global glycemic and insulinemic profiles, which is also in concordance with previous studies in healthy conditions [404, 498, 499].

Once orally consumed, BB phytochemicals are largely metabolized by resident gut microflora whose individual fingerprints distinctively shape bioactive compounds

bioavailability [500]. Besides this chief role on BB phytochemicals kinetics, GM is also modulated by BB prebiotic molecules (e.g. dietary fibers and unabsorbed polyphenols) with recognized benefits in distinct metabolic diseased conditions [329, 396, 500, 501]. Collectively, these reasons prompted us to characterize GM composition, SCFA-derived metabolites, and overall intestinal integrity once BB supplementation is orally provided. Our results do not depict any significant changes in microbiota composition, despite a significant increase in fecal succinic acid, an organic acid produced by bacterial fermentation of carbohydrates for which important roles on intestinal and hepatic gluconeogenesis as well as lipid metabolism are currently underlined [502-504]. Moreover, these observations are in line with studies of Haraguchi and colleagues, who reported enhanced cecal succinate levels upon consumption of some dietary polyphenols without major changes in propionate levels and succinate-producing gut bacteria (e.g. Bacteroidetes phylum) [505]. Albeit we acknowledge that modern analytical approaches available for microbiome assessment (e.g. 16S rRNA gene sequencing) could better discriminate subtle changes in GM upon BB supplementation [506], the increment in fecal succinic acid levels reported herein hints at a metabolic adaptive response of resident gut bacteria community towards enhanced fermentation of dietary fibers and intrinsic gut health-promoting effects [507-509]. The physiological levels of epithelial integrity modulators (e.g. occludin, ZO-1, mucin) along with a regular colonic ultrastructural morphology and the absence of systemic endotoxemia and inflammation further corroborates this assumption.

Blueberry's health benefits are closely related with antioxidant activity [290, 313, 510] mainly due to their high content in phenolic compounds (e.g. hydroxycinnamic acids, caffeic or ferulic acid derivatives, anthocyanins), which we have verified in our BJ samples, analogous to what has been previously reported [511]. FRAP and $ABTS^{\bullet+}$ assays denoted a higher antioxidant capacity in serum samples of BB-supplemented rats, which is consistent with currently available preclinical and human data [387, 512, 513]. To decipher whether a long-term intake of BB could induce systemic metabolic effects, we conducted a non-targeted ^{1}H NMR-based metabolomic approach. The resultant PCA did not discriminate any segregation between two experimental groups. Yet, an unexpected and marked decrease in serum 3-HB ketone bodies and 3-HB/acetoacetate ratio was observed in BB-supplemented rats. Circulating ketone body levels are main surrogates of energy metabolism and have been traditionally looked upon as an evolutionary ancient fasting

energy vector from liver to peripheral tissues (e.g. brain, skeletal muscle, heart) [514]. Nevertheless, their importance as vital metabolic and signaling mediators has been recently underscored, even under regular glucose supply conditions [515].

Bearing in mind that (i) ketogenesis is primarily carried out in the hepatic mitochondria [516] and (ii) BB-derived phytochemicals (e.g. antioxidant polyphenols) are able to regulate mitochondrial metabolism/biogenesis and are tagged as mitochondria-protecting agents [420, 517, 518], we next attempted to dissect the impact of this BB consumption in hepatic tissue, with a major focus on mitochondrial function. Livers displayed a similar macroscopic appearance, regular histomorphology, and lipid deposition, which is aligned with the normal serum lipid profile observed between experimental groups. Moreover, the comparable serum ALT and AST activities classically score for hepatic function preservation in a clinical perspective [519, 520]. However, at a subcellular level, long-term BB intake clearly triggered a mitochondrial-adaptive setting, featured by an accentuated bioenergetic remodeling in isolated hepatic mitochondria. Mitochondrial respiration supports an electric potential (measured with the TPP^+ electrode) and the decrease in this potential may result from an inhibitory effect at the level of the respiratory chain or due to an uncoupling effect (non-specific permeabilization of the membrane at the level of the internal membrane, or eventually the BJ has some ionophoretic property). These two effects can be simultaneous, and this is very likely, since respiratory state 4 rises significantly and V FCCP respiration is significantly inhibited. These two effects are reflected in an increase in the lag phase (the mitochondria take longer to phosphorylate the added ADP and do not phosphorylate it completely since the ADP/O lowers). Mitochondria are also important intracellular storage sites of the secondary messenger Ca^{2+}, which accumulates in a direct relation with membrane potential. High concentrations of Ca^{2+} added to mitochondria in the presence of Pi promote the assemblage of a pore (permeability transition pore) that leads to loss of functional integrity of mitochondria (e.g. uncoupling) that cannot efficiently produce ATP.

In sum, the (i) mitochondrial swelling, a surrogate parameter of calcium-induced mPTP opening [521]; (ii) dissipation of the transmembrane electric potential ($\Delta\Psi m$), probably reflecting proton leakage or inhibitory effects at the respiratory chain, or both [522, 523]; (iii) overall impaired phosphorylative system, stemmed from the increased lag phase time (reflecting more time spent for ADP phosphorylation), decreased uncoupled respiration (V FCCP), lower RCR (implying an inefficient capacity for substrate oxidation

and ATP turnover) and decreased ADP/O flux ratio [mol of ATP synthesized per mol of O (1/2 O_2)] [524], collectively emphasize BB-derived phytochemicals ability to strongly impact mitochondrial respiratory control, in line with several reports focused on polyphenolic phytochemicals [525-527]. Moreover, a concomitant downregulation of mitochondrial respiratory complex I (*Ndufb6*) and complex V (*Atp5c1*) encoding genes further corroborates, at a molecular level, the mitochondrial functional data and is closely aligned with the dose-dependent decrease of ECT activity and oxidative phosphorylation observed *in vitro* when rat heart mitochondria were exposed to bilberry fruit anthocyanin-rich extracts [528].

To further underscore the first insights of BB-derived hepatic remodeling at a molecular level, we broadened our study through a transcriptomic analysis of functionally diverse cellular targets of BB phytochemicals encompassing energy-derived metabolic pathways, transcription factors, and inflammatory mediators. From the lipidic perspective, BB oral consumption triggered a pair-decreased mRNA transcripts of fatty acid transporter 5 (FATP5/Slc27a5) and fatty-acid binding protein-1 (*FABP1*), impaired hepatocyte fatty acid uptake, along with a collective transcriptional repression of Acadl, Acox-1, Cpt1a and Cpt2a encoding-genes, suggesting mitochondrial acyl-CoA uptake decline and successive fatty acid β-oxidation deceleration, which is in accordance with previously published data [529]. Interestingly, a concerted downregulation of PPAR-α gene, a transcription factor responsible for the induction of fatty acid transport/oxidation along with ketone biosynthesis and import, was also recorded. Taking into consideration that hepatic ketogenesis is a proxy of total hepatic fat oxidation [129, 130], future work will be critical to better understand the possible correlation between the transcriptomic fatty acid β-oxidation repression and the decreased circulating 3-HB contents detected in BB-treated rats. Ensuing inhibition of the expression of the lipogenic enzyme DGAT-2 may also be postulated as a downstream event of PPAR-α transcription factor repression [530]. In opposition, the *SCD-1* gene, which encodes an enzyme that catalyzes the conversion of saturated into monounsaturated fatty acids, was found up-regulated in orally supplemented BB rats. Furthermore, a simultaneous up- and downregulation of *ApoA1* and *ApoC3* gene transcription, two major structural components of HDL and VLDL lipoproteins, respectively, were detected in the livers of BB experimental group without a significant translation in serum/liver lipid profile. Within the carbohydrate context, BB-induced transcriptomic remodeling also followed similar traits. Glucokinase (GCK), the

principal hexokinase acting as the gatekeeper for hepatic glycolysis, was found downregulated upon BB-supplementation [531, 532]. A similar fall in *Pdk4* gene transcription (probably due to *PPAR-α* downregulation) was also observed and may correlate with decreased pyruvate availability for gluconeogenic processes [531-533]. This assumption is further corroborated by *CYP2E1* gene repression for which an important role in the oxidative metabolism of gluconeogenic substrates is well-known [534, 535]. Moreover, Ptpn1 gene downregulation is compatible with insulin signaling improvement and is aligned with previous reports denoting the ability of polyphenol anthocyanins to exert their insulin-sensitizing properties [363, 511]. Collectively, BB-induced hepatic transcriptomic metabolic reprogramming observed herein hints at a repression of chief fuel sources and cellular energetic pathways (e.g. fatty acid β-oxidation, glycolysis, gluconeogenesis).

Finally, and on the basis of the anti-inflammatory potential of BB phytochemicals [536, 537], we also assessed the transcriptomic profile of a panoply of inflammatory-related genes. A combined gene repression of NFkb1, cytokines (e.g. IFN-g, *IL-1β*), adipokines (e.g. Adipor1 and Adipor2), and stress-element responses (e.g. *Hsp90ab1*) were unequivocally triggered by the BB supplementation. Besides their well-known antioxidant properties, BB phytochemicals also display the ability to modulate cellular pathways through epigenetic mechanisms encompassing DNA methylation, histone modifications, and posttranscriptional gene regulation of noncoding RNAs, constituting a link between external environmental cues and gene expression [443, 538]. In this regard, we cannot overlook the probable "noncanonical" signaling rearrangement arising from BB phytochemicals-altered ketone bodies homeostasis. In fact, decreased 3-HB levels may also trigger chromatin remodeling through histone hypoacetylation and subsequent transcriptional repression [129, 130]. Due to its reversibility, dietary-targeted epigenetics is an attractive approach for disease prevention and is often envisaged as a starting point of clinical intervention [539]. Taken together, and based on the marked transcriptomic repression in metabolic, transcription factors, and inflammatory-related genes, future experimental studies are warranted to underscore the impact of long-term intake of BB on the epigenetic phenomena and their potential for disease prevention and/or intervention.

One emergent mechanism of action of dietary phytochemicals relies on their ability to act as hormetins, triggering adaptive cellular stress response pathways in both plants

and humans [540]. Typically, the concept of hormesis has been adopted to describe the phenomenon where a given substance/condition is able to induce biologically opposite effects at different doses [541]. In other words, a hormetic response may occur when a mild stress (e.g. calorie restriction, exercise) activates intrinsic changes that enhance resistance to a more severe stress arising from higher doses of the same stressor or even from other less-specific stressors comprising oxidative, metabolic, and thermal stresses [542]. Altogether, the mitochondrial bioenergetic challenge and metabolic transcriptomic reprogramming presently disclosed portray a unified adaptive response to the nutritional challenge imposed by BB phytochemicals intake. Interestingly, mitohormesis is being widely disclosed in many model organisms and strongly implicated in metabolic health [543-546]. For instance, mitochondrial ECT modulation was found to reprogram energy metabolism towards cell survival and improved lifespan [547]. Remarkably, downregulation of ECT activity due to mitochondria-targeting xenobiotics in Caenorhabditis elegans resulted in cytoplasmic proteostasis restoration and suspected increased vitality later in life [548]. Moreover, mitochondrial bioenergetics remodeling may also encompass a central node in the fine-tuning of metabolic "switch" in fuel sources and catabolic/anabolic rates that optimize organism performance in varying nutrient states and physiological conditions [129, 549]. Interestingly, the global repression of genes enclosing distinct metabolic pathways collectively hint for a "hibernated" energetic state upon BB supplementation. Conceivable altered ketogenesis, classically viewed as a spillover pathway from fat combustion, further reinforces a quiescent mitochondrial-related metabolomics. Supported by the notion that ketone body metabolism may be beneficial even in carbohydrate-laden states, the ketohormetic hypothesis has been recently postulated [129] and may actually intertwine with mitohormesis [129, 550] within this BB phytochemical challenge.

Since the hormetic response is a phenomenon where low doses of a stressor trigger opposite responses to high ones, the beneficial or toxic outcomes should be interpreted in the light of the non-linear biphasic hormetic dose-effect relationship [551]. Accordingly, the amounts of phytochemical cell stressors in fruits and vegetables consumed by humans in a regular diet are considered to fall within the low dose stimulatory range of concentrations; however, once they are consumed in the form of concentrated supplements, the doses may exceed the toxic threshold with potential adverse health consequences [542, 552]. Remarkably, previous work also disclosed that hepatic tissue is

able to hormetically cope with chemical stressors (ethanol) through a transcriptional adaptive response [553]. Considering that we only assessed a unique dose in a single-hit stress paradigm (BB consumption) along with the ambivalent redox nature of polyphenols [525], the beneficial or noxious consequences arising from this BB hepatic remodeling should be carefully interpreted. In a global perspective, no evident toxic effects were recorded. However, at a molecular level, BB intake may trigger distinct consequences. For instance, if the transcriptomics remodeling of some of the analyzed genes are effectively translated into the protein level, one can envisage both positive [anti-atherogenic properties (e.g. *ApoA1* upregulation) and hepatic steatosis reversal (e.g. *SCD-1* upregulation)] and negative [altered xenobiotic metabolism (e.g. acetaminophen, isoniazid, coumarin, ethanol), due to *Cyp2E1* downregulation] outcomes [554-556]. Moreover, the mitochondrial "idle motion" observed herein may be beneficial at a basal condition and prelude optimal fitness but also be detrimental if not quickly reversed upon highly demanding operating challenges.

3.7 Conclusions

To the best of our knowledge, this is the first work providing molecular insights of the mitochondrial and metabolic effects of BB consumption in a healthy condition from a translational perspective. Collectively, data presented herein hint for mitochondrial-related metabolic transcriptomic reprogramming together with a concerted anti-inflammatory pre-conditioning. Future studies comprising prophylactic or interventional BB regimens in diseased conditions (two-hit experimental paradigms) and in both sexes will be of utmost importance to disclose tailored beneficial/toxic outcomes of this new dietary trend and guide evidence-based medicine.

Supplementary data

Table S3.1 | Serum metabolites identified by ^{1}H-NMR.

Serum metabolites	Chemical shift (ppm)	CTRL (n=7)	BJ (n=7)
Histidine	7.01 – 7.07	$0.001 \pm 1.15 \times 10^{-4}$	$0.001 \pm 1.1 \times 10^{-4}$
TGs	5.23 (5.244 - 5.34)	$0.010 \pm 1.56 \times 10^{-3}$	$0.010 \pm 8.72 \times 10^{-4}$
Glucose	5.189 - 5.235	$0.026 \pm 1.13 \times 10^{-3}$	$0.026 \pm 8.94 \times 10^{-4}$
Mannose	5.149 – 5.176	$0.001 \pm 5.90 \times 10^{-5}$	$0.001 \pm 1.0 \times 10^{-4}$
Malic acid	4.261 – 4.307	$0.010 \pm 1.96 \times 10^{-3}$	$0.014 \pm 9.82 \times 10^{-4}$
Serine	3.947 - 3.985	$0.004 \pm 3.86 \times 10^{-4}$	$0.004 \pm 3.16 \times 10^{4}$
Glycerol	3.64 - 3.635	$0.006 \pm 6.35 \times 10^{-4}$	$0.005 \pm 3.96 \times 10^{-4}$
Glycine	3.538 - 3.544	$0.007 \pm 8.81 \times 10^{-4}$	$0.007 \pm 8.89 \times 10^{-4}$
Choline	3.175 - 3.184	$0.005 \pm 9.86 \times 10^{-4}$	$0.004 \pm 7.31 \times 10^{-4}$
unknown (DMSO?)	3.124 - 3.134	$0.001 \pm 1.19 \times 10^{-4}$	$0.001 \pm 1.20 \times 10^{-4}$
Creatine	3.005 - 3.023	$0.003 \pm 4.16 \times 10^{-4}$	$0.004 \pm 4.22 \times 10^{-4}$
Glutamine	2.406 - 2.457	$0.016 \pm 9.70 \times 10^{-4}$	$0.017 \pm 1.29 \times 10^{-3}$
Succinate	2.379 - 2.387	$0.003 \pm 3.92 \times 10^{-4}$	$0.002 \pm 4.54 \times 10^{-4}$
Acetoacetate/Acetone[a]	2.195 - 2.217	$0.003 \pm 5.96 \times 10^{-4}$	$0.0026 \pm 5.06 \times 10^{-4}$
N-acetylproteins	2.004 – 2.045	$0.016 \pm 9.96 \times 10^{-4}$	$0.017 \pm 9.40 \times 10^{-4}$
Acetate	1.889 - 1.904	$0.003 \pm 3.56 \times 10^{-4}$	$0.002 \pm 1.20 \times 10^{-4}$
Alanine	1.457 - 1.475	$0.007 \pm 3.93 \times 10^{-4}$	$0.008 \pm 4.09 \times 10^{-4}$
Lactate	1.292 - 1.334	$0.083 \pm 8.06 \times 10^{-3}$	$0.073 \pm 5.70 \times 10^{-4}$
3-hydroxybutyrate	1.163 - 1.1896	$0.003 \pm 7.42 \times 10^{-4}$	$0.002 \pm 3.29 \times 10^{-4}$ *
Valine	1.004 - 1.035	$0.006 \pm 2.98 \times 10^{-4}$	$0.006 \pm 5.70 \times 10^{-4}$
Isoleucine	0.9977 - 1.001	$4.99 \times 10^{-5} \pm 8.50 \times 10^{-6}$	$4.03 \times 10^{-5} \pm 8.31 \times 10^{-6}$
TGs (0.87)	0.87 (0.825 - 0.893)	$0.01 \pm 1.71 \times 10^{-3}$	$0.015 \pm 1.66 \times 10^{-3}$
Betaine	3.26	$1.70 \times 10^{11} \pm 2.34 \times 10^{10}$	$1.21 \times 10^{11} \pm 1.47 \times 10^{10}$
Lactate /Alanine		13.19 ± 0.932	9.800 ± 1.221
3-HB / Acetoacetate		1.107 ± 0.124	0.587 ± 0.072 **

Data are presented as mean ± SEM (n=5-6/group). * $p<0.05$ and ** $p<0.01$ vs. CTRL group. [a]Putatively annotated as level 3 of identification according to Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative recommendations. All other metabolites identified as level 2.

CHAPTER 4

Blueberry counteracts prediabetes in a hypercaloric diet-induced rat model and rescues hepatic mitochondrial bioenergetics

(Original article published in *Nutrients 2021, 13(12):4192; https://doi.org/10.3390/nu13124192*)

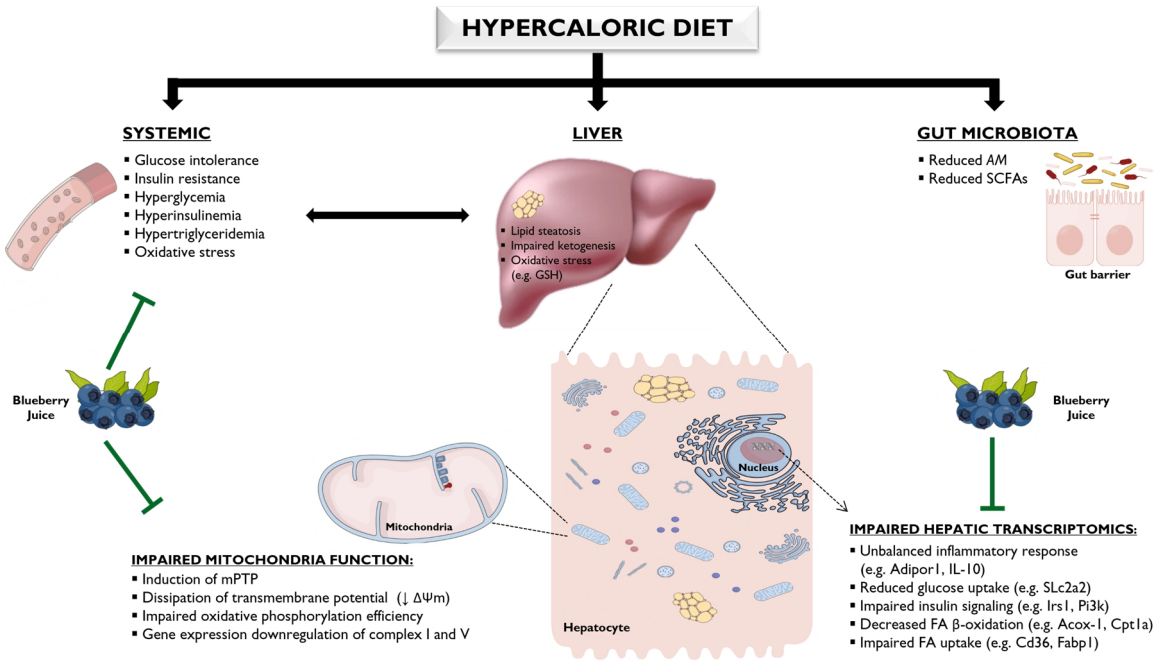
4.1 Abstract

The paramount importance of a healthy diet in the prevention of type 2 diabetes is now well recognized. Blueberry (BB) fruits have been described as attractive functional fruits for this purpose. This study aimed to elucidate the cellular and molecular mechanisms pertaining to the protective impact of blueberry juice (BJ) on prediabetes. Using a hypercaloric diet-induced prediabetic rat model, we evaluated the effects of BJ on glucose, insulin, and lipid profiles; gut microbiota composition; intestinal barrier integrity; and metabolic endotoxemia, as well as on hepatic metabolic surrogates, including several related to mitochondria bioenergetics. BJ supplementation for 14 weeks counteracted diet-evoked metabolic deregulation, improving glucose tolerance, insulin sensitivity, and hypertriglyceridemia, along with systemic and hepatic antioxidant properties, without a significant impact on the gut microbiota composition and related mechanisms. In addition, BJ treatment effectively alleviated hepatic steatosis and mitochondrial dysfunction observed in the prediabetic animals, as suggested by the amelioration of bioenergetics parameters and key targets of inflammation, insulin signaling, ketogenesis, and fatty acids oxidation. In conclusion, the beneficial metabolic impact of BJ in prediabetes may be mainly explained by the rescue of hepatic mitochondrial bioenergetics. These findings pave the way to support the use of BJ in prediabetes to prevent diabetes and its complications.

Keywords

Blueberries; hypercaloric diet-induced prediabetes; gut microbiota; hepatic energy metabolism; mitochondria function; hepatoprotection.

4.2 Graphical abstract



4.3 Introduction

Prediabetes, characterized by impaired glucose tolerance (IGT) and/or impaired fasting glucose (IFG) [557, 558], represents a high-risk state for type 2 diabetes mellitus (T2DM) development. Annually, around 5–10% of prediabetic patients progress to T2DM, and estimates indicate that up to 70% of patients with prediabetes will eventually develop overt diabetes within their lifetime [1, 2].

During the last decades, new studies have suggested a chief role for gut microbiota (GM) in the development of metabolic diseases, including T2DM. Although incongruent data subsist, emerging evidence underlines a dysbiotic GM community characterized by changes in bacterial abundance/diversity along with decreased short-chain fatty acids (SCFAs) levels (e.g., butyrate) early in the period of human prediabetes [86, 103, 559]. Moreover, the liver plays a predominant role in the development of insulin resistance since it is a key organ involved in glucose, lipid, and amino acid metabolism and crucial for maintain energy homeostasis [120]. Notably, both insulin resistance and hepatic glucose/lipid dysmetabolism start years before T2DM diagnosis, underpinning prediabetes' subclinical evolution [2, 237, 560, 561]. Likewise, hepatic energy dysmetabolism has been traced to mitochondria as early as prediabetes in both rodents [562] and humans [563]. However, a direct causal relationship between insulin resistance and mitochondrial dysfunction still lacks further elucidation [209, 564, 565]. Collectively, the aforesaid pathophysiological mechanisms often culminate in a state of chronic low-grade inflammation and oxidative stress that follows the progression of prediabetes to overt T2DM [52, 566]. In the view of the T2DM health burden, which has reached epidemic proportions [567, 568], it is crucial to establish interventions targeted to early pathophysiological mechanisms in the asymptomatic prediabetic phase, an opportunity window to prevent or attenuate disease progression and to reduce the risk of subsequent complications [569].

Lifestyle changes focused on improved diet quality can slow down or even halt (pre)diabetes progression [570-572]. For instance, an inverse association between plant-based diets (e.g., fresh vegetables, fruits) and T2DM evolution has been consistently highlighted [310, 573-576]. Blueberry (BB), one of the most popular berries due to their palatableness and nutritional/phytochemical composition, display a panoply of health-related properties in several metabolic diseases, including T2DM, as comprehensively reviewed elsewhere [290, 368, 577]. Briefly, their low caloric value pairs with an enriched

content of dietary fibers and an array of polyphenolic secondary metabolites (e.g., flavonols, anthocyanins, phenolic acids) that greatly dictate BB' antioxidant and anti-inflammatory profiles [280, 282, 290, 293]. Moreover, a wealth of experimental evidence openly points out the BB-derived hypoglycemic and insulin-sensitizing effects [30,32,36,37]. However, the precise pathophysiologic mechanisms counteracted by BB in early prediabetes progression are far from being disclosed. They are also consistently narrowed by the discrepancy in the experimental designs (e.g., disease stage, BB doses and presentation forms, treatment duration) in both preclinical studies and clinical settings.

Recently, our group underscored that long-term BB supplementation in a healthy condition triggered an expressive polyphenol-derived remodeling of hepatic mitochondrial bioenergetics [578]. Given the well-recognized hepatic mitochondrial impairments in overt T2DM, we hypothesized that BB' ability to counteract prediabetes would follow similar traits. This study aimed to shed light on cellular and molecular mechanisms paralleling the protective effects of BB on glucose tolerance, insulin resistance, and lipid profile as early as prediabetes. To this end, we carried out an integrative preclinical approach encompassing functional and molecular readouts of gut health, hepatic metabolomics, and oxidative/inflammatory status, focusing on mitochondria, upon a clinically relevant BB dose supplementation, in diet-induced prediabetes.

4.4 Materials and Methods

4.4.1 Blueberry juice preparation

Blueberries (*Vaccinium corymbosum* L., cultivar "Liberty") were supplied from the same variety and in the same maturation stage by COAPE (Farming Cooperative of Mangualde, Mangualde, Portugal) and stored at −80 °C until use.

BB juice (BJ) was obtained as described in a previous study [578]. Briefly, BB were weighed, blended with sucrose solution (35%), and converted into juice to assure consumption of whole fruit parts (peel, pulp, and seeds). The volume of sucrose solution added was corrected every day to guarantee that 25 g/kg of BW of BB were daily consumed by the animals. This BB dose was selected based on a previous study of our group [578].

4.4.2 Phytochemical screening of blueberry juice

The characterization of the phenolic compounds present in BJ was performed through HPLC-PAD-ESI/MSn analysis. The fresh BJ sample was analyzed by a liquid chromatography system equipped with a Spherisorb ODS-2 column (150 x 2.1 mm id; particle size, 3 µm; Waters Corp., Milford, MA, USA) with a Spherisorb ODS-2 guard cartridge (10 x 4.6 mm id; particle size, 5 µm; Waters Corp., Milford, MA, USA), maintained at 25 °C. The elution was performed using 1% aqueous formic acid (v/v) (A) and methanol (B) as mobile phase, with a gradient profile of 0–75 min (0%–100% B), at a flow rate of 200 µL/min. The detection was carried out using a photodiode spectrophotometer-PDA detector (Thermo Finnigan Surveyor, San Diego, CA, USA) interfaced with a linear ion trap mass spectrometer (LIT-MS) (LTQ XL, Thermo Scientific, Waltham, MA, USA). The PDA detection was recorded in a wavelength range of 200–600 nm, followed by the detection in the mass spectrometer. Mass spectra were acquired in a negative ion mode. The mass spectrometer performed three consecutive scans: full mass (m/z 100–2000), MS2 of the most abundant ion in the full mass, and MS3 of the most abundant ion in the MS2. Source and capillary voltage were 5.0 kV and -35.0 V, respectively and the capillary temperature was 275 °C. Nitrogen was used as sheath and auxiliary gas at 40 and 5 Finnigan arbitrary units, respectively, and helium as collision gas with a

normalized energy of 35%. Data treatment was carried out with the XCALIBUR software (Thermo Scientific, Waltham, MA, USA).

4.4.3 Animals and experimental design

Eight-week-old male Wistar rats (Charles River Laboratories, Barcelona, Spain) were pair-housed in ventilated cages, under controlled environmental conditions of temperature (22 ± 1 °C), humidity (50–60%), and light (12 h light-dark cycle) and *ad libitum* access to tap water and standard rodent chow. All animal procedures performed in this study were approved by the local (iCBR) Animal Welfare Body (ORBEA, #9/2018) and complied with the Animal Care National and European Directives and with the ARRIVE guidelines [579].

Following a one-week period of acclimatization, rats were arbitrarily assigned into 3 groups, in a 23-week protocol: control group, received standard chow and tap water (CTRL, n = 8); prediabetic group, received 35% sucrose (Hsu-84100; Sigma-Aldrich, St. Louis, MO, USA) in the drinking water plus standard chow until week 9, further supplemented by high-fat diet (HFD) (58Y1, TestDiet, St. Louis, MO, USA) until week 23 (HSuHF, n = 10); and prediabetic group supplemented with BJ (HSuHF + BJ; n = 10), submitted to the same dietary regimen as the prediabetic group but received 25 g/kg BW/day of BJ (in 35% sucrose solution) between weeks 9 and 23 (Figure 4.1). The development of prediabetes in rats, including the protocol of the dietary regimen and the duration, was performed based on our previous study [275].

Briefly, the high-fat diet contained 61.6% of energy as fat, 20.3% as carbohydrates, and 18.1% as protein, with a total of 5.10 kcal/g (58Y1, TestDiet, St. Louis, MO, USA), whereas standard rat chow contained 8.6% of energy as fat and 67.9% as carbohydrates, 23.5% as protein, with a total of 3.15 kcal/g (4RF21 Mucedola, Milan, Italy).

Feed and beverage were provided *ad libitum* during the entire experimental protocol, except for the fasting periods. BW was weekly monitored; food and beverage consumption were recorded daily per cage throughout the protocol and used to calculate energy intake. The estimated values (calculated by dividing the total intake by two rats per cage) are presented per week.

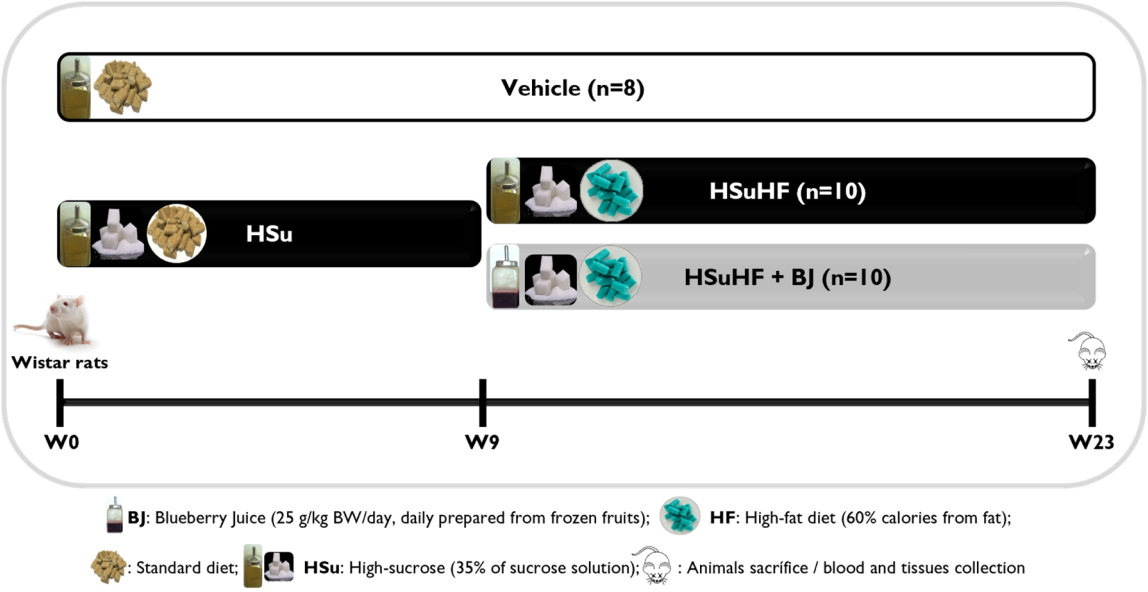


Figure 4.1 | Experimental groups and protocol design.

4.4.4 Glucose Tolerance Test (GTT) and Insulin Tolerance Test (ITT)

At the first day of week 22, GTT was performed to assess the rats' glucose tolerance. Rats submitted to a fasting period of 6 h were intraperitoneally (i.p.) injected with a solution of glucose (2 g/kg BW) and the blood glucose levels from the tail vein were recorded in samples taken immediately before (0 min) the injection and 30, 60, 90, and 120 min after, using an ACCU-CHEK® Aviva portable glucometer (Roche Diagnostics, Mannheim, Germany). In addition, blood samples (≈30 µL) were collected before glucose challenge to assess fasting insulin levels.

At the first day of week 23, the *in vivo* peripheral insulin sensitivity was evaluated by ITT performed through the i.p. injection of insulin solution (0.75 units/kg BW; Actrapid Novo Nordisk, Bagsvaerd, Denmark) in 6-h fasted rats. Blood glucose levels were measured in the tail vein blood collected immediately before (0 min) the injection and 15, 30, 45, 60, and 120 min after, using the same glucometer, as previously described [275]. The area under the curve (AUC) of GTT (AUC_{GTT}) and the rate constant for glucose clearance (K_{ITT}) were calculated as previously reported elsewhere [473, 474].

4.4.5 *In vivo* hepatic ultrasonographic analysis

Rat liver ultrasound examination was performed using e General Electrics LOGIQe (GE Healthcare, Milwaukee, WI, USA), with a linear-array transducer with multifrequency (7.5–12 MHz). Before ultrasound examination, the rats were anesthetized by inhalation of 1.5% isoflurane by using an inhalation anesthesia system, kept under mild anesthesia during the data acquisition, and the abdomen was shaved by an electric shaver to reduce artifacts in the ultrasonography images. The liver and portal vein were analyzed in animals positioned in the supine position and the spleen in the right posterior oblique position, through multiple transversal and longitudinal scanning. A sound-conducting gel was applied, and the liver was assessed by placing the transducer just distal to the last right costal ribs and angling its beam cranially and obliquely, obtaining multiple transversal and longitudinal scans. A fundamental brightness mode (B-mode) was applied to all imaging. The acoustic focus was placed in the center of the liver and in the largest transverse cross-section of the spleen.

The ultrasound examination was performed by one blinded expert and the criteria for ultrasound diagnosis, including changes in the liver parenchymal echogenicity, focal steatosis, and diameter of the portal vein, were analyzed as previously described [580].

4.4.6 Biological samples collection

Serum samples were obtained by centrifugation (2300 g/15 min/4 °C) of whole blood samples (immediately collected after animal sacrifice by venipuncture from the jugular vein) and stored at −20 °C until assay. Liver and gastrointestinal tissues (duodenum and colon) were immediately excised, dissected into small pieces, and stored in conditions according to the assay's requirements. The liver was firstly weighed and then divided into distinct pieces: one was immediately used for functional mitochondria assays; one was stored for NMR analysis; one was directly frozen in liquid nitrogen and stored at −80 °C until analysis for further protein and RNA extraction; one was kept in neutral buffered formalin solution (10%) to be used for histological analysis; and the remaining liver pieces were frozen by liquid nitrogen and stored at −80 °C. The liver weight relative to BW was calculated. During the last week of the experimental protocol, animals were put in

metabolic cages to collect fecal samples, which were weighed and stored at −80 °C until processing.

4.4.7 Determination of serum metabolic parameters

Serum samples were used to determine the postprandial glucose and triglycerides (TGs) by using automatic validated methods and equipment (Hitachi 717 analyzer, Roche Diagnostics GMBH, Mannheim, Germany), as previously described [237]. Serum insulin contents were quantified by using commercially available ELISA kits for rat samples (10-1250-01, Mercodia, Uppsala, Sweden).

4.4.8 Evaluation of serum redox status

Total antioxidant status (TAS) in serum samples was estimated through the ferric reducing antioxidant potential (FRAP) assay, as previously described [475], with slight modifications. This method is based on the ability of the antioxidants contained in a sample to reduce ferric-tripyridyltriazine (Fe^{3+}-TPTZ) to a ferrous form (Fe^{2+}) that develops an intense blue color, with maximum absorbance at 593 nm. In brief, the FRAP reagent was prepared freshly by the addition of acetate buffer (300 mM, pH 3.6), 10 mM of TPTZ (2.4.6-tripyridyl-s-triazine, T1253, Sigma-Aldrich, St. Louis, MO, USA) solution in 40 mM HCl and ferric chloride ($FeCl_3.6H_2O$, 236489, Sigma-Aldrich, St. Louis, MO, USA) solution (20 mM), at the ratio of 10:1:1 (*v*/*v*/*v*), respectively. In total, 10 µL of serum sample were added to 30 µL of distilled water and 300 µL of FRAP reagent in a 96-well microplate and incubated at 37 °C for 15 min; then, the absorbance was measured spectrophotometrically at 593 nm using a microplate reader (Synergy™ HT Multi-Detection Microplate Reader, BioTek®, Vermont, USA). The antioxidant capacity of the samples was quantified from the calibration curve plotted using Trolox solution as the standard reference. Malondialdehyde (MDA) levels were performed through the thiobarbituric acid reactive substances (TBARs) test. In total, 100 µL of serum were incubated (for 1 h) in a thiobarbituric acid solution, at room temperature and in dark conditions. The samples were then incubated at 90 °C for 60 min. Afterwards, the tubes were placed on ice for reaction cessation. In this assay, one MDA molecule chemically reacts with two thiobarbituric acid molecules, the final product being a molecule that can

be spectrophotometrically quantified at 532 nm (pink pigment). The MDA concentration was calculated against a calibration curve using 1,1,3,3-tetramethoxypropane (108383, Sigma-Aldrich, St. Louis, MO, USA) as the external standard (range: 0.1–83.5 μM). The results were expressed as MDA/TAS ratio, a marker of oxidative stress [581].

4.4.9 ^{1}H Nuclear Magnetic Resonance (NMR) spectroscopy

Serum samples were prepared for the NMR analysis as described previously [275]. Liver metabolites were extracted from liquid N_2 ground samples using a Folch extraction [582]. Polar extracts were lyophilized and dissolved in D_2O phosphate buffer (0.2 M, pH = 7) supplemented with sodium fumarate (2 mM) used as internal standard. Samples for high-resolution ^{1}H NMR analysis were loaded into 3 mm NMR-grade tubes.

1D-^{1}H cpmg (Carr-Purcell-Meiboom-Gill spin-echo pulse sequence) NMR spectra were recorded by a 600 MHz (14.1 T) spectrometer (Agilent, Santa Clara, CA, USA) using a 3 mm indirect detection probe with a z-gradient. The spectra acquisition and processing were performed as previously described [578].

Recorded spectra were compared to the reference data from public databases, such as the Human Metabolome Database (HMDM), for spectral assignment [477]. In order to help spectral assignment, 2D homonuclear total correlation spectroscopy (TOCSY) spectra were also recorded [478]. Metabolites were identified according to Metabolomics Standards Initiative (MSI) guidelines for metabolite identification [479] and the identification levels are indicated in supplementary data (Tables S4.2 and S4.3).

Processed 1D cpmg spectra were bucketed using one-point bucket (0.6–9.0 ppm, with signal-free, water, and fumarate regions excluded) using Amix Viewer (version 3.9.15, Bruker Biospin GmbH, Rheinstetten, Germany) and aligned using the icoshift algorithm [480]. The resulting matrix was normalized by the total spectral area and also analyzed. Multivariate statistical analysis was applied on unit variance scaled matrix (SIMCA 14, Umetrics, Sartorius Stedim Biotech, Gottingen, Germany). Information on the global data structure was obtained from principal component analysis (PCA); in addition, partial least square discriminant analysis (PLS-DA) was used to evaluate class separation and identify the main metabolites involved in the discrimination of class. The qualitative measure of the predictive power (Q2) and the degree of fit to the data (R2) were given by a 7-fold internal cross-validation of the PLS-DA models, which were validated by the permutation

test (*n* = 100) [481]. The corresponding PLS-DA loadings plots were obtained by multiplying the loading weight factors (w) by the standard deviation of the respective variables and were color-coded according to the variable importance in the projection (VIP). For quantitative assessment of metabolite variations between the groups, metabolite signals (VIP > 1) were integrated and normalized by the total spectral area. Outliers were excluded on the basis of the quality of the recorded NMR spectra according to the recommendations of MSI [479].

4.4.10 Extraction and quantification of gut microbiota in feces

4.4.10.1 DNA extraction from stool

An NZY Tissue gDNA Isolation Kit (NZYtech, Lisbon, Portugal) was used to extract and purify genomic DNA from fecal samples, as previously reported [482]. DNA purity and quantification were evaluated with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

4.4.10.2 Real-time PCR for microbial analysis of stool

Real-time PCR was performed by using a LightCycler FastStart DNA Master SYBR Green kit and a LightCycler instrument (Hoffman-La Roche Ltd., Basel, Switzerland) and the assay´s conditions were performed as previously described [482]. Primer sequences (Sigma-Aldrich, St. Louis, MO, USA) used to target the 16S rRNA gene of the bacteria and the conditions for PCR amplification reactions were previously described [578] and are listed in Table 3.1 in the Chapter 3. Data are presented as the mean values of duplicate PCR analysis.

4.4.10.3 Quantification of fecal SCFAs and organic acids

SCFAs and organic acids (lactic and succinic acid) were evaluated using an Agilent 1200 series HPLC system with a refractive index—RI detector and with a UV detector, as previously described [482]. Quantification of fecal SCFAs was achieved by using calibration curves, and concentrations were expressed as mean µg/g of wet weight.

4.4.11 Evaluation of intestinal permeability

Intestinal integrity was determined by measuring the permeability of FITC-dextran. Rats from each group were fasted for 6 h and then gavaged with FICT-dextran solution (600 mg/kg, 4 kDa, Sigma-Aldrich), as previously described [583]. After a 4 h dosage, animals were anesthetized, and blood collected from the jugular vein. Blood samples were then centrifuged (2300× *g*, 10 min, 4 °C) to obtain serum. Aliquots of serum were diluted in PBS (1:1 *v*/*v*) and then added in a black 96-well microplate (Costar 96 back opaque). Subsequently, FITC-dextran was measured using a fluorescence spectrophotometer (BioTek Synergy HT, Winooski, VT, USA,) at an excitation and emission wavelength of 485 and 535 nm, respectively. Each sample was quantified in triplicate and the standard curve was obtained by diluting the serial concentrations of FITC-dextran in non-treated serum diluted in PBS (1:1 *v*/*v*). The results are expressed as µg/mL.

4.4.12 Evaluation of serum Lipopolysaccharide (LPS) levels

Endotoxin LPS contents in serum samples were measured by using a Pyrochrome Lisate Mix, a quantitative chromogenic reagent, diluted in Glucashield® buffer, as previously reported by our group [578].

4.4.13 Colon and duodenum analysis by Transmission Electron Microscopy (TEM)

Duodenum and colon samples collected after the sacrifice were immediately sectioned in small fragments of about 1 mm^3 and fixed in 2.5% glutaraldehyde solution in 0.1 M phosphate buffer (pH = 7.2) for 2 h. Successive post-fixation was done in 1% osmium tetroxide (1.5 h) and in 1% aqueous uranyl acetate (1 h in the dark). After washing with distilled water, samples were dehydrated in a graded acetone series (30–100%) and embedded in an Epoxy resine (Fluka Analytical, Sigma-Aldrich, Darmstadt, Germany). Ultrathin sections obtained with a Leica EM UC6 (Leica Co, Vienna, Austria) ultramicrotome were then mounted on copper grids and stained with lead citrate 0.2% for 10 min. Observations were carried out on a TEM Tecnai G2 Spirit Bio Twin at 100 kV (FEI, Hillsboro, OR, USA), and images were processed using AnalySIS 3.2.

4.4.14 Immunohistochemical staining

Cross-sections of rat colon (10 μm) were cut with a cryostat (Leica CM3050S, Nussloch, Germany) and fixed with an acetone:methanol mixture (1:1) at 20 °C for 2 min and then rehydrated in phosphate-buffered saline (PBS) (3 × 5 min). After rinsing, sections were permeabilized with 0.5% Triton X-100 in PBS for 15 min and blocked for 40 min with 4% nonfat milk in 20 mM Tris, pH 7.2, and 150 mM NaCl. Then, samples were incubated with primary antibodies: rabbit polyclonal anti-ZO-1 (ab96587, Abcam, Cambridge, MA, USA) and mouse monoclonal anti-occludin (OC-3F10, 33-1500, Life Tecnologies, Carlsbad, CA, USA) in PBS containing 1% BSA (4°C overnight). After washing with PBS (3 × 5 min), the sections were incubated with the secondary fluorescent antibody Alexa Fluor 488-conjugated goat anti-rabbit IgG or Alexa Fluor 568-conjugated donkey anti-mouse IgG (1:200; Molecular Probes, Life Technologies, Paisley, UK) and 4′,6-diamidino-2-phenylindole (DAPI, nuclei dye, D1306, Invitrogen, Carlsbad, CA, USA), for 1h at room temperature. Samples were then washed with PBS (3 × 5 min), and the slides mounted using the Glycergel mouting medium (Dako, C0563, Agilent, Santa Clara, CA, USA). Anti-ZO-1 and anti-occludin immunostaining samples were imaged using a confocal fluorescence microscope (LSM 710, Carl Zeiss, Gottingen, Germany).

4.4.15 Hepatic histological analysis

Hematoxylin-eosin (H&E) and Oil Red O staining were performed as previously described [275, 483]. Sections were visualized with a Zeiss microscope Mod. Axioplan 2 (Zeiss, Jena, Germany).

4.4.16 Quantification of hepatic triglycerides

Triglycerides contents in the liver samples were quantified by using a Triglycerides Colorimetric Assay kit (1155010, Cromatest®, Linear Chemicals, Barcelona, Spain), as described previously [584].

4.4.17 Hepatic SEM analysis

Frozen liver sections were defrosted and mounted on an SEM stub using a double-sided carbon sticker. The specimens were analyzed using a compact variable-pressure scanning electron microscope (Hitachi, FlexSEM 1000, Tokyo, Japan) equipped with a cryostage at 15KV.

4.4.18 Hepatic mitochondria bioenergetics

Liver mitochondria were isolated in homogenization medium composed of 250 mM sucrose, 10 mM HEPES (pH 7.4), 0.5 mM EGTA, and 0.1% fat-free bovine serum albumin (BSA), as previously described [484, 485]. EGTA and BSA were omitted from the final washing medium, adjusted at pH 7.4. After homogenization of the minced blood-free hepatic tissue, homogenates were centrifuged at 800× *g* for 10 min at 4 °C. Supernatant was collected and centrifuged at 10,000× *g* for 10 min at 4 °C to pellet mitochondria, which were resuspended in washing medium (250 mM sucrose and 10 mM HEPES, pH 7.4) and centrifuged again at 10,000× *g* for 10 min at 4 °C. Final pellet were resuspended in a final washing medium and immediately used. The integrity of mitochondrial (93 ± 2.5%) was assessed by quantifying citrate synthase activity, in the absence and presence of detergent of. The protein concentration was measured by using the biuret method calibrated with BSA [486].

4.4.18.1 Mitochondrial membrane potential (ΔΨm)

The mitochondrial membrane potential (ΔΨm) was assessed using an ion-selective electrode to quantify the distribution of tetraphenylphosphonium (TPP^+) as previously described [485, 487] using an $Ag/AgCl_2$ electrode as the reference. The parameters evaluated were the membrane potential (-mV), lag phase (seconds), repolarization (-mV), respiratory rates, and ΔΨm.

4.4.18.2 Mitochondrial respiration (Oxygen consumption) and permeability transition (MPT)

Mitochondria oxygen consumption was polarographically monitored with a Clark oxygen electrode (Oxygraph, Hansatech Instruments Ltd., Cambridge, UK) as previously described [484]. Mitochondrial swelling was followed by changes in light scattering, as monitored spectrophotometrically at 540 nm, and the reaction conditions were performed as previously described [487]. All the experiments were performed in triplicate.

4.4.19 Gene expression by quantitative Real-Time PCR analysis

Total RNA samples from liver and colon tissue were extracted using a PureLink RNA Mini Kit (12183018A, Ambion, Thermo Fisher Scientific, Carlsbad, CA, USA) as well as a Trizol protocol (93289, Sigma Aldrich; St. Louis, MO, USA), following the manufacturer´s instructions and as previously described [487, 578].

4.4.20 Statistical analysis

The distribution of continuous variables was analyzed using the Kolmogorov–Smirnov test to assess significant deviations from normality. Differences between experimental groups were compared using the nonparametric test Kruskal–Wallis test (followed by the Dunn's test for multiple comparisons) for non-normally distributed data or the parametric test one-way ANOVA, followed by Bonferroni's test for multiple comparisons) for normally distributed data. Repeated measures ANOVA, followed by the Bonferroni post-hoc test, was used to compare glucose levels throughout the GTT and ITT assays.

The G*Power software (version 1.3.9.4) was used to calculate the statistical power concerning fasting insulin levels. p-values < 0.05 indicated statistical significance. Results are depicted as the mean ± standard error of the mean (SEM) and data analyses were performed using GraphPad Prism 6.0 software for Windows.

4.5 Results

4.5.1 Phenolic composition of BJ

The phenolic compounds were identified through HPLC-PDA-ESI/MS^n. Results are displayed in the Figure 4.2 and in supplementary data (Table S4.1). The classes of phenolic compounds detected were hydroxycinnamic acids, flavonoids, and anthocyanins [578]. The identification of phytochemicals was based on UV and MS^n spectral data, complemented with the available literature. Chlorogenic acid (5-CQA) was the most abundant compound (peak 5) in BJ. Other caffeic and ferulic acid derivatives were also identified. The flavonoids present in BJ were essentially quercetin and myricetin O-glycosides. The anthocyanins present in BJ showed UV spectra and fragmentation patterns characteristic of delphinidin, malvidin and petunidin derivatives.

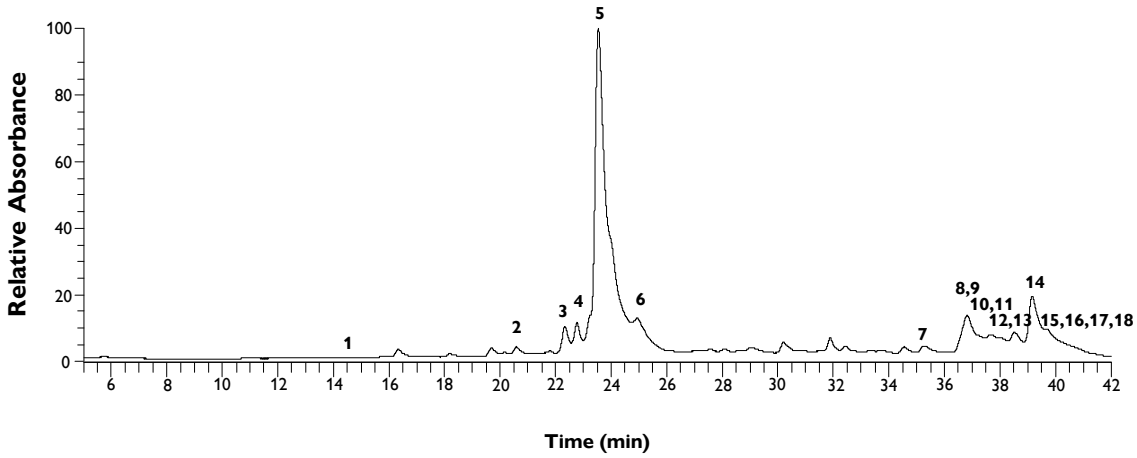


Figure 4.2 | Chromatographic profile of phenolic compounds in BJ, obtained with HPLC-PDA-ESI/MS^n (UV, 320nm).

4.5.2 Effects of BJ on BW and caloric intake

During the experimental protocol, the body weight (BW) of the animals was monitored weekly. Before feeding the HFD and BJ being provided, similar BW values were recorded among groups. Afterwards, the increase in BW over time was more pronounced in both groups fed with the HFD (HSuHF and HSuHF+BJ) than in the CTRL group. As shown in Table 4.1, the delta BW (final minus initial) was significantly higher in the HSuHF

group compared to the CTRL group (p<0.05). However, BJ supplementation had no significant effect on BW gain.

HSuHF showed a higher total caloric intake as compared to the CTRL group (p<0.0001). Prediabetic animals supplemented with BJ ingested an equivalent total amount of calories compared to the HSuHF group, which therefore supports the similar BW between these two experimental groups (Table 4.1). Significantly lower feed intake was observed in animals of HSuHF when compared to the CTRL group (p<0.0001). Meanwhile, HSuHF+BJ animals ingested more pellets of HFD diet than those fed with the HSuHF diet alone (p<0.001) (Table 4.1).

Table 4.1 | Body weight, cumulative total calories, food, drink, and nutrients intakes of the animals after 23 weeks of the experimental period.

Parameters	CTRL	HSuHF	HSuHF + BJ
Body weight (BW)			
Initial (g)	285.40 ± 19.80	287.30 ± 14.71	286.20 ± 16.68
Final (g)	510.00 ± 20.90	602.00 ± 32.89	589.40 ± 31.75
Delta BW (g)	224.60 ± 10.67	314.60 ± 23.62 *	303.20 ± 17.85 *
Cumulative intakes			
Total calories (Kcal/rat/week)	515.50 ± 5.55	715.20 ± 10.12 ****	742.00 ± 16.03 ****
Food (g/rat/week)	163.00 ± 1. 77	60.44 ± 1.63 ****	76.42 ± 1.94 ****, ###
Drink (mL/rat/week)	214.10 ± 4. 30	328.10 ± 7.51 ****	285.70 ± 12.98 ****, ###
Macronutrients cumulative intakes			
Carbohydrates (Kcal/rat/week)	350.20 ± 3.93	573.90 ± 14.01 ****	548.40 ± 21.73 ****
Lipids (Kcal/rat/week)	44.19 ± 0.47	98.73 ± 8.76	132.40 ± 12.35
Proteins (Kcal/rat/week)	121.10 ± 1.30	51.92 ± 1.61 ****	65.84 ± 1.90 ****, ####

Results are expressed as mean ± SEM. (n = 8 – 10/group); * p<0.05 and **** p<0.0001 vs CTRL; ### p<0.001 and #### p<0.0001 vs HSuHF.

Regarding cumulative drink intake, a higher liquid ingestion was found in the HSuHF group compared to CTRL group (p<0.0001) and, on the other hand, HSuHF+BJ decreased beverage intake, when compared to the HSuHF animals (p<0.001). Concerning the macronutrient composition, the HSuHF rats displayed a significantly higher intake of carbohydrates (p<0.0001) and lipids along with a lower protein (p<0.0001) intake when compared to the CTRL group. Owing to increased high-fat ingestion, the HSuHF+BJ groups consumed significantly more proteins (p<0.001) and

lipids, together with a slight reduction in carbohydrates contents (reflected by the lower BJ ingestion) when compared to the HSuHF group (Table 4.1).

4.5.3 Effects of BJ on glycemic and insulinemic profile

Fasting and postprandial blood glucose concentrations were significantly higher in the HSuHF group when compared to the CTRL rats ($p < 0.001$ and $p < 0.01$, respectively). However, although there was no statistical difference on fasting glycemia, BJ supplementation had a positive effect on fed glucose levels, being significantly lowered ($p < 0.05$) when compared to the HSuHF group (Figure 4.3A). Regarding serum insulin, there was a trend to increased fasting levels in the HSuHF group, although it did not reach statistical significance (Figure 4.3B), eventually due to the small sample size used for this parameter (statistical power value of 0.644). Still, in the fed state, an increase in insulin values was observed in the HSuHF group when compared to the CTRL rats ($p < 0.05$), an effect that was ameliorated in the HSuHF+BJ group. To further determine the impact of BJ supplementation on glucose tolerance and insulin sensitivity, we next performed the glucose and insulin tolerance tests.

As expected, HSuHF displayed glucose intolerance and insulin resistance as revealed by the higher glucose concentrations throughout the 2-hour of GTT and ITT (Figure 4.3C and 4.3D, respectively) and further supported by both the significantly higher area under the curve (AUC) of GTT ($p < 0.001$ vs CTRL group) (Figure 4.3E) and lower glucose disappearance rate (K_{ITT}), a measure of insulin sensitivity ($p < 0.01$ vs CTRL group) (Figure 4.3F). Otherwise, no statistical changes were found in the fasting glucose and insulin levels between the two experimental groups. However, in the HSuHF+BJ -treated rats, glucose intolerance and insulin sensitivity were alleviated when compared to the HSuHF group, as reflected by both the lower AUC of GTT ($p < 0.05$) and significantly higher K_{ITT} values ($p < 0.001$) (Figure 4.3C-F). Collectively, these findings indicate that BJ supplementation had significant protection against glucose intolerance and insulin resistance in the prediabetic rats.

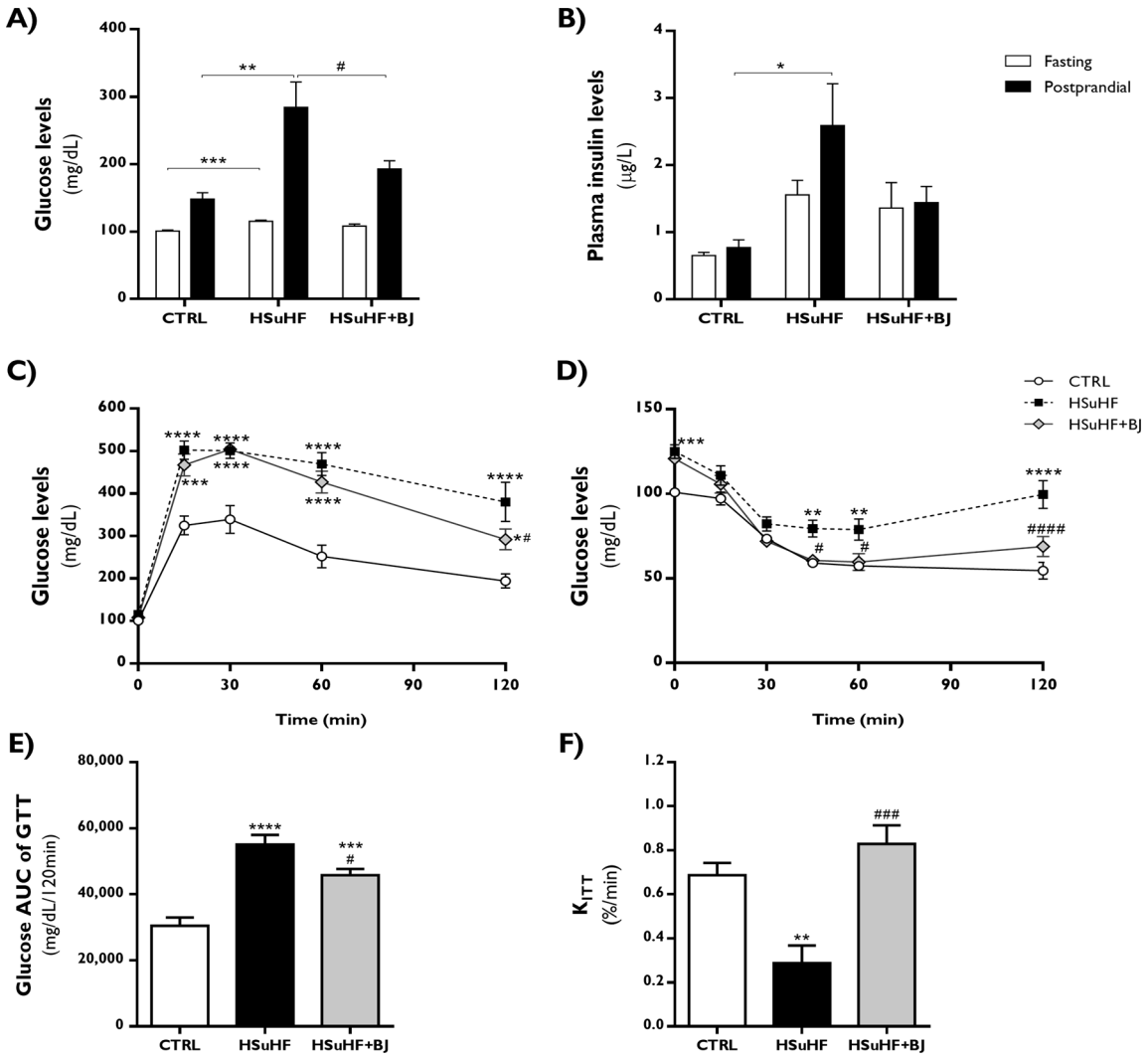


Figure 4.3 | Effects of BJ supplementation on the glycemic and insulinemic profile in HSuHF-fed rats. Fasting and postprandial glucose levels (A). Fasting and postprandial insulin levels (B). Blood glucose levels throughout the glucose tolerance test (GTT) (C); Blood glucose levels throughout the insulin tolerance test (ITT) performed through intraperitoneal injection of insulin (0.75 U / kg body weight) (D); area under the curve (AUC) of the blood glucose level during GTT, calculated between baseline and 120 min after glucose bolus (2 mg/kg) (E); K_{ITT} representing the glucose clearance rate (% min) (F) in CTRL, HSuHF and HSuHF+BJ groups. Results are expressed as mean ± SEM. (n = 8 – 10/group; in fasting insulin levels: n=3 – 4/group were used); * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$ vs CTRL; # $p<0.05$, ### $p<0.001$ and #### $p<0.0001$ vs HSuHF.

4.5.4 Effects of BJ on the serum lipid profile and redox status markers

Regarding serum TGs, significantly higher concentrations were found in the prediabetic group when compared to the CTRL group ($p<0.05$). The HSuHF-induced hypertriglyceridemia was reverted with BJ supplementation for 14 weeks, with significantly lower postprandial TGs values *versus* those found in HSuHF-fed animals ($p<0.01$) and were similar to those observed in the CTRL group (Figure 4.4A).

Serum TAS and MDA levels were used to assess the total antioxidant capacity and lipid peroxidation. We determined the serum MDA/TAS ratio as a redox status marker. The HSuHF group presented significantly higher values of the serum MDA/TAS ratio ($p<0.05$ vs CTRL group). This effect was restored by BJ supplementation in the HSuHF+BJ-treated group, which showed a significantly lower MDA/TAS ratio ($p<0.01$) compared to the HSuHF-fed animals, suggesting a restored antioxidant profile (Figure 4.4B).

Moreover, we further performed a nontargeted proton nuclear magnetic resonance spectroscopy (^{1}H NMR) - based metabolomic approach in serum samples to identify possible endogenous metabolite alterations and to define putative molecular mechanisms underpinning BJ protection. The identified serum metabolites as well as their chemical shifts are listed in the supplementary data (Table S4.2). To identify the key metabolite changes between the groups, a partial least squares-discriminate analysis (PLS-DA) was performed. Accordingly with the multivariate data analysis, the most pronounced findings were related to betaine and ketone bodies levels. There was a significant reduction in betaine levels in serum samples from HSuHF-fed animals compared to the CTRL group ($p<0.01$), which was slightly recovered in serum from HSuHF+BJ-treated animals ($p<0.05$ vs HSuHF group). The levels of 3-hydroxybutyrate (3-HB) and the 3-HB/acetoacetate ratio were significantly lowered in the serum of the HSuHF-fed group compared to the CTRL group. Conversely, a remarkable recovery in the contents of these metabolites was found in the serum of HSuHF+BJ-treated animals when compared to the prediabetic group ($p<0.05$ vs HSuHF group), reaching similar values from those found in the control group (Figure 4.4D and 4.4E). Moreover, the relative concentrations of valine, histidine, lactate and N-acetylglycoproteins were significantly lower in the HSuHF group, when

compared with control animals, however BJ supplementation had no significant impact on their contents in serum, as can be seen in the supplementary data (Table S4.2).

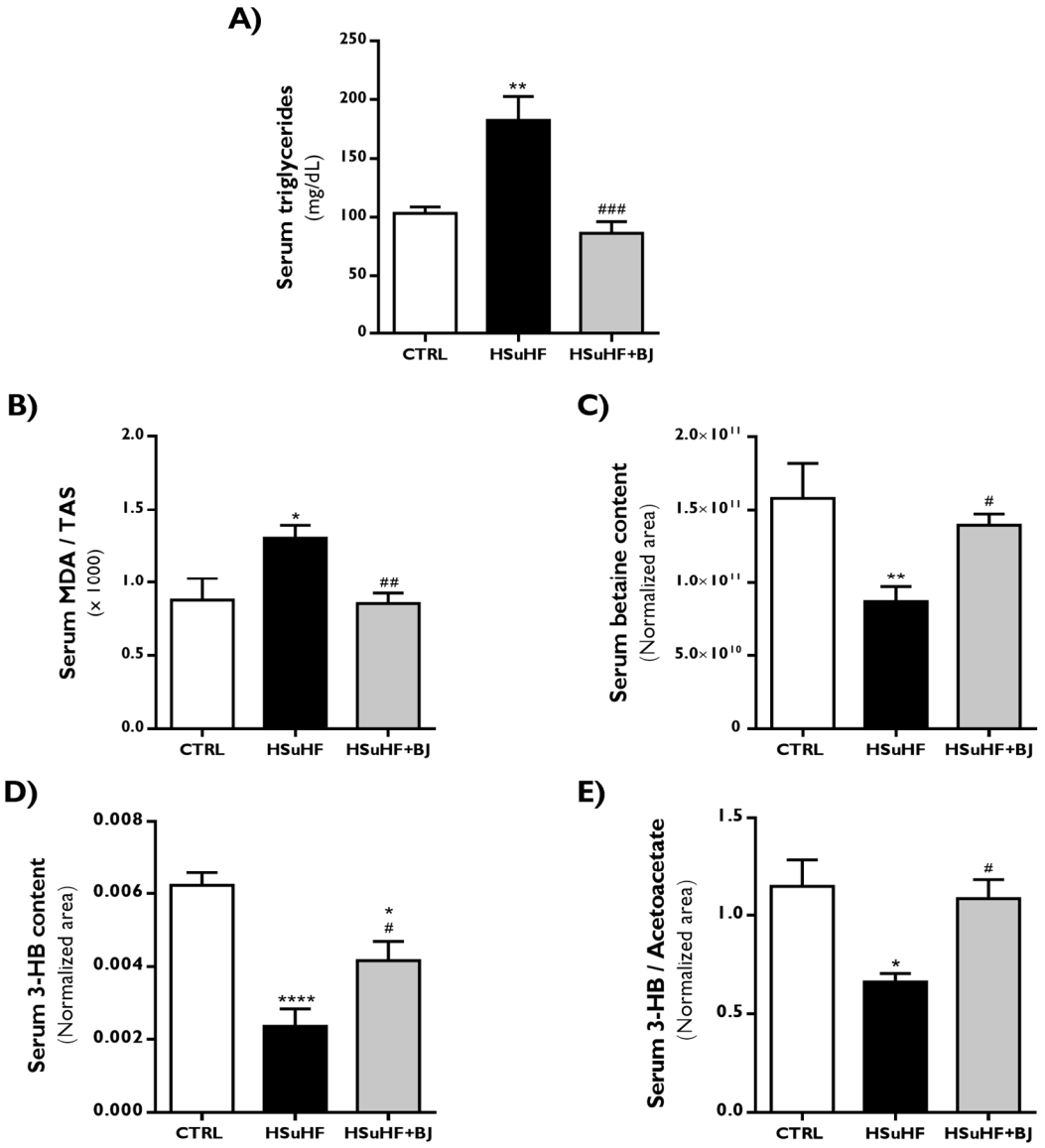


Figure 4.4 | Effects of BJ on serum lipidic profile, antioxidant and metabolomic profile. Serum triglycerides contents (A) and on serum redox marker (B). Serum contents of betaine (C). Serum levels of 3-hydroxybutyrate (3-HB) (D) and 3-HB/acetoacetate ratio (E) in CTRL, HSuHF and HSuHF+BJ groups. Results are expressed as mean ± SEM. (n = 8 – 10/group); * $p<0.05$, ** $p<0.01$ and **** $p<0.0001$ vs CTRL; # $p<0.05$, ## $p<0.01$ and ### $p<0.001$ vs HSuHF.

4.5.5 Effects of BJ on gut microbiota composition and intestinal integrity

We assessed the composition of the bacterial community by real-time PCR and the SCFAs and organic acids by HPLC in fecal samples that were collected at the end of the experimental protocol. As shown in Table 4.2, the GM composition of HSuHF-fed rats was modestly altered compared to the control´s GM. The hypercaloric diet-fed animals presented a trend of a decreased relative abundance of Firmicutes and Bacteroidetes phylum independently of the supplementation with BJ, albeit no significant differences were found when compared to the CTRL group. Moreover, a trend was observed towards an increase in the Firmicutes/Bacteroidetes ratio and *Enterococcus* in HSuHF-fed rats but without statistical significance when compared to the control animals ($p>0.05$).

The abundance of *Akkermansia muciniphila* was significantly lower in HSuHF-fed animals ($p<0.05$) compared to the control animals. Nonetheless, in spite of the BJ addition showed a trend to counteract the loss of the proportions of *Akkermansia muciniphila* induced by hypercaloric feeding, a modest antimicrobial effect of BJ was encountered in the fecal GM composition from BJ-treated animals. Interestingly, the GM of the HSuHF+BJ group was less abundant in *Bifidobacterium* and *Prevotella* when compared to the GM of untreated prediabetic animals (Table 4.2).

Table 4.2 | Gut bacterial microbiota groups and SCFAs contents in feces of CTRL, HSuHF and HSu+BJ rats.

Parameters	CTRL	HSuHF	HSuHF + BJ
Bacteria groups (Log10 copies /ng of DNA)			
Universal	6.188 ± 0.317	5.941 ± 0.178	5.140 ± 0.190 *, #
Firmicutes	7.316 ± 0.175	7.043 ± 0.123	6.785 ± 0.201
Bacteroidetes	4.641 ± 0.183	4.167 ± 0.262	4.142 ± 0.185
Firmicutes/Bacteroidetes	1.605 ± 0.042	1.778 ± 0.086	1.643 ± 0.033
Bifidobacterium	1.706 ± 0.119	2.53 ± 0.02	1.280 ± 0.156 ##
Prevotella	2.736 ± 0.286	2.817 ± 0.286	1.820 ± 0.225 #
Lactobacillus	4.087 ± 0.795	4.507 ± 0.350	4.317 ± 0.169
Akkermansia	4.823 ± 0.271	3.561 ± 0.373 *	3.859 ± 0.141
Clostridium leptum	4.817 ± 0.176	4.714 ± 0.195	4.604 ± 0.138
Roseburia	3.628 ± 0.277	3.829 ± 0.306	3.901 ± 0.322
Enterococcus	2.620 ± 0.494	3.096 ± 0. 240	2.496 ± 0.230
SCFAs (mg/g feces)			
Acetic acid	1.24 ± 0.11	0.97 ± 0.11	0.68 ± 0.07 **
Butyric acid	0.07 ± 0.01	0.02 ± 0.01 **	0.02 ± 0.00 **
Propionic acid	0.03 ± 0.00	0.01 ± 0.00 *	0.01 ± 0.00 *
Succinic acid	7.01 ± 0.79	8.86 ± 1.33	4.82 ± 0.76 #

Results are expressed as mean ± SEM. (n = 8 – 10/group); * $p<0.05$ and ** $p<0.01$ vs CTRL; # $p<0.05$ and ## $p<0.01$ vs HSuHF.

Despite these subtle changes observed in the bacterial community from HSuHF groups, the SCFAs profile revealed a lowered fecal content of butyric and propionic acids when compared to the CTRL group ($p<0.01$ and $p<0.05$, respectively). Moreover, the BJ supplementation was unable to alter the fecal SCFAs contents compared to the HSuHF-untreated rats (Table 4.2); however, the addition of BJ resulted in a significant decrease in the fecal contents of succinic acid when compared to the GM of the untreated HSuHF group ($p<0.05$), likely due to the loss of the succinate-producing *Prevotella* ssp.

The junctional complexes, namely tight junctions, adherens junctions, and desmosomes were visualized in the duodenum and colon sections through TEM and representative images of the ultrastructure of the duodenum and colon were showed in Figure 4.5A. Intact junctional complexes were found in all epithelial cells observed in each

experimental group. The data of the epithelial barrier integrity were further supported by the data of immunofluorescence staining of the tight junction proteins ZO-1 and occludin.

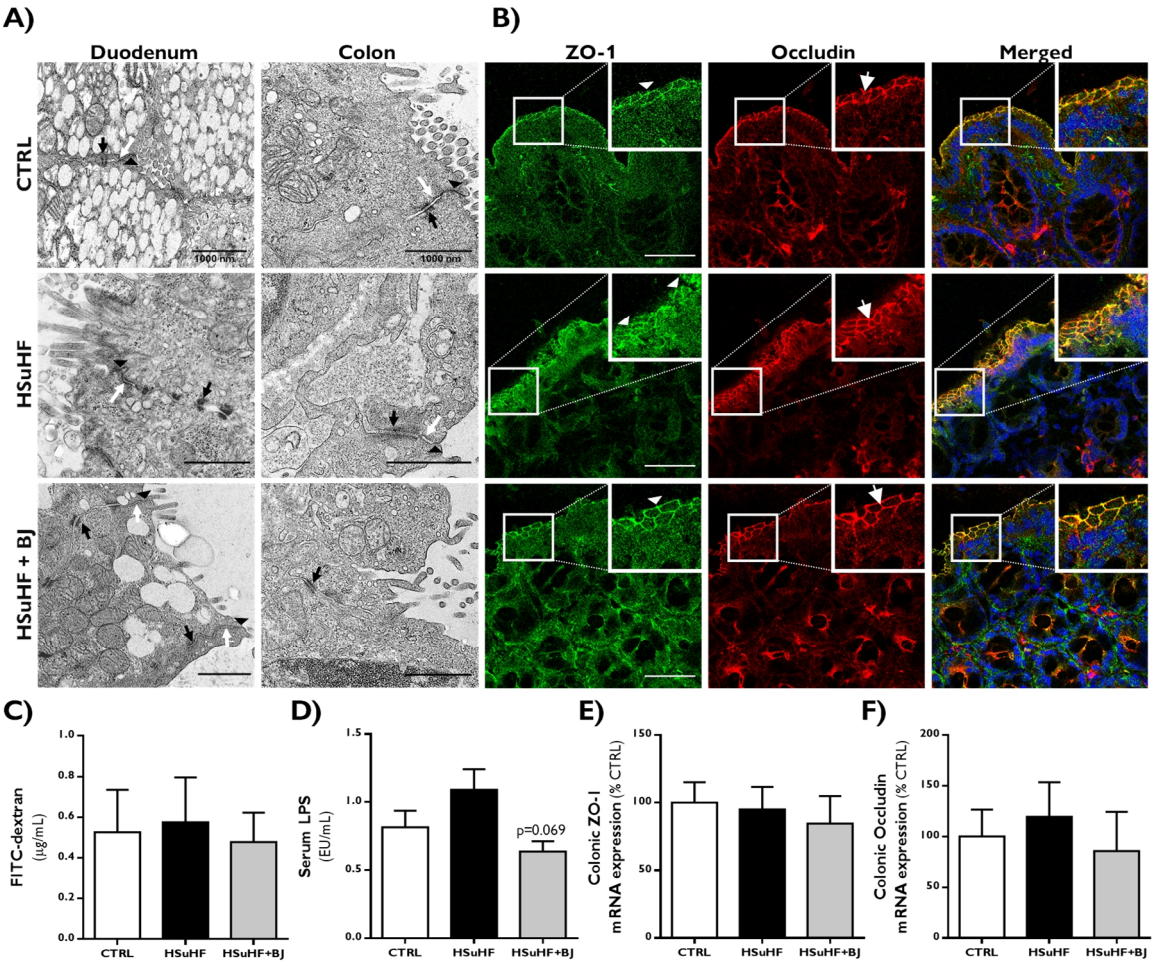


Figure 4.5 | Impact of BJ on gut barrier structure and intestinal permeability. Representative TEM images of ultrastructural analysis of tight junctions (black arrowhead), adherens junctions (white arrow) and desmosomes (black arrow) in duodenum and colon sections (A) (scale bar: 1000 nm). Representative confocal images of immunohistochemistry staining of ZO-1 (white arrowhead) and occludin (white arrow) in colon sections (B) (scale bar: 50 µm). Serum FITC-dextran concentrations (C); serum LPS levels (D); colonic mRNA expression of ZO-1 (E) and occluding (F) in CTRL, HSuHF and HSuHF+BJ groups. Results are expressed as mean ± SEM. (n = 8 – 10/group); p=0.069 vs HSuHF group.

At the epithelial cell's periphery, an identical distribution of ZO-1 and occludin was found between the groups (Figure 4.5B), which was also validated by the unchanged mRNA expression of colonic tight junctions, ZO-1 and occludin between the control and the experimental groups (Figure 4.5E and 4.5F, respectively). We also evaluated the intestinal permeability by measuring the paracellular passage of the 4 kDa-dextran labeled with FITC to serum and by quantifying the serum LPS contents, which is a membrane

component found in Gram-negative bacteria. There were no changes in the serum FITC-dextran concentrations 4 h after oral FITC dextran administration between the groups, suggesting the absence of intestinal paracellular permeability changes induced by the hypercaloric diet and by BJ (Figure 4.5C). These results were further corroborated by the unchanged serum LPS levels; despite a trend to increased serum LPS contents in the HSuHF group *versus* the CTRL, the differences found did not reach statistical significance. However, a trend of decreased LPS levels in animals supplemented with BJ was observed ($p=0.069$ vs HSuHF group) (Figure 4.5D).

4.5.6 Effects of BJ on hepatic lipid steatosis

The relative liver weight (normalized to BW) was unchanged between HSuHF and CTRL (28.84 mg / Kg ± 1.31 and 25.75 mg / Kg ± 0.57, respectively); however, in the HSuHF+BJ-treated rats, there was a reduced liver / BW value (24.24 mg / Kg ± 0.72) when compared to the prediabetic animals ($p<0.01$). Ultrasound imaging, macroscopic appearance, ultrastructure analysis and histological examination by H&E staining and Oil red O staining were performed to analyze hepatic changes between experimental groups. As shown in Figure 4.6A, through ultrasound imaging, we found that the HSuHF groups displayed signs of focal steatosis, which was almost absent in the HSuHF+BJ-fed animals. Moreover, during dissection and tissue collection, macroscopically changes were notorious in the liver of the HSuHF group, namely related to the color, which became slightly yellow along with abnormal enlargement and distinct texture when compared with CTRL and HSuHF+BJ animals´ livers (Figure 4.6B). This latter was also validated through the SEM technique, where ultrastructural changes were found in the livers of HSuHF animals (Figure 4.6C). As expected, while CTRL animals had normal livers, the HSuHF group showed hepatic fat vacuoles with inflammatory cell infiltration and enhanced lipid accumulation, as observed in H&E and Oil red O-stained slides. BJ supplementation improved the macroscopical appearance and markedly reduced the ballooning and lipid deposition in the hepatocytes of the HSuHF+BJ group (Figure 4.6D and 4.6E). Furthermore, these results were also supported by the assessment of hepatic TGs contents. In agreement with serum samples, we observed that the hypercaloric diet increased liver TGs levels ($p<0.0001$ in HSuHF-fed animals *vs* CTRL groups), while BJ supplementation resulted in significantly lowered hepatic TGs values ($p<0.001$) when

compared to the HSuHF group, despite remaining higher than the CTRL group ($p<0.05$) (Figure 4.6F). Nevertheless, these findings demonstrated that BJ has relieved lipid accumulation in hypercaloric diet- induced prediabetic rats.

Multivariate analysis was performed on ^{1}H NMR data for hepatic metabolic profile comparison between CTRL and experimental groups. Metabolomic analysis of the liver samples demonstrated a reduced hepatic glutathione level in HSuHF rats compared to the CTRL groups. Although missing statistical significance, there was a clear trend of increased hepatic GSH levels in the HSuHF+BJ rats ($p=0.07$ vs HSuHF-fed rats) (Figure 4.6G). In addition, the relative levels of betaine were significantly decreased in the liver of prediabetic animals, which were totally recovered in HSuHF+BJ-treated rats (Figure 4.6H).

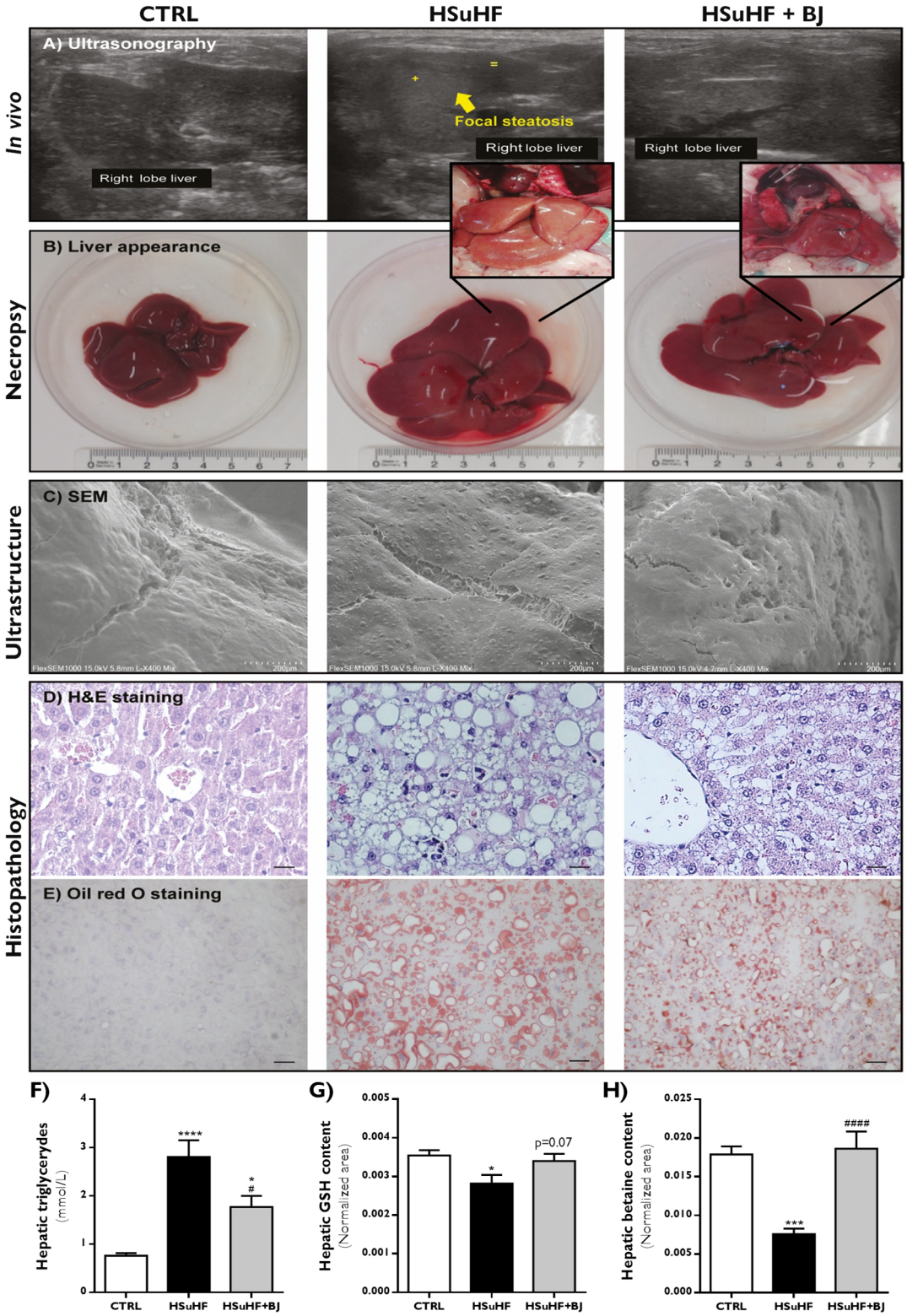


Figure 4.6 | Impact of BJ on liver appearance and hepatic findings. Representative photographs of *in vivo* ultrasonography (A), macroscopic liver appearance during dissection and tissue collection (B), SEM images of rat liver tissue (C), and Hematoxylin–eosin (H&E) (D) and Oil Red O staining (E) of liver sections from representative rats from each group (scale bar: 20µm); hepatic triglycerides contents (F); hepatic GSH (G) and betaine contents (H) per group. Results are expressed as the mean ± SEM. * $p<0.05$, *** $p<0.001$ and **** $p<0.0001$ vs CTRL group; # $p<0.05$, #### $p<0.0001$ and $p=0.07$ vs HSuHF group.

4.5.7 Impact of BJ on hepatic mitochondrial function

To elucidate whether BJ consumption induced protective effects on hepatic mitochondrial function, we assessed several bioenergetic parameters in isolated hepatic mitochondria. Oxidative phosphorylation relies on the generation of a transmembrane electrochemical potential (ΔΨm, in the form of a proton gradient), associated with molecular oxygen consumption. Compared to the CTRL group, the hepatic mitochondria from HSuHF groups showed a significant reduction in mitochondrial initial ΔΨm (after substrate addition) and ΔΨ after repolarization (mitochondrial capacity to establish ΔΨm after ADP phosphorylation). These parameters were clearly reverted by BJ supplementation in the HSuHF+BJ-treated rats. In addition, a significant increase in the lag phase time (which represents the time required for ADP phosphorylation) was noticed in hepatic mitochondria from HSuHF-fed animals compared to the CTRL groups ($p<0.05$), suggesting a compromised hepatic mitochondrial function in HSuHF-fed animals. In contrast, mitochondria from HSuHF+BJ-treated animals showed a shorter lag phase, which indicated faster phosphorylation activity (Figure 4.7A and 4.7B).

The putative alterations in the respiratory chains and oxidative phosphorylative system of mitochondria isolated from the CTRL and both experimental groups were also determined by following the oxygen consumption in the presence of succinate. Mitochondria isolated from HSuHF-fed rats displayed a significant decline in respiratory state 3 (ADP-stimulated respiration) and FCCP-uncoupled respiratory rates together with an increase in respiratory state 4 (in the ADP absence, corresponding to the resting state) ($p<0.0001$), when compared to mitochondria from the control group (Figure 4.7C). Consequently, the respiratory control ratio (RCR) was significantly lowered ($p<0.0001$) in mitochondria isolated from the livers of HSuHF-fed animals (Figure 4.7D). Additionally, the oxidative phosphorylation efficiency estimated by the ADP/O ratio was significantly decreased in mitochondria from HSuHF-fed rats as compared to control animals ($p<0.01$) (Figure 4.7D), reflecting an uncoupling between respiration and ADP phosphorylation. BJ supplementation effectively reversed the effects of the HSuHF diet on mitochondrial respiration parameters. When compared to rats fed simply with a hypercaloric diet, the results showed that mitochondria from HSuHF+BJ-treated rats had a significant rise in respiratory state 3, FCCP-uncoupled respiration, RCR, and the ADP/O ratio, as well as reduced state 4 respiration (Figure 4.7C and 4.7D).

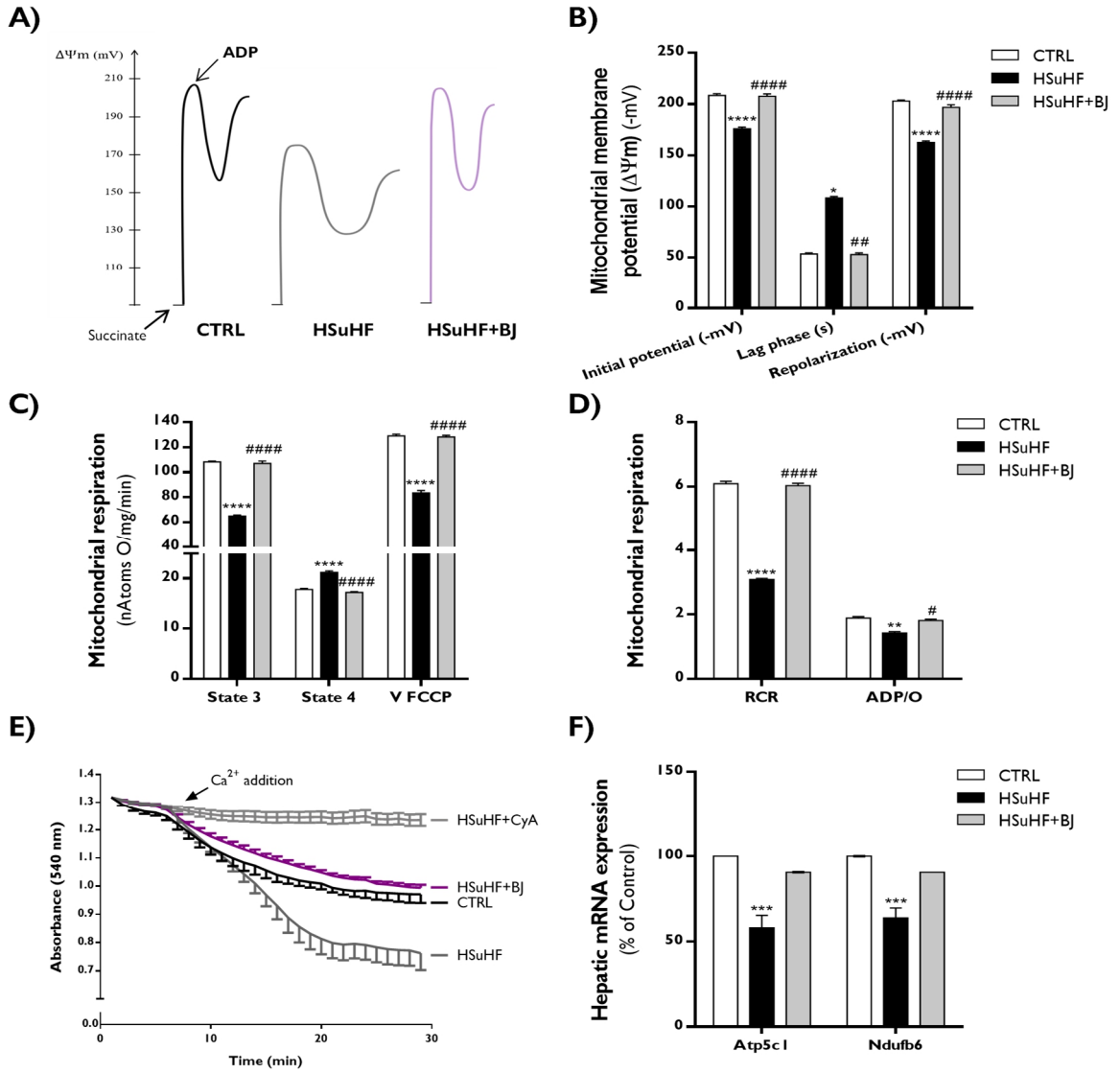


Figure 4.7 | Effects of BJ on hepatic mitochondrial function parameters. Mitochondrial membrane potentials and lag phase (A and B), mitochondrial respiration parameters (C and D), and susceptibility to the induction of mitochondrial permeability transition (MPT) (E); hepatic mRNA expression of genes involved in the mitochondrial electron transport chain (F). Results are expressed as the mean ± SEM. (n = 6–8/group). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$ vs CTRL group; # $p<0.05$, ## $p<0.01$ and #### $p<0.0001$ vs HSuHF group.

Figure 4.7E illustrates the induction of mitochondrial swelling as a marker for permeability transition pore induction. The isolated hepatic mitochondria from HSuHF in the presence of 20 nmol Ca^{2+} and 5 mM succinate underwent a pronounced decrease in light scattering, reflecting the ability to further induce the opening of the mitochondrial membrane permeability transition pore. The hypercaloric diet-induced mitochondrial swelling was completely prevented by BJ supplementation, as detected by an increase in

light scattering. Furthermore, hypercaloric diet-induced swelling was sensitive to cyclosporine A (Figure 4.7E), reflecting Ca^{2+}-dependent MPT induction.

Collectively, these findings prompted us to evaluate at the transcriptional level of genes regulating the mitochondrial energetics in the liver. We, therefore, analyzed the expression of genes encoding for subunits of the mitochondrial respiratory chain complexes by RT-PCR. The expression of *Ndufb6* and *ATp5c1*, which encode proteins of complex I (subunit NADH:ubiquinone oxidoreductase) and complex V (mitochondrial ATP synthase), respectively, was significantly downregulated ($p<0.001$) in the liver tissue from HSuHF-fed rats when compared to the CTRL animals. As shown in Figure 4.7F, even though it failed to reach statistical significance, in HSuHF+BJ-treated rats, there was a trend of increased expression of both mitochondrial phosphorylation-related genes, compared to the HSuHF-untreated group.

4.5.8 Effects of BJ on hepatic mRNA expression of inflammation-related genes

We assessed the impact of BJ supplementation on the hepatic mRNA expression of genes related to the inflammatory response. The mRNA expression of adiponectin receptors (*Adipor1* and *Adipor2*) and the anti-inflammatory gene interleukin-10 (IL-10) was significantly repressed in the liver of HSuHF-fed rats ($p<0.05$ and $p<0.01$) when compared to the CTRL group. Surprisingly, despite a trend to recover the mRNA expression of these genes, no statistical changes were detected in the rats of the HSuHF+BJ group when compared to the HSuHF ones. Similarly, the mRNA expression of *Cebpb*, which is an important transcription factor that regulates the expression of genes involved in the immune and inflammatory response, was not altered in HSuHF-fed rats ($p>0.05$ vs CTRL group); however, this tended to decrease in the liver of HSuHF+BJ treated rats. Furthermore, there was a slight but not significant increase in the mRNA levels of plasminogen activator inhibitor 1 precursor (*Serpine1*) and tumor necrosis factor-α (*Tnf-α*) in the HSuHF rats compared to the animals of the CTRL and HSuHF+BJ groups, despite a trend to be restored upon BJ treatment. The expression of the tumor necrosis factor receptor superfamily member 6 precursor Tnfrsf6 (*Fas*), *Ifn-γ*, interleukin-1 beta (*Il-1β*), *Nf-kB*, and *Stat3* mRNA was unaffected by either the hypercaloric diet or BJ treatment (Figure 4.8).

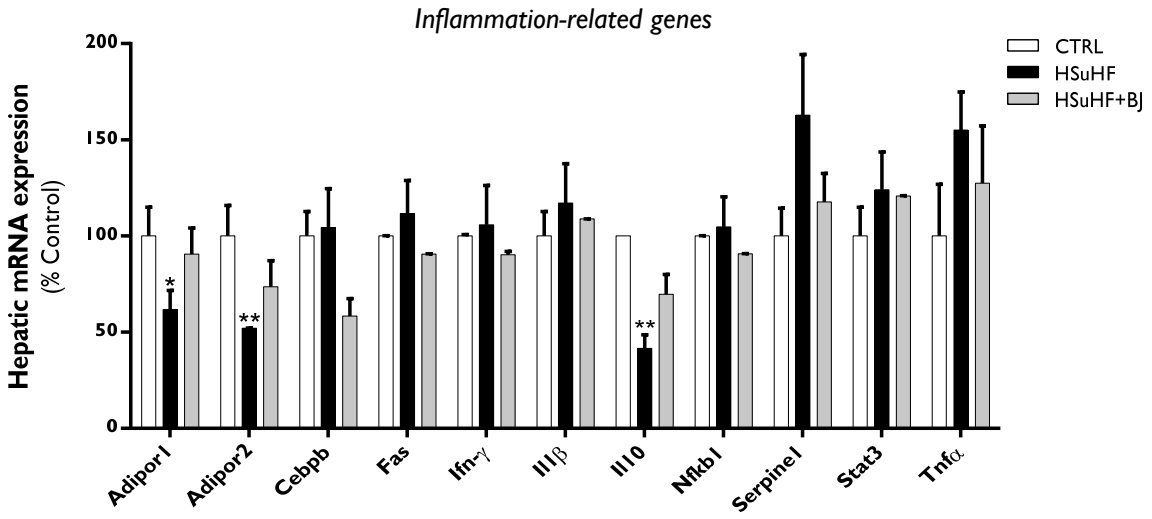


Figure 4.8 | Hepatic mRNA expression of genes related to the inflammatory response of the CTRL, HSuHF and HSuHF+BJ groups. Data are presented as mean ± SEM. (n = 6 – 8/group). * p<0.05 and ** p<0.01 vs CTRL group.

4.5.9 Effects of BJ on hepatic mRNA expression of glucose metabolism and insulin signaling-related genes

As glucose homeostasis and insulin sensitivity were compromised in prediabetic animals and improved upon BJ supplementation, the relative mRNA expression of genes involved in glucose metabolism as well as in the insulin-signaling pathway was determined (Figure 4.9). Concerning genes encoding rate-limiting glycolytic enzymes, the mRNA levels of glucokinase (*Gck*) and pyruvate kinase L/R (*Pklr*) were not significantly different between the three groups. In addition, no significant differences in the mRNA expression of the rate-limiting gluconeogenic enzyme, the mitochondrial isoform of phosphoenolpyruvate carboxylase 2 (*Pck2*) as well as in the mRNA of *Mlxipl* (gene encoding carbohydrate response element-binding protein, ChREBP), were identified in the liver of the experimental groups. Among the genes required for glucose uptake, the expression of *Slc2a2* (gene encoding GLUT2) was remarkably decreased in HSuHF-fed rats compared to the CTRL group ($p<0.001$). There was a slight increase of the *Slc2a2* mRNA expression in the livers of HSuHF+BJ-treated animals compared to the HSuHF-fed rats, although this was still lowered when compared to the CTRL group. Conversely, a slight increase in hepatic mRNA expression of *Slc2a1* (encoding glucose transporter GLUT1) was found in HSuHF-fed rats (p=0.066 vs CTRL group); however, it was unaltered upon BJ intake. Despite a trend of decreased the mRNA expression of *Slc2a4 (*gene encoding GLUT4)

when animals were fed with the hypercaloric diet, statistical significance was not detected between the experimental groups (Figure 4.9).

The expression of genes involved in insulin signaling, including *Insr* (insulin receptor), *IRS-1* (insulin receptor substrate-1) and *Pik3ca* (catalytic subunit alpha isoform of phosphoinositide-3-kinase - PI3K), was notably declined in prediabetic animals ($p<0.01$ and $p<0.001$ vs CTRL group, respectively). BJ provided to the HSuHF-fed animals partly reversed this reduction when compared to the untreated prediabetic animals. A similar tendency was found in the mRNA expression of *Akt1* (protein kinase B - isoform 1) and *Prkaa1* (protein kinase AMP-activated catalytic subunit alpha 1); however, no statistical differences were detected between the experimental groups. The mRNA expression of *Pik3r1* (gene encoding the p85 alpha regulatory subunit of PI3K) was significantly enhanced in HSuHF-fed animals ($p<0.05$ vs CTRL group), which was not restored upon BJ treatment (Figure 4.9).

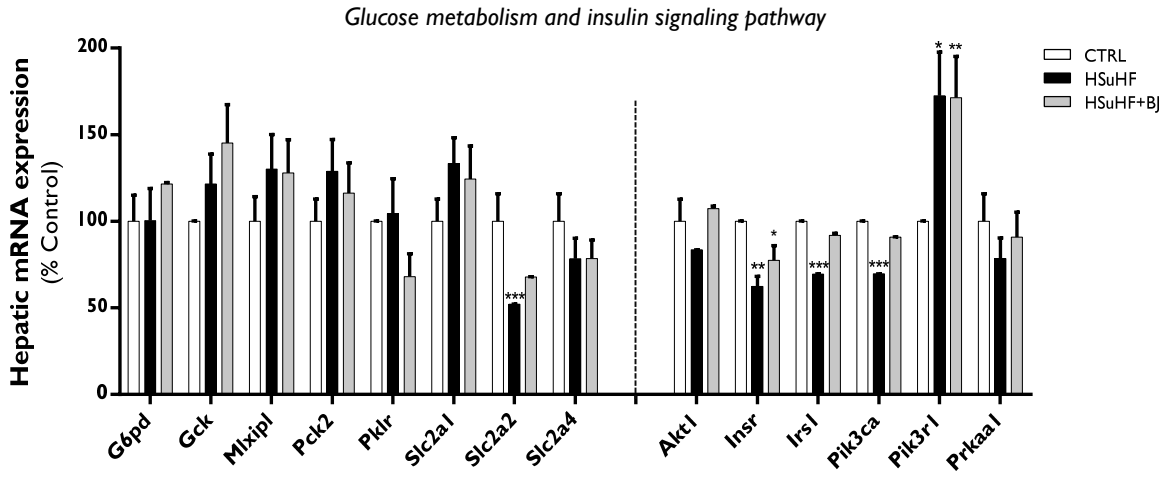


Figure 4.9 | Hepatic mRNA expression of genes related with glucose metabolism and the insulin signaling pathway of CTRL, HSuHF and HSuHF+BJ groups. Data are presented as mean ± SEM (n = 6 – 8/group). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs CTRL group.

4.5.10 Effects of BJ on hepatic mRNA expression of lipid metabolism-associated genes

To explore the molecular basis for the lipid-lowering effect hepatoprotection of BJ, the mRNA expression levels of several lipid metabolism-related genes, including those involved in hepatic fatty acids uptake, synthesis, and β-oxidation, were determined by RT-PCR in the liver tissue (Figure 4.10). The mRNA expression of fatty acid translocase *Cd36*, which mediates fatty acid uptake across the cell membrane, was significantly enhanced in the HSuHF-fed rats ($p<0.05$ HSuHF vs CTRL group), whereas the mRNA expression of hepatic fatty acid-binding protein 1 (*Fabp1*), and fatty acid transport protein 5 (*Slc27a5*) was markedly lowered in HSuHF-fed rats ($p<0.001$ vs CTRL). There were no statistical differences in the hepatic mRNA expression of *Cd36*, *FATP5*, and *Slc27a5* in the HSuHF+BJ-treated group when compared with the HSuHF-fed one, despite a trend of suppression of these hypercaloric diet-induced mRNA changes.

Regarding the genes involved in the fatty acid synthesis, the mRNA expression of *Acly*, which encodes ATP-citrate synthase that catalyzes the synthesis of cytosolic acetyl-CoA from citrate, was significantly decreased in HSuHF-fed rats compared to the CTRL ones ($p<0.01$) and remained lower when animals were treated with BJ. In contrast, in the HSuHF group, there was a trend to increased mRNA expression of *Scd1*, which encodes the stearoyl-coenzyme A desaturase 1, which catalyzes a rate-limiting step in the biosynthesis of monounsaturated fats. This increase tended to be abolished when HSuHF-fed animals were supplemented with BJ since the mRNA levels reached similar values to those of the CTRL group; however, no statistical significance was achieved when compared to the prediabetic group.

The expression of other genes involved in lipogenesis (*Acaca*, *Acsl5* and *Dgat2*) remained unchanged either by feeding with the hypercaloric diet or BJ supplementation. The mRNA expression of fatty acid synthase (*Fasn*) was significantly decreased in HSuHF+BJ-treated rats compared to the CTRL group ($p<0.05$), with no significant changes in HSuHF rats. Regarding lipogenic transcription regulator, the expression of *SREBF1* (encoding sterol regulatory element-binding protein-1c, SREBP-1c), remained unaltered in the hepatic tissue of animals from the HSuHF and HFuHF+BJ groups (Figure 4.10).

Compared with CTRL group, HSuHF-fed animals showed a significant decrease in the hepatic expression of fatty acid oxidation-related genes, including long-chain acyl

coenzyme A dehydrogenase (*Acadl*), acyl-CoA oxidase (*Acox-1*), carnitine palmitoyltransferase 1 (*Cpt1*) and carnitine palmitoyltransferase 2 (*Cpt2*) ($p<0.05$ vs CTRL group). Conversely, despite not reaching statistical differences, the HSuHF+BJ-treated rats showed a trend to normalize the mRNA expression of these key enzymes when compared to the HSuHF-fed rats. Surprisingly, the mRNA expression of *PPAR-α* (peroxisome proliferator-activated receptor-alpha), a nuclear receptor regulator of the fatty acid oxidation pathway in the liver, was unchanged between the experimental groups (Figure 4.10).

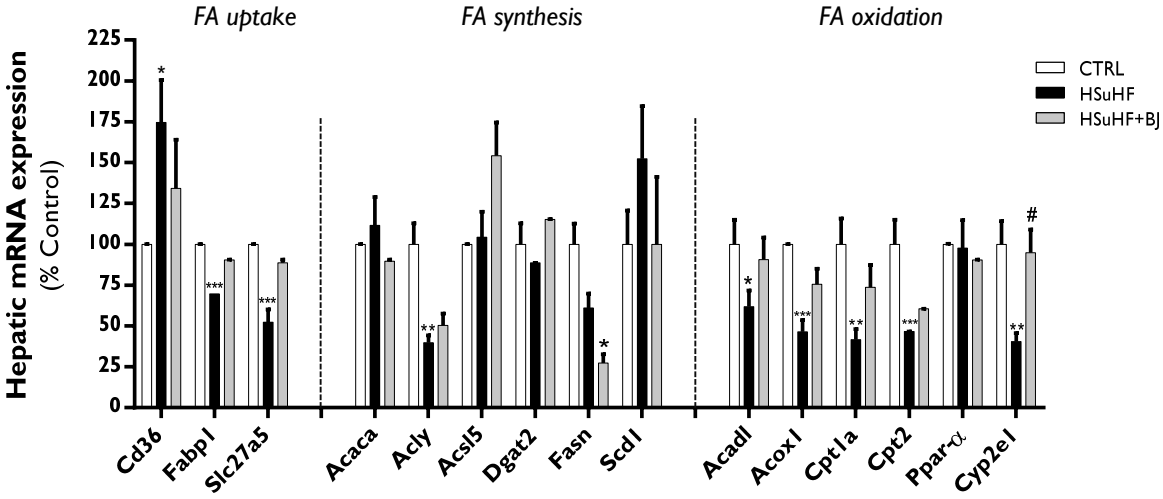


Figure 4.10 | Hepatic mRNA expression of genes related with lipid metabolism of the CTRL, HSuHF and HSuHF+BJ groups. Data are presented as mean ± SEM. (n = 6–8/group). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs CTRL group; # $p<0.05$ vs HSuHF group.

4.6 Discussion

In the present study, we dissected the putative mechanisms underpinning BJ protection in a prediabetic rat model, focusing on the gut microbiota composition and intestinal barrier integrity as well as on hepatic structural and functional data, with a particular emphasis on mitochondrial bioenergetics.

As expected, the hypercaloric diet induced an increase in BW gain in the prediabetic rats, as a result of a higher intake of calories, especially carbohydrates and lipids, when compared with the control animals. These changes were not prevented in the HSuHF rats under BJ supplementation, which presented unchanged BW and calorie consumption. Previous studies showed contradictory data regarding the impact of different sources of BB on BW gain in animal models, with some of them reporting reductions [396, 498, 585] and others no significant effect [329, 397, 457]. In our study, the use of a liquid form (juice) of BB consumption may explain the compensatory increase of food intake and the lack of an impact on animals' BW. In fact, it has been shown that fluid calories have lower satiating power and that fibers are lost during fruit-to-juice processing [586-588], which collectively may explain the reduced protective effect of BB against excess intake and weight gain. In this study, we also replicated the major metabolic features of rat models of diet-induced prediabetes [225, 275]. In fact, the HSuHF-fed rats presented postprandial hyperglycemia, glucose intolerance (as viewed by the GTT), postprandial hyperinsulinemia, and reduced insulin sensitivity (as suggested by the ITT), as well as hypertriglyceridemia. The addition of BJ to the hypercaloric diet-fed prediabetic animals promoted a reduction of fed glucose levels, ameliorated glucose intolerance, and enhanced insulin sensitivity. These findings agree with previous studies in animal models of obesity and T2DM [379, 410, 457, 589].

In addition, our results are consistent with those showing that BB prevents postprandial TGs accumulation in the serum in rats fed hypercaloric diets [423, 424, 590]. The phytochemical composition of BB, enriched in phenolic compounds (namely anthocyanins, among others) and fibers, is likely to explain the beneficial metabolic effects of BB [494, 496, 591] due to their antioxidant and prebiotic properties being very well documented [388, 577, 592-594]. However, the precise mechanisms underlying the protection of BB against deregulation of glucose, insulin, and lipids, especially in the prediabetic state, remain to be elucidated. In the present study, we chose to deepen some of the most promising hypotheses, starting from the potential to modulate the GM and related mechanisms.

Changes in the composition or diversity/richness of GM (called dysbiosis) have been associated with several extraintestinal disorders, including metabolic diseases, such as obesity and diabetes [78]. Several mechanisms have been proposed to explain that association, including the flow of bacterial constituents (namely LPS) through a more permeable intestinal barrier (leaky gut), causing systemic inflammation (endotoxemia) and inducing insulin resistance [78]. In addition, it has been shown that in states of obesity and diabetes there is an increment of pathogenic bacteria and reduction of symbiotic SCFAs-producing bacteria; since SCFAs (namely acetate and butyrate) are associated with beneficial metabolic properties, their reduction due to dysbiosis has been linked with metabolic disorders. Diet is a major modulator of GM, and hypercaloric diets evoke dysbiosis [595, 596]; on the contrary, fiber and polyphenols – which are abundant in BB – are promoters of symbiosis [296, 307].

Regarding the prediabetic rats, we did not obtain all the typical markers of dysbiosis described in other studies using both animal models and human samples [86, 103, 559, 597]. For example, the HSuHF-treated rats presented an unchanged relative abundance of Firmicutes to Bacteroidetes. Notwithstanding, consistent with our data, a similar unchanged Firmicutes:Bacteroidetes ratio was observed in Wistar rats fed with HFD or HSuHF diets for 10 weeks [598]. In addition, ultrastructural analysis of the gut by TEM revealed maintenance of the epithelial barrier integrity. The intestinal barrier's function is based on an intact epithelial lining, which is comprised of epithelial cells that are joined at different levels of the intercellular junction by cell-to-cell structural adhesions, such as tight junctions, adherens junctions, and desmosomes [599]. Tight junctions are dynamic and highly organized complexes, consisting of various proteins, including ZO-1 and occludin, which allow selective transport across the intestinal barrier. Moreover, the mRNA expression levels of ZO-1 and occludin and their distribution at the periphery of epithelial cells were unchanged. In line with these results, the measure of endotoxemia and the FITC-dextran permeability assay showed that the intestinal barrier was functionally still intact. These results could be eventually explained by the early disease stage, although some studies have already described dysbiosis in prediabetic states [559, 597, 600]. Even though, there was a reduced abundance of *Akkermansia muciniphila* in the HSuHF-treated animals, a bacterial species that has been negatively correlated with glucose tolerance, insulin resistance and obesity [601-603]. Furthermore, there was a reduced fecal content of SCFAs, which is in line with what has been described in states of

prediabetes and diabetes [81, 86, 597, 604]. Regarding the HSuHF animals treated with BJ, the prebiotic properties previously described in other conditions [392, 396, 403, 404, 605] were not replicated in our study. In fact, the reduced abundance of *Akkermansia muciniphila* found in the HSuHF-treated animals was not prevented, which was accompanied by a significant reduction of *Prevotella* spp. and *Bifidobacterium*. These distinct bacterial communities are typically associated with SCFAs production, which were not recovered by BJ supplementation in the HSuHF rats. In addition, fecal succinate was reduced in the BJ-fed animals; since *Prevotella* spp. belongs to a group of succinate-producing bacteria [502], the reduction in fecal succinate in BJ-treated prediabetic animals can be eventually derived from the suppressed *Prevotella* abundance. In addition, there was a reduced overall count of bacteria (universal), which might be eventually explained by the antimicrobial properties described for some polyphenols that are abundant in BB, such as quercetin and hesperidin [606-608]. It is known that GM can be deeply influenced by several factors associated with animal models, such as the species and the strain used, as well as the dietary regimen, namely the type of fat and carbohydrates (digestible and undigestible), among others, as we recently reviewed [225]. Although it is important in the future to study models with other characteristics, the results of our study, using this early disease model, show that GM modulation cannot explain the metabolic improvement observed in prediabetic animals when treated with BJ. In this sense, we moved forward to check the expected antioxidant properties of BJ and find possible crosslinks with other mechanisms that regulate the metabolism of glucose and lipids.

Hyperglycemia and hypertriglyceridemia are key drivers of glucotoxicity and lipotoxicity, which are among the main causes of oxidative stress and inflammation, two mechanisms closely linked with the (pre)diabetic state [49, 51]. In our model, the serum MDA/TAS ratio was higher in HSuHF rats and ameliorated in BJ-treated animals. In addition, we found a depletion of hepatic GSH levels in prediabetic rats, an effect that was prevented by BJ supplementation (HSuHF+BJ group). Another important finding of this study from NMR analysis was the increment of taurine in the hepatic tissue of BJ-treated animals. This endogenous metabolite has diverse cytoprotective actions, such as antioxidant activity linked to improved mitochondrial function (e.g. decreases the superoxide generation by mitochondria) [609-611]. Furthermore, we also found a decreased betaine content in the serum and liver of HSuHF rats, an effect that was prevented in BJ-treated animals. Betaine is a methyl donor that can be obtained directly

from the diet or by choline oxidation [612, 613]. This metabolite plays a role in the hepatic remethylation of the methionine-homocysteine cycle by betaine-homocysteine methyltransferase, producing carnitine, a key factor in fatty acid metabolism related to the transfer of long-chain fatty acids into mitochondria for subsequent β-oxidation [614]. In line with our results, lower levels of hepatic betaine were observed in Zucker rats [615] and in HFD-induced obese mice [616, 617]. Indeed, it has been shown that betaine supplementation improves hepatic steatosis and insulin resistance both in *in vivo* [617-620] and *in vitro* studies [617]. Betaine levels were restored in BJ-treated animals, which could also be explained by the enriched amount of betaine present in BB [613]. In addition, 3-hydroxibutyric acid (3-HB), a ketone body that represents the main alternative energy substrate to glucose and is a by-product of hepatic fatty acid oxidation, was recovered in the rats of the HSuHF+BJ group. In agreement with previous studies [621, 622], the reduced serum levels of 3-HB in HSuHF rats might suggest impaired fatty acid metabolism and a degree of hepatic mitochondrial dysfunction since ketone bodies are produced from acetyl-Coa via ketogenesis, which mainly occurs in the hepatic mitochondria [120]. The recovery of 3-HB levels and the 3-HB/acetoacetate ratio in the rats of the HSuHF group when supplemented with BJ suggests an improvement in fatty acid oxidation and mitochondrial function. Thus, our findings agree with the expected antioxidant properties of BB and further points to hepatic mechanisms regulating the metabolism, including at the mitochondrial level.

Several studies have indicated a close relationship between mitochondrial dysfunction or defects in mitochondrial biogenesis and the pathogenesis of insulin resistance and associated metabolic disorders [209, 214]. Albeit there is a controversial issue whether mitochondrial dysfunction is the cause or consequence of insulin resistance [209], mitochondria-targeted interventions could also counteract insulin resistance and improve glucose tolerance. Accordingly, it has been consistently considered that the health-promoting effects of some polyphenols extend to mitochondria and may represent a valuable and attractive nutritional strategy to prevent or hinder the metabolic impairments underpinning mitochondrial dysfunction [623, 624]. Indeed, some polyphenols can modulate several mitochondrial processes, including mitochondrial biogenesis, oxidative phosphorylation, mitochondrial membrane potential ($\Delta\Psi m$), and ATP synthesis, among others [625-627]. Our group previously showed that a long-term intake of BB phytochemicals triggered a hepatic mitochondrial-related metabolic transcriptomic

reprogramming in healthy rats [578]. There is also evidence that the variations in dietary macronutrient environments can differentially influence the rate of mitochondrial oxidative remodeling and subsequently can influence the hepatic insulin resistance severity [628]. In light of these considerations, we then focused our attention on the BJ effects on the hepatic tissue towards functional and molecular readouts from a mitochondria perspective in a prediabetic setting.

Our results revealed that the prediabetic animals displayed hepatic steatosis, viewed mainly by an increased hepatic content of TGs, which was paralleled by an impaired mitochondrial function and insulin resistance. Noticeably, BJ supplementation for 14 weeks alleviated hepatic steatosis, which is in accordance with other studies [423, 590]. The hepatic lipid overload represents a key driver for lipotoxicity, which together with glucotoxicity are closely associated with mitochondrial dysfunction [629]. Furthermore, hypercaloric diet-induced prediabetic animals display an impairment in hepatic mitochondrial bioenergetics machinery, which contributes to a redox imbalance and fosters ectopic lipid storage [214, 630]. Accordingly, we showed that the hypercaloric diet elicited considerable impairments in mitochondrial functional activity comprising disturbances in mitochondrial electric potentials and respiration, associated with decreased state 3 and V FCCP, which may result from dysfunction in the electron transport chain complexes or due to an uncoupling of oxidative phosphorylation, as suggested by the decreased RCR and ADP/O. Moreover, the hypercaloric diet-induced changes in respiratory parameters rendered mitochondria more susceptible to Ca^{2+}-induced mitochondrial permeability transition. mPTP opening, resulting from unbalanced cytoplasmic ion levels, mainly Ca^{2+}, which potentiates membrane potential dissipation and uncoupled oxidative phosphorylation, resulting in ATP depletion. Although discrepancies have been reported in other studies [631-634], several pieces of evidence are in line with our data showing impaired HFD-induced bioenergetics [214, 635, 636].

BJ supplementation restored these detrimental effects observed in the prediabetic animals, as viewed by: i) recovery of the transmembrane electrochemical potential indicating protection of passive permeability to protons (proton leakage); ii) increased oxidative phosphorylation efficiency (restored state 4 and increased RCR and ADP/O; and iii) restored activity of the electron transport chain (increase state 3 and V FCCP). The protection of mitochondrial function exerted by BJ is also sustained by the reduced mPTP opening, protecting mitochondrial from hypercaloric diet-induced swelling. This

effect can be mediated by antioxidant protection against oxidative damage in the mitochondria membrane [626]. Consistent with the mitochondrial dysfunction, prediabetic animals showed significantly decreased mRNA levels of *NDufb6* (a subunit of complex I) and *ATP5C1* (a subunit of complex V), which were recovered upon BJ supplementation, indicating that BJ improved hepatic mitochondrial integrity and function. Additionally, the recovery of mitochondrial function and activity induced by BJ ensures better ATP availability, particularly when there is a special need for energetic substrates to keep vital cellular processes and could predict the improved hepatic function. In models of NAFLD [418] and alcoholic fatty liver disease (AFLD) [637], BJ combined with probiotics was also able to improve hepatic mitochondrial dysfunction through reduced mitochondrial ultrastructure damage and swelling along with improved respiratory function and attenuated oxidative stress.

To gain further insight into the metabolic protection prompted by BJ, we also evaluated the expression of genes involved in glucose metabolism and insulin signaling. Significantly reduced hepatic expression of genes involved in insulin signaling (e.g. *Ins*, *Irs1*, *Pi3ca*) was observed in the HSuHF group, an effect that was partly restored in the liver of BJ-treated animals. Similar results were found in the liver, pancreas, and white adipose tissue of HFD diet-fed mice after blueberry-leaf extract intake [285]. Furthermore, we showed that BJ was able to normalize the hepatic mRNA expression levels of *Glut2*, the main glucose transporter in the liver that was downregulated in prediabetic rats. This effect may account for the normalization of hepatic glucose levels by promoting insulin-stimulated glucose uptake and by restoring insulin sensitivity.

Besides being the cell´s powerhouse, mitochondria are also pivotal for oxidative catabolism of aminoacids, ketone bodies synthesis, and fatty acid breakdown through β-oxidation [120, 629, 638]. With more efficient mitochondria, hepatocytes can handle more nutrients (namely lipids) and consequently avoid their accumulation. The reduced deposition of hepatic TGs along with high levels of ketone bodies in BJ-treated prediabetic animals pave the way to an efficient shift towards fatty acid β-oxidation and ketone synthesis. These effects are in line with the recovery of serum 3-HB levels and the 3-HB/acetoacetate ratio in the rats of the HSuHF+BJ group. To complement these observations, we analyzed the liver expression of a panel of genes related to lipid metabolism, including fatty acid uptake, synthesis, and oxidation. Previous studies have shown that FAT/CD36 overexpression increases hepatic TGs storage by increasing fatty

acid uptake [639], which is in line with our results showing increased mRNA CD36 expression in prediabetic animals. In the livers of BJ-treated animals, a trend toward normal values was obtained, which was probably due to decreased systemic FA. Conversely, the normalization of the mRNA expression of fatty acid-binding protein-1 (Fabp1) and fatty acid transporter 5 (FATP5/Slc27a5) in BJ-treated animals suggests a compensatory hepatocyte fatty acid uptake, preventing cytotoxicity promoted by binding of long-chain fatty acids. While most evidence shows the BB phytochemicals can protect from steatosis by suppressing the expression of genes involved in lipogenesis [285, 413, 455, 640, 641], in this study, we found unchanged mRNA expression of key genes involved in lipogenesis, such as Fasn, Acaca, Acsl5, and SREBP-1C, together with unaltered mRNA expression of Dgat2 and Scd1. Thus, considering the above data of fatty acid uptake and synthesis, our results suggested that the lipid-lowering activity of BJ cannot be explained by the ability to suppress fatty acid synthesis, contrasting with the effects of BJ on fatty acid β-oxidation. In fact, the HSuHF rats presented decreased mRNA expression of peroxisomal Acox1, as well as mitochondrial ACADL, CPT-1, and CPT-2, reflecting an imbalance from the substrate influx exceeding fat burning, thus favoring the ectopic lipid accumulation and storage in hepatocytes. BJ supplementation was able to rescue the mRNA expression of some of these genes, which collectively may contribute to the improvement of hepatic fatty acid β-oxidation in prediabetic rats. These results are consistent with other studies showing beneficial lipid homeostasis of BB polyphenols or anthocyanins extracts in mice models of metabolic diseases [396, 408, 423, 455, 640, 641]. Moreover, the prevention of serum and hepatic lipid accumulation in the BJ-treated animals of our study was accompanied by changes in some genes towards the improvement of the liver inflammatory profile, suggesting a protective effect of BJ against lipotoxicity. Indeed, the reduced lipid loading in the BJ-treated group could eventually explain the improved performance of hepatocytes, namely the slight changes in gene expression data.

Future studies are warranted to elucidate the open questions and to overcome specific limitations of this study. Microbial analysis should be performed using metagenomic sequencing technology to provide stronger information about the functional diversity of the bacterial community. In addition, further work should dissect the possibility that the decreased consumption of 35% sucrose in the BJ group might be partially responsible for the improved metabolic phenotype. In addition, alternative players

possibly involved in the impact of the consumption of sugar-sweetened beverages should be explored. For instance, it would be interesting to assess the effects of BJ on the activity of ChREBP and the expression of other key regulators of hepatic carbohydrates metabolism, such as PEPCK1 and G6PC.

4.7 Conclusions

In the current study, using a hypercaloric diet-induced rat model, we found that long-term BJ supplementation at 25 g/day for 14 weeks afforded protective effects against prediabetes by: i) improving glucose homeostasis and insulin sensitivity; ii) preventing hypertriglyceridemia and liver lipid deposition; iii) promoting strong antioxidant effects at serum and hepatic levels. The beneficial metabolic effects of BJ in this early disease stage could not be attributed to a correction of gut dysbiosis or prevention of leaky gut. Instead, the improved hepatic mitochondrial function, accompanied by restored fatty acid oxidation and ketogenesis, may contribute to explain the reduction of hepatic steatosis and hyperlipidemia, as well as the amelioration of insulin resistance and glucose metabolism. Our data provide evidence that amelioration of hepatic mitochondrial function could be a crucial mechanism by which BJ exerts antidiabetic properties in early stages (prediabetes). The present work paves the way to support the use of BB to counteract prediabetes progression.

Supplementary data

Table S4.1 | Polyphenol compounds identified in Blueberry juice by HPLC-PDA-ESI/MSn.

Peak	R_t (min)	λ_{max} (nm)	ESI-MSn[a] [*m/z* (relative abundance, %)]			Attempt to identify	Refs.
			Precursor Ion $[M-H]^-$	MS^2	MS^3		
1	14.22	248sh, 271, 280sh	315	153 (100)	123 (100)	Protocatechuic acid hexoside	[642]
2	20.59	250, 280, 299sh, 322sh	341	179 (100)	135 (100)	Caffeic acid hexoside	[642]
3	22.33	246, 285, 309, 320sh	341	179 (100)	135 (100)	Caffeic acid hexoside	[642]
4	23.23	248, 291, 299, 326	355	217 (55), 193 (100), 175 (20)	178 (22), 149 (50), 134 (100)	Ferulic acid hexoside	[643]
5	23.54	245, 291sh, 303sh, 326	353	191 (100)	173 (100), 171 (24)	5-CQA	[280]
6	24.93	249, 291, 321	353	191 (100)	173 (87), 171 (17), 155 (14), 127 (100), 111 (29), 109 (34), 93 (49), 87 (13), 85 (64)	*cis*-5-CQA	[280]
7	35.27	218, 249, 384sh, 347	463	317 (78), 316 (100)	287 (38), 272 (13), 271 (100), 270 (40), 179 (36), 151 (11)	Myricetin-O-deoxyhexoside	[644]
8	36.21	218, 250, 278sh, 335	463	337 (16), 318 (10), 317 (65), 316 (100), 315 (13), 301 (18), 300 (27)	288 (12), 287 (25), 271 (100), 270 (25)	Myricetin-O-deoxyhexoside	[644]
9	36.84	220, 253, 267sh, 287sh, 354	463	302 (12), 301 (100), 300 (21)	273 (17), 257 (16), 179 (100), 151 (60)	Quercetin-O-hexoside	[645]
10	37.33	219, 249, 267, 282sh, 353, 527	---	---	---	Delfinidin derivative	[646]
11	37.72	220, 248, 270, 281sh, 348, 529	569	523 (68), 491 (100)	343 (12), 331 (17), 329 (100), 328 (22)	Malvidin hexoside	[646]
12	38.50	219, 251, 267, 281sh, 352, 527	433	301 (100)	273 (13), 257 (10), 179 (100), 151 (71)	Quercetin-O-pentoside	[645]
13	38.67	219, 251, 267, 281sh, 352, 527	---	---	---	Delfinidin–pentoside	[646]
14	39.16	220, 254, 264sh, 284sh, 350, 530	447	302 (11), 301 (100), 300 (19)	283 (10), 273 (17), 179 (100), 151 (65)	Quercetin-O-deoxyhexoside	[645]
15	39.30	220, 254, 264sh, 284sh, 350, 530	---	---	---	Delfinidin	[646]
16	39.69	219, 253, 266sh, 283sh, 350, 530	447	302 (15), 301 (100), 300 (19)	283 (15), 273 (20), 257 (12), 229 (10), 179 (100), 151 (74)	Quercetin-O-deoxyhexoside	[645]
17	39.90	219, 253, 266sh, 283sh, 350, 530	---	---	---	Petunidin	[646]
18	39.90	219, 253, 266sh, 283sh, 350, 530	---	---	---	Malvidin	[646]

Identification based on the UV-Vis spectra, the molecular weight, and the fragmentation patterns, which are according to references (Refs).

Table S4.2 | Metabolites detected in serum samples by ^{1}H-NMR analysis.

Serum Metabolites	Chemical shift (ppm)	CTRL	HSuHF	HSuHF + BJ
Amino acids and BCAA metabolism				
Isoleucine	0.997 - 1.001(d)	$4.55 \times 10^{-5} \pm 9.02 \times 10^{-6}$	$4.07 \times 10^{-5} \pm 1.15 \times 10^{-5}$	$7.61 \times 10^{-5} \pm 1.64 \times 10^{-5}$
Valine	1.004 - 1.035(d)	0.0060 ± 0.0003	0.0046 ± 0.0004 *	0.0054 ± 0.0002
Glutamine	2.45 (m)	0.0166 ± 0.0011	0.0136 ± 0.0013	0.0139 ± 0.0008
Glycine	3.538 - 3.544	0.0068 ± 0.0009	0.0054 ± 0.0010	0.0074 ± 0.0008
Alanine	1.45 (d)	0.0070 ± 0.0004	0.0058 ± 0.0004	0.0064 ± 0.0005
Histidine	7.01 - 7.07 (s)	0.0011 ± 0.0001	0.0006 ± 0.0000 **	0.0007 ± 0.0000
Serine	3.947 - 3.985	0.0038 ± 0.0004	0.0041 ± 0.0005	0.0041 ± 0.0003
Ketogenesis				
Acetoacetate / Acetone[a]	2.195 - 2.217 (s)	0.0032 ± 0.0007	0.0033 ± 0.0006	0.0040 ± 0.0006
3-HB	1.16 - 1.189 (d)	0.0060 ± 0.0004	0.0024 ± 0.0005 ****	0.0042 ± 0.0005 *, #
3-HB / Acetoacetate		1.1250 ± 0.0892	0.7038 ± 0.0051 ***	1.086 ± 0.0655 ##
TCA cycle metabolism and intermediates				
Acetate	1.89 - 1.90 (s)	0.0027 ± 0.0004	0.0022 ± 0.0002	0.0023 ± 0.0003
Lactate	1.292 - 1.33 (d)	0.0800 ± 0.0094	0.0553 ± 0.0055 *	0.0604 ± 0.0048
Succinate	2.379 - 2.387 (s)	0.0024 ± 0.0004	0.0020 ± 0.0003	0.0026 ± 0.0003
Malic acid	4.261 - 4.307	0.0110 ± 0.002	0.0082 ± 0.002	0.0126 ± 0.001
Glucose	5.189 - 5.235	0.0261 ± 0.001	0.0321 ± 0.0001 **	0.0290 ±0.0000
Lactate /Alanine		12.5900 ± 0.8709	9.1790 ± 1.1030	9.8990 ± 1.2950
Plasma protein				
N-acetyl-glycoproteins	2.004- 2.045(s)	0.0165 ± 0.0010	0.0127 ± 0.011*	0.0109 ± 0.0007**
Creatine metabolism				
Creatine	3.005 - 3.023(s)	0.0034 ± 0.0004	0.0031 ± 0.0003	0.0030 ± 0.0004
Choline metabolism				
Choline	3.175 - 3.184(s)	0.0055 ± 0.0010	0.0041 ± 0.0008	0.0052 ± 0.0010
Betaine	3.26(s)	$1.58 \times 10^{11} \pm 2.38 \times 10^{11}$	$8.68 \times 10^{10} \pm 1.04 \times 10^{10}$ *	$1.40 \times 10^{11} \pm 7.64 \times 10^{11}$
Fatty acid metabolism				
Glycerol	3.64 - 3.635	0.0063 ± 0.0007	0.0049 ± 0.0005	0.0047 ± 0.0002
Others				
Unknown (DMSO?)	3.13 (s)	0.0010 ± 0.0001	$0.0003 \pm 7.98 \times 10^{-5}$ ****	$0.0006 \pm 7.89 \times 10^{-5}$ ***
Mannose	5.149 - 5.176	0.0009 ± 0.000	0.0011 ± 0.0002	0.0010 ± 0.000

Results are expressed as mean ± SEM. n = 8 – 10/group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ vs CTRL; # $p<0.05$ and ## $p<0.01$ vs HSuHF. [a] Putatively annotated as level 3 of identification according to Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative recommendations. All other metabolites identified as level 2.

Table S4.3 | Metabolites detected in liver samples by ^{1}H-NMR analysis.

Hepatic metabolites	Chemical shift (ppm)	CTRL	HSuHF	HSuHF + BJ
Amino acids and BCAA metabolism				
Alanine	1.48 (d)	0.0062 ± 0.0002	0.0058 ± 0.0002	0.0049 ± 0.0001 ****, ##
Aspartate	2.80 (m)	0.0007 ± 2.61 × 10^{-5}	0.0008 ± 6.53 × 10^{-5}	0.0007 ± 4.09 × 10^{-5}
Glutamine	2.46 (m)	0.0151 ± 0.0005	0.0159 ± 0.0006	0.0159 ± 0.0006
Glutamate	2.36 (m)	0.0059 ± 0.0005	0.0066 ± 0.0006	0.0051 ± 0.0003
Glycine	3.56 (s)	0.0052 ± 0.0002	0.0046 ± 0.0002	0.0046 ± 0.0001
Histidine	7.13 (s)	0.0002 ± 2.21 × 10^{-5}	0.0002 ± 2.77 × 10^{-5}	0.0002 ± 2.88 × 10^{-5}
Isoleucine	1.01 (d)	0.0012 ± 3.40 × 10^{-5}	0.0009 ± 4.59 × 10^{-5} ***	0.0010 ± 3.16 × 10^{-5}
Leucine	0.96(d)	0.0017 ± 3.31 × 10^{-5}	0.0016 ± 0.0001	0.0015 ± 3.41 × 10^{-5}
Phenylalanine	7.29-7.45 (m)	0.0012 ± 4.38 × 10^{-5}	0.0010 ± 0.0001	0.0009 ± 2.56 × 10^{-5}
Taurine	3.43 (t)	0.0024 ± 0.0004	0.0026 ± 0.0003	0.0045 ± 0.0005 **, #
Tyrosine	6.91(d),7.18 (d)	0.0004 ± 2.00 × 10^{-5}	0.0003 ± 3.34 × 10^{-5}	0.0003 ± 1.32 × 10^{-5}
Valine	1.05 (d)	0.0019 ± 7.40 × 10^{-5}	0.0015 ± 0.0001 *	0.0014 ± 4.12 × 10^{-5}
Ketogenesis				
3-HB	1.20 (d)	0.0011 ± 5.90 × 10^{-5}	0.0013 ± 0.0001	0.0014 ± 7.67 × 10^{-5}
TCA cycle metabolism and intermediates				
Acetate	1.92 (s)	0.0012 ± 5.58 × 10^{-5}	0.0011 ± 9.77 × 10^{-5}	0.0010 ± 2.97 × 10^{-5}
Lactate	1.33 (d)	0.0196 ± 0.0007	0.0189 ± 0.0009	0.0169 ± 0.0006
Succinate	2.41 (s)	0.0076 ± 0.0003	0.0064 ± 0.0003	0.0061 ± 0.0005
Glucose	5.24 (d)	0.0149 ± 0.0008	0.0182 ± 0.0009 *	0.0163 ± 0.0005
Creatine metabolism				
Creatine	3.03 (s)	0.0008 ± 3.73 × 10^{-5}	0.0005 ± 0.0001 *	0.0008 ± 7.66 × 10^{-5}
Choline metabolism				
Choline	3.20 (s)	0.0220 ± 0.0001	0.0022 ± 0.0008	0.0022 ± 0.0002
Phosphocholine	3.22(s)	0.0152 ±0.0008	0.0092 ± 0.0021	0.0108 ± 0.0034
Glycerolphos phocholine	3.67 (m)	0.0106 ± 0.0004	0.0092 ± 0.0003	0.0089 ± 0.0006
Betaine	3.25 (s)	0.0191 ± 0.0153	0.0076 ± 0.0007 **	0.0164 ± 0.0029 #
Microbial metabolism				
TMAO	3.27 (s)	0.0045 ± 0.0007	0.0039 ± 0.0006	0.0068 ±0.0007 #
Others				
Glutathione	2.97 (d)	0.0035 ± 0.0014	0.0028 ± 0.0002 *	0.0034 ± 0.0002
Nicotinurate	8.94 (m)	0.0004 ± 2.60 × 10^{-5}	0.0003 ± 3.46 × 10^{-5}	0.0003 ± 4.04 × 10^{-5}
NADP+	9.30 (s)	0.0004 ± 2.20 × 10^{-5}	0.0004 ± 2.59 × 10^{-5}	0.0004 ± 1.53 × 10^{-5}
NAD	9.34 (s)	0.0007 ± 3.10 × 10^{-5}	0.0008 ± 5.04 × 10^{-5}	0.0009 ± 5.20 × 10^{-5}

Results are expressed as mean ± SEM. n = 8 – 10/group; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$ vs CTRL; # $p<0.05$ and ## $p<0.01$ vs HSuHF.

CHAPTER 5

General discussion and concluding remarks

5.1 General discussion

This work is focused on the impact of long-term blueberry (BB) consumption, as juice, in two specific conditions with possible real life' implications. On the one hand, the preclinical simulation of consumption in a healthy situation, a context that has a human translation in people with a high concern about their diet and highly prone for taking supplements or other options (such as "detox juices") enriched in polyphenols (PP), including those derived from soft fruits such as BB. On the other hand, the preclinical simulation of consumption by someone in an early stage of metabolic disease (prediabetes), as a way of reverse or prevent progression through measures related to dietary modifications.

It is now well-recognized the adoption of healthy dietary habits entails several benefits for overall health. Consequently, during the recent years, we have witnessed a growing number of people concerned about the quality of their diet, either to prevent or revert the disease or just to improve their physical and mental health. This reality has pushed for an industry linked to functional nutrients and food supplements. It is worth noting that the focus of the present thesis was BB, by virtue of their claimed health benefits and by their richness in bioactive compounds. Indeed, BB has gained enormous popularity in recent decades, leading to a sector growth in terms of agri-food in several world regions, including Portugal, but also in terms of consumption, under diverse forms [647, 648].

The reasons for this popularity lie in its appreciable attributes – richness in polyphenolic compounds and fibers, antioxidant, anti-inflammatory, prebiotic properties –, which have been highly publicized by companies that commercialize fruits and other forms, but also increasingly scrutinized by science, with repercussions on the consumer. For instance, the daily diet has been extensively disclosed as one of the major driver shaping gut microbiota (GM) composition [649-651] in such a way that even healthy people are nowadays encouraged to regularly consume food components able to benefit the microbial community, such as PP and fibers, in order to reinforce intestinal health. However, there are existing doubts regarding the real impact of the continued consumption of appreciable amounts of BB, namely in the form of supplements. As for other antioxidant-rich foods and supplements, the whole spectrum of possible consequences at a metabolic level are unknown. While it is true that the main qualities of this type of food and supplements rich in PP and fibers are the antioxidant and prebiotic capacity, it is no less true that the amount consumed can lead to other effects as a result

of saturation of the capacity of some tissues, namely the liver, to manage these components. In fact, there are some works questioning the antioxidant vs pro-oxidative effect of phenolic compounds, and the hepatic mitochondria may play a decisive role. In pharmacology, and also in nutraceuticals and supplements field, the dose (and also the duration of exposure) can be decisive in defining the overall impact. Thus, in order to help clarify some of these doubts, we defined the first experimental approach by assessing the impact of continued BB supplementation in wild-type adult Wistar rats without any underlying pathology (healthy condition).

Importantly, it is now also established that the implementation of healthier dietary habits is one of the first-line approach to be recommended in metabolic dysregulation conditions, such as type 2 diabetes mellitus (T2DM), namely in early stages of the disease. In this context, prediabetes, as an elevated risk for T2DM and underlying complications, constitutes a key state to implement strategies to prevent or delay disease progression to more advanced stages associated with serious consequences [1, 2]. For this to be possible, it is necessary to continue to unravel the pathophysiological mechanisms that are at the genesis of the disease and its progression, as well as to identify new therapeutic or nutraceutical strategies with the potential to halt its progression.

Very exciting findings in recent years strongly suggest the involvement of new mechanisms and mediators in the onset and progression of diabetes, adding complexity to the existing knowledge. It is now known that chronic low-grade inflammation, already recognized as an essential actor in the pathophysiology of diabetes and its complications, is strictly related to the GM and its interaction with the host [652-654]. Additionally, significant variations in the composition of the intestinal flora have been described in diabetic patients and even in prediabetic subjects [103, 108, 559, 655]. The possibility of using the GM as a target for therapeutic and/or nutraceutical interventions has been a subject of extensive scientific debate in recent years [114-117].

Diet has certainly the greatest potential to influence the course of cardiometabolic disorders [452, 656]. Indeed, unbalanced diets are one of the key factors for the onset and progression of diabetes. Additionally, it is known that food strongly influences GM, which is highly benefited by foods enriched in fiber and phenolic compounds, among other phytochemicals and nutrients [307, 657]. As already described, BB is quite popular red fruits due to their pleasurable flavor and phytochemical composition, with low overall caloric content but high nutritional value due to their antioxidant, anti-inflammatory and

prebiotic properties, among others. The aforementioned and other attributes of BB have been held responsible for the beneficial impact in several cardiovascular and metabolic conditions. In the case of diabetes, preclinical and human studies have revealed hypoglycemic and insulin-sensitizing effect [276]. Furthermore, there is considerable evidence of protective biological actions against hyperlipidemia and hepatic steatosis, especially in animal models [413, 424, 594, 605]. These effects may be related to mechanisms that target processes involving mitochondrial dynamics and bioenergetics, including the fatty acids oxidation and the reduction of ROS, among others. Considering that many of the target mechanisms for the expression of BB antidiabetic effects are probably already affected in a prediabetic stage, the consumption of this fruit at this stage may be an appealing strategy to prevent disease progression, *per se* or in combination with pharmacotherapy. However, its efficiency at this early disease phase requires further studies and evidence. Indeed, it is relevant to elucidate the prebiotic component and the ability to prevent fat deposition, among other mechanisms that are expected to be positively affected, such as oxidative stress and inflammation. Thus, the present thesis also aimed to assess the impact of long-term supplementation of BB in a prediabetic condition. For this purpose, we used a high-sucrose and high-fat diet fed animal model, as our group described in previous studies [275].

The BB dose used in this study, in a juice form, was adapted from previous human and preclinical studies [332, 466, 469] and is equivalent to a daily intake of 240 g of fresh whole BB fruit (about 3/2 daily BB cups intake for a person with a body weight of 60 kg). Additionally, in a previous study of our group (unpublished data), we further estimate a daily intake of total phenolic content (TPC) corresponding approximately 50 mg of TPC/kg BW of rats, which was based on the TPC measured in BB juice (0.574 mg GAE/mL) and the daily volume (≈ 50 mL/day) ingested by the animals. Considering the body surface area [466, 470], the dose transposition to humans was estimated for an intake of 500 mg TPC per day. This amount is higher than the TPC obtained from BB fruit consumption by the general population, but readily achievable in the context of dietary supplements and food enriched in PP [430, 658], particularly in people with concerns and practices described above.

Impact of long-term BB supplementation in healthy conditions

As expected, the long-term BB supplementation in healthy animals did not exert significant effects in terms of BW as well as on global metabolic profile, namely in glycemic, insulinemic and lipid profiles, consistent with previous studies [492, 493].

Although the PP content can vary between BB cultivars [357], our study confirmed the overall expected phenolic profile, characterized by the presence of anthocyanins, flavonoids and hydroxycinnamic acids, being chlorogenic acid the most abundant compound, in line to what has been previously reported in the literature [280, 357, 359, 371, 374, 659]. Hence, it is presumed that these compounds are pivotal for the increased serum antioxidant activity highlighted in the present study.

It is now thoroughly established that GM has a pivotal role for human health, as result of immune, endocrine, and metabolic regulation [660, 661]. In particular, it is now well-recognized a reciprocal interaction between PP and GM; i.e. modulation of GM by PP and their catabolism by the GM is critical for the PP' effects [662-664]. Accumulating evidences from *in vivo* pre-clinical studies and human trials have reported positive effects of BB phytochemicals (e.g., dietary fibers and unabsorbed PP) in the digestive tract accompanied by beneficial GM modulation [328, 329, 389, 500, 665]. Considering this, we started by evaluating the impact of long-term BB supplementation on GM and SCFA composition in feces, and in overall intestinal integrity in healthy rats. No major changes were observed, which is a somewhat surprising finding when comparing previous studies showing that BB fruit has prebiotic activity [328, 500]. Different experimental conditions, namely the PP-rich supplementation source and form, may explain some of the discrepancies [500, 606, 655]. Indeed, a distinct impact on GM composition can be expected when comparing fresh vs dried fruit, juice, purified powder extracts or isolated phenolic components [500]. Interestingly, a recent study using Sprague Dawley rats showed that although an overall GM diversity was not changed in lyophilized BB compared with purified phenolics, differences in metabolic flux of phenolic metabolites were observed, suggesting that the food matrix distinctly influenced the gut microbial phenolic metabolism of whole foods vs dietary supplements [606].

We decided to use the entire fruit in the form of juice, including the pulp and the peel, thus using all the components with putative biological effects, such as dietary and functional fibers (non-digestible carbohydrates that have beneficial physiological effects) [666, 667], however, the possibility of exerting different effects from other forms tested

in other studies cannot be excluded. These questions should be elucidated in future works, accompanied by more powerful technologies, such as high-throughput sequencing and omics technologies, to better determine GM composition, as well as its functional activity.

As the liver is one of the key target tissue for PP metabolism and bioactivities [668], and PP can targeted the mitochondria which is a major source of ROS [438, 625, 626, 669], we next explored the impact of BB administration in this tissue, with a particular focus on mitochondria. When looking at the overall results from hepatic tissue, no histological changes as well as adverse effects viewed by the classical toxicological markers were encountered, suggesting that this dose can be apparently safe in terms of liver function. In fact, our findings are consistent with other studies that observed normal hepatic structure without changes in serum and hepatic parameters in rats fed with high doses of BB extracts [670, 671].

The current consensus assumes that natural PP have powerful antioxidant properties, with radical scavenging action that directly confer a cellular protection against oxidative stress, mainly via the modulation of mitochondrial redox state [438, 626]. On the other hand, consistent lines of evidence suggest that PP might exhibit a dual behavior, becoming pro-oxidants, a feature that might be related with their structure, concentrations, and the cellular redox contexts, which are often dismissed [348, 672]. The open hypothesis regarding this issue prompted us to evaluate the impact of long-term BB supplementation on the hepatic mitochondria function in the present study.

Unexpectedly, here we showed that long-term BB intake induced: i) mitochondrial swelling; ii) dissipation of the transmembrane electric potential; and iii) impaired oxidative phosphorylation efficiency linked with inhibitory effects at the respiratory chain or with an uncoupling effect. Collectively, these unforeseen findings advertised BB-derived PP´ ability to strongly impact mitochondrial respiratory control. To our knowledge, although there are some *in vitro* and *in vivo* studies using a few popular BB phytochemicals (isolated phenolics or in extract-rich forms), this is the first study reporting results pertaining BB effects (a juice form obtained from the whole fruit) on hepatic mitochondria bioenergetics in healthy experimental setting. Nevertheless, consistent with our data, other study revealed that aqueous and ethanolic extract of BB reduced, in a dose-dependent manner, the electron transport chain (ECT) activity and oxidative phosphorylation on rat heart mitochondria *in vitro* [528]. In fact, the inhibition of mitochondrial respiration along with

a mild uncoupling activity can represent putative mechanisms pertaining natural PP pro-oxidant properties [438, 673]. Many studies have showed the ability of plant-derived compounds to interact with the mitochondrial machinery, in a dose-, time and cell-type-specific manner [674]. In fact, there are a few studies showing pro-oxidant activity of PP *in vivo* [349-351]. Wang and colleagues have shown that tea polyphenol (-)-epigallocatehin-3-gallate (EGCG) acts as a pro-oxidant in mice at higher doses [349], which is in line with other studies suggesting that some of the biological effects of high dose of EGCG are due to the induction of oxidative stress [675]. Moreover, dose-dependent treatment of EGCG induced mitochondrial oxidative stress that was disclosed by reduced mitochondria DNA copy number, density, and complex expression along with decreased mRNA and protein expression of antioxidant enzymes' response, suggesting that mitochondria could be a key mechanistic target underlying EGCG-induced hepatotoxicity [351, 676].

Of notice, although potential adverse effects inherent of high doses of PP intake have been recorded, there was little evidence upon which to determine a dose threshold for different phenolics and/or food sources of complex phenolics [352]. In this study, using a single dose of BB, the absence of functional and structural changes on the liver tissue (viewed by hepatic enzymes' activity and histomorphology) suggests, at a first glimpse, a safety profile. Interestingly, in parallel with our findings at hepatic mitochondria bioenergetics, the long-term BB supplementation resulted in a marked repression in metabolic, transcriptomic factors and inflammatory-related genes. Globally, this surprising pattern of accentuated mitochondria bioenergetic challenge, metabolic transcriptomic reprogramming along with a marked anti-inflammatory preconditioning, suggest the existence of a unified adaptive response to a nutritional challenge advocated by long-term BB phytochemicals intake, that can be envisaged as an hormetic effect. These findings are consistent with the fact that apart from their well-known antioxidant properties, BB phytochemicals have been shown to interfere with epigenetic machinery through acting on DNA methylation, histone modifications, and posttranscriptional gene regulation by noncoding RNAs [443, 538, 539]. These effects of PP suggest that dietary-targeted epigenetics could be an attractive approach for disease prevention, which have been often envisaged as a starting point of clinical intervention [539].

The signaling remodeling arising from BB phytochemicals can be strictly implicit in the hormesis paradigm [542]. This theoretical hypothesis has been supported by many studies reporting that dietary PP may have an ambiguous dual role [542, 677]. Hormesis can be

defined as the duality in response by a cell/individual in reply to an endogenous or exogenous impetus that evoke favorable effects at a low dose and harmful effects at higher doses [678]. This duality of response may depend on dose, exposure times, pharmacokinetic properties of the PP, environment of the target system and target cell´ biological status [678]. The basis for these compounds acting as hormetins was initially described in *in vitro* studies, where they exhibited an antioxidant effect (either directly or indirectly through signaling pathways) at low doses, but a pro-oxidant effect at higher doses [679]. In general, it has been suggested that hormetic effects evoked by the physiologically relevant pro-oxidant activity of PP might represent a powerful approach to protect cells against physiological stress and to counteract some diseases [679, 680].

This surprising effect disclosed in this study has raises further questions. Not having found other evidence of toxicity in the animals treated with BB, including at the hepatic level, the key question remains as to whether we are facing: i) a cytotoxic effect that over time will cause liver dysfunction and then injury (hepatitis) or ii) a beneficial antioxidant effect expressed in a type of "cell rest or numbness", capable to protect against subsequent metabolic insult. Both hypotheses find some support in previous evidence showing that certain phenolic compounds can exert a harmful character, including at the liver level, namely in high doses, by distorting their traditional antioxidant potential into pro-oxidant. Many reports have showed that mitochondria can, indeed, convert external stimuli into alteration in antioxidant defense mechanism and in energy demands, thus contributing to higher resistance to stress [681]. As above mentioned, this effect may be viewed as hormesis (or more specifically as mitohormesis), a theory according to which there is a cellular adjustment, involving the mitochondria, in the sense of altering its antioxidant versus pro-oxidant response as a function of the intensity and duration of a stimulus. The open question of a toxicological effect can only be clarified through a longer study, using different forms and doses of BB and/or its phenolic compounds. The question of a beneficial "cell rest or numbness" (a type of preconditioning) to withstand a subsequent metabolic insult more efficiently can only be clarified with a protocol that simulates this condition. In this regard, these are interesting aspects raised by the current work that deserve additional effort in future work.

In this regard, in this particular study, the notes to be retained for a healthy condition in humans need additional caution given the impossibility of pointing out at this moment a clear definition of the beneficial or harmful nature of the prolonged use of BB. However,

it is worth noting the strong impact on the liver, namely on the mitochondria, which clearly deserves further elucidation.

Impact of long-term BB supplementation in prediabetic conditions

In the second study of this thesis, we were interested to determine the effects and mechanism of a long-term supplementation with aforesaid dose of BB on an experimental animal model of prediabetes. To achieve this goal, we chose an animal model that closely replicate the phenotype and early pathophysiological features of prediabetic subjects. Based on previous studies of our and other groups, we have used a prediabetic rat model that was induced by the intake of high sucrose (35%) solution during 9 weeks in drink water [236, 248, 682]. In the current study, we decided to accelerate and aggravate the prediabetic phenotype, by feeding the animals with a high-fat diet (60%) for additional 14 weeks (after the previous 9 weeks of sucrose solution). Diet-evoked animal models can recapitulate the human disease associated with unhealthy dietary pattern, as our group recently reviewed [225]. In fact, apart from the excessive consumption of huge amounts of simple sugars in soft drinks, the increasingly regular intake of hypercaloric diets, rich in fats, is strongly associated with an increased risk of developing prediabetes and T2DM [683, 684].

Beyond the expected rise in weight gain, the prediabetic animals presented postprandial hyperglycemia, glucose intolerance, postprandial hyperinsulinemia and insulin resistance that were accompanied by hypertriglyceridemia. These results are in line with other studies reporting that the chronic consumption of a combined high sucrose and high-fat diet can be a suitable experimental approach to establish an animal model of prediabetes and insulin resistance [264, 379, 410, 457, 589].

As already stated throughout this thesis, nowadays there are an increasing number of animal studies and clinical research data reporting the association of GM dysbiosis in several metabolic diseases, including T2DM [81, 655, 685, 686]. Several mechanisms have been proposed to explain the direct link between the gut dysbiosis and the onset of diabetes and insulin resistance, that are yet to be completely unravel [685, 686]. These mechanisms have been discussed and include the production of metabolites during fermentation and its resulting secondary effects; activation of inflammatory cascades leading to the release of cytokines; disrupting the permeability of the intestinal mucosal barrier allowing the influx of toxin and the direct signaling effect through incretin secretion

[687]. T2DM is well understood to have significantly enhanced permeability in the gut barrier allowing for the translocation of bacteria across the gut epithelium resulting in host metabolic endotoxemia triggering low-grade inflammation [78, 79]. The resulting effects can initiate β-cell destruction and insulin resistance [688]. T2DM has been widely associated with a poor species richness and diversity and loss of butyrate-producing bacteria [86]. Importantly, it has been recently suggested either in animal models or in humans that GM impairment could be linked with prediabetes development [86, 103, 689, 690]. This hypothesis is supported by some evidence that patients with prediabetes are associated with an aberrant intestinal microbiota characterized by a decreased microbial diversity and a depletion in the numbers of the genera *Akkermansia muciniphila* and *Clostridium*, together with increases in *Ruminococcus* and *Streptococcus* [103]. However, although alterations in the population of major phyla in the prediabetic state have been described in several animal and human studies, a specific microbiome pattern for prediabetes has not been reported.

Additionally, several studies have reported that high-sugar diet (HSD) and HFD promote an imbalance in the GM composition which can be associated with the onset of metabolic disorders [596, 691]. Most studies have shown that hypercaloric diets lead to a reduced Bacteroidetes-to-Firmicutes ratio [397, 691-693]. This dysbiotic microbial pattern has been linked to a higher harvest energy from dietary intake along with insulin resistance and obesity [693]. Similar microbial community pattern can be also found in both obese and diabetic subjects [559], albeit there is a lack of uniformity regarding the GM profile in both prediabetic animals and humans [103, 559, 694-696].

In our study, the prediabetic animal model displayed subtle changes in GM composition evidenced by the unchanged Firmicutes/Bacteroidetes ratio, the decline in *Akkermansia muciniphila* abundance and the reduction in fecal short-chain fatty acids (SCFAs) contents, namely propionate and butyrate, which is partially in line with some previous studies in prediabetes and T2DM [103, 598]. Moreover, our findings were accompanied by functional and structural markers of an intact intestinal barrier. Collins and colleagues have reported that dynamic alterations in GM composition of Sprague Dawley rats were evident as early as 3-days exposure to a high-fat/high-sugar diet (HFHSD) challenge [697]. Specifically, these animals displayed a decreased relative abundance of Bacteroides/*Prevotella* spp. which remained decreased after 14- and 28-days and a decreased abundance of *Clostridium* cluster IV; in addition, there were no differences

detected in the abundance of *Bifidobacterium* spp., *Roseburia* spp., and *Akkermansia muciniphila* in the 14- and 28-day upon HFHS dietary regimen in rats [697]. Nevertheless, some reports, including the study from Collins and colleagues, have highlighted that diet can induce rapid changes in GM composition, which may be transient and rapidly returning to the stable state due to the high resiliency of the GM community composition [661, 697]. Indeed, despite the similarity of methodology used (qPCR), our study did not show dramatic changes on GM composition. Coherently, very recent studies have indicated that different hypercaloric dietary models distinctively influence GM composition [692, 698, 699]. Moreover, dietary fat sources may be linked to distinct impact on compositional and functional GM profiles [698]. It is worth noting that, as above mentioned, we used an animal model that initially received a sucrose solution for 9 weeks, then changed to a HFD for additional 14 weeks to aggravate the prediabetic condition. This dietary regimen protocol may eventually contribute to explain the differences between our results and those reported by previous studies [697].

Accumulating evidence suggests that T2DM, obesity, and insulin resistance are closely associated with ectopic fat accumulation especially in the liver. Hepatic steatosis is often accompanied by hepatic insulin resistance [146, 700]. However, the initial events triggering the development of insulin resistance and its causal relations with dysregulation of glucose and fatty acids metabolism remain unclear [700, 701]. In this regard, we focused our attention in putative changes on hepatic tissue to address the role of liver in the onset of prediabetes. Other aspects that we were interested in the current study are related with mitochondria bioenergetics and energy metabolism. Indeed, it has been speculated that mitochondrial abnormalities may be involved in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) [702]. However, little is known about the spectrum of changes in mitochondrial content and function and their potential role in the prediabetes and insulin resistance development [703]. In this study, we observed in the prediabetic animals the presence of hepatic steatosis evidenced by an increased tissue content of TGs, accompanied by impaired liver antioxidant system, as viewed by a higher MDA/TAS ratio and a decline in hepatic levels of GSH - a powerful antioxidant. Accordingly, the consumption of high-fat and high carbohydrate diet is frequently associated with the presence of hepatic steatosis and liver damage; moreover, the excessive fat accumulation in hepatocytes can trigger lipid oxidative stress, reduce antioxidant enzymes production that can lead to hepatic dysfunction, and then to NAFLD [704, 705].

It is important to note that there are diverse findings in the studies that have examined the association between mitochondrial dysfunction and insulin resistance development in the hepatic tissue [214, 706-709]. Some studies have shown unchanged [710] or increased hepatic mitochondrial oxidative capacity in mice exposed to HFD [709], namely in the adult ob/ob mice [711] and in the insulin resistant and diabetic Goto-Kakizaki (GK) rat [712]. Some of these inconsistent data may be related with different diets, magnitude of metabolic disease and experimental methods used to assess mitochondrial function (i.e. mitochondrial respiration, mtDNA analysis) or perhaps induction of early compensatory mechanisms that mask the detrimental effects observed latter on.

In our study, we observed that hypercaloric diet-induced prediabetic animals exhibited hepatic mitochondrial bioenergetics impairments which comprised disturbances of the electric potentials, decreased phosphorylation efficiency and increased susceptibility to Ca^{2+}-induced mitochondrial permeability transition pore (mPTP) opening. These results are in line with other data showing impaired hypercaloric diet-induced bioenergetics [214, 706, 707, 713]. Wistar rats with obesity and insulin resistant induced by HFD presented hepatic steatosis together with increased oxidative stress and reduced respiratory capacity of isolated hepatic mitochondrial [706]. In general, these studies certainly argue with the hypothesis that impaired mitochondrial activity plays a causal role of in the onset of insulin resistance as well as in hepatic steatosis, leading to hepatic oxidative stress, changes in insulin sensitivity and facilitating the intracellular lipid accumulation [222-224].

Nevertheless, our data regarding the prediabetic rat model suggest that impairments of hepatic mitochondrial bioenergetics could be implicated in hypercaloric diet-induced insulin resistance, which should therefore be considered as a key target to studies exploring mechanisms underlying the onset of prediabetes. In fact, the changes related with hepatic mitochondrial function found in the prediabetic animals were accompanied by reduced liver expression of several genes involved in insulin signaling and in lipid metabolism, namely associated with fatty acid oxidation, along with a decrease in key mediators of ketogenesis. These alterations reflect a compromised insulin signaling and an imbalance from the substrate influx exceeding fat burning, which foster the ectopic lipid accumulation and storage in hepatocytes. The intracellular lipid accumulation and the impaired hepatic mitochondrial oxidative capacity could be detrimental (lipotoxicity) and trigger insulin resistance. This hypothesis postulates that reduced mitochondrial fatty acid

oxidation would facilitate the intracellular accumulation of different intermediates of lipid metabolism (e.g. diacylglycerols or ceramides), which, as discussed earlier, would contribute to impairment in insulin signaling pathway and consequently be involved in the development of insulin resistance.

Unsurprisingly, our data revealed BB protective effects in the overall metabolic profile, namely expressed by improvement of glucose tolerance and insulin sensitivity along with a remarkable reduction of serum TGs when compared with prediabetic animals without BB administration. The data disclosed herein are in concordance with several preclinical and human studies reporting antidiabetic effects of BB [334, 357, 494, 496, 497], which have been largely attributed to the presence of phenolic compounds (namely anthocyanins). As previously mentioned, a number of molecular mechanisms underlying the antidiabetic effects of BB or its bioactive compounds have been extensively investigated and disclose, apart from their antioxidant and anti-inflammatory activities, for instance, its ability to: i) suppress the activity of digestive enzymes (α amylase and α-glucosidase); ii) promote prebiotic effects; and iii) modulate the gene expression related to β-cell proliferation, insulin signaling and glucose uptake and metabolism, among others [365, 408, 714]. However, the precise mechanisms behind these effects remain unclear [494, 714], especially in prediabetic conditions.

Unlike the metabolic parameters associated with glucose tolerance and insulin sensitivity, this study showed that long-term BB supplementation did not prevent the BW gain observed in prediabetic animals. Our finding is consistent with some previous studies which demonstrated that BB were ineffective in reducing BW gain and adiposity [329, 379, 457, 498], but in disagreement with other studies reporting antiobesity effects of BB in diet-induced animal models of obesity [396, 585, 589, 715]. Song and colleagues showed that the supplementation of BB peel extract (150 mg/kg) for 5 weeks effectively inhibited the HFD-induced BW gain and the increase of adipose tissue mass in obese Sprague Dawley rats [585]. Likewise, the consumption of BB juice for 12 weeks suppressed the BW gain of the C57BL/6 mice fed with an HFD (with 45% energy from fat and 35% carbohydrates [715]. More recently, Jiao and colleagues also showed that 12 weeks of BB polyphenol extract (200 mg/kg/day) was able to reduce the BW gain and the increment of TGs and total cholesterol in liver and fecal samples from HFD-fed C57BL/6 mice [396]. Conversely, the supplementation of BB powder for 8 weeks did not show protective effects against HFD-induced BW gain in Wistar rats [329] and in C57BL/6 mice [379].

Moreover, the incorporation of BB in the diet reduced the abdominal fat and increased insulin sensitivity in the obese Zucker rats; however, the total fat mass and BW gain were unchanged during the 12 weeks [457]**.** Thus, factors contributing to these inconsistencies encompass the differential sources and doses of BB and their bioactive compounds, distinct processing methods as well as diverse dietary regimen used (e.g. dietary amounts of fats contents).

A putative reason for the divergent outcome between our study and previous reports can be related with the fact that we used whole BB fruits in a liquid form (juice), which led to a compensatory intake of solid food, for covering energy requirements. In this sense, the rise in fat energy load observed could have blunted the protective effect of BB on BW. Indeed, despite some inconsistent evidence, it has been suggested that energy from liquids is less satiating than energy from solid food [716, 717], which may have contributed to increased HFD consumption. An alternative explanation could be the increased beverage´s viscosity derived from juice processing together with the addition of sucrose, thus evoking a less palatable beverage.

Next, we have explored the potential role of GM as a putative key mediator of the metabolic benefits of BB in prediabetic animals. As already described, there are several factors associated with animal models, such as the species and the strain used, as well as the dietary regimen, namely the type of fat and carbohydrates (digestible and indigestible), among others, that can greatly influence GM composition and diversity [225]. In this study, we found that prediabetic animals supplemented with BB displayed weak changes on GM composition, mainly reflected by a reduction in the abundance of *Bifidobacterium* and *Prevotella*, together with decreased succinate contents in fecal samples. In addition, these subtle changes were accompanied by a reduced overall count of bacterial community (reflected by a decreased Universal abundance), which might be eventually explained by antimicrobial properties described for some polyphenols that are abundant in BB, such as quercetin [606-608]. Moreover, the long-term BB supplementation did not significantly impact the gut barrier integrity in this prediabetic rat model. Our data are in contrast with the expected outcomes in GM remodeling based on earlier studies, concerning the prebiotic effect of BB [396, 397]. As previous described, there are several studies reporting significant prebiotic effects of BB and its bioactive compounds on GM in hypercaloric diet-fed animals. The increase of fecal SCFAs contents (acetic, propionic, and butyric acids) was reported in mice fed with a HFD (45% of calories from fat) under BB

anthocyanin treatment at dose of 200 mg/kg in daily food for 12 weeks [594]. Furthermore, Wistar rats fed with the same HFD and supplemented with BB powder for 8 weeks significantly increased serum acetate, which was negatively correlated with Bacteroidetes and positively with *Bacilli* and *Lactobacillus* [329]. Interestingly, in a recent study, it was showed that polyphenols-rich whole BB fruit powder and of its fibrous fractions increased GM diversity and modestly improved colonic mucus thickness in mice fed HFHS diet for 8 weeks; however BB powders and fibrous fractions failed to reduced BW gain, to improve HFHS-induced glucose intolerance and to lower hepatic triglycerides levels [296].

Comparing with our study, a possibility for this discrepancy can be related with the fact that, in most previous studies, the impact in GM of other type of hypercaloric diets are used and compared without changing the macronutrients during the experimental protocol [718]. It is also interesting to note that the BB-treated prediabetic animals changed the dietary macronutrients pattern compared with prediabetic rats without BB administration, which may justify the absence of a profound impact on GM, as we will further discuss.

Since *Prevotella* spp. belongs to a group of succinate-producing bacteria [502], the reduction in fecal succinate in BJ-treated prediabetic animals can be eventually derived from the suppressed *Prevotella* abundance. In general, the role of members of the *Prevotella* genus within the intestinal microbiota and their effects on the host are not completely understood and somewhat conflicting data have been reported. High prevalence and relative abundance of *Prevotella* is found in consumers with dietary habits enriched in high carbohydrate and plant diet [719, 720]. Conversely, reduced abundance of *Prevotella* have been most noticeable in hypercaloric diets [698, 721]. It has been shown that *Prevotella copri* is positively correlated with branched-chain amino acids biosynthesis, insulin resistance and aggravation of glucose tolerance [111, 722]. In contrast, other studies have suggested that *Prevotella* and other members of the *Prevotella*-dominated microbiome are more prone to evoke inflammation and intestinal dysbiosis [723]. Specifically, *Prevotella* intestinalis, a representative member of the genus *Prevotella*, has been associated with host inflammation [724].

Bifidobacterium also use fibers (namely frutooligosaccharides) and carbohydrates (such as fructose and sucrose) as substrate for energy [725]. In our study, the reduction in drink intake in the prediabetic animals supplemented with BB was associated with a shift in

macronutrients consumption (more specifically, was translated in an increase in fat and a decline in carbohydrates intake) which may justify the reduction in *Bifidobacterium*. In fact, it was recently shown that restriction of carbohydrates associated with a high-fat ketogenic diets may contribute to the marked reduction in *Bifidobacterium* [726]. Importantly, it has been also reported that very low-carbohydrates diets (0%–1%) are distinctive from low-carbohydrates diets (5%–15%) in both GM and immune responses [726].

Notably, while compositional changes in the microbiome due to dietary change have been observed in several studies, few of them have compared the influence on the microbiota composition of diets rich in sugars with different fat contents [692]. Interestingly, in rats fed combinations of high-/low-fat and high-/low-sucrose diets, the animals fed high sucrose, irrespective of fat content, presented a reduction in fecal microbial diversity and compositional changes in select gut microbes [727]. Thus, the effects of BB on GM and SCFAs composition were diverse from what was expected considering the previously published evidence. In our study, the diet protocol used to induce the prediabetes model, supported by the group's previous work, may eventually contribute to justify some discrepancies. Indeed, animals fed with BB showed a distinct consumption profile of macromolecules that perhaps justifies the loss of some bacteria usually considered symbiotic and the consequent loss of SCFAs production.

Overall, our results clearly suggest that the beneficial metabolic effects prompted by the long-term BB supplementation in this prediabetic condition may not be attributed to a correction of gut dysbiosis or prevention of leaky gut. Nevertheless, before definitive conclusions can be drawn regarding the impact of BB on GM in this model of hypercaloric diet-induced prediabetes, we believed that the characterization of GM and related markers should be improved. Indeed, a detailed assessment of bacterial communities should be performed by using high-throughput metagenomic sequencing technology, complemented with other markers of GM and barrier functionality, as well as with quantification of toxic metabolites derived from the metaorganism degradation (involving bacterial and host enzymes and pathways) of food components (namely aromatic and branched-chain amino acids, carnitine, phosphatidylcholine, among other components).

We then moved towards the assessment of putative antioxidant properties of BB and find possible crosslinks with other mechanisms that regulate the metabolism of glucose and lipids. The long-term BB supplementation remarkably alleviated hepatic steatosis,

exhibiting favorable hepatoprotective effects in prediabetic animals, which is in line with other previous studies [423, 590, 728]. Besides hypolipidemic effects, BB has been recognized for its high antioxidant activity and the corresponding benefit outcomes [282]. Our findings showed that BB supplementation restored serum MDA/TAS ratio (a marker of oxidative stress), improved the antioxidant defense system (by normalizing hepatic GSH and enhancing taurine contents), and effectively restored hepatic mitochondrial dysfunction observed in prediabetic rats. Indeed, there was a clear improvement in all processes associated with mitochondria bioenergetics, encompassing a recovery of electrochemical potential, increased oxidative phosphorylation efficiency, restored activity of electron transport chain together with the inhibition of mPTP opening and recovered in genes expression of encoding subunits of mitochondrial respiratory complexes I and V. In alignment with our findings, it was recently reported that a combination of BB juice with probiotics could improve hepatic mitochondrial dysfunction through reduction of mitochondrial ultrastructure damage and swelling along with amelioration of respiratory function and attenuation of mitochondrial oxidative stress in NAFLD [418] and AFLD [637]. Indeed, during the last decade compelling evidence has emerged revealing the potential of certain polyphenols to modulate a diversity of processes which pertain to the mitochondria structural integrity and its metabolic functionality [627, 729, 730].

Apart from antioxidant-related mechanisms, PP are being recognized for their ability to modulate the capacity of mitochondria to undergo biogenesis (i.e. inducing sirtuins) [627, 729, 731]; to control its membrane potential (i.e. mPTP opening and uncoupling effects) [730] and its electron transport chain and ATP synthesis (i.e. through complexes I to V activity) [732, 733]; and ultimately, to trigger cell death (i.e. inhibiting/inducing intrinsic-apoptosis) [734]. We assume that the recovery of mitochondrial function and activity induced by BB ensures better ATP availability, particularly when there is a special need for energetic substrates to keep vital cellular processes, thus reflecting improved hepatic function. In fact, with more efficient mitochondria, hepatocytes can handle more nutrients (namely lipids) and consequently avoid their accumulation. Indeed, this remarkable BB impact on mitochondria bioenergetics should be highlighted. Actually, we found a prevention of serum and hepatic accumulation of TGs in the prediabetic animals treated with BB, accompanied by beneficial modifications of genes involved in hepatic pathways for regulation of glucose metabolism, insulin sensitivity and fatty acid β-oxidation. Since it is not possible to precisely identify the molecular mechanism able to

better explain hepatoprotection, a mitochondrial component eventually linked to energy and glucose and lipid management could be suggested as a major hypothesis, with a beneficial impact also in terms of oxidative stress and inflammation, to be explored in works futures.

Overall, evidences point to beneficial metabolic effects, apparently not explained by benefits on GM but rather by the correction of defects in mitochondrial dynamics and bioenergetics at the hepatic level. It is expected that the use of this type of approach concomitantly with antidiabetic medication, in the early stages of the disease or even in a slightly more advanced disease stage, may allow lowering the quantity and/or the dosage of antidiabetic drugs or even delay of its prescription. This may bring benefits on tolerance and safety, without losing the effectiveness of glycemic management and, perhaps, helping to control the progression of the disease and of its vascular complications by protecting the target-organs.

Collectively, our findings indicate that long-term BB supplementation in prediabetic rats, in a dose with opposite readouts in healthy animals, was able to improve hepatic fatty acid oxidation and reduce lipid loading, thus improving the performance of hepatic cells.

5.2 Concluding remarks

This thesis was carried out in order to elucidate the impact of long-term supplementation of BB in healthy and prediabetes experimental settings. The key findings of this work could be summarized as follows:

A – Regarding the **impact of BB in healthy** rats:

- There was an unchanged global metabolic profile and an increased serum antioxidant activity, which might be due to the polyphenolic composition of BB;
- The unaffected gut bacterial community along with a regular intestinal barrier integrity may suggest a specific influence of the form of BB administration which, however, requires further clarification with additional tools;
- The preservation of hepatic structure and function was accompanied by an accentuated hepatic mitochondrial bioenergetics challenge, a metabolic transcriptomic reprogramming, along with an anti-inflammatory preconditioning, that collectively may point to a hormetic response to be further dissected.

Due to claimed beneficial effects of BB-derived phytochemicals in several diseases, it has become a popular and frequently consumed dietary supplement readily available for sale in different types of stores. Our work, using healthy animals exposed to long-term supplementation of BB, unravel novel findings and concomitantly raised new questions that require further studies before beneficial or deleterious impact of BB in this condition could be claimed.

B – Regarding the **impact of BB in a prediabetic rat model**:

- Concerning the **rat model of prediabetes**:

  – An hypercaloric diet for 23 weeks was able to foster a prediabetic phenotype, ascertained by postprandial hyperglycemia, glucose intolerance, postprandial hyperinsulinemia and insulin resistance along with increased BW gain and hypertriglyceridemia;

  – The prediabetic rats showed subtle changes in fecal GM and a reduction of SCFAs, together with preservation of the epithelial barrier integrity, suggesting the inexistence of markers changes on this early disease phenotype, eventually due to the type of diet used to induce this model;

  – This model developed hepatic steatosis, most probably as a result of excessive fatty acids flux from circulation to the liver, which may have contributed to the impaired antioxidant defense system;

  – Also in the liver, there was a notorious impairment of the mitochondrial parameters, suggesting that the alterations in hepatic mitochondrial bioenergetics may eventually precede insulin resistance, thus, suggesting that this organelle might be considered as a key target for future studies elucidating the mechanisms underlying prediabetes progression;

  – These changes were accompanied by a downregulation of important mediators involved in the insulin signaling and lipid metabolism, particularly in fatty acid oxidation and ketogenesis, suggesting a reduced fatty acids oxidation at the mitochondrial level, which may contribute to the impaired insulin signaling.

- Concerning the **effects of BB supplementation in prediabetes**:

– There was a consistent protection of hypercaloric diet-evoked metabolic deregulations, as viewed by the amelioration of glucose tolerance and insulin sensitivity, accompanying by correction of hypertriglyceridemia;

– The antimicrobial properties of BB-derived polyphenols may have contributed to the slight changes in fecal GM composition and SCFAs contents observed in the prediabetes animals supplemented with BB; however, concomitant changes in macronutrients intake may also contribute to explain such disclosed findings, highlighting that BB-induced metabolic improvement in this study could not be reasoned to a correction of gut dysbiosis or prevention of leaky gut;

– As expected, the long-term BB supplementation promoted strong antioxidant effects at serum and hepatic levels that were accompanied by an improved hepatic mitochondrial function, which point out that mitochondria could be eventually viewed as a potential attractive target for the prediabetic state;

– Moreover, BB apparently restored hepatic fatty acid oxidation and ketogenesis, suggested by the positive modulation of genes involved in fatty acid metabolism towards a metabolically efficient shift to fatty acid oxidation; these effects can enlighten the reduction of hepatic steatosis and hyperlipidemia, as well as the amelioration of insulin resistance and glucose metabolism;

– Additionally, BB also partially restored the expression of genes involved in insulin signaling and glucose transporter, which may contribute to the improvement in insulin resistance and glucose homeostasis in the prediabetic animals.

Collectively, our data provide evidence that the amelioration of hepatic mitochondrial function may be a crucial mechanism by which BB exerts antidiabetic properties at early stages. Thus, this thesis highlight that BB supplementation could be a useful non-pharmacological approach to counteract prediabetes progression.

CHAPTER 6

References

References

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