Publicaciones derivadas de la Tesis Doctoral "Combinación de metodologías ómicas y meta-ómicas para estudiar el efecto de la suplementación de selenio y su interacción con la microbiota intestinal"

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Programa de Doctorado: Ciencia y Tecnología Industrial y Ambiental
Línea de investigación: Análisis Medioambiental, Bioanálisis y Biotecnología

1. Omic methodologies for assessing metal(-loid)s-host-microbiota interplay: A review

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2020 JOURNAL IMPACT FACTOR	2020 JOURNAL IMPACT FACTOR WITHOUT SELF CITATIONS
6.558	6.157

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EDITION

Science Citation Index Expanded (SCIE)

CATEGORY

CHEMISTRY, ANALYTICAL

10/87

JCR YEAR	JIF RANK	QUARTILE	JIF PERCENTILE
2020	10/87	Q1	89.08
2019	10/86	Q1	88.95
2018	10/84	Q1	88.69
2017	8/81	Q1	90.74
2016	7/76	Q1	91.45
2015	8/75	Q1	90.00
2014	5/74	Q1	93.92
2013	5/76	Q1	94.08
2012	7/75	Q1	91.33
2011	5/73	Q1	93.84

2. Selenium supplementation influences mice testicular selenoproteins driven by gut microbiota

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Science Citation Index Expanded (SCIE)	MULTIDISCIPLINARY SCIENCES - SCIE

LANGUAGES	REGION	1ST ELECTRONIC JCR YEAR
English	ENGLAND	2011

Publisher Information

PUBLISHER	ADDRESS	PUBLICATION FREQUENCY
NATURE RESEARCH	HEIDELBERGER PLATZ 3, BERLIN 14197, GERMANY	1 issues/year

Journal Impact Factor

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2020 JOURNAL IMPACT FACTOR	2020 JOURNAL IMPACT FACTOR WITHOUT SELF CITATIONS
4.380	4.170

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EDITION

Science Citation Index Expanded (SCIE)

CATEGORY

MULTIDISCIPLINARY SCIENCES

17/72

JCR YEAR	JIF RANK	QUARTILE	JIF PERCENTILE
2020	17/72	Q1	77.08
2019	17/71	Q1	76.76
2018	15/69	Q1	78.99
2017	12/64	Q1	82.03
2016	10/64	Q1	85.16
2015	7/63	Q1	89.68
2014	5/57	Q1	92.11
2013	5/55	Q1	91.82
2012	8/56	Q1	86.61
2011	56/56	Q4	n/a

3. Antagonistic interaction of selenium and cadmium in human hepatic cells through selenoproteins

Autores: Sara Ramírez-Acosta, Renata Uhlírová, Francisco Navarro, José Luis Gómez-Ariza, Tamara García Barrera

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2020 JOURNAL IMPACT FACTOR
5.221

2020 JOURNAL IMPACT FACTOR WITHOUT SELF CITATIONS
5.030

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EDITION
Science Citation Index Expanded (SCIE)

CATEGORY
CHEMISTRY, MULTIDISCIPLINARY

53/178

JCR YEAR	JIF RANK	QUARTILE	JIF PERCENTILE
2020	53/178	Q2	70.51
2019	57/177	Q2	68.08
2018	54/172	Q2	68.90
2017	44/171	Q2	74.56
2016	44/166	Q2	73.80

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Omic methodologies for assessing metal(-loid)s-host-microbiota interplay: A review

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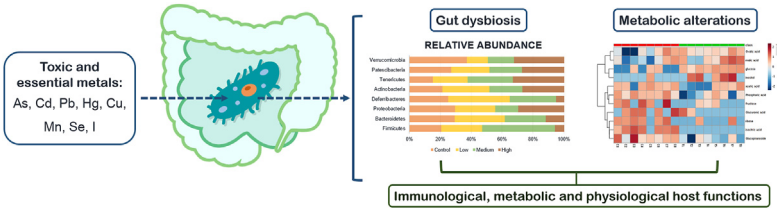
HIGHLIGHTS

- The influence of metals and their species on the gut microbiota-brain axis has been pointed out.
- The mother-offspring microbiota transference is important to seeding the infant gut.
- The hypothalamic-pituitary-thyroid axis can also be affected by environmental pollution.
- The chemical form of a metal(loid) determines their toxicity or essentiality.
- Omic methodologies for assessing the impact of metal(loid)s in the host microbiota are reviewed.

GRAPHICAL ABSTRACT



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ABSTRACT

Omic methodologies have become key analytical tools in a wide number of research topics such as systems biology, environmental analysis, biomedicine or food analysis. They are especially useful when they are combined providing a new perspective and a holistic view of the analytical problem. Methodologies for microbiota analysis have been mostly focused on genome sequencing. However, information provided by these metagenomic studies is limited to the identification of the presence of genes, taxa and their inferred functionality. To achieve a deeper knowledge of microbial functionality in health and disease, especially in dysbiosis conditions related to metal and metalloid exposure, the introduction of additional *meta*-omic approaches including metabolomics, metallomics, metatranscriptomics and metaproteomics results essential. The possible impact of metals and metalloids on the gut microbiota and their effects on gut-brain axis (GBA) only begin to be figured out. To this end new analytical workflows combining powerful tools are claimed such as high resolution mass spectrometry and heteroatom-tagged proteomics for the absolute quantification of metal-containing biomolecules using

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Heteroatom-tagged proteomics
Metabolomics

the metal as a "tag" in a sensitive and selective detector (*e.g.* ICP-MS). This review focus on current analytical methodologies related with the analytical techniques and procedures available for metallomics and microbiota analysis with a special attention on their advantages and drawbacks.

Contents

Abbreviations

2-DE	2-dimensional gel electrophoresis
5-HIAA	5-hydroxyindole acetic acid
5-HT	5- hydroxytryptamine, serotonin
AAs	amino acids
AAS	atomic absorption spectroscopy
AEC	anion exchange chromatography
AFLPs	amplified fragment length polymorphism
AFS	atomic fluorescence spectroscopy
Aβ	β-amyloid
BAs	bile acids
CAs	catecholamines
Cbl	cyanocobalamin
CE	capillary electrophoresis
CEC	cation exchange chromatography
CE-LIF	capillary electrophoresis coupled to laser induced fluorescence
DA	dopamine
DGGE	denaturing gradient gel electrophoresis
DMA	dimethylarsinic
DMSO	dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	dithiothreitol
ECD	electrochemical detector
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESI-QTOF-MS	electrospray ionization quadrupole time of flight mass spectrometry
FAAS	flame atomic absorption spectroscopy
ETAAS	electrothermal atomic absorption spectroscopy
FAES	flame atomic emission spectroscopy
FCM	flow-cytometer
FISH	fluorescence in situ hybridization
FLD	fluorescence detector
GABA	γ-aminobutyric acid
GBA	gut-brain axis
GC	gas chromatography
GPx	glutathione-peroxidse
HILIC	hydrophilic interaction liquid chromatography
HITChip	human intestinal tract chip
HMP	Human Microbiome Project
HPLC	high performance liquid chromatography
iAs	inorganic arsenic
ICP-AES	inductively coupled plasma with atomic emission spectroscopy
ICP-MS	inductively coupled plasma mass spectrometry
IDA	isotopic dilution analysis
LA	laser ablation
LC	liquid chromatography
LIF	laser induced fluorescence
LLE	liquid-liquid extraction
LMWaas	low molecular weight amino acids
Mkn	menaquinones
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MsrB1	methionine-R-sulfoxide reductase 1
NGS	next-generation sequencing
NMR	nuclear magnetic resonance
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PFGE	pulse-field gel electrophoresis
PMA	propidium monoazide
PMSF	phenylmethylsulfonyl fluoride
PolyAs	polyamines
PTMs	post-translational modifications qPCR, quantitative polymerase chain reaction in real time
RBC	red blood cells
RFLP	fragment length polymorphism
RIP3	receptor-interacting protein kinase 3

RP	reverse phase	SLE	solid-liquid extraction
rRNA	Ribosomal ribonucleic acid	SSID	species specific isotopic dilution
SAM	S-Adenosyl methionine	SUID	species unspecific isotopic dilution
SCFAs	short chain fatty acids	TCEP	tris(2-carboxyethyl)phosphine
SDS	sodium dodecyl sulfate	TGF-β	transforming growth factor beta
SEC	size exclusion chromatography	TGGE	temperature gradient gel electrophoresis
SeMet	selenomethionine	TNF-α	tumour necrosis factor
SEPP1	selenoprotein P	TrxR	thioredoxin reductase
SFC	supercritical fluid chromatography	UMIs	unique molecular identifiers
sIgA	secretoryimmunoglobulin A	VOCs	volatile organic compounds

1. Human microbiota, environmental pollutants and health

The human microbiota is a dynamic, complex, and wide ecological community consisting of bacteria, viruses, archaea, and fungi, which inhabit the human body, and participate in immunological, metabolic and physiological host functions. The term microbiome refers to the collection of genes within microbiota and their functions. Accumulating evidence shows the key impact of microbiota on human health. Alterations in microbial composition, diversity and functionality, defined as dysbiosis, have been associated with the risk of developing certain diseases, such as intestinal, inflammatory, and immune problems; diabetes; allergies; metabolic syndrome-related problems; and neurological and cognitive disorders through the gut-brain axis (GBA) [1]. Recent research advances have described the (GBA), which is a bidirectional communication between intestinal functions and the central and enteric nervous system of the host [2] and alterations of the microbiota-GBA have also been associated with stress-related disorders, mood, behavior and food preferences [3].

There is a growing interest in the impact of breastfeeding on infant gut colonization. It is considered the main postnatal microbial source and a key maternal-neonatal relationship with consequences for the development of the gut-brain axis and the immune system [4].

Beyond nutrition-related functions, human breast milk also contains an exclusive and personalised combination of specific bioactive components such as immunoglobulins and cytokines, bioactive lipids, microRNAs, hormones, oligosaccharides, and microbiota [5]. Breast milk would also contains essential and toxic metals as well as other pollutants [6,7] with a potential impact on infant microbiota and brain development [8,9]. Together with other toxic and essential elements, selenium is transferred from the mother to the offspring by the placenta and cord blood as well as during breastfeeding as selenometabolites and selenoproteins, such as the recently discovered selenoprotein P in human breast milk [10], which is a selenoprotein with a multifaceted role in the nervous system [11]. Based on current evidence, there are indirect links between breastfeeding and the microbiota-brain axis, although they still need to be investigated.

Gut microbiota have a crucial role in human protection against pathogens and toxic compounds [12] and their potential role in the GBA has been pointed out [13], but little is known about the possible effects of environmental pollutants on both axes (Fig. 1). The estimation of the interactions between metabolic shifts caused by environmental pollutants and gut microbiota can provide new evidence regarding the roles of gut microbiota. Animal studies have shown the impact of oral exposure of metals on the gut microbiota [14], which exert a barrier effect against chronic exposure to heavy metals [15]. Other elements, such as selenium, are essential and can counteract the toxic action of metals [16]. The interlink between the status of selenium and the expression of selenoproteins and the gut microbiota has also been shown in mice [17,18] and other mammal [19]. On the other hand, toxic and essentials elements can interact with other environmental pollutants, such as persistent organic pollutants, which can also be present in breast milk. These pollutants can alter the homeostasis of iodinated thyroid hormones due to the similarity in their chemical structure, impacting on the **hypothalamic-pituitary-thyroid axis**, which is a third axis involving the essential element iodine that can be affected by environmental pollution (Fig. 1) [20].

The significance of metalloenzymes in human gut microbial metabolism has also been described [21]. An analysis of the prevalence and distribution of different classes of metalloenzymes has revealed that about 40% of the genes categorised from the metagenomic data from the stool of the healthy humans (Human Microbiome Project (HMP)) [22] can be assigned to a metal cofactor using the Pfam protein family database [23]. The radical S-adenosyl-L-methionine (SAM) enzyme family is the most predominant metalloenzyme related family in the human gut microbiome (PF04055) [21], and it plays a wide range of roles in gut microbial

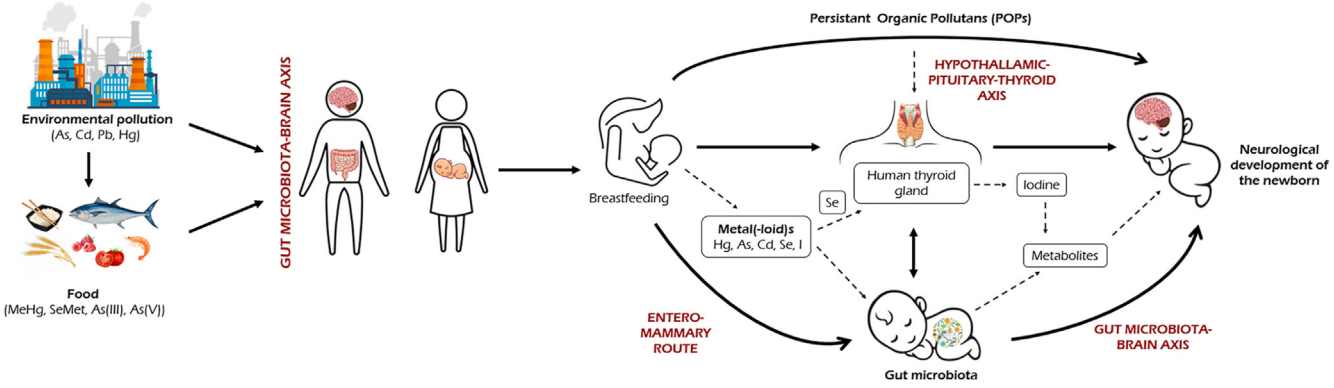

Fig. 1. Interplay between metal(-loid)s and gut microbiota-brain, breast milk-microbiota-brain and hypothalamic-pituitary-thyroid axes.

metabolism. However, the characterisation of the different metalloenzymes in stool samples or in other tissues (*e.g.* brain) or biofluids that could be correlated with specific microbial metabolites or bacteria is challenging and requires robust omic analytical methodologies and a holistic view.

Remarkably, our current knowledge of the metal-microbiota-brain interaction in humans is limited and uncovered. Thus, this review explores the current knowledge of the influence of toxic and essential elements in the gut-(mammary gland)-brain microbiota axis and the actual state-of-the-art analytical techniques and procedures available for metallomics (heteroatom tagged proteomics), and microbiota analysis (from classical microbiology to next-generation sequencing).

2. How to study the metal-microbiota interactions

2.1. Models used to study metal-microbiota interactions

The evaluation of the impact of environmental pollution on living organisms requires the use of model organisms as bioindicators (*e.g. Mus musculus*) and the measurement of biomarkers (*e.g.* proteins, transcripts, metabolites, bacteria) [24]. *In vitro* models allow for the simplification, standardisation and analysis of specific parameters as well as reducing *in vivo* assays. Thus, several *in vitro* studies have been performed to evaluate the bioaccessibility of metal(-oid)s and their interplay with food components or gut microbiota, such as those related to arsenic speciation in rice after *in vitro* gastrointestinal digestion assays [25,26]. *In vitro* studies have demonstrated that salivary and gut microbiomes affect the bioaccessibility and arsenical species profile of food matrices (rice, mussels and nori seaweed) [26].

In vivo studies with mammalian species are useful for integrating the digestive tract as a biological filter present in humans in order to regulate the transport of contaminants to the bloodstream [27]. However, ethical considerations and limitation of such experiments to the essential minimum are very convenient. In this sense, mouse model is one of the most widely used since mice shares 95% of their genes with humans [28]. Rats seem to be a good model, but, for example, arsenic metabolism in rats is different from humans [29]. Finally, studies in human populations exposed to pollutants or free-living animals are the last step to validate the biological responses observed in model organisms, in which it is easier to isolate the impact of a particular pollutant [30].

Several model organisms have been used in connection with metal(-loid)s-gut microbiota interplay including mice, rats, pigs and humans. In this sense, the connection between gut microbiota and metal(-loid)s has been explored in mice after arsenic [31–36], mercury [37–39], copper [38,40], cadmium [41–45], lead [40,41,46,47], iodine [48] and selenium [17,18,49] exposure. Rats have been used as model organisms to assess the interaction of gut microbiota with arsenic [50,51], mercury [52], copper [53,54], cadmium [50,52], iodine [55], manganese [56], lead [57,58], and selenium [59]. Human gut microbiota have been linked to arsenic [60–62], mercury [63,64], heavy metals [57,58] and lead [65]. Finally, pigs gut microbiota has been correlated with selenium [19].

2.2. Sample preparation for the characterisation of gut microbiota-metal(-loid) interactions

2.2.1. Metals and metalloproteins

The determination of metal(-loid)s can be performed in any tissue or biofluid to explore the distribution of elements, metabolism, and homeostasis in the body. Usually, serum and plasma are the most commonly used samples because they are easy to collect. Typically, they are accessible and respond quickly to dietary intake or physiological disorders. Both biofluids can be obtained from whole blood at room temperature to allow natural clot after centrifugation (serum) or using anti-coagulants (heparin, citrate, ethylenediaminetetraacetic acid (EDTA)) to avoid the fibrinogen-clotting cascade, followed by centrifugation for separation into red blood cells (RBCs) and plasma (supernatant) [66]. Serum samples are easy to collect and do not require a refrigerated centrifugation step, but plasma samples lead to greater sample volume per volume of blood withdrawn. The use of anti-coagulants for plasma samples may be a problem because they can sequester the metal(-loid) and prevent the identification of the metallobiomolecule. Metal(-loid)s can be measured in each organ or biofluid. After excision of the organs, they are rapidly transferred to dry ice, cleaned with 0.9% NaCl (w/w) solution, frozen in liquid nitrogen, and stored at −80 °C until they are used for mineralisation or metalloproteins extraction [24]. Atomic analytical techniques can determine the total content of the elements after mineralisation with acids using, for example, a microwave digestion system [24], but for metalloproteins determination, a modified proteomic procedure is usually applied to preserve the integrity of the metal-molecule bond during the extraction. This metal-biomolecule bond could be covalent (*e.g.* selenoproteins) but is sometimes labile, and the metal can be released from the biomolecule (*e.g.* metal complexes with proteins) [67]. In this sense, tris(2-carboxyethyl)phosphine (TCEP) can be used instead of dithiothreitol (DTT) as a reducing agent, and phenylmethylsulfonyl fluoride (PMSF) instead of EDTA as proteases inhibitor [68].

In connection with the gut microbiota-elements interplay research, the majority of studies are based on metal exposure or food supplementation in the model organism and microbiome analysis [33,69]. However, other studies typically determine the total content of metals (not chemical species) in a number of biofluids and tissues and link this with gut microbiota. In this sense, arsenic has been determined in plasma [33,36,70], urine [61,70] and the liver [33,70] after arsenic exposure and correlated with gut microbiota. Mercury has been determined in blood [37,64] and also in the hair [57,63,64], a good reflect of Hg body burden [71].

Copper has been determined in serum [53,54], the spleen and the kidney [54], while lead has been measured in urine [65] serum and the liver [72] and cadmium has been measured in serum [42,73], plasma [74], the liver [43,44,73–76] whole blood [44,76] and the kidney [44,75,76]. Remarkably, gut microbiota have been linked to the manganese concentration in brain tissue and plasma [56] and to iodine in thyroid tissue, urine and plasma [48,55]. Different selenium supplementation experiments have been carried out to associate gut microbiota and selenium concentrations in serum [19,59], plasma [17], the kidney and the liver [17,18,59].

2.2.2. Metabolome and microbiota

The biological samples typically analysed for metabolomics are plasma, serum, urine, cerebrospinal fluid, saliva, exhaled breaths, and tissues from target organs [77], even though the liver and kidney are the most metabolically active organs and the brain is of utmost interest when exploring the correlation between alterations in metabolic routes and neurological activity. Similarly, for microbial metabolomics, faeces is the most frequent analysed sample due to its non-invasive character and because it may directly probe the connection between intestinal bacteria and the physiology of the holobiont [78–80]. In relation to the metal-gut microbiota interplay, the metabolomic analysis of samples besides faeces is scarce. Thus, plasma metabolomics has been correlated with gut microbiota and arsenic in mice [70], as well as with manganese [56] and iodine [55] in rats; liver metabolomics with lead in rats [72]; and urine metabolomics with arsenic in mice [70] and iodine in rats [55]. Faecal metabolomics and microbiome analysis has been correlated with arsenic [70] and cadmium [45] in mice and with

copper in rats [54]. These works are discussed in more detail in section 2.4. (metabolomics and microbial metabolomics).

Faeces samples are the most used for microbial metabolomics and taxonomical identification of gut microbiota [81], but several studies have found differences between faecal and mucosal-associated microbiota. In this sense, it has been observed that different parts of the gastrointestinal tract have different microbial composition driven by environmental conditions such as pH [82] or oxygen content [83]. Therefore, the choice of the sample source could trigger huge variability in the results and conclusions of gut microbiota identification.

Even though faeces is the sample chosen for most microbiome studies (metataxonomics and microbial metabolomics), other sources could also be used after an endoscopy procedure, including biopsy [84], luminal brush [85], laser capture microdissection, or catheter aspiration, among others [81]. However, all of these are invasive and the collection of the sample is difficult, it is sometimes affected by the contamination of the instrument used in the procedure and by the bowel preparation method [86]. These reasons limit the use of these methods in large-scale studies, which is, on the contrary, essential for reliable conclusions in microbiome studies due to the variability among individuals in terms of microbiota composition [87]. Therefore, although it is becoming clear that faecal microbiota could not be representative of the mucosal-associated microbiota [88], they are the most used for pragmatic reasons.

Regarding faeces storage for microbial metabolomics, samples should be kept at 4 °C and extracted within 1–24 h upon collection [89]. However, since this is not always possible, different storage conditions have been assayed with immediate freezing, lyophilisation and the addition of 95–97% ethanol being the most important, emphasising compatibility between the analysis of gut microbiota by 16S rRNA gene-based sequencing and non-targeted and targeted metabolomics analyses [90]. Immediate faecal freezing with liquid nitrogen and storage at −40 °C has been successfully carried out due to the absence of bacteria activity compared to the reduction caused by cooling at 4 °C [91,92]. However, the gold standard for the combined analysis of faecal metabolites and gut microbiota is immediate storage at −80 °C upon collection [93–95].

The addition of antioxidants, such as ascorbate, mercaptoethanol or 2,3-dimercapto-1-propanol has been demonstrated to be a good choice for a number of metabolites including folate [96]. Recently, the preservation of faecal samples for both metabolome and microbiome studies has been performed, with no differences compared to the procedure involving immediate freezing [97]. However, the addition of buffers or stabilisers is not always recommended due to the possible modification of gut microbiota and interference during later analysis [98–100]. Furthermore, other methods have been studied including the use of RNAlater, 95% ethanol solution and commercially available collection systems [98]. However, the possible effect of these preservation systems is still controversial [98,99]. A summary of the options for the optimisation of the pre-processing of faecal samples for microbiome studies was provided by Wu et al. [79]. The general principles for faecal sample processing include avoiding freeze-thaw cycles repeated temperature fluctuation, and short transportation time [79].

Gut microbiota metabolites can be extracted from faeces by several procedures, namely: (i) solid-phase liquid extraction (short-chain fatty acids-SCFAs and low molecular weight amino acids-LMWAAs), liquid-liquid extraction (SCFAs and volatile organic compounds-VOCs), solid -phase microextraction (SCFAs, LMWAAs and VOCs), deconjugation (bile acids-BAs, catecholamines-CAs, folate, phenols), solid-phase extraction (BAs, folate, cyanocobalamin-Cbl, menaquinones-Mkn) and derivatisation (SCFAs, AAs-amino acids, polyamines-PolyAs) [96].

Solid liquid extraction (SLE) is used to obtain the “faecal water”, which contains the gut microbiota metabolites. Since faecal samples contain about 60–80% of water, it can be a source of variability and should be eliminated. Thus, samples are freeze-dried to calculate for accurate quantification of the metabolites [101]. A typical workflow applied for the extraction of gut microbiota metabolites from faeces by SLE can be: weighing, lyophilisation (when possible), homogenisation with solvent extraction, centrifugation, and storage at −80 °C before analysis by high performance liquid chromatography (HPLC), gas chromatography (GC) or supercritical fluid chromatography (SFC) coupled to mass spectrometry (MS), with a preceding derivatisation step, when required [102]. In comprehensive untargeted metabolomics, the selection of solvent extraction is driven by the coverage of a wide number metabolites. In this sense, water [103] sodium hydroxide, methanol [102] and phosphate-buffered saline (PBS) [104] have been used. Extraction with water, allows for the identification of about 100 microbial metabolites including amino acids, fatty acids, hydroxylic acids, carboxylic acids and phenolic acids [103]. Extraction with PBS is recommended for SCFAs and PolyAs [96]. Before centrifugation, the supernatant is usually acidified with perchloric acid (for SCFAs analysis) and trichloroacetic acid (biogenic amines, AAs and PolyAs). The addition of water (cold)-miscible polar solvents (2-propanol, methanol, acetonitrile, ethanol) is recommended for protein denaturation and quenching of metabolic activity [105]. Thus, metabolites have been extracted by SLE from faeces using water followed by centrifugation/filtration and analysis [106], combined with protein precipitation with sulfosalicylic acid, centrifugation and pH adjustment [107], using methanol, incubation and centrifugation [105,108–110], with trichloroacetic acid and diethyl ether [111] and by a two-step extraction with PBS and acetonitrile [112]. Targeted metabolomics has also been applied for 5-HIAA and tryptophan extraction from colon content of piglets by SLE with aqueous methanol and acetonitrile, dried and reconstituted with methanol [113]. In contrast tryptophan and indole metabolites have been determined by SLE with dimethyl sulfoxide (DMSO) and acetonitrile precipitation [114].

Liquid-liquid extraction (LLE) is frequently carried out after ultracentrifugation by adding diethyl ether, tert-buthyl ether (for the extraction of both, short and medium-chain fatty acids) or butanol [96]. After that, the addition of chloroform is usually recommended to remove lipophilic compounds that contain long chain fatty acids and that can influence the derivatisation process, as in the case of esterification of SCFAs [115]. However, the most efficient extraction methods for faecal metabolomics are based on the combination of SLE and LLE, such as, those described for rodents faeces by SLE with PBS followed by centrifugation and LLE with methanol [116], mice ileum, cecum and colon contents by SLE with PBS and LLE on supernatant with $HClO_4$ [117]. Also, the application of SLE with cold chloroform/methanol has been described for tryptamine and indole metabolites [118], while tryptophan, kynurenine, tryptamine and indole 3-acetic acid have been determined by SLE with water and LLE on supernatant with hexane [119].

The most used approach for microbiome studies is microbiome sequencing, which requires a prior extraction of DNA from stool. It is widely accepted that the choice of extraction protocols, especially the addition of a preliminary step of mechanical disruption [120], as well as the commercially available kits for nucleic acid extractions affect the obtained results [121]. Moreover, it has been suggested that each commercial DNA extraction kit has a specific microbial community that is identified as contaminants, known as “kitome”, which are then detected in the sequencing analysis [122]. All of these factors contribute to the observed variability among

Table 1
Analytical techniques for the determination of metals, chemical species and metalloproteins.

Total concentration of metal(-loid)s				
Atomic spectroscopic detectors (*e.g.*, FAAS, FAES, AFS, ETAAS)		ICP-AES	ICP-MS	
Pros				
Low cost Simplicity High experience is not required High precision		Multielemental Rapid High tolerance to dissolved solids	Multielemental Rapid Good control of interferences Allows isotopic dilution analysis for quantification High sensitivity Distribution of elements in tissues (LA-ICP-MS)	
Cons				
Time consuming High sample volume required (not for ETAAS) Poor sensitivity (not for AFS) Single element determinations Limited dynamic range Interferences		Lots of interferences Highly complex spectra Relatively poor sensitivity	High cost of instrument acquisition, operation and maintenance High experience is required Limited tolerance to dissolved solids	
(Metallo-)proteins				
Hyphenated ICP-MS	Western Blot	qPCR	UV/Vis spectrophotometry	ELISA
Pros				
Absolute quantification of elements and chemical species Protein standards are not required Allows for the determination of phosphorylation of proteins High sensitivity High accuracy and precision	Relative quantification Allows for the determination of molecular weight	Targeted relative or absolute quantification of transcripts	Enzymatic activities Quantification of total protein Low cost	Absolute quantification Simplicity High specificity Easily automated It can be used as a diagnostic or forecast method Low sample volume consumption
Cons				
Enzymatic activities cannot be estimated, but it can be combined with metabolomics An heteroatom in the molecule is required	Not absolute quantification Requires specific antibodies for each protein	Possible lack of correlation between transcripts and proteins Only for DNA or RNA	Requires combining multiple preparative techniques High sample volume consuming	The antigen must be well known (purified and isolated) Enzymatic activities cannot be estimated

microbiome studies, and the election of each pre-processing option needs to be investigated and carefully planned in a microbiome study design depending on the research objective.

2.3. Analytical techniques to study metal-microbiota interactions

2.3.1. Heteroatom tagged proteomics and biomolecular analytical techniques

It is noteworthy that approximately one-third of all proteins require metals as cofactors to perform their functions [123–125] and that metals have an influence on more than 50% of the proteins [126]. The metallome refers to the identities and/or quantities of metals/metalloids and their species in cellular compartments, cells or organisms [127]. Thus, metallomics is the research field that elucidates the identification, distribution, dynamics, role, and impact of metals and metalloids in biological systems [126]. The complexity of the biological molecules analysed in metallomic studies is much higher than those studied in classical speciation analysis (*e.g.* selenoproteins in biological fluids *vs* inorganic selenium speciation in soils), and the main pitfall is the absence of standards for the identification, the labile character of the metal(loid)-biomolecule link, and the usually low concentrations of elements in biological samples (in the range of sub-ng g^{-1}). However, undoubtedly, the speciation of elements is mandatory, besides the total content measurement. This chemical species can be defined as "specific forms of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure" [128]. The chemical form of the metal(-loid) is crucial because it determines its toxicity (*e.g.* chromium (VI), methylmercury, inorganic arsenic), essential role (*e.g.* selenomethionine, chromium (III)) or innocuous character (*e.g.* arsenobetaine).

Table 1 shows the most common analytical techniques that can be used for the determination of metals, chemical species, and metalloproteins. As can be seen, the most classical spectroscopic methods such as flame atomic absorption spectroscopy (FAAS), flame atomic emission spectroscopy (FAES) or electrothermal atomic absorption spectroscopy (ETAAS) are simpler, cheap, and highly precise. Atomic fluorescence spectroscopy (AFS) is much more sensitive, and the hyphenation with high performance liquid chromatography is feasible, thus allowing for chemical speciation, but it is only applicable to several elements that are measured independently after hydride generation (*e.g.* antimony, arsenic, bismuth, cadmium, germanium, lead, selenium, tin, tellurium, zinc) or cold vapour technique (mercury) [129]. The inductively coupled plasma with atomic emission spectroscopy (ICP-AES) is multielemental and more sensitive, but the obtained spectra are complex, with many atomic emission lines and interferences. Undoubtedly, inductively coupled plasma mass spectrometry (ICP-MS) is of great importance in metallomics due to its high selectivity and sensitivity at trace and ultra-trace levels that allows the detection of the element in the biomolecule (when ICP-MS is coupled to a separation unit), which is usually at a very low concentration. Moreover, the use of ICP-MS allows for multielemental determinations [16,130] and the absolute quantification of the metallobiomolecule using species-specific isotopic dilution (SSID) or species-unspecific isotopic dilution analysis (SUID), which overcome matrix effects and, problems derived from instrumental

drift and avoid the correction with dilution or preconcentration factors [131]. In isotopic dilution analysis (IDA), the quantification is performed by the addition of known amounts of an isotopically-enriched standard to the sample. When the enriched-isotopic standard is mixed with the sample "dilutes", the isotopic enrichment of the standard and the isotopic ratios are measured. Isotope dilution is classified as a method of internal standardisation, because the standard (isotopically-enriched form of the analyte) is added directly to the sample. In addition, unlike traditional analytical methods, which depend on signal intensity, isotope dilution employs signal ratios [131]. In metallomics, the source laser ablation (LA) coupled to ICP-MS can be used to determine the distribution of elements in thin sections of tissues (*e.g.* brain) or electrophoresis spots after separation of proteins [132]. ICP-MS with reaction/collision cell is highly recommended due to elimination of the polyatomic interferences (*e.g.*, $^{40}Ar_2^+$ and $^{79}Br^1H^+$ for selenium) [133,134].

The quantitative dimension is very important in life sciences research since any other biomolecular technique does not easily achieve it. Thus, as can be seen in Table 1, hyphenated ICP-MS (coupled to HPLC or capillary electrophoresis) allows for the determination of chemical species and metalloproteins using the heteroelement (an atom different to C, H, N, O or F, *e.g.* selenium) in the biomolecule as a "tag" (heteroatom tagged proteomics) [135]. As the determination of the metal-containing molecule is performed by the measurement of the element, using a generic standard for this element, protein standards are not required for the absolute quantification of the biomolecule, and only the number of heteroatoms in the molecule should be known (*e.g.* selenoprotein P has 10 selenium atoms) [136]. This is important because protein standards are usually not available, or the purity assessment is not easy. This approach, based on the intact protein analysis by hyphenated ICP-MS is more sensitive than the typical proteomic approaches that involve tryptic digestion and further analysis of peptides that are usually difficult to separate [137]. A shortcoming of the hyphenated ICP-MS can be the possible loss of the metal(-loid) from the metallobiomolecule during sample preparation or chromatographic separation. This is not the case of selenoproteins in which Se is covalently bond to the protein, but when the metal(-loid)-biomolecule bond is labile, special care should be taken to keep the union.

Western blot analysis, which is based on the use of an antibody that binds to the protein, only allows for relative quantification, while quantitative polymerase chain reaction in real time (qPCR) quantifies the amount of an expressed gene by determining its transcript copy number [138–140]. Enzyme-linked immunosorbent assay (ELISA) allows for absolute quantification, but the antigen must be well known (purified and isolated), and the precision and accuracy are poor and far from that of hyphenated ICP-MS. Nonetheless, immunoassays are widely used in the clinical laboratory, either because no other type of assay system is feasible or because they are often the simplest and most suitable analytical methods. The major trend has been to avoid the use of liquid-phase assays involving radioisotopic labels to select fast, homogeneous, or solid-phase assays capable of operation anywhere, as well as precise and reliable nonisotopic, automated or semi-automated laboratory assays, often with detection limits measured in pico- or attomoles [141,142]. UV–Vis spectrophotometry is a low cost analytical technique that allows for the determination of the total content of proteins in a sample and the enzymatic activities [143]. The determination of the enzymatic activity is very important because the cell does not necessarily synthesise more enzymatic units or degrade existing ones in response to a stimulus, which will be slow and energy-consuming. In this case, when the cell might provide a rapid response, it simply activates or desactivates

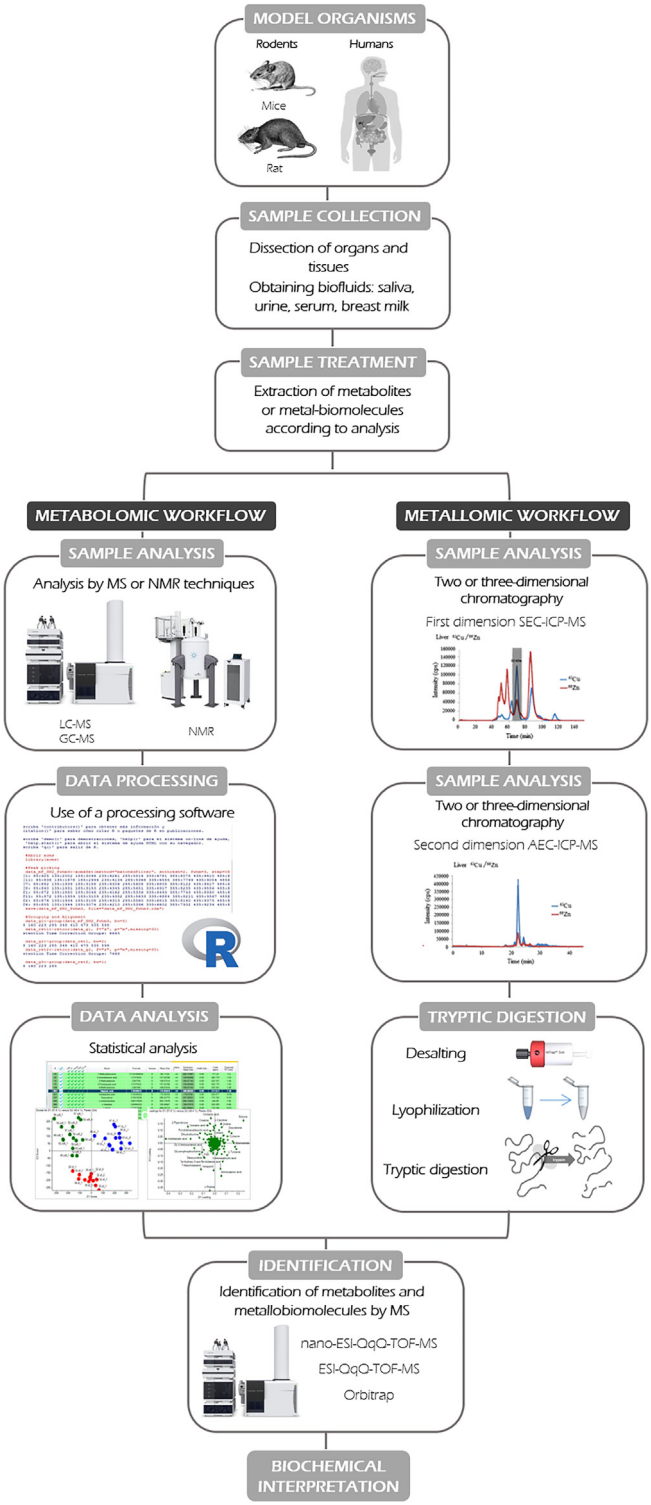

Fig. 2. Workflow for metallomic and metabolomic analysis.

enzymes existing according to their metabolic needs, being the phosphorylative pathway one of the most used [144]. The activity of a metalloenzyme cannot be measured by hyphenated ICP-MS, but it can be combined with metabolomics that is a most powerful analytical approach. ICP-MS also allows for the determination of protein phosphorylations using the phosphorous element as a "tag" in ICP-MS [145].

In metallomic studies, a two or three-dimensional approach is used (Fig. 2). After selection of the model organism (*e.g.* cellular systems, environmental biomarkers) and the samples (tissues, faeces, biofluids), the metal-containing molecules would be extracted following specific protocols adapted to the organisms and sample-type. After sample treatment, the first step of analysis usually involves the use of size exclusion chromatography (SEC) coupled to an elemental detector, such as, ICP-MS, for the sensitive and selective detection of the metallobiomolecules using the metal as a "tag". Several sequential and orthogonal chromatographic steps can be applied to the eluted fractions of interest to purify the metallobiomolecules and to increase the low resolution of SEC (*e.g.* anion exchange chromatography (AEC), hydrophilic interaction liquid chromatography (HILIC), cation exchange chromatography (CEC), reversed phase (RP), etc). In addition, electrophoresis can be used as a separation step instead of chromatography [146]. Selenium and its diverse species are among the most studied elements, due to their important biological roles, and specific metallomic methodologies have been developed to study Se-containing biomolecules such as selenoproteins.

In this sense, the combination of different chromatographic steps can be carried out in only one chromatographic run using a column switching system as the proposed method for determining of selenoproteins and selenometabolites in human serum [147] and breast milk [10]. Organic mass spectrometry (*e.g.*, electrospray ionization quadrupole time of flight mass spectrometry (ESI-QTOF-MS)) is usually applied for the unequivocal identification of the metallobiomolecules, after desalting, lyophilisation and tryptic digestion in the case of metalloproteins and the structure is elucidated using a database search (Fig. 2A). Metallometabolites (*e.g.* dimethylarsinic acid (DMA), selenomethionine (SeMet)) can also be identified by organic mass spectrometry and the biochemical interpretation is performed by metabolomics pathway analysis.

In the field of metal-gut microbiota interplay, ICP-MS is the most widely used technique. It has been applied to heavy metals [57], arsenic [31,36,61], lead [46,65] and selenium [17,18]. In contrast, atomic absorption spectroscopy (AAS) has been used for the determination of arsenic [62], copper [53], mercury [52,63] and cadmium [43,44]. ETAAS has been applied to cadmium determination in mice serum and tissues and correlated with gut microbiota [42,75,76]. Due to the reduced number of elements that can be measured by AFS, it has only been applied in a few studies related to selenium [19] and mercury [57] in connection with gut microbiota.

2.3.2. Analytical approaches for metabolomics and microbial metabolomics

Metabolomics, the last step in the omics cascade and closer to the phenotype, was defined by J. Nicholson in 1999 as the measurement of all the metabolites (molecules <1500 Da) in a specified biological sample [148]. The approach is characterised by the following series of steps (workflow) [149] (Fig. 2): (i) the selection of a statistically significant number of biological samples, usually tissues, biofluids or faeces; (ii) sample treatment for metabolites extraction, depending on the subsequent analysis and type of sample; (iii) targeted or untargeted metabolomic analysis; (iv) data processing, to reduce complexity; (v) data analysis, using statistical tools to separate groups, (vi) identification of metabolites as biomarkers by combining databases and tandem mass spectrometric identification; (vii) metabolomic pathway analysis and interpretation of the results. This workflow can easily be integrated with other omic methodologies. For example, metabolomics can easily be integrated with metallomics to study metal containing metabolites or the metabolic impairments caused by a xenobiotic [27]. Microorganisms are a good model for conducting systems biology studies since they are easy to handle and have key human health roles. Microbial metabolomics can integrate biological information into systems microbiology to understand microbial interactions and cellular functions. The analytical approaches for metabolomics can also be integrated with those for metataxonomics, combining the sample extraction procedures, as commented in section 2.2.

Regarding the analytical techniques, nuclear magnetic resonance (NMR) and mass spectrometry (MS) are the most frequently used in metabolomics. The former allows higher reproducibility of the results and precise quantification of complex mixtures. It is a non-invasive technique, but its main shortcomings are low sensitivity and high cost. By contrast, organic MS presents high sensitivity, specificity, wide metabolite range coverage, selectivity, and the possibility of coupling to CE, liquid chromatography (LC) and gas chromatography (GC). There is now a timely possibility to unravel some unanswered challenges due to the advancements of these techniques and bioinformatic analysis [77]. The compassion of the different analytical techniques that can be used in metabolomics has been previously reviewed in detail [77].

Metabolomics has a pivotal importance in the assessment of both, the relationship between the host and its gut commensal microbiota [150] and the risk of environmental pollution [151]. In this case, non-targeted metabolomics has been demonstrated to be more effective than other traditional hypothesis-led approaches [152]. However, the measurement of many metabolites related to brain impairments and environmental pollution would be crucial to validate results.

Considering the gut microbiota-brain axis, the most important metabolites are neurotransmitters, amino acids and related compounds. An important amino acid is γ-aminobutyric acid (GABA), which is a neurotransmitter in the central nervous system. Several neurohormones have also been identified in faecal metabolomics analysis, such as acetylcholine, 5-hydroxytryptamine (5-HT, serotonin), noradrenaline and dopamine (DA). The hypothesis is that these metabolites interact with the nervous system through several routs including endocrine, immune, neural, vagus nerve, and metabolic pathways [96]. Thus, gut microbiota produces substances such as tryptophan related metabolites, kynurenic acid, short chain fatty acids, neurometabolites, GABA, noradrenaline and dopamine which potentially target and influence our central nervous systems [96].

Several metabolomic methods have been proposed for the determination of amino acids and related metabolites in faeces. The detection system most frequently used is mass spectrometry, due to its capability to obtain mass spectra and its unequivocal identification of analytes. MS has been frequently coupled to HPLC [118] or UHPLC [105,108–110,113,114], offering higher resolution. However, a number of works have used HPLC with fluorescence detector (FLD), which is more sensitive, but unequivocal identification is not possible [112,116]. Few works describe the use of an amino acid analyser [106,107], HPLC-ECD (electrochemical detector) [117,119] or capillary electrophoresis coupled to laser induced fluorescence (CE-LIF) [111].

In connection with metals, metabolomics and gut microbiota, HPLC-MS has been the most widely used technique for metabolomics in mice exposed to arsenic [70], cadmium [45] and in rats in relation to copper [54] manganese [56] and iodine [55]. NMR has been applied in rats in relation with the lead-gut microbiota interplay [72].

2.3.3. Technical approaches for microbiota analysis

The study of human microbiota could be addressed by different technical approaches depending on the research objectives that can also be integrated with metabolomics (microbial metabolomics) (Table 2).

Table 2
Technical approaches for microbiota analysis.

Metataxonomics/Metagenomics - Who/what is there?		
Pros	*Cons*	Approaches/Techniques
Offer taxonomical information	Only targeted amplification and qPCR: biased by PCR amplification	Sanquer sequencing
Cheaper than other omics approach (specially targeted amplification)	Only metagenomics: required more bioinformatics than other approaches	Targeted amplification (16S rRNA)
Only metagenomics: permit the assessment of functional capacity		
Higher number of bioinformatics tools available	No information about bacterial viability and activity	Metagenomics
Only qPCR: offers quantification of targeted data	Sensitive to contaminant and DNA degradation	Quantitative PCR (qPCR)
Metatranscriptomics - What they can do?		
Pros	*Cons*	Approaches/Techniques
Provided information of gene expression and viability	Elevated cost Experimental issues (instability of RNA and enzymatic degradation)	Microarrays
Study of function and metabolic acitivity of microbiota	Poor database quality	qPCR
High sensitivity and repeability compared to metagenomic	Sensitive to host RNA: difficult in data analysis Poor predictor of protein level	RNA-seq with NGS
Metaproteomics- What are they doing?		
Pros	*Cons*	Approaches/Techniques
Great amount of data generated	Still expensive techniques	Nuclear magnetic resonance spectroscopy (NMR)
Only metaproteomics: permit taxonomy, function and metabolic pathway identification	Complex analysis Poor database quality	High resolution mass spectrometry (MS)
Global metabolite/proteomic profile or measurement of specific metabolites (targeted)	Problems in low peptide identifications	hyphenated with LC, GC and CE

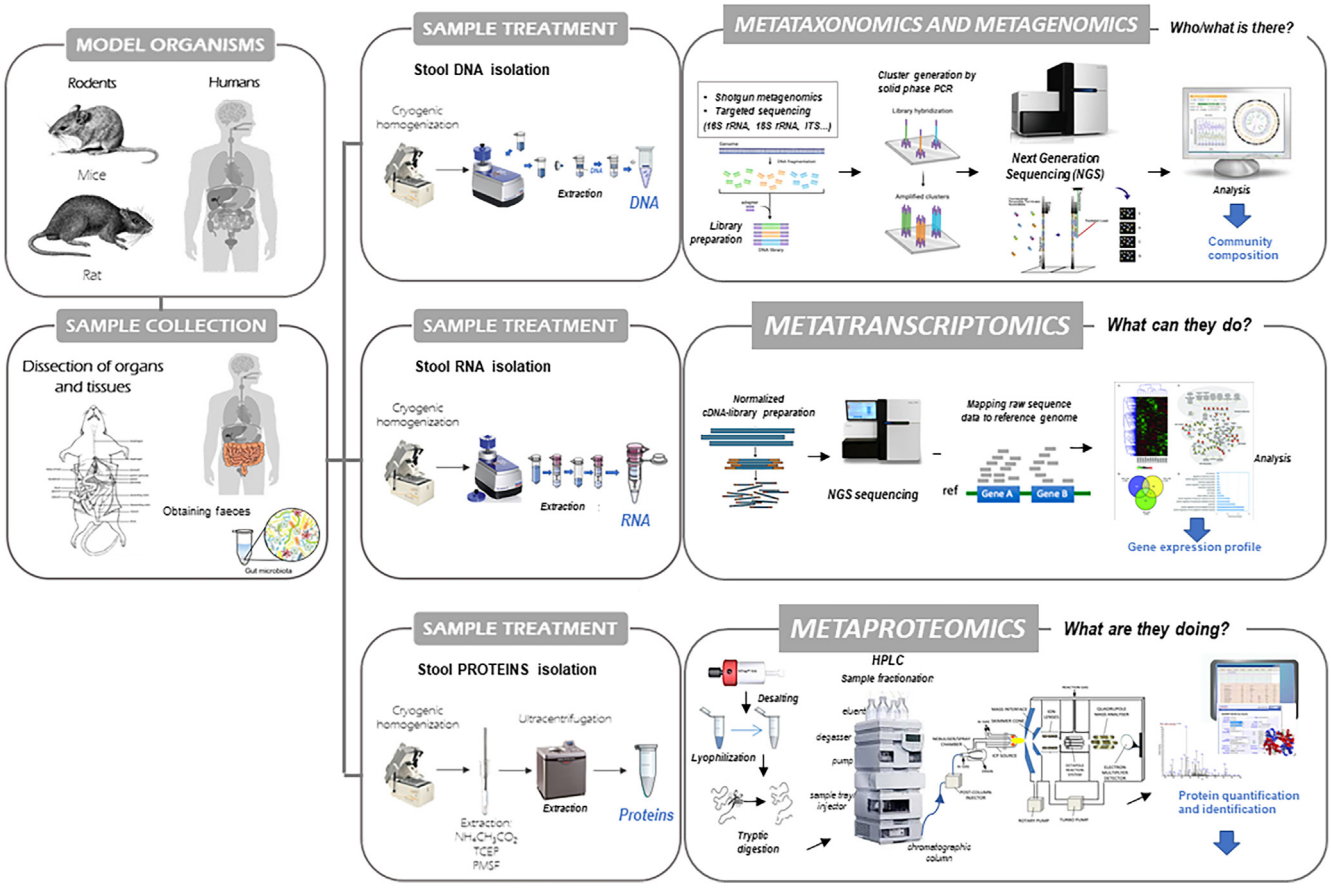

Fig. 3. Workflow for microbial analysis: metataxonomics, metagenomics, metatranscriptomics and metaproteomics.

The combination of these technologies is of great interest to answering the following questions: "who/what is there?" (metataxonomics/metagenomics); "what can they do?" (metagenomics/metatranscriptomics); and "what are they doing? (metaproteomics and metabolomics) (Fig. 3).

2.3.3.1. Microbiota studies: from traditional methodologies to next generation sequencing. The traditional microbiological methods for culturing human bacteria have usually been employed to isolate and identify some bacteria; however, there are species and strains that require complex culture conditions that are difficult to reproduce in a synthetic culture grown media or, some strains are not culturable [153]. During recent decades, microbial analysis methods are mainly based on next generation sequencing (NGS) techniques, which, together with bioinformatics pipelines and tools, have increased knowledge of microbial communities in a reliable and fast way [154]. Targeted amplicon sequencing, mainly targeted to a specific region of the hypervariable 16S rRNA gene [155], represents the current standard for microbial communities profiling. Several platforms with different methodologies have been used in the last decade, from Roche 454 pyrosequencing, sequencing by ligation SOLID platform, sequencing by synthesis Solexa platform, Illumina systems such as HiSeq and MiSeq, Ion Torrent from Life Technologies and the recently Nanopore sequencing with MinIon, PacBio and Heliscope [156]. Metagenomic analysis or "shotgun sequencing" is based on the direct DNA sequencing from the sample without the previous PCR amplification requirement reducing the potential PCR-amplicon bias [157]. It should also be noted that DNA sequencing techniques have some limitations and particularities, such as the microbial viability cannot be analysed, total bacteria counts would be over- and/or underestimated because cell-wall composition and DNA extraction method and also, by microbial 16S gene copy numbers. In addition to these problems, DNA contamination from laboratory personnel and molecular biology reagents have been reported [158]. To study microbial viability, RNA-based methods can be used [159] as well as DNA-based methods based on the discrimination of viable microbes from the total community by using specific compounds such as propidium monoazide (PMA), which intercalates into double-stranded DNA, preventing it from being amplified by PCR and removing free DNA from dead/non-viable microbes [160].

Metatranscriptomics informs us about the global expression of RNA by the microbial community ("what can they do"), providing a view of the putative active functional profile of a given sample at a given moment under specific conditions. This approach can also be used to estimate the taxonomic composition of the microbial population and to identify novel sites of transcription and/or translation from microbial genomes. In the last decade, dozens of studies have demonstrated that disease, dietary interventions and xenobiotics [161,162] significantly alter the microbial gene-expression profile.

2.3.3.2. Metaproteomics. Although, metagenomics undoubtedly highlights the importance of microbiome gene expression profiling to understand the microbiome response to the environment, it is important to gain deep information about important microbiome functions and the microbiota/host relationship, and metaproteomics plays a big part. This technique quantifies the sum of all proteins of a cell, tissue, or organism under a particular condition, including their structure and physiological functions, and accounts for protein abundance, localisation, posttranslational modifications, isoforms, and molecular interactions [163], helping to determine the structure function (activity, "how are they doing it"), and dynamics of the gut microbiota, providing information about the true functionality of the gut microbiota [164].

Metaproteomics relies on MS to provide information on the metabolic and signalling pathways operative in the gut microbiota. However, metaproteomics faces important limitations, such as the lack of suitable metaproteomic computational workflows or the insufficient resolution of MS to measure low-abundance proteins present in the complex microbial communities [165].

Protein extraction procedure can be accomplished by direct cellular lysis of faecal material, protein precipitation and clean-up processes. This procedure limits the depth of microbial proteome measurement because of the presence of highly abundant host proteins [166].

The protein extraction procedure markedly impacts metaproteomics results. A recent study recommends mechanical cell disruption and extraction protocols combining sodium dodecyl sulfate (SDS) and ultrasonication for complete protein extractions for metaproteomic analysis [167]. Protein extracts from microbiota samples are extremely complex and several procedures (desalting, fractionation, labelling, etc) are usually included in the metaproteomics workflow to increase protein/peptide identifications. Before the widespread use of shotgun methods, metaproteomic studies used 2-dimensional gel electrophoresis (2-DE) for protein fractionation according to the *isoelectric point* and molecular weight. This process is laborious and suffers from low throughput, limited dynamic range, and bias against membrane proteins [168]. 2-DE has gradually been supplanted by LC to fractionate the peptide mixtures obtained after proteolytical digestion of the protein extract to simplify the sample complexity before a mass spectrometric measurement [168]. The combination of LC and tandem mass spectrometry (LC-MS/MS) has become the preferred method for the simultaneous identification and quantification of proteins in stool samples.

Instrument sensitivity, mass accuracy and resolution, protein coverage and dynamic range affect LC-MS data in any proteomic study. These factors become serious challenges in metaproteomics as it faces the need to quantify low-abundance proteins of complex microbial communities. The accurate protein identification of microbiota requires knowledge of its taxonomic composition and, undoubtedly, the availability of databases containing exactly those sequences, since small changes in the protein sequence between related microorganisms will impact protein identification [169].

Usually, metaproteomics studies rely on the estimation of the protein amount by counting identified peptides or spectra and normalising these results. Mass spectrometry measurements needs internal calibration to convert arbitrary signal intensities in protein quantities. The use of internal standards, an exogenous protein, or peptides spiked into the experimental sample itself, introduces quantitative biases that affect comparison across studies. External standard reference materials, in contrast, enhances the precision and standardisation of measurements [170]. Even so, these approaches are inaccurate and possess a small dynamic range, since they depend on the data-dependent selection of precursor ions and successful peptide identification.

3. Applications related to metal(-loid)-gut microbiota interplay

Limited data is available on the impact of metal(-loid)s on gut microbiota in animal and human studies. Most of the evidence is focused on arsenic, cadmium, copper, lead and mercury. They represent the main toxic compounds that humans are exposed to through diet and environment. Different studies have shown associations between specific bacterial composition and toxic metals or metalloids (*e.g.* arsenic) after exposure or supplementation with essential elements (*e.g.* selenium and iodine) (Table 3). In the majority of these studies, metal(loid)s not only affected the bacterial composition, but also the diversity and richness of gut microbiota.

Remarkably, an integrated analytical approach based on metagenomics and metabolomics has been proposed to demonstrate that arsenic exposure to mice perturbs gut microbiota and plasma metabolites [70]. Another study combining microbiome analysis with fecal metabolomics in mice also demonstrated that arsenic

Table 3
Studies related with the metal(-loid)-gut microbiota interplay. ASD: autism spectrum disorder, HMs: heavy metals, AAS: atomic absorption spectroscopy, ALP: alkaline phosphatase, ALT: alanine aminotransferase, AST: aspartate aminotransferase, FFA: free fatty acid, GF-AAS: graphite furnace-atomic absorption spectroscopy, GLU: glucose HDL: high density lipoproteins, IL: interleukin, LDL: low density lipoprotein, LPS: lipopolysaccharide, SCFAs: short chain fatty acids, sIgA: secretory immunoglobulin A, TC: total cholesterol, TG: triglycerides, TGF- β: transforming growth factor beta, TNF-α: tumor necrosis factor-alpha.

TOXIC OR ESSENTIAL ELEMENT	MODEL ORGANISM	GUT BACTERIA	CORRELATIONS	REFERENCE
Pb, Cd, As, Cu, Zn, Fe, Hg, Ca, Mg	Humans	**ASD children:** ↑ *Bacteroides, Parabacteroides, Sutterella, Lachnospira, Bacillus, Bilophila, Lactococcus, Lachnobacterium* and *Oscillospira* genus	Carbon fixation pathways and the citrate cycle were positively associated with *Bacteroides*, *Oscillospira* and *Sutterella*. Ether lipid metabolism and sporulation were negatively related to *ParabacteroidesLachnobacterium, Oscillospira* and *Parabacteroides* have a relatively positive correlation with the level of As. *Oscillospira, Parabacteroides* and *Sutterella* are positively correlated with the level of Hg	[57]
As, Cd, Cu, Pb, Zn	Humans	**HMs exposure:** ↑ *Lachnospiraceae, Eubacterium eligens, Ruminococcaceae UGG-014, Erysipelotrichaceae UCG-003, Tyzzerella 3, Bacteroides, Slackia, italics,* and *Roseburia* ↓ *Prevotella 9*	–	[58]
–	Humans	↑ Hg parameters ↑ *Faecalibacterium, Lachnospiraceae_nk4a136, Megasphaera, Ruminococcaceae_ucg013, and Parabacteroides* genus	54% of the correlations between phyla and Hg parameters changed direction of association between early and late gestation. Early gestation: Associations (positive/negative) between Hg parameters and *Lachnoclostridium, Collinsella, Ruminococcaceae_ucg002, Ruminococcaceae_ucg013, Prevotella_9*	[64]
$HgCl_2$	Mice	**Cecal portion:** ↑ [Hg] ↑*Bilophila, Coprococcus, Dehalobacterium, Oscillospira* and *Butyricimonas* ↓ *Staphilococcus, Jeotgailcoccus, Acinetobacter* and *Sporosarcina* **Rectal portion:** ↑ [Hg] ↑ *Helicobacter, Oscillospira, Butyricimonas, Streptococcus, Dehalobacterium* and *Coprococcus* ↓*Lgnatzschineria, Salinococcus, Jeotgailcoccus, Staphylococcus, Corynebacterium, Lactococcus* and *Bacillus* genus	Acidobacteria, Bacteroidetes, Cyanobacteria, Firmicutes and Proteobacteria phyla were signigicantly correlated with diverses apoptotic genes (caspase3, TNF-α, ASK1, Bax and JNK)	[37]
$HgCl_2$	Mice	↑[Hg] ↑ *Butyricimonas, Dehalobacterium, Coprococcus, Oscillospira* and *Bilophila* ↓ *Sporosarcina, Jeotgailcoccus, Staphylococcus* and *Acinetobacter* genus	–	[38]
$HgCl_2 + CuCl_2$	Mice	↑ [Hg + Cu] ↑*Tenericutes, Dehalobacterium, Coprococcus, Anaeroplasma* ↓ *Sporocarcina, Jeotgailcoccus, Staphylococcus* and *Acinetobacter* genus	–	[38]
α-HgS	Mice	**α-HgS:** ↑Firmicutes, Proteobacteria, *Rikenellaceae, Rikenellaceae_massiliensis, Clostridium perfringens, Lachnospiracea_Ruminococcus, Peptococcaceae_rc4-4, Erysipelotrichaceae_dolicum, Verrucomicrobiaceae_Akkermansia, Coriobacteriaceae-Adlercreutzia* ↓ *Bacteroidetes*	–	[39]
β-HgS	Mice	**β-HgS**: ↑*Streptococcaceae_garvieae, Clostridiales, Coprococcus* and *Ruminococcus*, Proteobacteria *(Neisseriaceae, Rickettsiales, Enterobacteriaceae*) *Deferribacteraceae* (*Mucispirillum*) ↓Bacteroidetes	–	[39]
$HgCl_2$	Mice	**$HgCl_2$:** ↑Bacteroidetes (*B. uniformis*), *Porphyromonadaceae (P.gordonii),Ruminococcaaceae* (*R.flavefaciens*), *Erysipelotrichaceae*_p-75-a5, Proteobacteria ↓ Firmicutes	–	[39]
MeHg	Mice	**MeHg:** ↑*Prevotella, Odoribacter, Mollicutes, Erysipelotrichaceae, Lactobacillus, Fusobacterium, Alcaligenaceae* ↓Firmicutes	–	[39]
$HgCl_2$	Rats	**Hg group:** ↓total aerobic, anaerobic and lactic acid bacteria. ↓ *L. plantarum* and *B. coagulans* counts **Hg + probiotic feeding:** ↑ total aerobic, anaerobic bacteria and lactic acid bacteria. ↑ *L. plantarum* and *B. coagulans* counts	Hg accumulation affects the gastrointestinal tract and impaired the gut barrier. The use of synbiotics decrease the repressive effect on population of gut bacteria.	[52]
THg MeHg	Humans	**Phyla composition:** Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Verrucomicrobia **Genera:** *Bifidobacterium, Bacteroides, Alistipes, Subdoligranulum, Blautia, Lachnospiraceae*, *Akkermansia* spp.	Hair THg and cord blood MeHg are highly correlated. Stool MeHg is positive correlated with cord blood MeHg. *Lachnospiraceae* and *Moryella* are inversely correlated with stool IHg. *Clostridiales, Erysipelotrichaceae, Faecalibacterium, Peptostreptococcaceae, Ruminococcaceae* and *Turicibacter* are positive correlated with Hair THg. *Akkermansia, Lachnospiraceae* and *Ruminococcaceae* are positive correlated with Stool MeHg and *Streptococcus* is inversely correlated. *Subdoligranulum* is positive correlated with stool MeHg and hair THg Microbial richness is significantly higher for mothers with high stool MeHg	[63]
iAs	Mice	**As group:** ↑ Bacteroidetes ↓ Firmicutes	Bacteroidetes phylum and other, members from Firmicutes phylum as Bacillales order, were positive correlated with iAsV and negative correlated with DMAs *Clostridiales, Dehalobacteriaceae*; and *Ruminococcaceae*	[31]

(*continued on next page*)

Table 3 (*continued*)

TOXIC OR ESSENTIAL ELEMENT	MODEL ORGANISM	GUT BACTERIA	CORRELATIONS	REFERENCE
			families were negative correlated with iAsV and positively with DMAs	
As_2O_3 +/-$FeCl_3$	Mice	**As, Fe As + Fe treated mice:** ↑ Firmicutes, Tenericutes, Proteobacteria ↓ Bacteroidetes and TM7 phyla **As-treated mice:** ↑ *Acidobacteria* and *Cyanobacteria/ Chloroplast* genus Fe, Fe + As treated mice: ↑ Verrucomicrobia phylum *Lactobacillus* genus ↑ in Fe + As treated mice ↓ in Fe-treated and As-treated mice *Barnesiella* and *Bacteroides* genus ↓ in Fe-treated and As-treated mice	–	[32]
$NaAsO_2$	Mice	↑ [As] ↑ Bacteroidia ↓Clostridia	–	[33]
–	Mice	**As- treated females:** ↑*Akkermansia.* ↓*Dorea* genus **As-treated males:** ↑*Dorea* genus	As-treated female mice are correlated with metabolic pathways involved in metal resistance (including mercury resistance operon, zinc resistance, and the mdtABCD multidrug resistance cluster) and cell transport system (such as glutathione regulated potassium-efflux system, ATP-dependent efflux pump transporter Ybh, general secretion pathway, and iron acquisition in *Streptococcus*. As-treated male mice were associated with nitrogen, carbon, and sulfate metabolism	[34]
$NaAsO_2$	Mice	**As group:** ↑ *Bifidobacterium*, *Akkermansia* and *Anaerostipes* ↓ *Lactococcus, Ruminococcus, Coprococcus, Dorea, Oscillospira,*	–	[35]
–	Humans	**High As group:** ↑ Proteobacteria phylum	↑Proteobacteria ↑ As concentration in drinking water samples	[60]
$NaAsO_2$	Mice	**Single and repeated dose of as:** ↑ *Bilophila* ↓ *Intestimonas* genus **Postnatal mice single dose:** ↑ *Butyricicoccus, Phyllobacterium* and *Parasporobacterium* genus	–	[36]
$NaAsO_2$	Rats	**Low dose As:** ↓ *Turicibacteriaceae, Verrucomicrobiaceae, Erysipelotrichaceae, S24-7* families **Medium dose As:** ↑ *Porphyromonadaceae, Streptococcaceae* ↓ *Bifidobacteriaceae, Corynebacteriaceae, Lactobacillaceae, Verrucomicrobiaceae* families **High dose As:** ↑ *Porphyromonadaceae, Enterobacteriaceae, Lachnospiraceae, Ruminococcaceae, Bacteroidaceae, Peptococcaceae* ↓ *Bifidobacteriaceae, Corynebacteriaceae, Lactobacillaceae, Verrucomicrobiaceae, Eryspelotrichaceae, Turicibacteraceae, Clostridiaceae*	–	[50]
–	Humans	**High As exposure:** ↑ *Streptococcus*, Lachnospiraceae, ↓*Clostridiaceae, Bacteroides, Bifidobacterum and Lactobacillus.*	↑urinary As ↑*Ruminococcus* ↓ *Clostridiaceae Bacteroides, Bifidobacterium*	[61]
–	Humans	**High As content in urine:** ↑ *Bacillaceae*, *Collinsela* **Moderate As content in urine:** ↑ *Lactobacillus*	↑**As concentration in urine:** ↓*Catenibacterium, Ruminococcus, Clostridiaceae*, *Haemophilus, Luteimonas*, Proteobacteria, *Desulfovibrionaceae, Bilophila, Succinovibrio and* ↑ *Bacillaceae*	[62]
$NaAsO_2$	Mice	**As-treated group:** ↑ *Acetivibrio, Peptococcaceae, Clostridiales incertaesedis_XIII, Bacillales, Bacteroidia, Rhizobiales, Burkholderiales* ↓ *Lachnospiraceae, Ruminococcaceae, Clostridiaceae, Veillonellaceae, Enterobacteriacea, Sutterellaceae, Anaeroplasmataceae*	*Clostridium_XIVa, Marvinbryantia* and *Eisenbergiella* presented signnificant changes in the number of metabolites interactions	[45]
$NaAsO_2$ + NaF	Rats	**As group:** ↑ *Barnesiellaceae*, *Peptostreptococcaceae Desulfovibrionaceae, Helicobacteraceae* and *Prevotellaceae* ↓ *Lactobacillaceae* families **As + F:** ↑ *Muribaculaceae* ↓ *Clostridiaceae*, *Peptostreptococcaceae* families	*Clostridium sensu stricto 1* and an unclassified genus in family *Lachnospiraceae* positively correlated with latency to first reaching the platform. *Coprococcus 3*, *Romboutsia* and *Adlercreutzia* were positively correlated in terms of residence time spent at the target quadrant, but *Phascolarctobacterium*, *Muribaculaceae* and *Peptococcaceae* were negatively correlated. *Clostridium sensu stricto* 1 was positively correlated with number of times crossing the platform, while *Erysipelotrichaceae UCG*-003 had an inverse correlation time spent at the target quadrant, showed significant positive correlation with four genera, which were *Adlercreutzia, Clostridium sensu stricto 1, Coprococcus 3, Romboutsia* and were inversely correlated with *Desulfovibrio*, *Phascolarctobacterium* and *Ruminococcaceae 4* and *Muribaculaceae.*	[51]
$NaAsO_2$	Mice	**As group:** ↑ Bacillales, *Clostridiales* Family XIII *incertae sedis* ↓ Cyanobacteria and Tenericutes phyla; Clostridiales order, and the families *Catabacteriaceae, Clostridiaceae, Erysipelotrichaceae*	↑Indolelactic acid correlated with ↑Tenericutes phyla and *Erysipelotrichaceae* family and ↓*Clostridiales* Family XIII. ↑Daidzein ↑Firmicutes phylum; and *Clostridiaceae* family. ↑ Phenylpyruvic acid, indole-3-carbinol and glycholic acid ↑ Cyanobacteria phyla and, *Clostridiaceae* family. ↑Dihydrodaidzein↓ *Clostridiales* Family XIII incertae sedis	[70]
$CdCl_2$	Rats	**Cd group:** ↓total aerobic, lactic acid and anaerobic bacteria. ↓ *L. plantarum* and *B. coagulans* counts		[52]

Table 3 (*continued*)

TOXIC OR ESSENTIAL ELEMENT	MODEL ORGANISM	GUT BACTERIA	CORRELATIONS	REFERENCE
		Cd + probiotic feeding: ↑ total aerobic, anaerobic and lactic acid bacteria. ↑ *B. coagulans* and *L. plantarum* counts	Cd accumulation cases impairments in the gut barrier affecting the gastrointestinal tract. The use of symbiotic decrease the repressive effect on population of gut bacteria.	
$CdCl_2$	Mice	**Cecalmicrobiota:** ↑[Cd] ↑*Lactobacillaceae, Coriobacteriaceae* and *Eryspelotrichaceae*↓ *Lachnospiraceae* **Fecal microbiota:** ↑ [Cd] ↑*Eryspelotrichaceace* and *Clostridiaceae* ↓ *Lachnospiraceae*	–	[41]
$CdCl_2$	Mice	↑ [Cd] ↑ *Sphingomonas*↓ *Bifidobacterium* and *Prevotella* genus	Low Cd exposure-induced gut microbiota alterations contributes to disorder in hepatic lipid metabolism and accumulation of fat in adults mice	[74]
$CdCl_2$	Mice	**Cd-treated group:** ↑ *Alistipes, Odoribacter,* ↓ *Tenericutes, Verrucomicrobia (Akkermansia* genus*), Rominococcaceae*	–	[40]
$CdCl_2$	Rats	**Cd-treated groups:** ↑ *Bifidobacterium, Akkermansia, and Allobaculum* ↓ *Ruminococcus* genus	–	[50]
$CdCl_2$	Mice	**Acute Cd exposure:** ↑ *Lactobacillus, Bacteroides* ↓ *Turicibacter, Akkermansia* **Chronic Cd exposure:** changes in *Cryocola, Ruminocaccus, Anaeroplasma* and an unknown genus in F16 family of TM7 phylum	–	[75]
$CdCl_2$	Mice	**Cd-treated group:** ↑ Firmicutes, Proteobacteria ↓ Bacteroidetes phylum	↑*Akkermansia mucinipbila* ↓intestinal permeability	[42]
CdTe QDs	Mice	**Cd-treated group:** ↑ *Bacteroides, Alloprevotella*↓ *Proteobacteria, Roseburia* genus **Low [Cd] exposure:** ↑ *Deferribacteres, Bacteroidales S24-7, Odoribacter* ↓ *Lachnospiraceae, Ruminococcacea families and, Lactobacillus* genus **High [Cd] exposure:** ↑ Firmicutes, and Bacteroidetes phyla	A negative correlation was obtained between the Firmicutes/ Bacteroidetes ratio and LDL, TC and TG levels while the correlation was positive with HDL. ↑*Bacteroidales* S24-7 and *Lactobacillus*, ↑LDL, TC and TG ↑*Lachnospiraceae*. *Bacteroides* and *Alloprevotella* ↓IL-12, IL-6,sIgA and TNF-α ↑IL-10. ↑*Roseburia* genus ↑IL-12, IL-6, TNF-α and sIgA, ↓IL-10	[73]
$CdCl_2$	Mice	**Cd-treated groups:** ↑ Erysipelotrichia*Bacteroidales_S24-7_group, Erysipelotrichaceae, Bifidobacteriaceae,), Lactobacillus, Turicibacter*↓ *Clostridiales, Lachnospiraceae, Ruminococcaceae, Bacteroide*s, *Unclassified Coriobacteriaceae, Ruminococcaceae_UCG-014, Ruminiclostridium*	–	[69]
$CdCl_2$	Mice	**Cd-treated group:** ↑ *Bacteroidales, Candidatus_Saccharibacteria, Acidaminobacter, Anaerosporobacter* ↓ *Lachnospiraceae, Ruminococcaceae, Clostridiaceae, Peptostrepcoccaceae, Gracillibacteraceae, Peptococcaceae, Erysipelotrichaceae, Veillonellaceae, Proteobacteria, Deferribacterales, Tenericutes, Actinobacteria*	*Eisenbergiella, Anaerostipes, Syntrophococcus, Oribacterium, Hespellia, Clostridium_XIVa* and *Blautia* presented signnificant changes in the number of metabolites interactions	[45]
$CdCl_2$	Mice	**Cd-treated group:** ↑ Verrucomicrobia	-	[76]
$CdCl_2$	Mice	**Cd-treated group:** ↓ Firmicutes and Bacteroidetes, phyla and also, ↓ *Bifidobacterium* and *Lactobacillus* genus	Cd exposure disturbs the capabilities of the microbiome, especially with respect to SCFAs	[44]
$CdCl_2$	Mice	**Cd-treated group:** ↑ Bacteroidetes (*Paraprevotellaceae*) ↓ Firmicutes and Proteobacteria (*Helicobacteraceae*)	–	[43]
$CuCl_2$	Mice	↑[Cu] ↑ *Corynebacterium* ↓ *Rikenella, Odoribacter, Jeotgailcoccus, Staphylococcus* genus	–	[38]
$CuSO_4$	Mice	Cu-treated group: ↑ *Tenericutes, Actinobacteria, phyla and Alistipes, Bacteroides, Ruminococcaceae_UCG-014, Ruminococcaceae_unclassified* genus ↓ *Akkermansia, Mollicutes, Turicibacter, Allobaculum, Rikenellaceae* and *RC9_gut_group* genus	–	[40]
–	Rats	**High-Cu level (*vs* low-Cu level):** ↑ *Oscillibacter, Anaerotruncus, Peptococcus, Dorea, Ruminococcaceae, Defluviitaleaceae, Turicibacter, Coprococcus, Blautia (*OTU368) *Peptococcaceae, Peptostreptococcaceae, Rikenella, Barnesiella, Bacteroides* (OTU64), *Barnesiella, Rikenella, and Alistipes*-related OTUs ↓ *Allobaculum, Flavinifractor, Oscillopira, Christensenellaceae, Lachnospiraceae, Allobaculum, Flavonifractor, Oscillospira, Blautia*-related OTUs, *Alistipes* (OTU273), *Parabacteroides* related OTUs	↑Serum TNF-α ↑ Firmicutes and↓ Proteobacteria phyla and ↑ OTU425 (*Erysipelotrichaceae* family) ↑serum IL-6 and ↓IL-8	[53]
$CuSO_4$	Rats	↑ [Cu] ↑*Treponema_2 Erysipelatoclostridium* ↓ Romboutsia, *Chlamydia, Bifidobacterium, Lactobacillus* genus **Low-Cu level:** ↑ *Alloprevotella, Lachnospiraceae_NK4A136, Ruminiclostridium_5,* and *Ruminococcaceae_UCG-013*genus **High-Cu level:** ↓ *Alloprevotella, Lachnospiraceae_NK4A136, Ruminiclostridium_5,* and *Ruminococcaceae_UCG-013* genus	–	[54]
$MnCl_2$	Rats	**Mn group:** ↑*Clostridiales noname,, Faecalibacterium prausnitzii, Ruminococcus gnavus* ↓ *Prevotellaceae, Fusobacteriaceae, Lactobacillaceae, Clostridium celatum, Lactobacillus johnsonii, Fusobacterium* sp. *CAG:815, Clostridium* sp. *CAG:813, Clostridium* sp. *JCC,* Firmicutes *bacterium CAG:475, Clostridium* sp. *CAG:349*	↑Taurodeoxycholic acid and tryptamine ↑*Faecalibacterium prausnitzii*, *Firmicutes bacterium* ASF500 and *R. gnavus*, ↓β-Hydroxypyruvic acid, ↑*Clostridium celatum* and urocanic ↓*F. prausnitzii*, *Firmicutes B.* ASF500 and *R. gnavus,* ↑ *C. celatum.*	[56]

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Table 3 (*continued*)

TOXIC OR ESSENTIAL ELEMENT	MODEL ORGANISM	GUT BACTERIA	CORRELATIONS	REFERENCE
$PbCl_2$	Mice	**Cecal microbiota: ↑[**Pb] ↑*Lactobacillaceae, Coriobacteriaceae* and *Eryspelotrichaceae*↓ *Lachnospiraceae* **Fecal microbiota:** ↑ [Pb] ↑*Eryspelotrichaceace* and *Clostridiaceae,* ↓ *Lachnospiraceae*	–	[41]
$(CH_3COO)_2Pb·3H_2O$	Mice	**Pb-treated group:** ↓ Proteobacteria, Verrucomicrobia phyla *and Ruminococcaceae*family as well as the following genus: *Akkermansia, Rikenellaceae_RC9_gut_group, Lachnospiraceae, Oscillibacter, Ruminiclostridium_9, Lachnoclostridium* and *Anaerotruncus*	–	[40]
_	Humans	**According to levels of urine Pb:** ↑ Proteobacteria phyla and the genus:, *Desulfovibrio, Eubacterium, Ruminococcus* and ↓ *Clostridium, Coprococcus, Pediococcus* genus	–	[65]
$(CH_3COO)_2Pb·3H_2O$	Mice	**Pb-treated group:** ↑ Firmicutes, Proteobacteria *(Desolfovibrionales)*↓ *Bacteroidetes*	Bacteroides and Lactococcus displayed significant negative correlation with Pb exposure	[46]
$(CH_3COO)_2Pb·3H_2O$	Mice	**Cecal content:** ↓Bacteroidetes, Firmicutes **Faeces:** ↓ Firmicutes ↑ Bacteroidetes **Gut microbiota:** ↑ Bacteroidetes, Proteobacteria ↓ Firmicutes	TCA cycle, Amino acid metabolism, energy metabolism and other pathways (chronic Pb exposure)	[47]
_	Mice	**Se-deficient diet:** ↑*Dorea* genus **Se-enriched diet:** ↑*Turicibacter, Akkermansia* ↓ *Mucispirillum* genus	–	[49]
_	Rats	**Se-deficient black tea + high fat diet (compared to high fat diet):** ↓ *Erysipelotrichaceae incertae sedis, Flavonifractor, Holdemania, Bilophila, Allobaculum, Akkermansia* genus	–	[59]
_	Mice	**Se-diet:** ↑ *Porphyromonadaceae* family and *Tanarella* genus ↓ *Alistipes, Parabacteroides* genus	Higher levels and activities of GPx and MsrB in germ-free mice	[18]
	Mice	Intestinal bacteria might withdraw Se from mice, which in turn decrease two- to threefold the level of selenoproteins under Se-limiting conditions	–	[17]
Na_2SO_3 Se enriched probiotics (SeP)	Pig	**SeP diet:** ↑ *Lactobacillus* ↓ *E.coli* **iSe diet:** no changes on intestinal microbiota	SeP diet enhance antioxidative capacity, thyroid function, produce a more stable and healthy gastrointestinal ecosystem	[19]
KIO_3	Mice	**High-fat diet + KIO_3 group (*vs*. HFD group):** ↑Bacteroidetes phylum *and Clostridia, Oscillibacter, Allobaculum* genus ↓Firmicutes Proteobacteria and Actinobacteria phyla and *Deltaproteobacteria* and *Candidatus Saccharibacteria* families and the following genus: *Roseburia, Bifidobacterium, Faecalibacterium prausnizii, Lactobacillus, Blautia* **KIO_3 group (*vs* control):** ↑ Proteobacteria, Firmicutes phyla and the genus*Clostridium, Bacilli, Roseburia, Bifidobacterium, Faecalibacterium prausnizii, Lactobacillus, Oscillibacter, Allobaculum*↓ Bacteroidetes and Actinobacteria phyla, and also member of *Candidatus Saccaribacteria, Deltaproteobacteria, Bacteroidia, Blautia*	Negative correlation between phenotypes of thyroid disease and 14 OTUs from *Lactobacillus, Blautia, F. prausnizii, Roseburia and Enterococcus*. Positive correlation between phenotypes of thyroid disease and 50 OTUs from *Turicibacter, Prevotella, Alloprevotella, Barnesiella, Faecalibaculum rodentium, Burkholderiales and Peptostreptococcus*. 31 OTUs were related TT4 biochemical index. Two serum biochemical indicators were correlated with 26 OTUs	[48]
KI	Rats	-	1,3 -Acetonedicarboxylic acid, arabinose, xylose, citramalate, deoxycholate, hippurate, *o*-acetylserine, shikimate, 3-methyl-2-oxindole, pepecolate, 2,6-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, xanthosine, 2- hydroxicaproic acid, 5-hydroxyindole-3-acetic acid, indole-3-acetamide, 3-amino-4-hydroxybenzoate (Gut microbiota-derived metabolites)	[55]

and cadmium treatment perturbed gut microbiota and fecal metabolome significantly [45]. Interestingly, exposure to the neurotoxic metal manganese in rats decreased gut bacterial richness, and altered the metabolism [56]. The authors found correlations between metabolites and gut bacterial composition and demonstrated that faecal microbiome transplantation from normal rats could alleviate the neurotoxicity of manganese exposure by shaping the gut microbiota [56].

Regarding metal-gut microbiota interplay, there are few works related to chemical speciation or metalloproteins, and most of them describe correlations between gut bacteria and the total metal content. Total mercury in hair and methylmercury in stool and cord blood have been correlated with human microbiota [64] and in the case of selenium, mice models fed a selenium supplemented diet have been used to measure the expression of several selenoproteins by activity, protein, and mRNA levels in plasma, liver, and intestinal sections of mice fed a selenium supplemented diet [17,18] by western blots and activity assays in liver and kidney and in plasma by quantitative RT-PCR [18].

An interesting study in rats applied Western blot analysis and ELISA in the brain after manganese exposure to explore the impact of this element on gut microbiota and the brain [56].

It is noteworthy that studies related to metal(-loid)-gut bacteria interplay are scare, and the quality of the experimental design is not always the best. Most experimental design in the works included in Table 3 rely on the comparison of two different experimental conditions. However, the microbiome is intrinsically dynamic, and comparing two different treatment groups at a single time point dismissed the myriad of host and environmental factors that can affect the microbiome status at the beginning of the treatment time, and, consequently, they will also affect the obtained results. Longitudinal studies are more informative that those end-point experiments since they consider the inherent ordering of samples and their interdependence, which are both linked to time course [171]. Recent advances in high-performance experimental technologies allow researchers to measure microbiota dynamics on an

unprecedented scale. However, a number of practical concerns often complicate analysis of longitudinal microbiome data. In addition to pitfalls related to time point synchronization or the inherent variation, not following a normal distribution, inherent to longitudinal studies, proper experimental designs and powerful computational tools are needed to analyse the results of these longitudinal microbiome studies with multiple time points per subject. Hence, longitudinal studies testing hypotheses over time become a statistical challenge [172]. New computational techniques that appropriately model the particular properties of these studies are required to undoubtedly provide a deep vision of the complex dynamics of the microbiomes and their multiple types of interaction with the host and the environment.

4. Conclusions

Actually, there is the timely possibility of disentangling some unanswered challenges related to the role of metals and metalloproteins in the gut microbiota-brain axis due to the advancements in omic methodologies, such as metaomic, metabolomics, metallomics and heteroatom-tagged proteomics. A holistic view of the problem is critical to fully understanding the biological impairments caused by metal exposure at different levels: transcripts, metabolites, metal-containing molecules (metallometabolites and metalloproteins) and microbiota, but also the role of essential elements, such as iodine and selenium. In summary, although the link between gut microbiota and the brain is evident, a more detailed mechanistic understanding is needed to fully realise the impact of metals, metalloids, and their species in the gut microbiota-brain, breastmilk-microbiota-brain and hypothalamic-pituitary-thyroid axes. Metaproteomics, combined with metagenomics, metabolomics and heteroatom-tagged proteomics, might be an excellent tool to determine the potential causative mechanisms, requiring further animal and clinical research as well as the development of analytical approaches.

Authors contributions

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Selenium supplementation influences mice testicular selenoproteins driven by gut microbiota

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Selenium is a well-known essential element with important roles in human reproductive health mainly due to its antioxidant character. This study aimed to investigate the potential role of selenoproteins on gut microbiota and male reproductive health. A new assay for the absolute quantification of selenoproteins in testicular tissue based on two dimensional chromatography with inductively coupled plasma mass spectrometry was performed for the first time. The gut microbiota profile was obtained by 16S rRNA gene sequencing. Numerous associations were found between testicular selenoproteins and gut microbiota (e.g. *Mucispirillum*, related with sperm activity and testosterone, was associated with glutathione peroxidase (GPx) and selenoalbumin (SeAlb), while *Escherichia/Shigella*, related to sex hormones, correlated with GPx, selenoprotein P (SelP) and SeAlb). The effects of Se-supplementation on testicular selenoproteins only occur in conventional mice, suggesting a potential selenoproteins-microbiota interplay that underlies testicular function. The selenoproteins GPx and SelP have been quantified for the first time in the testicles, and the novel identification of SeAlb, a protein with nonspecifically incorporated Se, is also reported. These findings demonstrate the significant impact of Se-supplementation on gut microbiota and male reproductive health. In addition, the analytical methodology applied here in selenoprotein quantification in testicular tissue opens new possibilities to evaluate their role in gut microbiota and reproductive health axis.

Selenium (Se) is an essential trace element with important roles in immune function, the metabolism of thyroid hormones[1] and cancer chemoprevention[2]. Se deficiency has been related to heart failure, nutritional myodegeneration (white muscle disease)[3] and Keshan disease[4] among other pathologies. Se dietary supplementation leads to the formation of specific selenoproteins, in which Se occupies the active center; hence, influencing the redox-regulated genes and helping the cell to convert with reactive oxygen species (ROS) into less reactive molecules[5]. Se is also important in reproductive health[6], being essential for gonadal development, gametogenesis and fertilization[7], likely as a result of its ability to modulate antioxidant defense mechanisms and redox sensitive pathways. There is clear evidence that a deficiency of Se and selenoproteins can lead to several reproductive health and obstetric complications as well as infertility, preeclampsia, miscarriage, preterm labor, fetal growth restriction, gestational diabetes and obstetric cholestasis[6]. Mammalian Se-containing proteins can be divided into three groups: (1) proteins containing non-specifically incorporated Se, in which sulfur is replaced by Se in amino acids such as methionine (SeMet) (e.g. selenoalbumin (SeAlb), which is not considered as a "real" selenoprotein), (2) specific Se-binding proteins (e.g. Se-binding protein 1, SBP1), in which Se is tightly associated with a cysteine (Cys) residue in the peptide but not as a component of selenocysteine (SeCys) and (iii) specific selenocysteine-containing selenoproteins (e.g. selenoprotein P (SELENOP))[8]. The role of Se in mammal

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spermatogenesis is mainly mediated by two selenoproteins, namely phospholipid hydroperoxide glutathione peroxidase (PHGPx/GPx4) related to sperm quality and male fertility and SELENOP, a plasma protein required for Se supply to the gonads where it is used as a reservoir of Se[9]. Other selenoprotein transcripts (~ tenfold lower level than PHGPx and the majority of them with unknown function) have also been identified in male gonads (Thioredoxin/Glutathione Reductase (TGR), selenoprotein V, selenoprotein W, selenoprotein K, selenoproteins 15 and selenoprotein S)[9].

Recent studies have pointed out the potential role of dietary Se in shaping the gut microbiota and, subsequently, exerting effects on host metabolism and immunity[10–13]. Diet is considered a key regulator of gut microbiota with effects at local and systemic levels[14,15] and also, on reproductive hormones levels[16]. Several reports have shown that high-fat diet induced gut dysbiosis can affect the system's health even causing neurological disorders[17,18] and spermatogenesis impairments[19]. Recent studies suggest the impact of gut microbiota on fertility and reproductive health in both, males and females[20–22]. However, little is known about the mechanisms underlying the shifts in gut microbiota across reproductive states. An increase in dietary Se intake has also been implicated in enhancing the antioxidant GPX activity, thereby improving male fertility[23]. The encouraging results in the last years suggest that the combination of Se with other essential micronutrients may improve reproductive efficiency in males[23]. However, to date there is not sufficient nor consistent findings upon which to draw solid conclusions. Indeed, previous studies regarding selenoproteins in the testicles applied non quantitative methods, such as transcriptomics or enzymatic assays[9].

The aim of this work is to investigate the potential role of selenoproteins in the gut microbiota-reproductive health axis. For this purpose, the absolute quantification of selenoproteins by intact protein analysis will be performed using a metallomic approach based on inductively coupled plasma mass spectrometry (ICP-MS), a methodology used for analyses of serum, plasma[24] and the liver[25]. To this end, mice testicular selenoproteome has been determined after Se-supplementation of conventional mice and mice with microbiota depleted by antibiotics. The total metal content in testicles has been also measured to evaluate the possible impact of Se-supplementation and microbiota on the homeostasis of elements in testicles, as well as their traffic.

Results and discussion

Preliminary observations and histopathology evaluation. This study analyzed the impact and effect of a Se-enriched diet on selenoproteins, total Se concentration and metal homeostasis in the testes for 8 week-old male *Mus musculus* mice as well as the relationships of these parameters with the gut microbiota composition. To study the influence on the gut microbiota, half of the mice received a cocktail of antibiotics (200 mg kg^{-1} per body weight (bw) of ampicillin, neomycin and metronidazole, 100 mg kg^{-1} bw of vancomycin and 2 mg kg^{-1} bw of amphotericin B)[26] for one week, they were later fed with either a regular or a Se-supplemented diet for a further weeks. The Se-enriched diet provided the mice with a daily intake of 120 µg kg^{-1} bw, three times the regular mouse intake of Se[27]. This dose of Se is no-toxic, but able to modify some biological parameters[28]. The animals showed no external evidence of illness or discomfort, and all survived the treatment. No differences were found between the body weights of the mice at the end of the treatment. However, pretreatment with Abx caused a decrease in the testicular weight of mice (Fig. S2), albeit statistically non-significant one.

Studies in animal models clearly demonstrate the deleterious effects of antibiotics on testicular function[29]. The antibiotic cocktail we used here to deplete the intestinal microbiota included the aminoglycoside neomycin, an antibiotic that adversely affects spermatogenesis by cessation of meiosis at the level of primary spermatocytes[30]. Our histopathological study confirms this effect (Fig. 1), as we found a greater number of spermatogonia, the diploid undifferentiated germ cells, in the seminiferous tubules of the Abx group. Although the differences between the Abx group and the control group were not statistically significant, these results suggest a possible arrest of the cell division processes that convert spermatogonia into spermatozoa. The Abx group's histological samples also showed an alteration of the normal structure of the seminiferous tubes with mild and punctual degenerative changes at the level of the basement membrane. This morphological layout is essential for the process of spermatogenesis since it is the structural and hormonal support of the spermatogonia in the different stages of the seminiferous epithelial cycle[31] and abnormal basement membrane structures have been associated with spermatogenesis[32]. Although no significant decrease in the number of sperm per seminiferous tube was observed in any sample, our data suggests that Abx treatment may alter male fertility. The intake of a Se supplement after pretreatment with Abx (Abx-Se group) did not completely prevent the effect of Abx on the meiotic process but prevented the basement membrane abnormalities observed in the Abx group. These results suggest that Se supplementation likely improves the fertility of mice, in agreement with previous reports[9].

Selenoproteins and total selenium in testicles. Selenoprotein extraction protocols from mammalian tissues requires high efficiency, avoiding interferences and protein degradation during the procedure. For total protein extraction we used here the CelLytic™ MT extraction reagent for total protein extraction, which contains a low concentration of a dialyzable mild detergent for minimal interference with protein interactions and biological activity[33]. Selenoproteins obtained from mice testicles from each study group were clearly identified in the typical mass flow chromatogram (Fig. 2). These chromatograms represent the mass of Se (µg) vs time (min), and, thus, they do not reflect the abundance of different selenoproteins, but the Se accounted for by each one. When converting chromatograms peaks to selenoproteins abundance, it must be taken into consideration that both mice and human SELENOP contains 10 selenocysteine molecules ($C_3H_7NO_2Se$)[34], while GPx has 4 g atoms of Se per mole[35]. In addition, we quantified SeAlb, which is not a "real selenoprotein", but a protein that incorporates Se post-translationally in the form of selenomethionine (SeMet)[36]. Furthermore, the relative concentration of selenoprotein (in terms of Se) in the testicles is SELENOP > GPx + unretained (unr) ~ SeAlb (Fig. 2). It is noteworthy that GPx elutes in the void of the column and this peak should be assigned to GPx and

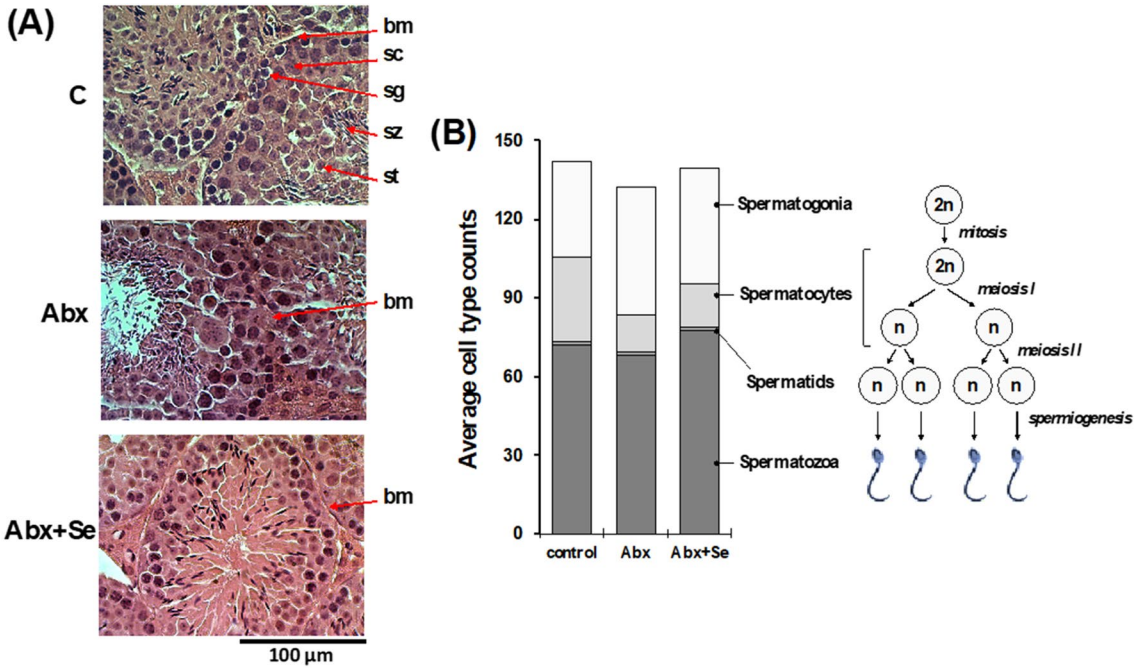

Figure 1. (**A**) Photomicrograph showing the cross section of H&E staining of the testes of a representative mouse from groups Control, C-Se, Abx and Abx + Se. The basement membrane (bm), spermatogonia cells (sg), spermatocytes (sc), spermatids (st) and spermatozoids (sz) are indicated by arrows. (400 × magnification). (**B**) Bar plots showing the average counts for each germ cell type in the seminiferous tubule; the scheme illustrates the process of generation of the different types of germ cells during spermatogenesis.

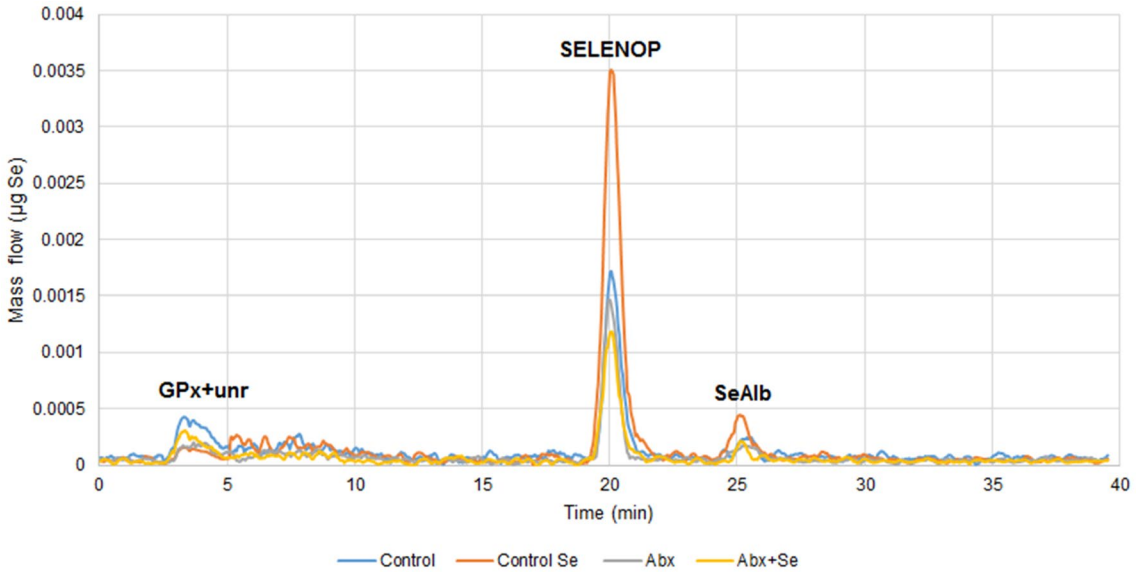

Figure 2. Selenium mass flow chromatogram obtained from testicular tissues of mice.

other unretained selenoproteins (they have not been identified in mice testicles by organic mass spectrometry and will be the object of future work).

Significant changes in both selenoprotein and total Se concentrations were observed between groups (Fig. 3 and Table S1). The total Se content in the testicles is affected by Se-supplementation. In conventional mice fed with a Se-supplemented diet (C-Se group), the total Se content was similar to the control group, and there were no statistical differences between groups. In contrast, mice fed Se-supplemented diet after microbiota depletion (Abx + Se group) presented the highest concentrations of Se in the testicles, showing statistical differences with the control group (C, 1.24-fold↑, $p = 0.01$), with the Se-supplemented conventional mice (C-Se, 1.19-fold↑, $p = 0.005$) and with the microbiota-depleted mice that were fed a rodent diet (Abx, 1.15-fold↑, $p = 0.01$).

Regarding selenoproteins, the concentrations of SELENOP and SeAlb were highest in the testicles of conventional mice fed a Se-supplemented diet, while the concentration of GPx + unr was the lowest in this group.

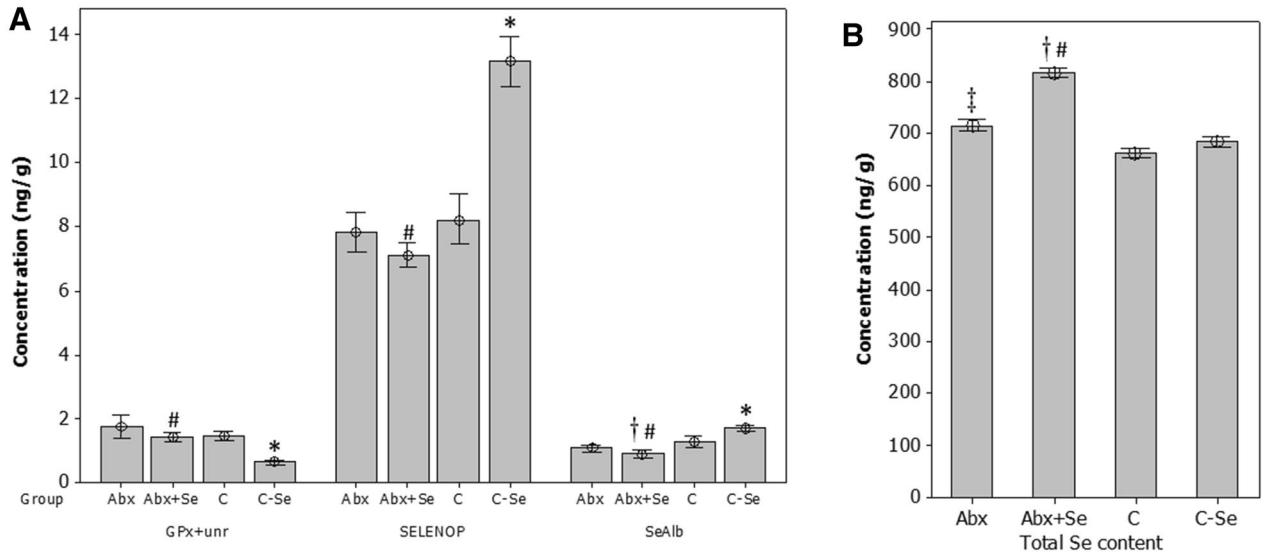

Figure 3. Selenoproteins (**A**) and total Se concentration (**B**) in the mouse testis. Data are expressed as mean ± SD (n = 10). (*) Statistically different in C-Se vs C comparison. (†) Statistically different in Abx + Se vs C comparison. (#) Statistically different in Abx + Se vs C-Se comparison. (‡) Statistically different in Abx + Se vs Abx comparison.

Thus, Se supplementation increases the concentration of SELENOP (1.61-fold↑, $p = 0.000$) and SeAlb (1.30-fold↑, $p = 0.013$) in the testicles of conventional mice (C-Se vs C), while decreasing the concentration of GPx + unr (2.32-fold↓, $p = 0.000$). The SeAlb concentration in the testicles was significantly different between Abx + Se and C groups (1.54-fold↓, $p = 0.017$). Se-supplementation of microbiota depleted mice (Abx + Se vs C-Se) increased the concentration of GPx + unr in testicles (2.22-fold↑, $p = 0.000$) and decreased that of SELENOP (1.85-fold↓, $p = 0.000$) and SeAlb (2.00-fold↓, $p = 0.000$). As mentioned in a previous study PHGPx/GPx4 (most abundant) and SELENOP have been previously identified in testicles along with other proteins with unknown function or lower abundance[8]. However, to our knowledge, this is the first identification of SeAlb in the testicles, likely due to either the methodologies previously used (transcriptomics and enzymatic activities[37,38] or a lack of information about its presence and role in the testicles. Testosterone is produced by the Leydig cell and secreted into the interstitial fluid from where it is taken up by the Sertoli or diffuses into the interstitial capillaries to bind to albumin for transport through the body. If the presence of Se in albumin favors testosterone binding and distribution to other organs and tissues, this is an interesting issue to be addressed in further studies. In addition, the absolute quantification of selenoproteins in the testicles has not been reported before. Moreover, the selenoproteins determined in this work accounts for the highest Se content linked to proteins in the testicles as concluded from the mass flow chromatogram (Fig. 2). Selenometabolites (SeO_4^{2-}, SeO_3^{2-}, SeMet, SeCys, SeMetSeCys) that elute between GPx and SELENOP were under the detection limits in all analyzed samples (LD = 0.5 ng Se g^{-1}).

Thus, we suggest that Se-supplementation in conventional mice influences the selenoprotome, but not the total concentration of Se in the testicles (Fig. 3). Indeed, SELENOP and SeAlb patterns are parallel, as they both increase in concentration after Se-supplementation. However, the concentration of GPx + unr decreased in the testicles in Se-supplemented conventional mice. Nevertheless, Se-supplementation of microbiota-depleted mice (Abx + Se vs. Abx) has no effect on the testicular selenoproteome, but the total concentration of Se is significantly increased. These findings suggest that the effect of Se-supplementation on the selenoproteome of the testicles could be influenced and mediated by microbiota although the exact mechanisms remain unknown.

Se-supplementation has proven the beneficial effects of Se in male fertility reproduction and testicular damage[23,39–41].The main function of SELENOP is the transport and distribution of Se to other tissues, but it also possesses antioxidant action and it is involved in Se homeostasis[42], while the GPx family are antioxidant selenoproteins[43] and SeAlb is a Se transporter[44]. In the testicles, SELENOP is located in the Leydig cells[45] and can influence sperm quality and, hence, male fertility[38,46]. PHGPx/GPx4 has also been related to sperm midpiece, mitochondrial sheath and sperm chromatin condensation[8].

In previous work, the expression levels of GPx and SELENOP in the testicles of mice were not affected with dietary Se deficiency or excess selenomethionine[47], however, in rats with Se deficiency the expression levels of SELENOP were decreased[37]. Other authors reported that Se-supplementation sharply increase the activity of testicular SELENOP[46] and, in studies of co-exposure to Cd and Se, Se ameliorates the effects of Cd by increasing SelP and GPX4 gene expression[48]. The results obtained from studies of Se-supplementation in human prostate adenocarcinoma cells (F-9 and Du-145 cells) revealed an increase in mRNA expression levels on the glutathione peroxidases GPX1, GPX2 and GPX3, SelS and SEP15, while some selenoproteins located in the testes, such as SelW and SelV changes slightly and the TRXR3 selenoprotein decreased sharply[49].

To summarize, our results suggest that the use of antibiotics (Abx) may affect the ability of the host to incorporate Se into SELENOP, which, as we have mentioned, may influence testicular activity and reproductive function, in good agreement with the above results.

Element	Control	C-Se	Abx	Abx + Se
Al	1337 ± 380	1239 ± 31	1196 ± 75	829 ± 54
V	9.3 ± 7.3	6.9 ± 0.2	4.5 ± 1.3	6.4 ± 0.2
Cr	65 ± 8	161 ± 4	33 ± 0.2	20 ± 6
Mn	467 ± 16	470 ± 3	448 ± 10	463 ± 1
Fe	31,843 ± 147	25,557 ± 44	28,281 ± 266	32,312 ± 96
Co	17.1 ± 6.7	13.8 ± 1.7	16.3 ± 1.7	21.6 ± 0.8
Cu	1702 ± 17	1429 ± 12	1426 ± 16	1482 ± 30
Zn	3815 ± 31	3662 ± 2	3723 ± 1	4143 ± 42
As	15.4 ± 1.6	40.2 ± 2.7	2.9 ± 1.1	2.4 ± 0.7
Mo	63 ± 4	54 ± 3	130 ± 4	68 ± 1

Table 1. Concentration of elements (ng g^{-1}) expressed as mean ± SD (n = 3) in mice testicles from the different groups.

Metals and metalloids are very important in biology since as one-third of all proteins in the human body require a metal cofactor for functionality[50]. Metallomics can be defined as the research field that elucidates the identification, distribution, dynamics, role and impact of metals and metalloids in biological systems[51,52]. The methodology for a metallomic analysis usually involve the use of an inductively coupled plasma mass spectrometer (ICP-MS) hyphenated to high performance liquid chromatography (HPLC), gas chromatography (GC–MS) or capillary electrophoresis (CE)) using the heteroelement (an atom different to C, H, N, O or F, e.g. Se) in the biomolecule as a "tag" (heteroatom-tagged proteomics)[53]. Thus, this approach, is more sensitive than the typical proteomic approaches which involve tryptic digestion and further analysis of peptides that are usually difficult to separate and it has not been previously applied for the absolute quantification of selenoproteins in testicular tissue[54]. Other techniques such as UV–Vis spectrophotometry allow determination of the total content of proteins or their activities, but not the absolute quantification of specific proteins.

Influence of selenium supplementation on testicular metal homeostasis. The concentration of toxic and essential metals (Al, V, Cr, Mn, Fe, Co, Cu, Zn, As, Mo Cd, Sb, Tl, and Pb) has been determined in the testicles of mice from the different groups to evaluate the metal homeostasis. The results are presented in Table 1. The concentrations of Cd, Sb, Tl and Pb in the testicles were all below the limit of detection (0.02–0.05 ng g^{-1}) in all mice groups.

The statistical analysis showed numerous differences in the concentration of metals between groups. The significant differences for each comparison are summarized in Table S2. Se-supplementation of conventional mice (C-Se vs C) increased the levels of Cr ($p = 0.004$) and As ($p = 0.008$) and decreased the levels of Fe ($p = 0.000$), Cu ($p = 0.003$) and Zn ($p = 0.020$) in the testicles. The apparent paradox of Se increasing the As concentration in the testicles but contributing to As detoxification can be explained by the ability of Se to additionally increase the concentration of betaine in testicles, which sequester As in the non-toxic form of arsenobetaine[55]. The toxicity of Cr is determined by its chemical form, Cr(III) is essential while Cr(VI) is carcinogenic[56]. The effect of Se on metal homeostasis in the testicles was different after microbiota depletion. The concentration of Cr ($p = 0.023$), Cu ($p = 0.013$) and As ($p = 0.009$) were lower in the Abx + Se group when compared to the control group, while the concentration of Zn was higher ($p = 0.013$). However, when Abx + Se is compared with C-Se, the levels of Fe ($p = 0.000$), Co ($p = 0.028$), Zn ($p = 0.004$) and Mo ($p = 0.024$) increased and the levels of Al ($p = 0.012$), Cr ($p = 0.001$) and As ($p = 0.003$) decreased. Finally, Se-supplementation in microbiota depleted mice (Abx + Se vs Abx) reduces the concentrations of Al ($p = 0.031$) and Mo ($p = 0.003$) in the testicles whereas the concentration of Fe ($p = 0.002$) and Zn ($p = 0.005$) were augmented. The cytosol of most eukaryotic cells contains the enzyme superoxide dismutase (SOD), which contains Cu and Zn. After exposure to antibiotics, tissues are subjected to oxidative stress, which likely led to an increase in SOD and therefore, increased Zn levels[57,58].

Essential metals like Mn, Cu and Zn are crucial for maintaining male reproductive functions, as they are involved in spermatogenesis and sperm motility[59,60]. Moreover, their interaction with toxic elements (As, Cd, Hg, Pb, and others) may change the toxicity of these metals[61]. The synergistic/antagonistic interactions between elements through metal traffic and homeostasis in the different organs and tissues have been reported[62]. The antagonistic role of Se has been proven with toxic elements such as Hg[63], Cd[64] and As[65] and also with organic pollutants[66].

Selenoproteins and Gut Microbiota. It is well-known that gut microbiota play important roles in host health, modulating physiological, immunological and metabolic functions, but they also participate in the regulation of hormones related to reproductive functions[16] through the hypothalamic-pituitary–testicular axis[67]. Recently, the subject of the effects of dietary and supplemented Se on gut microbiota is has been receiving growing attention, but the interplay between testicular selenoproteins and microbiota has not been previously reported. As detailed elsewhere[28], Se-supplementation shape the gut microbiota composition as well as the effect of the antibiotics treatment (Fig. S3). In brief, Se-supplemented groups showed an increase in members of the *Lachnospiraceae* and *Ruminococcaceae* families as well as *Christensenellaceae* family and *Lactobacillus* genus.

The microbial richness (Chao1 index) and diversity (Shannon index) indeces were associated with selenoproteins in the testicles despite the impact of Abx on the microbial composition. In control group, higher microbial diversity ($R = 0.67$, $p \leq 0.05$) and richness ($R = 0.71$, $p \leq 0.05$) were associated with higher levels of SeAlb. In the Se-supplemented group, higher microbial diversity was also associated to SeAlb ($R = 0.81$, $p \leq 0.05$), but not with microbial richness. Furthermore, the relationship between SeAlb and microbial diversity disappear after the Abx treatment; with one exception: in the Abx + Se group, Chao1 index showed a positive association with SELENOP ($R = 0.64$, $p \leq 0.05$). These data suggest a potential effect of the microbiota on the specific selenoproteins in mice testes. To further explore the gut microbiota-testicular selenoproteome interplay, specific associations at the genus level in each group were determined (Fig. 4). The associations at phylum and family levels are detailed in Tables S3–S6.

As shown in Fig. 4, an elevated number of correlations between selenoproteins in the testicles and microbiota composition appeared after Se-supplementation of conventional mice (C-Se) and/or microbiota depleted mice (Abx + Se). It is also noteworthy that the correlations between total Se and SELENOP with microbiota were highly similar in the control mice, and always in the opposite manner than those of GPx + unr. Moreover, this behavior was dependent on the group/treatment. As previously discussed, these findings suggest that the effect of Se-supplementation on the selenoproteome of testicles is influenced by microbiota. In the control group, a higher total Se concentration was positively linked to several groups from the *Ruminococcaceae* and *Lachnospiraceae* families as well as the *Butyricoccus* genus, all known as short chain fatty acids (SCFAs) producers with beneficial impacts on intestinal homeostasis and promoting health benefits. Furthermore, members from the *Lachnospiraceae* and *Ruminococcaceae* families were also found to be associated with testicular functions[68]. Indeed, after the supplementation with Se, some SCFA producers such as *Faecalibacterium* genus as well as *Lachnospiraceae_UCG001* were correlated with SELENOP and *Ruminococceae_UCG009* was correlated with SeAlb. Moreover, in the Abx group these correlations were lost and most of the relations between microbiota and selenoproteome components were found with the total Se and SeAlb (Tables S4–S7). However, in the Abx + Se group, several new associations between all components of selenoproteome were observed, including positive associations between previously mentioned families such as *Lachnospiraceae* groups and *Ruminococcus_1* with Total Se and SeAlb (Fig. 4). It is noteworthy that in the control group these bacteria correlated positively with total Se, while in Se-supplemented mice groups they correlated with specific selenoproteins such as *Mucispirillim*. This observation may indicate that Se-supplementation aids specific functions such as transport (SeAlb and SELENOP) or sperm quality and male fertility (SELENOP)[38,46].

A higher relative abundance of the *Mucispirillum* genus was associated with higher total Se content in the control group. However, after Se-supplementation, higher relative abundance of this genus was associated with lower GPx + unr and higher SeAlb concentrations in the testicular tissue of conventional mice. A decrease in the level of this genus in mice fed with supranutritional Se has been previously reported[69]. Other authors indicated numerous associations between bacterial taxa and testicular function, but specially showed that *Mucispirillum* were positively correlated with testosterone and sperm activity[68]. *Escherichia/Shigella* has been related with sex hormones in the reproductive endocrine system[70]. This genus correlated positively with SELENOP and negatively with total Se in conventional mice after Se supplementation, while correlating positively with SeAlb in the Abx group. After Se supplementation of this group (Abx + Se), *Escherichia/Shigella* correlated positively with GPx + unr and SELENOP, and negatively with SeAlb.

In summary, Se-supplementation has an impact on the selenoproteome and mineral homeostasis in the testes, and also, on the gut microbiota, suggesting a pivotal key interplay between Se-microbiota and male reproductive health. The metallomic analytical approach, based on the quantification of selenoproteins using an atomic spectrometric detector such as ICP-MS coupled to HPLC, allowed for the first time the absolute quantification of the selenoproteins containing most of the bonded Se in the testicles as well as the novel identification of SeAlb in testicles. Our data indicate that Se-supplementation of conventional mice did not change the total level of Se in the testicles, but significantly changed the selenoproteome profile. Moreover, the opposite situation was observed in microbiota depleted mice suggesting that the effect of Se-supplementation on the selenoproteome of the testicles could be influenced by microbiota. Specific associations between selenoproteins in the testicles and gut microbiota composition and diversity have been observed, some of them related to sperm activity and sex hormones, demonstrating the interplay of Se supplementation with microbiota and the impact on reproductive health. More studies are needed to ascertain the mechanisms behind the Se-microbiota-reproductive health.

Materials and methods

Animal experimental design. After a three day acclimation period, forty mice (male *Mus musculus* BALB/c, 8 weeks old) were randomly divided into two groups, one receiving water and the other, receiving water with a mixture of antibiotics (ampicillin 1%, metronidazole 1%, neomycin 1%, vancomycin 0.5%) and an antifungal (amphotericin B, 10 mg/L) for one week. After this pretreatment time, half of the mice in each group ($n = 10$) were fed for an additional two weeks (treatment period) with the same regular diet used in the previous days, and the other half, were fed with a Se-enriched diet (0.65 mg/kg of sodium selenite). The four groups, C (control), C-Se (Se-enriched diet during treatment); Abx (antibiotics in the water during pretreatment) and Abx + Se (Antibiotics in the water during pretreatment and Se-enriched diet during treatment) were caged in pairs, with free access to water and food, which were changed every other day. Figure S1 summarizes the design of the experiment. At the end of the experiment, mice were anesthetized (isoflurane) and sacrificed by cervical dislocation, and organs were immediately removed, cleaned in NaCl (0.9% w/w) solution, cryo-homogenized in liquid nitrogen and stored at -80°C until analysis.

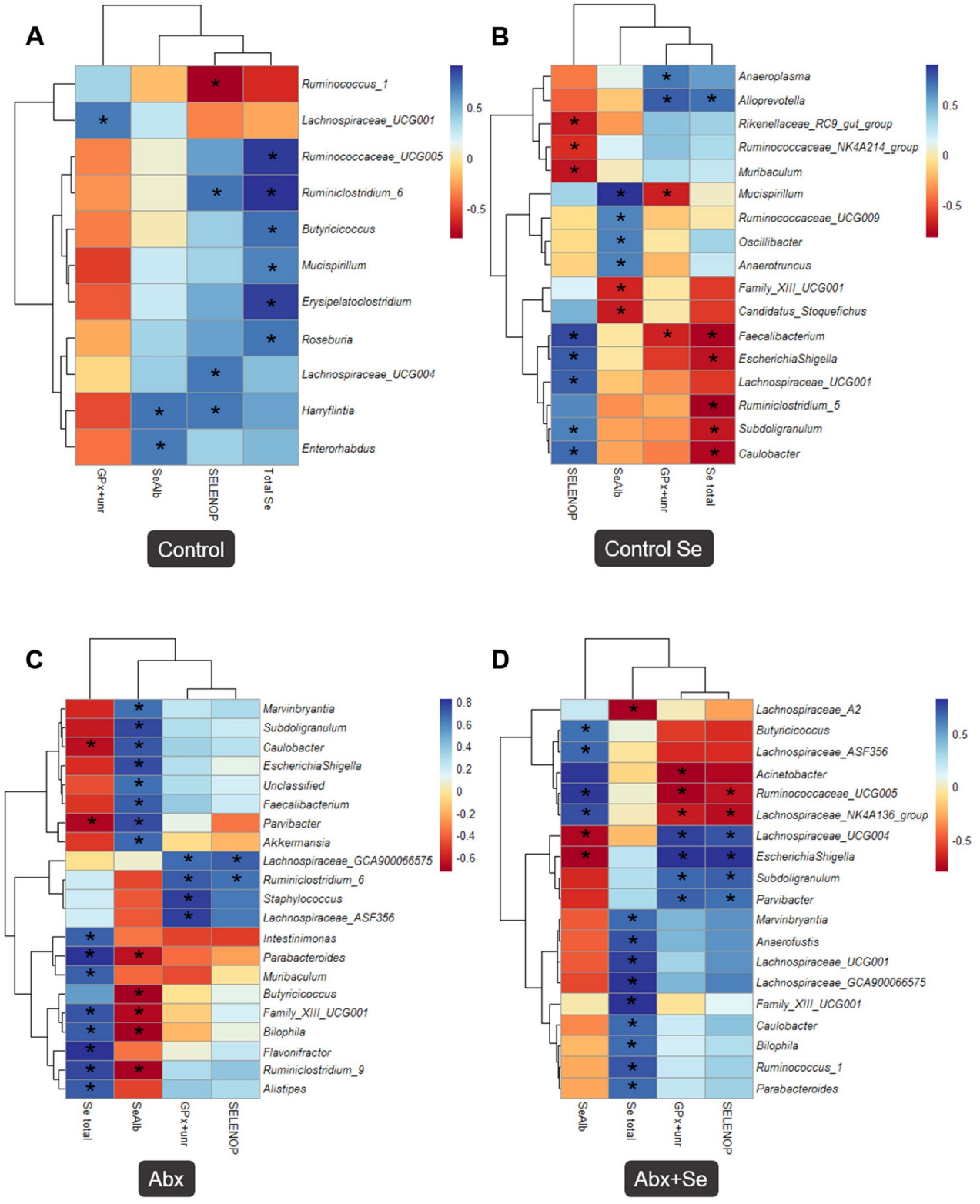

Figure 4. Heatmap showing the correlations between the testicular selenoproteome, total Se content and gut microbiota composition at genus level in the groups: Control (**A**), C-Se (**B**), Abx (**C**), Abx + Se (**D**). The colors range from blue (positive correlation) to red (negative correlation) and (*) indicates a p-value ≤ 0.05.

Ethics statement. The experimental procedures were carried out at the Animal Experimentation Service of the University of Cordoba (SAEX-UCO), after approval by the bioethics committee of the university and the regional government (Code Num. 02-01-2019-001), in accordance with current European Union regulations. Furthermore, the study was carried out in compliance with the ARRIVE Guideline.

Histopathological evaluation. Testicle tissues were fixed in 10% neutral buffered formalin for 24 h and then embedded in paraffin wax. 4 µm-thick paraffin serial sections were obtained using a rotary microtome (SAKURA Tissue Tek Accu Cut SRM 119 200) and stained with hematoxylin–eosin according to routine protocols. Photomicrographs were obtained with a Nikon Eclipse E400 photomicroscope at 400 magnifications.

Speciation of selenoproteins in mice testicles. To isolate selenoproteins, testicles were cryo-homogenized with a mortar and pestle in the presence of liquid nitrogen. Selenoproteins were extracted using the CelLytic™ MT extraction reagent (Sigma-Aldrich, Steinheim, Germany) (3 Ml g^{-1}) into a glass/teflon homogenizer at 4 °C. Protease inhibitor cocktail (Sigma-Aldrich, Steinheim, Germany) was added to CelLytic MT reagent to avoid protein degradation. Then, the mixture was centrifuged at 15,500*g* for 20 min at 4 °C. The supernatant was collected and filtered through low protein absorption Iso-Disc poly(vinylidene difluoride) filters (PVDF, 25 mm diameter, 0.45 µm pore size). A preconcentration step is necessary due to the low concentration of selenospecies. To this end, the extracts were completely evaporated under a nitrogen stream and re-dissolved in 0.1 mL of MilliQ water prior to the analysis.

Selenoproteins were separated from the obtained extracts using a previously described method[71]. Briefly, the chromatographic separation of GPx, SELENOP and SeAlb were performed with an ultra-high performance liquid chromatograph (model 1260 Infinity Quaternary LC, Agilent Technologies) using two size exclusion columns (5 ml HiTrap ®Desalting Columns, GE Healthcare, Uppsala, Sweden) and two different affinity chromatography columns (AFC, GE Healthcare, Uppsala, Sweden) with stationary phases of heparine-sepharose column (HEP-HP) and blue-sepharose column (BLUE-HP). The HEP-HP column retains only SELENOP, while the BLUE-HP column retains SELENOP and SeAlb. The column switching method allows the simultaneous separation of selenoproteins and selenometabolites: GPx and selenometabolites elute when the column switching system is in position 1 (0–20 min), while SELENOP and SeAlb are retained in the AFC columns. The valve switches to position 2 (20–24 min) to elute the SELENOP, and then returns to position 1 for the elution of SeAlb. Polyether ether ketone (PEEK) tubing (30 cm × 0.6 mm i.d.) and a T-connector were used to connect the eluent of the chromatograph to the Micromist nebulizer (Glass Expansion, Switzerland) of the triple quadrupole inductively coupled plasma mass spectrometer (ICP-QqQ-MS, model Agilent 8800 Triple Quad, Agilent Technologies, Tokyo, Japan) (2D-SEC-SEC-AFxAF-ICP-MS)[74]. Se (Cambridge Isotope Laboratories, Andover, MA, USA) was also introduced into the system via a T-connector for isotope dilution analysis. The absolute quantification of selenoproteins by 2D-SEC-AF-SUID-ICP-MS was carried out using the operational conditions as summarized in Table S7.

Total elements determination in mice testicles. For total elemental analysis, testicular tissue samples from mice in each group were pooled, and approximately, 0.1000 g of sample were digested in a microwave reaction system MARS 6 (CEM Corporation, Matthews, NC, USA) with a mixture of nitric acid and hydrogen peroxide (4:1, v/v). The mineralization was carried out from room temperature to 160 °C over 15 min, then maintaining at 400 W for 40 min. Then, the samples were diluted fivefold in 5% HNO_3 containing 100 µg L^{-1} of rhodium, and filtered using 0.45 µm PTFE syringe filters prior to the analysis by ICP-QqQ-MS. The operational conditions for ICP-QqQ-MS are listed in Table S7. The validation of the methodology was carried out using a fish protein certified reference material for trace element DORM-4 (National Research Council of Canada) (Table S8).

Gut microbiota analysis. Fecal samples were collected from the colon and immediately frozen in liquid nitrogen. DNA from fecal samples (approx. 100 mg) was obtained with the Master-Pure DNA extraction kit (Epicentre, Madison, WI, United States) following the manufacturer's instructions. Specific modifications were included as described elsewhere[72]. DNA concentration was measured using a Qubit® 2.0 Fluorometer (Life Technology, Carlsbad, CA, United States). A specific 16S rRNA amplicon (V3-V4 variable region of the 16S rRNA gene) was amplified and sequenced following Illumina protocols. Briefly, a multiplexing step was conducted using the NextEra Index Kit (Illumina, San Diego, CA, United States) and amplicons were checked with a Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA, United States). Libraries were sequenced (2 × 300 bp paired-end run, MiSeq Reagent kit v3) on a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain). Controls (DNA extraction procedure and libraries amplification) were included. A DADA2 pipeline was used to achieve quality filtering, sequence joining and chimera removal[73]. Taxonomy assignment was performed using Silva v132 database[74,75]. Sample with less than 1000 reads as well as specific taxa present at levels less than 0.01% and those present less than 3 times in at least 20% of the samples were filtered and removed from the analysis. Furthermore, sequences classified as Chloroplast and Cyanobacteria were filtered from the final dataset as they are associated with potential contaminants.

Statistical analysis. Statistical analysis was performed using Minitab16 Statistical Software (State College, PA, United States) and STATISTICA 8 Software. Firstly, Anderson–Darling normality test was used to determine whether or not data are normally distributed. Differences between groups were tested using the Kruskal–Wallis test (non-parametric statistics) and one-way ANOVA (parametric statistics). The Spearman correlation test was

performed for correlation analysis between gut microbiota abundance and selenoproteins concentrations. The level of $p < 0.05$ was considered statistically significant. Heatmaps were generated in R Project software (version 4.0.2) (R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria). For the microbiota analyses, total sum normalization (TSS) for the statistical analysis, multivariate test and data mining were performed with Calypso web platform v. 8.56[76]. Alpha- diversity metrics (Chao1 and Shannon indeces) and beta diversity analysis (based on Bray Curtis distance) were obtained. Briefly, Permutational multivariate analysis of variance (ADONIS) and Redundancy Discriminant Analysis (RDA) were obtained. Relative abundance (%) differences between groups at different taxonomical levels were tested using the Krustal-Wallis test with False discovery test rate (FDR) for multiple test correction. Alpha diversity indexes were obtained after a rarefaction to 93,525 sequences (minimum number of reads per sample). The level of statistical significance for all tests was fixed to $p < 0.05$.

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Author contributions

S.R.A: formal analysis, investigation, data curation, writing-original draft, writing—review and editing, visualization. M.S.R.: formal analysis, investigation, data curation, software, writing—review and editing, visualization. M.C.C.: conceptualization, methodology, resources, supervision, writing—reviewing and editing. F.N.R.: conceptualization, investigation, writing—review and editing. N.A.: conceptualization, methodology, data curation, writing—original draft, writing—review and editing. T.G.B.: conceptualization, methodology, resources, writing—original draft, writing—review and editing, visualization, supervision, funding acquisition.

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Competing interests

The authors declare no competing interests.

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Antagonistic Interaction of Selenium and Cadmium in Human Hepatic Cells Through Selenoproteins

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Cadmium (Cd) is a highly toxic heavy metal for humans and animals, which is associated with acute hepatotoxicity. Selenium (Se) confers protection against Cd-induced toxicity in cells, diminishing the levels of ROS and increasing the activity of antioxidant selenoproteins such as glutathione peroxidase (GPx). The aim of this study was to evaluate the antagonistic effect of selenomethionine (SeMet) against Cd toxicity in HepG2 cells, through the modulation of selenoproteins. To this end, the cells were cultured in the presence of 100 μM SeMet and 5 μM, 15 μM, and 25 μM $CdCl_2$ and a combination of both species for 24 h. At the end of the experiment, cell viability was determined by MTT assay. The total metal content of Cd and Se was analyzed by triple-quadrupole inductively coupled plasma–mass spectrometry (ICP-QqQ-MS). To quantify the concentration of three selenoproteins [GPx, selenoprotein P (SELENOP), and selenoalbumin (SeAlb)] and selenometabolites, an analytical methodology based on column switching and a species-unspecific isotopic dilution approach using two-dimensional size exclusion and affinity chromatography coupled to ICP-QqQ-MS was applied. The co-exposure of SeMet and Cd in HepG2 cells enhanced the cell viability and diminished the Cd accumulation in cells. Se supplementation increased the levels of selenometabolites, GPx, SELENOP, and SeAlb; however, the presence of Cd resulted in a significant diminution of selenometabolites and SELENOP. These results suggested that SeMet may affect the accumulation of Cd in cells, as well as the suppression of selenoprotein synthesis induced by Cd.

Keywords: cadmium, selenoprotein, HepG2, ICP-QqQ-MS, column switching, isotopic dilution, selenomethionine

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INTRODUCTION

Cadmium (Cd) is a highly toxic metal present in the environment as a consequence of natural and anthropogenic processes, causing its entry and accumulation in the food chain (Rigby and Smith, 2020). Cd toxicity depends on the dose, route, and duration of exposure producing numerous disorders in humans, including reproductive failure (Nasiadek et al., 2019) and DNA damage (Jia et al., 2011), and it is classified as a human carcinogen (Chen et al., 2019). Selenium (Se) is an essential trace element in mammals that can be presented in organic species [selenoamino acids such as selenocysteine (SeCys) and selenomethionine (SeMet) and methylated species such as dimethyl selenide (DMSe) or methyl selenol (CH_3SeH) and Se-containing proteins like selenoalbumin

(SeAlb)] and inorganic species (SeO_3^{2-} and SeO_4^{-2}) (Fairweather-Tait et al., 2010a). The main source of Se comes from food and nutritional supplements (Wang et al., 2016). Se bioavailability depends on many factors, but it is generally attributed to its chemical form. The absorption of all Se species is relatively high, between 70 and 95%, but differs on the source and the Se status of the individual (Finley, 2006). Inorganic species are better absorbed but less retained by the body than the organic forms (Fairweather-Tait et al., 2010b). Food supplements are based on the use of selenium-enriched yeast since they are the main source of SeMet. Most of the Se ingested is used for the synthesis of selenoenzymes, including selenoproteins from families of glutathione peroxidases (GPXs), thioredoxin reductases (TRXRs), and deiodinases (DIOs), which are involved in numerous metabolic processes (Schomburg et al., 2004). Animal studies have revealed the protective role of Se against Cd in the liver and kidney—the most sensitive organs to the toxicity of this element. The positive effect of Se is mainly attributed to selenoproteins (Huang et al., 2012; Boukhzar et al., 2016). *In vitro* models have become an effective alternative to the use of animal experiments and allow the elucidation of the mechanism of action of Se against toxic metals. The Cd/Se interaction has been studied in different cell lines. For example, the results from the SH-SY5Y catecholaminergic neuroblastoma cell line showed that treatment with 10 μM $CdCl_2$ and 100 nM sodium selenite (Na_2SeO_3) attenuates the changes in terms of oxidative stress and neuronal sprouting caused by Cd (Branca et al., 2018). Ren et al. (2020) reported that Se pretreatment (Na_2SeO_3) markedly represses Cd-induced apoptosis in Leydig TM3 cells. In addition, the level of reactive oxygen species (ROS) decreases, and the c-jun N-terminal kinase (JNK) signaling pathway is blocked. On the other hand, in the avian leghorn male hepatoma (LMH) cell line, Se intervention, in the form of Na_2SeO_3, inhibited the Cd-induced lactate dehydrogenase (LDH) release and endoplasmic reticulum (ER) stress crosstalk and autophagy by regulating intracellular Ca^{2+} homeostasis (Zhang et al., 2020b). Also, Cd-induced intracellular Ca^{2+} overload was mitigated by the Ca^{2+}/calmodulin (CaM)/calmodulin kinase IV (CaMK-IV) signaling pathway (Zhang et al., 2020b).

However, although the interaction of Se and Cd has been previously reported in human cells (Bianga et al., 2014; Marschall et al., 2017), the information about the influence of Cd exposure and Se supplementation on Se metabolites and expression profiles of selenoproteins is limited. To this end, the selenoproteome of hepatic carcinoma cells was quantified after Cd and/or Se exposure by column-switching combining affinity chromatography and size exclusion chromatography coupled to triple-quadrupole inductively coupled plasma–mass spectrometry (ICP-QqQ-MS).

MATERIALS AND METHODS

Preparation of Cadmium and Selenium Solution

Stock standard solutions of $CdCl_2$ (Sigma-Aldrich, St. Louis, MO, United States) and SeMet (Sigma-Aldrich, St. Louis, MO, United States) were prepared in deionized water using a Milli-Q system (Millipore, Burlington, MA, United States) at a concentration of 10 mM. This solution was sterilized using a syringe filter with a 0.22 μM pore size and stored in darkness at 4°C. A working solution of 1,000 μM of Cd and SeMet was prepared freshly in culture media before each exposure experiment.

Cell Culture

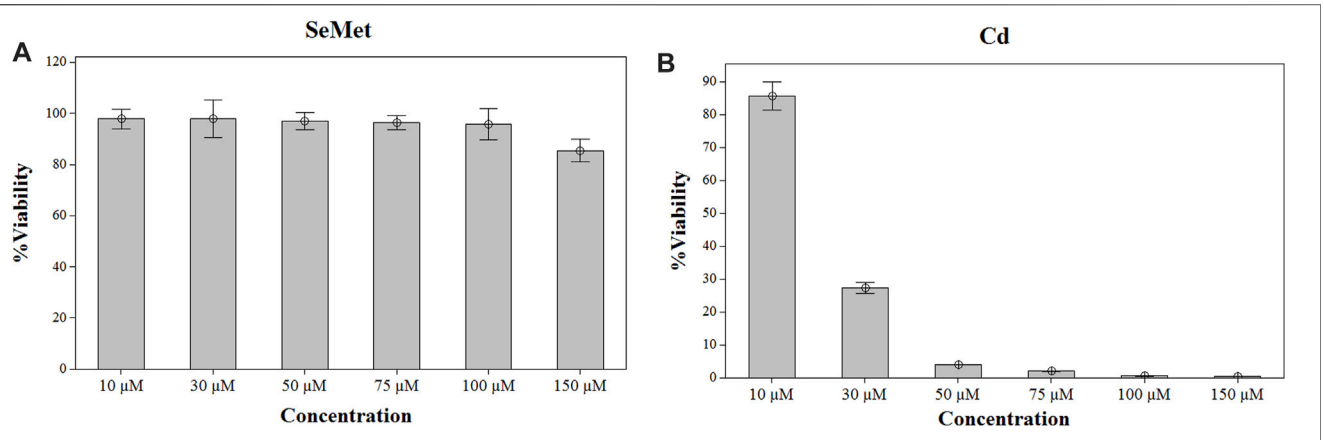
The hepatocellular carcinoma (HepG2) cell line was purchased from the European Collection of Cell Cultures (ECACC) (Sigma-Aldrich, St. Louis, MO, United States). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM/F12) (Gibco Life Technologies, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Gibco Life Technologies, Grand Island, NY), 1% non-essential amino acids (NEAA) (Gibco Life Technologies, Grand Island, NY), and 1% penicillin-streptomycin (Gibco Life Technologies, Grand Island, NY) and maintained at 37°C in an atmosphere with 5% CO_2 and 95% relative humidity. The medium was changed every 48 h. Once it reached 80% of confluence, the cells were detached using TrypLE Express (trypsin replacement) (Gibco Life Technologies, Grand Island, NY) and sub-cultured once a week.

Cytotoxicity Assay

Cell cytotoxicity induced by Cd and SeMet on HepG2 cells was evaluated by MTT [3-(4,5-dimethylthiazol-2 yl)-2,5-diphenyl tetrazolium bromide] (Sigma-Aldrich, St. Louis, MO, United States) assay. Briefly, HepG2 cells were cultured in a 6-well plate at a density of 5×10^5 cells/well and incubated for 24 h. After growth in the cell cycle, cells were exposed for 24 h to increasing Cd and Se concentrations, ranging from 0 to 100 μM, in order to determine their effects on cell viability. At the end of time exposure, the culture medium was removed, and 400 μl of MTT solution (2.5 mg mL^{-1}) was added and incubated for another 3 h at 37°C and 5% CO_2 atmosphere. The MTT solution was discarded, and the reaction product obtained, formazan, is solubilized with sodium dodecyl sulfate (SDS) (Fisher Scientific Co., Nepean, Ontario, Canada) solution (10% p/v). Finally, the absorbance was measured at a wavelength of 540 nm using a Helios Gamma UV-Vis spectrophotometer (Thermo Fisher Scientific, Bremen, Germany). Cell viability is expressed as a percentage relative to the control group. The cells cultured in a standard medium were used as the positive control (100% of viability).

Cell Exposure

Cell viability was not affected after selenium exposure in the entire range of concentrations studied (**Figure 1**). Therefore, a final concentration of 100 μM Se was chosen for the subsequent experiment exposure. In the case of Cd, a significant reduction of > 70% in cell viability was observed at concentrations higher than 30 μM (**Figure 1**). Consequently, three different Cd concentrations lower than 30 μM were employed. To determine the interactions of these elements in the *in vitro* model of HepG2 cells, they were exposed during 24 h to 100 μM SeMet, alone or in combination with different Cd



Q18 **FIGURE 1 |** Effect of SeMet **(A)** and Cd **(B)** on cell viability in HepG2 cells. Cells were exposed to the indicated concentrations of SeMet and $CdCl_2$ for 24 h.

concentrations, as follows: 1) 0 µM Cd + 0 µM Se (the control group), 2) 100 µM Se (the Se group), 3) non-cytotoxic concentration of 5 µM Cd (the Cd5 group), 4) 5 µM Cd + 100 µM Se (the Cd5+Se group), 5) lowest effective dose of 15 µM (the Cd15 group), 6) 15 µM Cd + 100 µM Se (the Cd15 + Se group), 7) high effective dose of 25 µM Cd (the Cd25 group), and 8) 25 µM Cd + 100 µM Se (the Cd25 + Se group).

At the end of the experiment, cells were washed with phosphate-buffered saline (PBS) (Gibco Life Technologies, Grand Island, NY) three times and then collected by mechanical harvesting using a cell scraper to ensure the integrity of the cells and to avoid chemical interferences with other reagents.

Determination of Selenium and Cadmium Contents

The total metal content in cell pellets and culture media were analyzed in an ICP-QqQ-MS model Agilent 8800 Triple Quad apparatus (Agilent Technologies, Tokyo, Japan). Harvested cells were previously homogenized with a buffer solution containing 150 mM NaCl (Sigma-Aldrich, St. Louis, MO, United States), 20 mM HEPES (Sigma-Aldrich, St. Louis, MO, United States), 1 mM EDTA (Sigma-Aldrich, St. Louis, MO, United States), 10% glycerol (Sigma-Aldrich, St. Louis, MO, United States), 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, United States), and 1% sodium dodecyl sulfate (Fisher Scientific Co., Nepean, ON, Canada). The cell homogenate was submitted to microwave-assisted acid digestion using a Mars 6 reaction system (CEM Corporation, Matthewa, NC, United States). For this, an aliquot of 100 µl of the cell sample was digested in 490 µl of HNO_3 (Fisher Scientific Co., Nepean, ON, Canada) and 10 µl of HCl (Fisher Scientific Co., Nepean, ON, Canada). Mineralization was carried out at 200 W from room temperature ramped to 180°C for 30 min and held for 10 min. A second ramp was performed from 200W to 400 W for 20 min and held for 20 min. After digestion, cell samples were 5-fold diluted to achieve a final concentration of 5% HNO_3 and 100 µg L^{-1} of Rh (internal standard) (Sigma-Aldrich, St. Louis, MO, United States) and filtered through 0.45 µM PTFE syringe filters.

For the analysis of culture media, an aliquot of 1 ml of the sample was collected and diluted using the same procedure as cell samples. The ICP-QqQ-MS operational conditions are shown in **Supplementary Table S1**. The certified reference material BCR-274 Single Cell Protein (Sigma-Aldrich, St. Louis, MO, United States) was used to validate the methodology (**Supplementary Table S2**).

Selenoprotein Speciation

Selenoproteins were extracted from HepG2 cells using the CelLytic™ MT extraction reagent (Sigma-Aldrich, St. Louis, MO, United States), following the manufacturer's instructions with some brief modifications. A cell pellet of 15 F0B4·10^6 cells was lysed with 100 µl of CelLytic™ MT containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, United States)) in a shaker for 15 min. Then, the lysed cells were centrifuged at 15,500 *g* for 10 min at 4°C, and the supernatant was collected for the subsequent analysis. The chromatographic separation of the selenoproteins (GPx, SELENOP, and SeAlb) was performed, as described elsewhere (Callejón-Leblic et al., 2018), using the column switching method that allows the simultaneous separation of selenoproteins and selenometabolites. The separation consists of two 5-ml HiTrap® desalting columns (GE Healthcare, Uppsala, Sweden) connected in series with a 1-ml heparin-sepharose (HEP-HP) column (GE Healthcare, Uppsala, Sweden) and a 1-ml blue-sepharose (BLUE-HP) column (GE Healthcare, Uppsala, Sweden) by ultra-high performance liquid chromatography (model 1,260 Infinity Quaternary LC, Agilent Technologies, Tokyo, Japan). The absolute quantification of selenoproteins was carried out in an ICP-QqQ-MS model Agilent 8800 Triple Quad apparatus (Agilent Technologies, Tokyo, Japan), employing the conditions from **Supplementary Table S1**. For the isotope dilution analysis, ^{74}Se (Cambridge Isotope Laboratories, Andover, MA, United States) was also introduced *via* T-connector into the system.

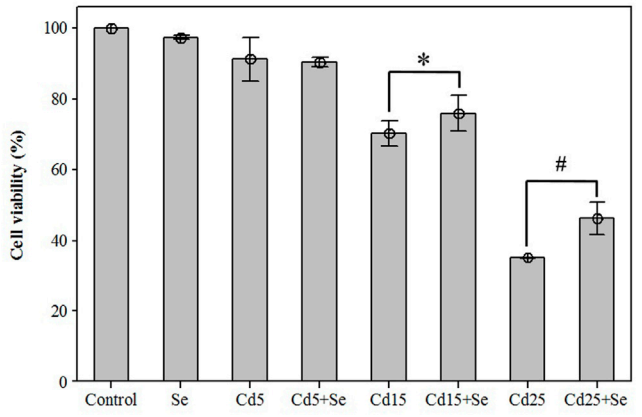


FIGURE 2 | Effects of different concentrations of $CdCl_2$ and/in combination with SeMet on cell viability in HepG2 cells. Values are expressed as mean ± S.D. (n = 3) (*): significant differences between groups Cd15 and Cd15+Se; (#): significant differences between groups Cd25 and Cd25+Se.

Statistical Analysis

Statistical analyses were performed by Minitab 21 Statistical Software (State College, PA, United States). The results are expressed as means ± SD of at least three replicates of each group. All experiments were repeated three times. The Anderson–Darling normality test was used to determine whether data are not normally distributed. Differences between groups were tested using the Kruskal–Wallis test (no normal distribution) and ANOVA (normal distribution). The level of $p < 0.05$ was considered statistically significant.

RESULTS

Effects of $CdCl_2$ and SeMet on Cell Viability

To select the dosage for the exposure, HepG2 cells were treated with a range of concentrations from 0 to 150 μM of SeMet and CdC_2 for 24 h. After the exposure, cell viability was measured by the MTT method. Selenomethionine exposure did not affect the cell viability of HepG2 cells in the range of concentrations from 10 to 100 μM, but a slight decrease was noticed at a concentration of 150 μM (**Figure 1**). Therefore, a final concentration of 100 μM SeMet was chosen for the subsequent experiment exposure. In the case of Cd, a reduction of >15% in cell viability was observed at concentrations higher than 10 μM (**Figure 1**), and at a concentration of 30 μM of $CdCl_2$, a significant cell reduction of 70% was detected. Consequently, three different concentrations of $CdCl_2$ were used (5, 15, and 25 μM).

To evaluate the effects on cell viability of the selected $CdCl_2$ concentrations alone and in combination with SeMet, a new MTT assay was carried out. The results are presented in **Figure 2**. The lowest concentration of $CdCl_2$ (Cd5 group) reduced cell viability to 91.19 ± 2.50%, but the combination with SeMet (Cd5+Se group) did not significantly affect cell viability (p = 0.591). The result for the Cd15 group was 70.27 ± 1.44%, and the presence of SeMet (Cd15 + Se) increased the cell survival to 75.82 ± 2.02% (p = 0.018). In the Cd25+Se group, a very significant increase to 46.01 ± 1.89% compared to the Cd25 group (35.04 ± 0.09%) was detected. Our results showed that Cd hepatotoxicity could be mitigated by SeMet supplementation.

Cadmium and Selenium Concentrations in Hepatocellular Carcinoma Cells and Culture Media

The analysis of Se and Cd by ICP-QqQ-MS in cell pellets and culture media is summarized in (**Table 1**). The detection limits obtained for the analytical procedure (LOD) were 0.008 ng g^{-1} for Se and 0.001 ng g^{-1} for Cd. The basal concentration (control group) of Se in HepG2 cells is 0.052 μM, while the concentration of Cd was <LOD. In the culture medium, the concentrations of both elements were <LOD. The groups not exposed to SeMet resulted in similar concentrations in all of them, varying in the range of 0.032–0.041 μM. The addition of SeMet in the culture medium resulted in an uptake by HepG2 cells ranging between 11.02 and 15.494 μM. The concentrations of Cd in the Cd5 and Cd5+Se groups were 3.851 μM and 3.916 μM, respectively, and no statistically significant differences were observed (**Table 2**). This assumes an absorption of approximately 78% of the exposed Cd by the cells. However, the cells exposed to a concentration of 15 μM of Cd absorbed between 56 and 58% of the exposed Cd, showing a slight but significant increase in the Cd15 + Se group (p = 0.014). At the maximum concentration, 25 μM Cd, HepG2 cells only absorbed between 34 and 36% of the total Cd. The total Cd content in the Cd25 + Se group was slightly high (p = 0.02).

Selenoprotein Speciation

To elucidate whether Cd interferes with selenoprotein synthesis, the concentration of selenometabolites and selenoproteins (GPx, SELENOP, and SeAlb) has been quantified using column switching and a species-unspecific isotopic dilution approach. **Figure 3** shows the typical chromatogram for the Se, Cd5+Se, Cd15+Se, and Cd25+Se groups. The relative concentration of the selenoproteins and selenospecies in HepG2 cells is SELENOP > GPx > Se-metabolites > SeAlb. **Table 2** summarizes the results obtained from the quantification of selenoproteins and selenometabolites for the different study groups. The results of the statistical analysis are included in **Table 3**.

As can be observed in **Table 2**, the concentrations of GPx, selenometabolites, SELENOP, and SeAlb in control HepG2 cells are 0.010, 0.022, 0.03, and 0.004 μg Se/g, respectively. After exposure for 24 h with SeMet, the concentration of the species increases significantly (**Table 3**), reaching values of 20,495 μg Se/g for GPx, 16,378 μg Se/g for selenometabolites, 25,200 μg Se/g for SELENOP, and 3.041 μg Se/g for SeAlb.

The concentration of selenometabolites was also slightly increased in cells cultured with different doses of Cd. Regarding selenoproteins, only the SELENOP concentration increased in cells that were exposed to 5 μM Cd but not at higher concentrations (15 μM and 25 μM). The co-exposure of SeMet and Cd provokes a decrease in the concentration of GPx in

TABLE 1 | Total Se and Cd contents (μM) in cell pellets and culture media expressed as mean ± standard deviation (n = 3) and Cd and Se absorption percentage (%).

Group	(Se) (μM)		Absorption	(Cd) (μM)		Absorption
	Cells	Culture media	Se (%)	Cells	Culture media	Cd (%)
Control	0.052 ± 0.010	<LOD	—	<LOD	<LOD	—
Se	15.630 ± 0.157	75.559 ± 1.296	15.6	<LOD	<LOD	—
Cd5	0.033 ± 0.003	<LOD	—	3.851 ± 0.006	0.304 ± 0.012	77
Cd5 + Se	15.493 ± 0.220	82.040 ± 0.180	15.5	3.916 ± 0.061	0.294 ± 0.17	78.3
Cd15	0.032 ± 0.006	<LOD	—	8.404 ± 0.045	5.869 ± 0.173	56
Cd15 + Se	13.058 ± 0.376	90.565 ± 0.882	13.1	8.799 ± 0.316	6.451 ± 2.815	58.7
Cd25	0.041 ± 0.005	<LOD	—	8.569 ± 0.002	15.594 ± 0.424	34.3
Cd25 + Se	11.020 ± 0.311	84.138 ± 1.382	11	8.999 ± 0.199	15.694 ± 0.296	36

TABLE 2 | Concentration of selenoproteins and selenometabolites in HepG2 cells.

Group	GPx (μg Se/g)	Se metabolites (μg Se/g)	SELENOP (μg Se/g)	SeAlb (μg Se/g)
Control	0.010 ± 0.002	0.022 ± 0.002	0.03 ± 0.001	0.004 ± 0.0005
Se	20.495 ± 2.067	16.378 ± 3.995	25.200 ± 0.065	3.041 ± 0.002
Cd5	0.010 ± 0.005	0.168 ± 0.003	0.043 ± 0.001	0.004 ± 0.0004
Cd5+Se	14.120 ± 0.827	4.889 ± 1.243	21.642 ± 1.085	2.6647 ± 0.8357
Cd15	0.019 ± 0.006	0.069 ± 0.001	0.028 ± 0.002	0.005 ± 0.001
Cd15+Se	10.392 ± 1.802	1.773 ± 0.351	13.405 ± 0.400	1.494 ± 0.100
Cd25	0.010 ± 0.001	0.035 ± 0.002	0.025 ± 0.001	0.004 ± 0.001
Cd25+Se	12.392 ± 0.150	2.269 ± 0.291	15.665 ± 0.894	2.749 ± 0.265

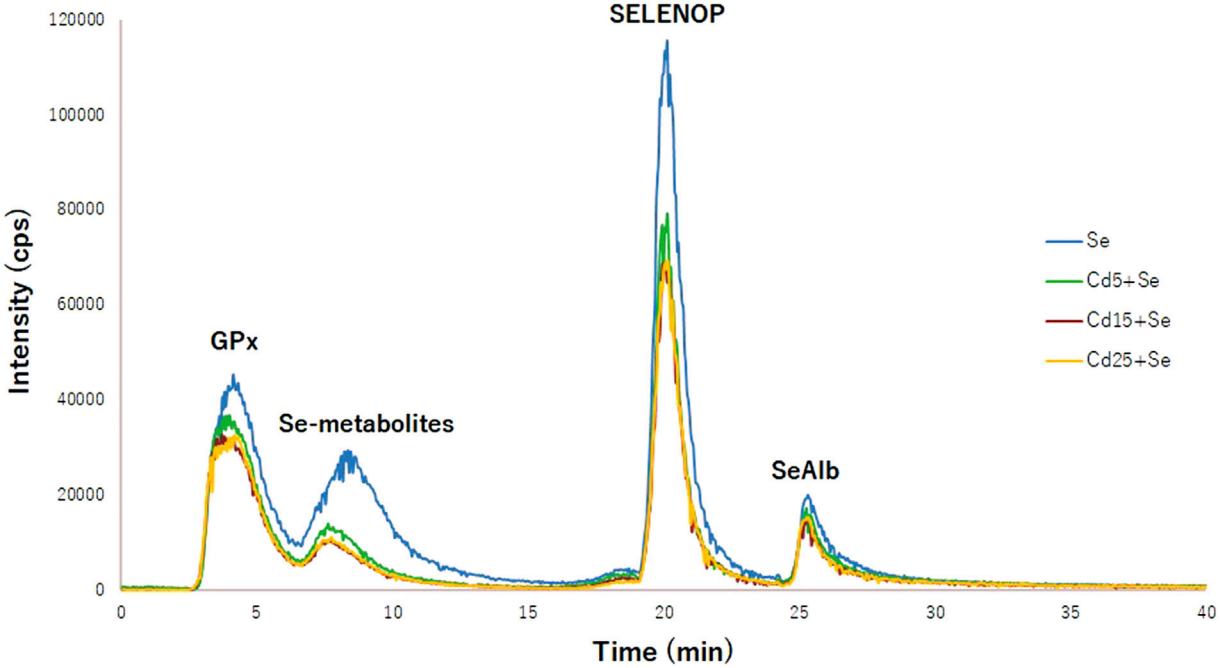


FIGURE 3 | Chromatogram of selenoproteins obtained by 2D-HPLC-SEC-AF-SUID-ICP-QqQ-MS. Blue (Se group); green (Cd5+Se group); red (Cd15+Se group); yellow (Cd25+Se group).

all study groups. However, no statistically significant changes are observed (**Table 3**). The concentration of selenometabolites in the Se group was 16.378 μg Se/g. Cd exposure significantly decreases the concentration to values of 4.889 μg Se/g (Cd5+Se), 1.773 μg Se/g (Cd15+Se), and 2.269 μg Se/g (Cd25+Se). The SELENOP concentration was also decreased to values of 21.642 μg Se/g (Cd5+Se), 13.405 μg Se/g (Cd15+Se), and 15.665 μg Se/g (Cd25+Se), with respect to the value of the Se group

TABLE 3 | *p*-values obtained for each comparison. Significance level $p < 0.05$.

	Selenoproteins and Selenoespecies (μg Se/g)			
Comparison	**GPx**	**Se metabolites**	**SELENOP**	**SeAlb**
Se *vs*. control	0.007	0.002	0.000	0.000
Cd5 *vs*. control	0.942	0.000	0.000	0.752
Cd15*vs*. control	0.062	0.000	0.479	0.486
Cd25*vs*. control	0.909	0.001	0.094	0.656
Cd5+Se *vs*. Se	0.196	0.009	0.005	0.478
Cd15+Se *vs*. Se	0.074	0.003	0.000	0.000
Cd25+Se *vs*. Se	0.118	0.004	0.000	0.129

(25.200 μg Se/g). SeAlb concentration was only diminished in cells from the Cd15+Se group.

DISCUSSION

The results obtained from this study indicated that intracellular Cd concentrations of the groups Cd15+Se and Cd25+Se were significantly lower than those exposed to Cd, and the total Se content was also decreased. Frisk et al. (2002) reported a similar study using K-562 cells that were either pre-treated or simultaneously treated with selenite or selenomethionine (5 or 50 μM) and cadmium nitrate (60 or 75 μM). Their results showed that the pre-treatment with Se caused a lower concentration of Cd in the cells when they were exposed to the higher concentration of Cd (75 μM) but not at the lower concentration (60 μM). On the other hand, the simultaneous treatment of both elements caused an increase in the intracellular Cd at all concentrations, suggesting that Se may affect Cd transport to cells.

In mammals, depending on the bioavailability of Cd, it can behave like other essential elements and can be better assimilated, inducing an imbalance in the normal balance of trace elements and destroying the structure and function of cells (Martelli et al., 2006). Our results indicated that the Cd content was reduced, pointing out that the interaction between Se and Cd produced a Cd-Se complex that affects the composition of these metals in the hepatocyte and presumably in the liver since this same effect has been seen in the liver of rabbits exposed to Cd and Se (Zhang et al., 2021); however, the opposite effect has been observed in mice that were exposed to low and high levels of Se simultaneously with Cd, resulting in a higher Cd concentration in the liver (Rodríguez-Moro et al., 2020).

On the other hand, the effects ofCd-induced hepatotoxicity in birds have also been quantified (Li et al., 2013). Results showed that dietary selenite decreased Cd accumulation and improved antioxidant defense, which attenuated adverse Cd-mediated morphological changes and oxidative stress in liver tissue (Li et al., 2013).

The protective role of Se has been demonstrated by restoring hepatic blood markers such as alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyl transferase (GGT), and total bilirubin, (Renugadevi and Prabu, 2010; Abu-El-Zahab et al., 2019; Wang et al., 2020) and antioxidant enzymes such as catalase (CAT) and glutathione peroxidase (GPx) (Ognjanović et al., 1995; Štajn et al., 1997; Jihen et al., 2009); the mechanisms by which trace metal Se suppresses Cd hepatotoxicity in mammals remain unclear.

Regarding selenoprotein concentration, the Se absorption efficiency in HepG2 cells depends on its chemical form, where organic species are more easily transported and assimilated than inorganic species (Wang et al., 2016; Takahashi et al., 2017). The results obtained from this study indicated that the concentration of GPx was not affected by the presence of Cd compared to control cells, but there was a slight decrease in the cells cultured with SeMet. GPx is employed as an oxidative biomarker as its activity is generally induced in response to mild oxidative stress as a defense mechanism. However, a rapid increase in intracellular ROS generation can overwhelm antioxidant mechanisms, resulting in a suppression of antioxidant enzymes (Cozzari et al., 2015).

As seen in both *in vivo* and *in vitro* studies, Se supplementation and exposure results in increased activity, expression, and concentration of most selenoproteins (Stoedter et al., 2010; Zwolak and Zaporowska, 2012; Nelson et al., 2016; Sun et al., 2020; Liu et al., 2021). In liver cells, the expressions of SELENOP and GPx3 exposed to SeMet, MeSeCys, and Na_2SeO_3 were increased compared to control cells (Takahashi et al., 2017). An increase in the expression of other selenoproteins such as GPx1 and SELENOK has also been correlated with a higher concentration of sodium selenite (Rusolo et al., 2013). In the study by Jamwal et al. (2016), the GPx activity was significantly (~35%) decreased in trout hepatocytes treated with Cd alone or in combination with a high concentration of selenite or SeMet (250 μM) relative to control cells. However, a partial recovery of the GPx activity was recorded in hepatocytes exposed to 100 μM Cd in combination with 25 μM selenite or SeMet.

The expression of 24 selenoproteins was determined in chicken hepatocytes that were cultured for 24 h with 5 μM Cd, 1 μM Se, and a mixture of both concentrations (Zhang et al., 2020a). The results presented that 18 of 24 selenoprotein transcripts (GPX1, GPX2, GPX3, GPX4, SelT, Sel15, SelP1, SelP2, TXNRD2, TXNRD3, DIO2, DIO3, SelS, SelK, SelN, SelO, SelU, and SelH) were over-upregulated, and SelW, TXNRD1, and SelX transcripts were downregulated in the Se group compared to the control group. When cells were cultured with Cd and Se, only nine transcripts were overexpressed (GPX1, SelT, SelP1, SelP2, SelS, SelK, SelN, SelO, and SelM), and two were decreased (SelW and SelU) when compared with the group only exposed to Cd (Zhang et al., 2020a).

Selenoproteins may be involved in the antagonistic process against Cd toxicity. In kidney and testicular tissues, it has been seen that Se protected against Cd toxicity by increasing the expression of most of the selenoproteins, including SELENOP and GPX (Messaoudi et al., 2010; Lin et al., 2018). However, our results showed a decrease in the concentration of selenoproteins and selenometabolites when cells are exposed to Cd and Se simultaneously. The recent study by Schwarz et al. (2020) has provided a new perspective on the behavior of selenoproteins in the presence of other metals. In his study, HepG2 cells were

exposed to variable concentrations of Cu and Se, revealing that Cu suppresses the mRNA levels of the selenoproteins GPX1 and SELENOW and decreases the activities of GPX and TXNRD. The effects of Cu were inhibited by applying copper chelators, showing that this element interferes with the synthesis and activity of selenoproteins through the limitation of UGA coding (Schwarz et al., 2020). The suppression of selenoprotein synthesis by Cd may also be due to the formation of the above-mentioned Cd-Se complex (Zhang et al., 2021).

Finally, SeAlb has been quantified for the first time in HepG2 cells. SeAlb is a Se transporter protein that is formed in the serum by the replacement of methionine with selenomethionine and transferred to the liver for the synthesis of selenoproteins. The concentration of SeAlb was higher in all Se-supplemented groups. Comparable results were reported from liver cytosolic extracts of mice exposed to Hg and Se simultaneously (García-Sevillano et al., 2015).

CONCLUSION

We can conclude that our study has provided new insights into the mechanisms behind the antagonistic interactions between Se against Cd. Se protects HepG2 cells by increasing their cell viability and inhibiting the transport of Cd into cells. The concentration of selenoproteins is also affected by the presence of Cd, interfering with its synthesis and resulting in a lower concentration of selenoproteins. For a better understanding of the Cd-Se interaction in HepG2 cells, it will be necessary to address new studies involving the study of selenometabolites, markers of oxidative stress, and the quantification and characterization of other selenoproteins and the Cd-Se complex.

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DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors. Q11

AUTHOR CONTRIBUTIONS

TG-B, FN, and JG-A conceived the idea. SR-A and RP performed the experimental work. SR-A and TG-B drafted the manuscript, and SR-A prepared the artwork. TG-B and FN supervised the whole work. TG-B acquired the funding and administrated the project. All the authors crucially revised the manuscript for important intellectual content and approved the final version to be published. Q12 Q13

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SUPPLEMENTARY MATERIAL Q17

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fchem.2022.891933/full#supplementary-material

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