Human Gut Microbial Taxa Metabolizing Dietary Obesogens: A BPA 1 directed-culturing and Bioinformatics Combined Approach

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Research

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Human gut microbial taxa metabolizing dietary obesogens: A BPA directed-culturing and bioinformatics combined approach

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Abstract

Background: Integrated data from culturomics and functional omics may depict holistic understanding on gut microbiome eubiosis or dysbiosis, and microbial isolates can become a source of differential enzymes and useful bioactive compounds. Culturing methods developed during last decade swift increases the importance of gut microbial isolates, focusing on media, modifications and conditions that propitiate cultured taxa that previously were considered fastidious or unculturable. In this context and focusing on gut microbiota dysbiosis triggered by obesogens and microbiota disrupting chemicals (MDC), we have conducted a directed-culturing and bioinformatics combined approach, adding bisphenol A (BPA) and specific treatments to find resistant spore-forming bacteria, to obtain isolated strains for further explore their molecular BPA metabolizing or neutralizing capacities.

Results: Overall microbiota culturing media and conditions have been retrieved and organized according to main gut taxa isolated during last decade. Furthermore, a catalogue of BPA directed-cultured microorganisms has been obtained from 46 fecal samples from two populations, children with obesity and normo-weight. A total of 235 BPA tolerating and potentially BPA biodegrading microorganisms were mainly grouped to strictly anaerobic sporuled/non-sporuled, anaerobic facultative sporuled/non-sporuled. Firmicutes, Enterobacteria and Actinobacteria species showed the major representation in both groups. However, differential BPA tolerant microbiota composition from the populations was detected. Bioinformatics analysis disclosed and predicted the variability of harboring genes encoding specific enzyme for BPA biodegradation pathways that corroborated from directed-culturing microbiota consortia obtained.

Conclusions: Strains from *Staphylococcus*, *Bacillus* and *Enterococcus* genera represented the majority of the successfully cultured bacteria in both population specimens. From them, the bioinformatics prediction assigned to *Bacillus spp.* the higher potential for BPA biodegradation.

Therefore, extensive directed-culturomics approaches could be designed for different MDC with common biodegradation pathways, such as parabens, phthalates, and benzophenones.

Keywords: culturomics, directed-culturing, obesogens, endocrine disruptors (ED), BPA, next-generation probiotics (NGP).

Background

Microbiota dysbiosis in obesity-related disorders triggered by exposure to ED and obesogens

Currently, the exposure to obesogens and ED can lead to a microbial dysbiosis [1,2]. The dysbiosis are based on misbalanced taxa compositions and associated to several metabolic diseases, such as type 2 diabetes, obesity, and other endocrine disorders [3, 4, 5]. To isolate, culture and analyze the microbial taxa components that can lead towards altered functional effects would allow a better understanding of the pathophysiological mechanisms and its prevention through the administration of beneficial microbes, helping to regulate the physiological hormonal axis [6]. Directed-culturing of microorganisms from obese and non-obese microbiota may lead to identify potential metabolizing and detoxifying strains, which could be used as NGP [7, 8].

The importance of culturomics for the human microbiome description is advancing towards more effective isolations via sophisticated culture methods of the human microbiome [9]. This method relies on intensively culturing human samples with different growth media under different conditions, along with identifying any isolated bacterial colonies with matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) and 16S rRNA gene sequencing [9, 10]. It showed its success in the isolation, description and characterization of new bacterial species from the human microbiota [9, 11, 12]. This enabled the expansion of the current human microbial database by reporting the isolation of a significant number of

novel bacterial species and rendered the identification of previously considered "unclassified organisms" possible in clinical settings [12].

Microorganisms detoxifying dietary obesogens: ED-Bisphenols

EDs are considered as MDC [13]. Concretely, BPA is used in polycarbonate and epoxy resins and packages. Its cumulative contamination reaches all kinds of environments, such as soils, sediments, and aquatic environments, water, air and dust particles [14]. Several routes of human exposure to BPA have been described, including the digestive system (ingestion) through exposure to food packaging, drinking containers, dental monomers [15, 16]; the vertical transmission (maternofetal) [17]; the respiratory system (inhalation) [18]; and the integumentary system (skin and eye contact) though the thermal paper of the receipts, eyeglass lenses and feminine hygiene products [19, 20]. The presence of this obesogen or MDC in humans has been confirmed by detecting it in human serum, urine, saliva, hair, tissue and blood [21, 22]. Thus, BPA removal from the natural environment is an increasing worldwide concern and several studies identified biological effective via to remove BPA from the environment through organisms such as bacteria, fungi, algae and plants [23, 24]. However, there are still no clear clinical studies aimed at eliminating or reducing the amount of exposure to BPA in the human body. The demonstrated evidence of the effects of BPA as an ED and its transfer to foods has led the industry to use analogous compounds such as bisphenol S (BPS). However, recently studies have shown that some of these analogues may be even more harmful than BPA [25]. In this case, BPS has also been shown to act as an ED but investigation in this field has remained limited [26]. Moreover, the use of NGP is increasing due to the specific knowledge of the human intestinal microbiota and the possibility of intervening and modulating the dysbiosis determined by certain diseases. Culturomics remains as main strategy for the isolation of new gut microorganisms.

The BPA-degradation capabilities from some microorganisms, like *Bacillus spp*., have been studied as an environmental and bioremediation resource [27, 28]. Furthermore, species from this genus have been isolated from infant fecal samples with the four complete molecular pathways of BPA degradation [29]. However, while the use of BPA-degrading microorganisms is widely extended in bioremediation, based on a previous review [30] there were no clinical trials involving beneficial microorganisms, metabolic diseases and xenobiotic obesogens. This fact may indicate a new area of research where NGP with the ability to modulate the microbiota are used, counteracting the impact of xenobiotics ingested through the diet.

This work focuses on promoting the knowledge regarding culturomics data searching and directed culturing through different microbial culture techniques to increase the catalogue of isolated microorganisms from human gut microbiota, more specifically, the approach focus on the BPA tolerant and/or biodegrader bacteria.

Material and Methods

Culturomics review data for increasing the microbiota taxa isolates

Literature search and review of studies were developed in collaboration with Granada librarian support using medical subject headings (MeSH) and the key words (see below) under a stepwise procedure search and adapted to each database's tutorials. The following electronic databases were searched from October 2020 to July 2021: PubMed, Web of Science (Thomson Reuters Scientific) and Scopus (Elsevier). The reviewers revised titles and abstracts, then full-text publications with reference to the inclusion criteria that were all the studies about culturomics or culturing from human gut microbiota, the key word were (Culturomics* AND microbiota), Culturing* AND microbiota AND obesity AND "endocrine disrupt*"; Culturomics* and microbiota and obesity and xenobiotic*; Culturing * and microbiota and obesity and hormon*; Culturing * and microbiota and obesity and "drug metabol*"; Culturing *

and microbiota and "metabolic syndrome" "endocrine disrupt*"; Culturomics * and microbiota and "metabolic syndrome" and xenobiotic*; Culturomics * and microbiota and "metabolic syndrome" and hormon*; Culturomics * and microbiota and "metabolic syndrome" and "drug metabol*"; Culturomics * and microbiota and diabetes and "endocrine disrupt*"; Culturomics * and microbiota and diabetes and xenobiotic*; Culturomics * and microbiota and diabetes and hormon*; Culturomics * and microbiota and diabetes and "drug metabol*"; Culturomics * and microbiota and fertility.

Experimental Culturomics approach to isolate gut microbes metabolizing obesogenic ED

BPA Directed-Culturing approach for the isolation of microbiota strain catalogue

A common approach to isolate microbial strains from microbiota has been pursued in our research team [29]. For this study, 235 microbial isolates from fecal human microbiota collections of 6–12 years-old children (Isolates-Project OBEMIRISK) appropriately maintained at -80 ºC underwent a directed culturing approach adding BPA to searching tolerant and potentially BPA biodegrading microorganism by a serial dilution method, and exposition to different BPA concentrations [0.5, 10, 20, and 50 ppm] during 72 h at 37ºC and further spreading in different media and incubated under aerobic and under anaerobic cultivation performed with Anaerocult® A system (Merck, Darmstadt, Germany) at 72 h and 37 ºC. Different conditions and culture mediums were used for optimizing the uncultured bacterial growth including Brain Heart Infusion (BHI), Man, Rogosa and Sharpe (MRS), Reinforced Clostridial Medium (RCM), Gifu Anaerobic modified Medium (GAMm) agar/gellan [31]. Isolated BPA-tolerant bacterial colonies with distinguishing features were isolated as pure culture for subsequent morphological, phenotypic and genotypic identifications: bacterial cell counts, gram staining, spore staining, capsule staining, catalase activity, oxidase, and motility tests.

BPA Directed-Culturing and Spore-forming searching taxa components: *Clostridium spp.* and *Bacillus spp.*

In parallel, a specific treatment was carried out to favor the isolation of spore-forming bacteria. For this, after the exposure to BPA and before the spread on the media, the samples were homogenized in 70% ethanol for 4h and treated with a bile acids solution (0,1mg/ml of bile bovine in PBS) for the metabolic activation of the spores. Then, the samples were processed and analyzed as described above. The 16s rRNA from all the isolated colonies were analyzed.

Genomic DNA extraction, Taxonomy Identification and Phylogenetic Analysis

Genomic DNA was extracted using DNAeasy columns (Qiagen®, Germany) following the manufacturing instructions. The isolated DNA was quantified using Nanodrop (Thermo Scientific) and biophotometer (Eppendorf® D30). The quality of DNA was monitored through gel electrophoreses. Complete 16S RNA gene sequencing of selected bacterial strains was done by Sanger method (Institute of Parasitology and Biomedicine "López-Neyra" (IPBLN) Service). Forward and reverse sequences were provided separately. Reverse sequence was converted to complementary sequence with Chromas Pro 2.0 software (Technelysium Pty Ltd., Tewantin, Australia). Sequences were examined for maximum homology against GenBank using National Center of Biotechnology Information's (NCBI) BLASTn program. The collection and phylogenetic comparison of 16S RNA partial gene sequences was done using the Ezbiocloud platform [32].

Genome data mining tools for prediction of BPA metabolic maps and enzymatic pathways in whole genome sequencing (WGS) Type strains from the closest isolated species and isolated from microbiota

In order to discover the presence of BPA biodegradation gene potential of cultured microbiota, several bioinformatics tools were used to perform genome mining. A data retrieving program

has been specifically computed using Pascal programming language to obtain the BPA pathways enzymes ID and the corresponding Loci from the microbial genomes.

Type strains genomes from the closest species isolated were retrieved from NCBI Genome Data Bank in GenBank file format in order to list the proteins that they were able to potentially encode the enzymes.

A more detailed prediction of the clusters was performed by checking the downstream and upstream genes of those involved in BPA biodegradation using NCBI genome map viewer.

The identification of BPA genes encoding enzymes involved on the four biodegradation pathways was carried out by the analysis of the WGS^T of type strains, following the same approach explained above.

Results and Discussion

Microbiota culturing approaches, media and conditions for isolation of gut microbial taxa

Theoretical searching on culturomics data, which were thoroughly analyzed, allowed retrieving main culturing media and conditions used for isolation of relevant gut microbiota taxa components are summarized in Table 1. This data extraction analysis displays at once a batery of media for susscessful isolating of specific species belonging to genera from phyla Firmicutes, Bacteroidetes, Actinobacteria, and alpha-Proteobacteria and information on their oxygen tolerance: aerobic, aerotolerant anaerobe; strictly anaerobic; and facultative anaerobe. Main media retrieved were: BCB (Blood Culture Bottle), BHI, BRU (Brucella medium), CBA (Columbia Blood Agar), CHRIS (Christensenella medium), CNA (Columbia NaladixicAcid Agar), COS (Columbia agar liquid medium + 5% sheep blood), CPVX (Chocolate agar + PolyViteX), GAM (Gifu Anaerobic Media), MB (Marine Broth), MRS (Man, Rogosa and Sharpe), RM (R-Medium), RCA (Reinforced Clostridial Agar), SCM (Schaedler Medium), TSB (Trypticase Soy Broth), YCFA (Yeast extract-casein hydrolysate-fatty acids), WC (Wilkins

188 Chalgren) with several modifications with supplements such as vitamins, blood, rumen fluid,
189 biliary salts, ethanol and several conditions collected in additional files (Supplementary
190 Material Excel Table 1S; Excel Table 2S).

191 Similarly, useful information on favoured cultured isolates from gut microbiota acting as
192 beneficial microorganisms or potential NGP was previously retrieved. Main media and
193 pertinent modifications for isolating obesity and anti-obesity probiotics were: BHI, GAM, Gut
194 Microbiota Medium (GMM), *Lactobacillus* selection (LB), MRS, YCFA, and BPA-added media
195 [8]. Therefore, culturomics efforts contributed to enlarge the repertoire of isolated bacterial
196 species from humans by 28% and provided biological material to the scientific community that
197 can be further studied for its role and interaction with other bacterial species and host [33].
198 Conversely, the efficient molecular methods, such as metagenomics, which aims to describe the
199 human microbiota with no culture efforts, needed complementary developing fields. However,
200 some drawbacks are encountered that require the use and development of comparing culture
201 approaches [34], such as sequencing depth bias [34, 35], incomplete genomic databases [12, 33,
202 36] or the ability to distinguish between live and dead bacterial DNA in the studied samples
203 [36]. In a recent study that examined the gut microbiota composition of 8 healthy individuals, it
204 was shown that culturomics enabled 20% higher bacterial richness in comparison to
205 metagenomics [37]. Interestingly, isolated species' genome sequences enlarged by 22% the data
206 obtained by metagenomics analyses and showed that the number of species recovered by
207 culture is higher than the number of species detected by metagenomics [37].

208

Table 1. Culturing media and conditions for isolation microbiota taxa components (Aer: Aerobic; Aan: Aerotolerant Anaerobe; SAn: Strictly Anaerobic; FAn: Facultative Anaerobe)

	Species / Oxygen Tolerance	Culturing Media and Conditions
Firmicutes	***Bacillus spp.*** / Aer / AAn /FAn [9, 38, 39, 40, 41]	BCB38; BCB02; BCB03; BCB04; BCB05; BCB06; BCB08; BCB09; BCB10; COS01; COS03; MB01; MB02; TSB01; BHI01; BCB19; YCFA06; BCB23; COS09; TSB04; BCB07; YCFA02; MB03; TSB03; BCB18; BCB01; COS02; COS04; BHI02; CBA01; MRS02; BCB37; BCB33; BHI07; BCB36; BCB46; BCB14; BCB15; BCB12; BBCB22; BHI04; BCB13; YCFA01; MB04; BCB55
	Blautia spp. / SAn [38, 39, 41, 42, 43, 44, 45]	BHI05; BCB13; BCB15; BCB01; BCB03; BCB05; BCB07; BCB09; BCB10; COS02; MB02; TSB04; YCFA05; CBA01; BCB52; RM01; BCB11; CNA01; YCFA01; BCB28; BCB19; COS09; YCFA03; WC02; CBA02; GAM02; RCA02
	Clostridium spp. / SAn [11, 40, 41, 42, 46, 47, 48]	BCB01; BCB03; BCB05; BCB07; BCB09; BCB10; COS04; MB02; BCB15; COS02; RM01; RCA01; BCB34; BCB39; CHRIS01; CBA01; BCB19; COS09; YCFA05; BCB13; TSB04; CBA02; YCFA01; SCM04; YCFA04; MB04; RM02; BCB49; BCB28; BCB25; CNA01; BCB17; BCB21; BCB50; BCB32; BCB02; BCB04; BCB11; WC01; BHI02; YCFA03; RCA02; WC02; BHI03; BCB33; BCB30; TSB02; BCB31; YCFA02; MRS02; RM03; COS03; TSB01; BCB22; COS08; MB03; TSB03; BCB06; MRS01; BHI01; BCB23; BCB12; BCB14; BCB16; BCB20
	Dialister spp. / SAn [41]	BCB07; CHRIS01; SCM04; RM01; RM02; BCB11; BCB19; COS02; BCB01; BCB03; BCB05; BCB09; BCB10; COS04; MB02; YCFA01; MRS01
	Enterococcus spp. / FAn [40, 41, 49]	CBA03; YCFA04; YCFA06; BCB04; BCB07; BCB06; BCB08; COS01; COS03; COS04; MB01; MB02; TSB01; YCFA01; CHRIS01; MRS01; SCM04; RM01; BCB23; BCB11; BCB17; BCB22; BCB19; BCB20; COS09; MB03; TSB03; TSB04; BCB10; RM02; BCB01; BCB03; COS02; BCB05; BCB09; BHI01; BCB02; CNA01; RM03; SCM01; YCFA02; RCA01; BCB15; BCB21; TSB02; WC01; BHI02; CBA01; MRS02; BCB13; BCB14; COS08; MB04; BCB12; BCB16; YCFA03; RCA02; WC02; BHI03; MRS03
	Eubacterium spp. / SAn [40, 41]	BCB07; SCM04; BCB15; MB02; BCB19; BCB01; BCB05; BCB09; COS02; COS04; RM01; RCA01; YCFA04; BCB03; BCB11; WC01; CBA01; COS09; BCB13; MB04
	Lactobacillus spp. / AAn [41]	BCB07; COS04; SCM04; CNA01; BCB10; COS02; YCFA01; MRS01; RM01; BCB11; YCFA02; CHRIS01; BCB15; BCB19; COS09; BHI03; BCB02; BCB03; BCB04; BCB06; COS01; COS03; MB01; MB02; TSB01; BHI01; BCB13; RCA01; RM02; BCB23; RM03; SCM01; CBA01; MRS02; BCB01; BCB09; BCB05; WC01; BHI02
	Megasphaera spp. / SAn [41, 50]	COS02; COS04; RM01; BCB07; YCFA01; BCB09; BCB10; SCM04; BCB31
	Peptoniphilus spp. / San [9, 11, 41]	CHRIS01; BCB01; BCB05; BCB07; MB02; BCB10; COS02; COS04; RM02; BCB35; YCFA01; MRS01; RM01; BCB53; BCB15; BCB03; BCB09; SCM04; BCB11; BCB38; BCB40; YCFA03
	Ruminococcus spp. / SAn [9, 11, 41, 42]	YCFA05; BCB11; RM03; SCM01; YCFA02; CBA01; BCB13; BCB15; BCB19; BCB03; BCB07; BCB09; COS02; RCA02; BHI03; BCB40; BCB41; RM01; RM02; TSB04; YCFA01; CHRIS01; SCM04; CNA01; BCB05
	Staphylococcus spp. / FAn [40, 41]	YCFA06; BCB01; BCB02; BCB03; BCB07; BCB06, BCB10; COS01; COS04; MB01; MB02; YCFA01; RM01; RM02; BCB11; BCB15; BCB19; COS09; MB04; BCB05; BCB14; BCB17; BCB20; COS08; MB03; TSB03; BCB08; BCB09; CHRIS01; BCB04; COS02; COS03; BHI01; CBA01
	Streptococcus spp. / FAn [40, 41]	BCB07; YCFA04; BCB04; BCB05; BCB10; MB02; RM01; CBA01; BCB06; COS02; BHI01: YCFA01; BCB02; BCB09; COS01; COS03; BCB03; COS04; BCB23; CNA01; BCB01; CHRIS01; SCM04; BHI02; BCB08; TSB01; MRS01; RCA01; WC01; YCFA06; BCB11
Bacteroidetes	***Alistipes spp.*** / SAn [9, 11, 40, 41, 42, 51]	YCFA05; BCB01; BCB03; BCB05; BCB07; BCB09; BCB10; COS02; COS04; RM02; BCB11; CNA01; YCFA02; WC01; CBA01; BCB19; YCFA01; CHRIS01; SCM04; BCB48; MB02; BHI02; CPVX01; BCB13; BCB24; MRS01; TSB04; YCFA04; RCA01; BRU02; SCM01; BCB15; COS09; MB04; SCM02; RM03; BCB27
	Bacteroides spp. / SAn [11, 40, 41]	BCB01; BCB03; BCB05; BCB07; BCB09; BCB10; COS02; COS04; MB02; BCB11; SCM01; YCFA02; RCA01; WC01; BHI02; CBA01; MRS02; BCB19; RM03; BCB13; CBA02; SCM04; YCFA04; CNA01; RM01; TSB04; BCB15; RM02; YCFA01; CHRIS01; MB04; TSB03; WC02; COS09; YCFA03; TSB02
	Butyricimonas spp. / SAn [11, 40, 41]	BCB41; BCB01; BCB03; BCB05; BCB07; BCB09; BCB10; COS02; MB02; CBA01; YCFA04; CHRIS01; SCM04; RM02; BCB11; SCM01; COS09; YCFA02; CNA01; BCB19
	Parabacteroides spp. / SAn / [40, 41]	BCB05; BCB07; COS02; COS04; SCM04; RM02; CBA01; BCB19; YCFA04; CHRIS01; RM01; BCB11; CNA01; SCM01; BCB15; TSB04; BCB01; BCB03; BCB09; BCB10; BHI01; WC01; BHI02; YCFA01; MB02; YCFA02; RCA01
	Prevotella spp. / SAn [40, 41, 42, 52]	BCB10; COS02; RM01; BCB05; BCB01; BCB07; BCB09; YCFA01; CHRIS01; WC01; BCB11; CNA01; CBA01; YCFA05; SCM01; SCM04; CBA04; BRU03; BCB19; BCB03
Actinobacteria	***Actinomyces spp.*** AAn [9, 11, 42]	BCB03; BCB09; YCFA02; CBA01; COS09; BCB19; BCB07; MB02; BCB48; BCB11; BCB42
	Bifidobacterium spp. / An/SAn [40, 41]	BCB07; BCB10; YCFA01; MRS01; SCM04; RM01; RM02; BCB11; CNA01; RM03; SCM01; CBA01; BCB15; BCB19; COS09; BCB01; BCB03; BCB05; YCFA02; RCA01; WC01; BHI02; COS02; MB02; CHRIS01; MRS02; BCB13; BCB17; YCFA03; WC02; BHI03; CBA02; MRS03; RCA02; BCB09; COS04; YCFA04; BCB23
	Collinsella spp. / SAn [11, 40, 41]	YCFA04; BCB05; BCB07; COS02; YCFA01; CHRIS01; RM01; BCB11; CNA01; RM03; SCM01; CBA01; BCB13; BCB15; BCB19; SCM04; RM02; BCB01; CBA02; MRS02; BCB41; MB07; BCB10; YCFA02; BCB23; COS09
	Corynebacterium spp. /AAn [11,41, 51]	SCM04; RM02; BCB11; BCB23; CPVX02; COS02; BCB44; BHI01; BCB07; COS04; BCB10; MRS01; CBA03
	Propionibacterium spp. / AAn [41]	BCB07; YCFA01; RM02; BCB11; BCB19; MB04; TSB04; YCFA03; CHRIS01; MRS01; SCM04; RM01; BCB09; MRS02; BCB02; BCB06; BCB10; MB01; MB02
α-P	***Enterobacter spp.*** / AAn [40, 41, 53]	BHI08; COS04; MB02; RM01; RM02; YCFA04; MRS01; BCB23; YCFA06; BCB11; BCB02; BCB03; BCB04; BCB07; BCB09; COS01; COS02; MB01; BHI01

BPA Directed-Culturomics approach

We identify 192 bacteria isolates from human gut microbiota with high BPA tolerance [>20 ppm]. They were isolated from general media, supplemented with BPA, without searching for associated taxa, in following order: BHI (80 isolates), MRS (49 isolates), RCM (30 isolates), GAMa (18 isolates) and GAMg (15 isolates) without any specific media for associated taxa. The overall mean values estimated for colony counts were BHI + BPA 20 ppm $7X10^7$ CFU/ml, BHI + BPA 50 ppm $2X10^8$ CFU/ml GAMa, MRS + BPA 20 ppm $8X10^7$ CFU/ml, MRS + BPA 50 ppm $4X10^7$ CFU/ml; RCM + BPA 20 ppm $5X10^7$ CFU/ml, RCM + BPA 50 ppm $1X10^7$ CFU/ml; GAMa + BPA 20 ppm $1X10^6$ CFU/ml, GAMa + BPA 50 ppm $5X10^5$ CFU/ml; GAMa + BPA 20 ppm $5X10^6$ CFU/ml, GAMa + BPA 50 ppm $2X10^6$ CFU/ml. It is interesting to highlight that taxa from Actinobacteria phylum with high BPA tolerance were isolated only in BHI medium. The relative abundance of these isolates, together with taxonomically closest species, maximum BPA concentration tolerated and specific media for isolation are detailed in Table 2 for normo-weight children specimens analyzed and Table 3 for specimens from children with obesity. A phylum grouping data analysis showed differences in relative abundance of cultured Firmicutes, Proteobacteria and Actinobacteria between both populations. Firmicutes were the most abundant phylum with BPA tolerance found, representing 72% in normo-weight children and 73% in children with obesity. Proteobacteria was differentially represented in both groups by 17% and 20%, respectively. However, dataset showed differences in Actinobacteria and uncultured bacteria groups, Actinobacteria group represented 6% of the bacteria isolated in normo-weight children and 5% in children with obesity, in comparison to uncultured bacteria that represented 5% of the total bacteria isolated in normo-weight group, and 3% in population with obesity. Similarly, xenobiotics and specifically BPA tolerance by specific gut microorganisms was previously described for the traditional probiotics *Bifidobacterium breve* strain Yakult (BbY) and *Lactobacillus casei* strain Shirota (LcS) that showed protective effects against BPA dietary exposure in rats by reducing the intestinal absorption of BPA and facilitating its excretion [54]. Similarly, *Lactococcus lactis* strains adsorbed BPA but not degrade it [55]. Bioaccessible BPA decreased after

digestion and this exposure changed microbial community, up-regulating the abundance of BPA-degrading bacteria, such as *Microbacterium* and *Alcaligenes* [56].

Table 2. BPA tolerant cultured bacteria taxa from **normo-weight** microbiota.

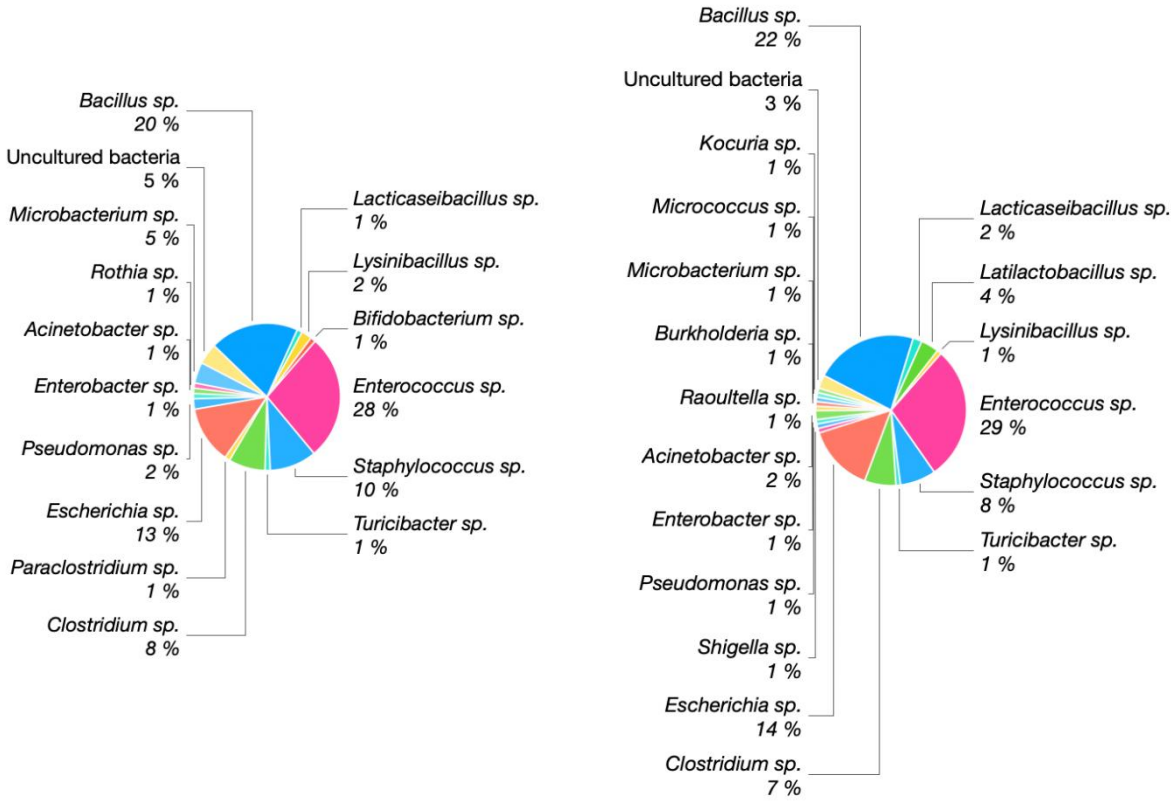
Representative Isolates (%)	Closest species Accession Number	Similarity of partial 16S rRNA (%)	BPA [ppm] Treatments	Media
	Firmicutes			
11	*Bacillus velezensis* |NC_009725.1|	100	50	BHI/MRS
1	*Bacillus amyloliquefaciens* |MW363310.1|	100	100	BHI
3	*Bacillus subtilis* |HQ333016.1|	99.54	50	BHI/MRS
1	*Bacillus nealsonii* |NR_044546.1|	99.66	50	GAMg
2	*Bacillus altitudinis* |MT627439.1|	100	50	BHI
1	*Lacticaseibacillus paracasei* |CP039707.1|	99.74	10	RCM
2	*Lysinibacillus fusiformis* |CP026120.1|	100	50	MRS
1	*Bifidobacterium animalis* |MT613598.1|	98.65	20	MRS
15	*Enterococcus faecium* |MW816627.1|	100	50	BHI /MRS/GAMg/RCM
6	*Enterococcus faecalis* |NR_113902.1|	100	20	BHI
1	*Enterococcus durans* |MT545097.1|	99.89	50	GAMg
1	*Enterococcus mundtii* |AP019810.1|	99.83	20	GAMg
1	*Enterococcus lactis* |MZ475096.1|	100	20	RCM
3	*Enterococcus hirae* |KX752868.1|	99.85	50	RCM
1	*Staphylococcus capitis* |CP053957.1|	100	20	BHI
2	*Staphylococcus caprae* |NR_119252.1|	99.39	50	MRS
2	*Staphylococcus cohnii* |MK465351.1|	99.88	50	BHI/GAMa
3	*Staphylococcus epidermidis* |CP040883.1|	100	50	RCM
1	*Staphylococcus saprophyticus* |CP054831.1|	100	50	BHI
1	*Turicibacter sanguinis* |CP053187.1|	91.26	50	BHI
1	*Clostridium tertium* |JX267105.1|	99.59	20	BHI
1	*Clostridium symbiosis* |KR364763.1|	99.49	20	BHI
6	*Clostridium paraputrificum* |NR_113021.1|	99.65	50	BHI/GAMa
1	*Paraclostridium bifermentans* |MT604800.1|	100	50	RCM
	Proteobacteria			
9	*Escherichia coli* |MH511549.1|	99.87	20/50	BHI/MRS/GAMa
1	*Escherichia coli* |CP059988.1|	93.09	50	BHI
1	*Escherichia coli* |CP046009.1|	91.35	50	BHI
1	*Escherichia fergusonii* |NR_074902.1|	99.79	20	BHI
1	*Pseudomonas synxantha* |CP074078.1|	77.83*	20	GAMg
1	*Pseudomonas parafulva* ||MT367815.1	100	50	GAMa
1	*Enterobacter hormaechei* |CP027111.1|	99.80	50	RCM
1	*Acinetobacter radioresistens* |MT367790.1|	99.76	20	RCM
	Actinobacteria			
1	*Rothia dentocariosa* |CP054018.1|	99.86	100	BHI
5	*Microbacterium paraoxydans* |NR_115540.1|	98.76	20/50	BHI
5	Uncultured bacteria		10/20/100	RCM/BHI

Table 3. BPA tolerant cultured bacteria taxa from **obese** microbiota.

Representative Isolates (%)	Closest species Accession Number	Similarity of partial 16S rRNA (%)	BPA [ppm] Treatments	Media
	Firmicutes			
12	*Bacillus velezensis* |NR_075005.2|	99.40	20/50/100	BHI/MRS/GAMa
1	*Bacillus amyloliquefaciens* |MW363310.1|	100	20	RCM
2	*Bacillus subtilis* |MN393073.1|	100	50	GAMg
1	*Bacillus pacificus* |CP041979.1|	99.74	50	GAMa
1	*Bacillus cereus* |KX161843.1|	99.11	20	MRS
1	*Bacillus paramycoides* |MT538529.1|	99.89	20	RCM
1	*Bacillus altitudinis* |MT627439.1|	99.84	20	GAMg
1	*Bacillus circulans* |MT294022.1|	99.87	50	MRS
2	*Bacillus safensis* |MT377905.1|	99.10	20	BHI
1	*Bacillus licheniformis* |MT642945.1|	100	20	MRS
2	*Lacticaseibacillus casei* |KF673514.1|	99.77	20	MRS
4	*Latilactobacillus sakei* |NR_113821.1|	99.85	10	MRS
1	*Lysinibacillus fusiformis* |MT605500.1|	99.71	50	BHI
18	*Enterococcus faecium* |MN453594.1|	97.95	20/50	BHI/MRS/GAMa
3	*Enterococcus faecalis* |MT611694.1|	99.73	20	BHI/RCM
4	*Enterococcus lactis* |MZ475096.1|	100	50	BHI
3	*Enterococcus hirae* |NR_114743.1|	99.85	20	BHI
1	*Enterococcus gallinarum* |NR_104559.2|	99.77	50	BHI
5	*Staphylococcus epidermidis* |CP043804.1|	99.87	20/50	BHI/RCM/GAMa
1	*Staphylococcus caprae* |NR_119252.1|	100	50	BHI
1	*Staphylococcus capitis* |NR_027519.1|	100	10	MRS
1	*Staphylococcus saprophyticus* |NR_041324.1|	99.55	20	BHI
1	*Turicibacter sanguinis* |CP053187.1|	100	50	BHI
5	*Clostridium paraputrificum* |MN055965.1|	97.47	50	GAMg
1	*Clostridium desporicum* |NR_026491.1|	99.46	20	GAMg
1	*Clostridium tertium* |MT539087.1|	100	50	BHI
	Proteobacteria			
13	*Escherichia coli* |CP053231.1|	99.87	20/50	MRS/BHI
1	*Escherichia fergusonii* |MT912775.1|	99.25	10	BHI
1	*Shigella flexneri* |JX307691.1|	99.87	50	RCM
1	*Pseudomonas parafulva* |MT367815.1|	100	50	MRS
1	*Enterobacter cancerogenus* |MT557032.1|	100	20	RCM
1	*Raoultella ornithinolytica* |MF462255.1|	100	50	BHI
2	*Acinetobacter radioresistens* |NR_114074.1|	99.80	10	BHI
1	*Burkholderia contaminans* |HQ746879.1|	99.18	20	MRS
	Actinobacteria			
1	*Microbacterium oxydans* |MT533951.1|	100	50	BHI
1	*Micrococcus luteus* |CP043842.1|	99.13	20	BHI
1	*Kocuria rhizophila* |NR_027193.1|	100	20	BHI
3	Uncultured bacteria		20/50	BHI

All the sequences were submitted to GenBank under the Accession Numbers: MZ614066-MZ614252.

Most dominant BPA tolerant genera were *Enterococcus* sp., *Bacillus* sp., *Escherichia* sp., *Staphylococcus* sp. in both populations (Fig. 1) representing near 75% of the taxa found. However, we can see differences between both groups in the minority BPA tolerant genera, some of these genera are exclusive of each population, conforming differential microbiota composition according to normo-weight children or children with obesity. The minority BPA tolerant genera found exclusively in normo-weight children were *Rothia* sp., *Paraclostridium* sp. and *Bifidobacterium* sp. However, *Kocuria* sp., *Micrococcus* sp., *Burholderia* sp., *Raoultella* sp., *Shigella* sp., and *Latilactobacillus* sp. were found exclusively in overweight and obese children.



(**a**) Normo-weight microbiota specimens. (**b**) Overweight and Obesity microbiota specimens.

Fig. 1 Relative abundance of genera in microbiota from 22 normo-weight children compared to 24 Overweight or Obesity treated with BPA. Numbers given in the pie chart correspond to this percentage.

Microbial community from the culturomics approach and the statistical analyses showed that the obese group had more diversity, richness (Chao1, Observe) and evenness (Shannon, InvSimpson) than the normo-weight group. As for other culturomics studies, these results lead to complement already adapted

approaches by highlighting the bacteria that were considered "un-cultivable" as they might be playing an important role in the health balance and disease development [36].

Importantly, culturomics for isolating new bacterial species included toxicogenomics approach to describe novel organisms able to metabolize toxicants [6]. New bacterial species are subjected to a series of phenotypic, biochemical and genomic characterization (habitat, sporulation, shape, antibiotics profile, metabolism, fatty acids contents, genome sequencing/ assembly and annotation).

BPA Directed-Culturomics and Spore-forming microbiota taxa: *Clostridium spp.* y *Bacillus spp.*

We identify 43 spore-forming bacteria isolates from human gut microbiota with high BPA tolerance [>20 ppm]. They were isolated from general media, supplemented with BPA, in following order: GAMg (14 isolates), GAMa (18 isolates) and RCM (13 isolates) without any specific media for associated taxa. The overall mean values for colony counts were for GAMa + BPA 20 ppm $9X10^4$ CFU/ml, GAMa + BPA 50 ppm $4X10^4$ CFU/ml, GAMg + BPA 20 ppm $1X10^5$ CFU/ml, GAMg + BPA 50 ppm $6X10^4$ CFU/ml, RCM + BPA 20 ppm $9X10^4$ CFU/ml, RCM + BPA 50 ppm $5X10^4$ CFU/ml. The relative abundance of these spore-forming bacteria isolates, together with taxonomically closest species, maximum BPA concentration tolerated and specific media for isolation are detailed in Table 4 for normo-weight children specimens analyzed and Table 5 for specimens from children with obesity.

Table 4. BPA tolerant spore-forming bacteria taxa cultured from **normo-weigth** microbiota.

Representative Isolates (%)	Closest species Accession Number	Similarity of partial 16S rRNA (%)	BPA [ppm] Treatments	Media
	Firmicutes			
9	*Bacillus amyloliquefaciens* |MZ359899.1|	100	50	GAMa/RCM
4	*Bacillus vallismortis* |KX462780.1|	99.54	50	RCM
4	*Clostridium disporicum* |LC515630.1|	99.53	50	GAMg
22	*Clostridium paraputrificum* |MN913836.1|	100	20/50	GAMa/GAMg/RCM
4	*Clostridium perfringens* |MT613499.1|	99.75	20	GAMg
9	*Paraclostridium benzoelyticum* |AB973393.1|	99.51	20/50	GAMg
39	*Paeniclostridium sordellii* |CP014150.1|	99.88	20/50	GAMa/GAMg/RCM
4	*Uncultured bacterium* |GQ159075.1|	97.30	20	GAMa
4	*Uncultured bacterium* |KF110610.1|	99.88	50	GAMa

All sequences were submitted to GenBank under the Accession Numbers: MZ612806-MZ612850.

Table 5. BPA tolerant spore-forming bacteria taxa cultured from **obese** microbiota.

Representative Isolates (%)	Closest species Accession Number	Similarity of partial 16S rRNA (%)	BPA [ppm] Treatments	Media
	Firmicutes			
14	*Bacillus amyloliquefaciens* |KM853034.1|	99.76	20/50	GAMa/GAMg
10	*Bacillus velezensis* |MZ474622.1|	99.88	20/50	GAMa
5	*Bacillus pumilus* |HM055978.1|	99.42	50	GAMa
5	*Bacillus subtilis* |KX950665.1|	98.99	50	GAMa
5	*Bacillus licheniformis* |HQ290087.1|	100	20	RCM
5	*Bacillus paralicheniformis* |MT645610.1|	99.04	50	GAMg
5	*Clostridium perfringens* |MH69435.1|	99.65	50	GAMa
24	*Clostridium paraputrificum* |MN913836.1|	99.88	20/50	GAMa/GAMg/RCM
5	*Clostridium symbiosum* |LC515566.1|	100	20	RCM
5	*Clostridium tepidum* |MF581527.1|	98.12	20	GAMa
14	*Paraclostridium benzoelyticum* |MT510437.1|	99.88	50	GAMa/GAMg
5	*Uncultured bacterium* |HQ541237.1|	99.12	20	GAMg

* Pretreatment ethanol and bile acids. All sequences were submitted to GenBank under Accession Numbers: MZ612806-MZ612850.

In this catalogue of spore-forming isolates from normo-weight children, *Clostridium spp*. represented 30.44% and *Bacillus spp*. 13.05%. In contrast, from obese children higher percentages were found, *Clostridium spp*. constituted 38.09% and *Bacillus spp*. 42.85%. It is interesting to highlight that *Paeniclostridium sordellii* specimens with high BPA tolerance were isolated only from normo-weight children, where it is the most representative specie (39.13%). If we focus on biodiversity at the species level, a total of 7 different species and 2 isolates categorized like Uncultured bacterium have been isolated from samples belonging to normal-weight children. In the case of the isolates belonging to children with obesity, a total of 11 species and an Uncultured bacterium have been cultured, highlighting the cultivation of 6 different species of the *Bacillus* genus compared to the 2 isolated from the samples of children with normo-weight.

BPA biodegradation metabolic maps through WGS^T data mining

The bioinformatics analysis carried out on the WGS of Type strains of closest species identified as cultivable species from microbiota showed a differential potential of BPA biodegradation and specific enzymes arsenal involved (Table 6). The genome mining allowed identifying specific clusters prone to degrade bisphenols. Bioinformatics tools and Pascal ad hoc programme allowed the exhaustive analysis

of genomes making it a powerful prediction toxicomicrobiomics tool. According to the theoretical predictive results, overall microbiota naturally possessed an intermediate degree of BPA biodegradation potential by the different enzymatic pathways disclosed (BPA (I) 41%, BPA (II) 36%, BPA (III) 41%, and BPA (IV) 39%). *Burkolderia*, *Bacillus*, *Raoultella*, *Acinetobacter*, *Micrococcus* and *Microbacterium* species were clustered as biodegrader. The analysis showed that they harboured the more complete BPA biodegradation genetic clusters (> 50%), while species of *Bifidobacterium*, *Lactobacillus, Enterococcus*, *Clostridium, Paeniclostridium* and *Turicibacter* did not contain representative percentages of the gene loci for BPA biodegradation encoding enzymes and were clustered as tolerant or resistant to BPA.

Table 6. Microbiota representative genera harboring gene loci for encoding differential BPA enzyme pathways

BPA PATHWAYS DATA ANALYSIS	BPA (I)	BPA (II)	BPA (III)	BPA (IV)	Mean	BPA capacity
Gut Representative Taxa (Genera)	40%	34%	42%	38%	38%	**Threshold***
Acidaminococcus	18%	31%	38%	14%	24%	Tolerant
Acinetobacter	52%	52%	55%	51%	52%	Biodegrader
Actinomyces	35%	38%	38%	29%	36%	Tolerant
Akkermansia	29%	23%	38%	43%	31%	Tolerant
Anaerostipes	24%	31%	38%	14%	27%	Tolerant
Bacillus	61%	53%	53%	54%	56%	Biodegrader
Bifidobacterium	24%	23%	38%	29%	27%	Tolerant
Burkholderia	76%	62%	75%	71%	71%	Biodegrader
Clostridium	13%	17%	22%	14%	16%	Resistant
Desulfovibrio	18%	15%	25%	43%	22%	Tolerant
Eggerthella	18%	15%	38%	14%	20%	Tolerant
Enterococcus	25%	15%	21%	14%	20%	Tolerant
Escherichia	56%	23%	38%	43%	41%	Biodegrader
Flavonifractor	29%	31%	38%	14%	29%	Tolerant
Kocuria	53%	38%	63%	71%	53%	Biodegrader
Lactobacillus	24%	12%	19%	29%	20%	Tolerant
Lysinibacillus	65%	58%	56%	64%	61%	Biodegrader
Micrococcus	53%	46%	50%	71%	53%	Biodegrader
Microbacterium	53%	54%	54%	43%	52%	Biodegrader
Paraclostridium	12%	8%	25%	14%	13%	Resistant
Pseudomonas	41%	38%	63%	57%	47%	Biodegrader
Raoultella	71%	46%	75%	57%	62%	Biodegrader
Roseburia	18%	31%	25%	14%	22%	Tolerant
Rothia	47%	31%	38%	71%	44%	Biodegrader
Shigella	41%	8%	38%	43%	31%	Tolerant
Slackia	29%	31%	50%	43%	36%	Tolerant
Staphylococcus	47%	23%	50%	43%	40%	Biodegrader
Turicibacter	12%	0%	25%	14%	11%	Resistant

*BPA Biodegrader>39%-71%; BPA Tolerant>20%-38%; BPA Resistant<19%; * Data analysis in Supplemental material

Genome mining data based on WGS^{T} representative BPA biodegradation analyses was achieved through the advances in next generation sequencing (NGS) and *in silico* tools allows performing an appropriate screening of genes of concern or interest in microbiota, such as biodegradation capacities or toxicomicrobiomics potential through bioinformatics, metagenomics or in silico analysis of cultivable

isolates WGS [58, 59]. A better understanding of the microbiota ecology driven by the bioactive compounds, which are released by gut microbial components may drive towards better clinical interventions [60]. Genome mining done in the present study allowed BLAST driven searching for predicted BPA pathways. Pascal ad hoc programme analysed the type strain genomes making it a powerful prediction tool. Similarly, another useful prediction tool could be used as well as for BPA biodegradation pathways [61].

Interestingly, the species found exclusively in normo-weight children microbiota (*Paraclostridium* sp. and *Bifidobacterium* sp.) had a low BPA biodegradation potential, being clustered as BPA tolerant or resistant. However, the species from obese children (*Kouria* sp., *Micrococcus* sp., *Burkolderia* sp., *Raoultella* sp. and *Shigella* sp.) showed higher BPA degradation potential, being grouped as BPA biodegrader. Thus, a first trend of this analysis showed that microorganisms from obese children seemed to present more BPA biodegradation potential than normo-weight children.

Moreover, comparative data from wide metagenomics analysis regarding the variability of taxa composition in individuals with obesity and normo-weight, Firmicutes/Bacteroidetes (F/B) ratio constitutes a recognized biomarker for comparisons, as well as Actinobacteria and Proteobacteria relative abundances. F/B ratio showed higher values in obese than normo-weight individuals [62] as Actinobacteria appeared usually also higher in obese population. Conversely, Bacteroides and Proteobacteria were slightly higher in normo-weight populations [62]. In parallel, our BPA directed-culturomics approach have demonstrated that Firmicutes was one of the more predominant populated taxa able to grow in BPA and showing biodegrader-like profiles (*Bacillus*, *Staphylococcus*, *Micrococcus*). However, we could not compare data from Bacteroidetes as no cultivable taxa were obtained through this approach. On the other hand, isolated Proteobacteria taxa able to grow from no-obese specimens were different and they harboured lower capacity of BPA biodegradation compared to those obtained in children with obesity, which compiled species with the highest percentage of BPA enzymatic gene loci (*Escherichia coli, Escherichia fergusonii, Shigella flexneri, Pseudomonas parafulva, Enterobacter cancerogenus, Raoultella ornithinolytica, Acinetobacter radioresistens* and *Burkholderia contaminans*).

In this sense, it is important to consider the ecological role of those enzymes and their impact on the gut microbiota composition may have a huge influence on metabolizing and neutralizing BPA, by releasing metabolites that contribute to the modification of individual taxa microbial components on long-term basis [63].

Interestingly, specific transitory gut taxa identified with high potential of BPA biodegradation could be also used for environmental bioremediation purposes or plant probiotics. Several authors investigated the BPA removal capacity using bacterial strains from dessert soil that belong to *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Klebsiella* sp. and *Pantoea* sp. [64]. Degradation of BPA by *Pseudomonas putida* YC-AE1 was considered as a low cost effective and eco-friendly method compared to physical and chemical methods [65]. Similarly, a consortium isolated from river sediment (*Terrimonas pekingensi*s and *Pseudomona*s sp.) was able to use BPS as the sole carbon source and was highly efficient to degrade 99% with an initial concentration of 50 mg/L in 10 days [66]. Gut bacteria harbouring laccases could be used for detoxification of several hazardous dietary contaminants and emerging ED through bioreactor with novel biocatalytic system based on active membranes and immobilized laccase technology [67].

Conclusions

We are exposed to obesogenic MDC, such as bisphenols and concretely to BPA. The pathophysiological impact of these obesogens seem to depend on inter-individual and diverse microbial gut composition, and we are just starting to understand how these microbiota consortia interact with host and how their enzymatic arsenals would shape those communities to build a functional human microbiome. Our results indicate that specific and differential gut enriched microbial isolates or consortia that resist, tolerate or biodegrade BPA were present in human-associated microbial communities and they harboured the specific gene encoding enzymes involved in biodegrading BPA and other obesogens, and that such enhancing enzymatic properties of the gut communities could perpetuate their modulation ecological actions, even after the exposure to obesogens or BPA should be present, impacting in health and disease host status.

Availability of supporting data

Sequence files and metadata for all samples used in this study have been deposited in Genbank under the GenBank submission numbers: SUB10046802; SUB10052679. A full record of all raw analysis for culturomics and bioinformatics BPA-biodegradation prediction is included as Additional files.

Availability of data and materials: Additional files and Special Files

All data generated or analysed during the study are included in **Additional files:** Excel 1. Culturomics retrieving information; Excel 2. Culturing media and conditions; Excel 3. Bioinformatics for BPA Loci Prediction.

Special files are available under request to the Authors. Excel 4. Complete Bioinformatics for BPA Loci Prediction.

Abbreviations

MDC: Microbiota Disrupting Chemicals; BPA: Bisphenol A; ED: Endocrine Disruptors; NGP: Next-Generation Probiotics; MALDI-TOF MS**:** Matrix-Assisted Laser Desorption/Ionization Time Of Flight Mass Spectrometry**;** BPS: Bisphenol S; MeSH: Medical Subject Headings; BHI: Brain Heart Infusion; MRS: Man, Rogosa and Sharpe; RCM: Reinforced Clostridial Medium; GAMm: Gifu Anaerobic modified Medium; IPBLN: Institute of Parasitology and Biomedicine "López-Neyra"; NCBI: National Center of Biotechnology Information; WGS: Whole Genome Sequencing; BCB: Blood Culture Bottle; BRU: Brucella Medium; CBA: Columbia Blood Agar; CHRIS: Christensenella Medium; CNA: Columbia Naladixicacid Agar; COS: Columbia Agar Liquid Medium + 5% Sheep Blood; CPVX: Chocolate agar + PolyViteX; GAM: Gifu Anaerobic Media; MB: Marine Broth; RM: R-Medium; RCA: Reinforced Clostridial Agar; SCM: Schaedler Medium; TSB: Trypticase Soy Broth; YCFA: Yeast Extract-Casein Hydrolysate-Fatty Acids; WC: Wilkins Chalgren; GMM: gut microbiota medium; LBS: *Lactobacillus* selection; Aer: Aerobic; AAn: Aerotolerant Anaerobe; SAn: Strictly Anaerobic; FAn: Facultative Anaerobe; CFU: Colony Forming Unit; GAMa: GAM agar; GAMg: GAM gelano; BbY: *Bifidobacterium breve* strain Yakult; LcS: *Lactobacillus casei* strain Shirota; NGS: Next Generation Sequencing.

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Authors' contributions

MA conceptualized the rational of the Manuscript; ARM, PO and MU performed the core work of the culturomics literature review. ALM and ARM performed the experimental directed-culturing for the bacteria catalogue. ALM drafted and prepared the initial manuscript. JP performed the BPA bioinformatics and WGS data mining. All assessed the content of the manuscript and discussion and performed a critical comparison of full data. MA revised and commented on the final draft of the manuscript.

Ethics approval and consent to participate

Fecal sample library was obtained after corresponding approval of CEIC 20/12/2019.

Consent for publication

Not Applicable.

Competing interests

The authors declare that they have no competing interests.

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