FEMS Microbiology Letters, 367, 2020, fnaa041

https://doi.org/10.1093/femsle/fnaa041
Advance Access Publication Date: 3 March 2020
Minireview

MINIREVIEW – Food Microbiology

Probiotic antigenotoxic activity as a DNA bioprotective tool: a minireview with focus on endocrine disruptors

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One sentence summary: This review deals with the ability of probiotics and/or food associated bacteria to counteract the DNA damage induced by food and environmental genotoxic compounds with focus on endocrine disruptors.

Editor: Cinzia Randazzo

ABSTRACT

Nowadays, the interest in the role of dietary components able to influence the composition and the activity of the intestinal microbiota and, consequently, to modulate the risk of genotoxicity and colon cancer is increasing in the scientific community. Within this topic, the microbial ability to have a protective role at gastrointestinal level by counteracting the biological activity of genotoxic compounds, and thus preventing the DNA damage, is deemed important in reducing gut pathologies and is considered a new tool for probiotics and functional foods. A variety of genotoxic compounds can be found in the gut and, besides food-related mutagens and other DNA-reacting compounds, there is a group of pollutants commonly used in food packaging and/or in thousands of everyday products called endocrine disruptors (EDs). EDs are exogenous substances that alter the functions of the endocrine system through estrogenic and anti-estrogenic activity, which interfere with normal hormonal function in human and wildlife. Thus, this paper summarizes the main applications of probiotics, mainly lactobacilli, as a bio-protective tool to counteract genotoxic and mutagenic agents, by biologically inhibiting the related DNA damage in the gut and highlights the emerging perspectives to enlarge and further investigate the microbial bio-protective role at intestinal level.

Keywords: probiotics; antigenotoxicity; SOS-Chromotest; endocrine disruptors; zebrafish

INTRODUCTION

In recent years, the field of nutritional science has enjoyed great successes, defining the essential requirements for specific nutrients to assume during each phase of human life and for the maintenance of a health status and well-being. The quality of food and the magnitude of the diet-health relationship have now attracted extreme interest with important scientific discoveries and with commercial and technological implications. According to the World Health Organization (WHO), the state of health of the population is strongly influenced by the diet. The human gut hosts a complex ecosystem generated by the integrity and stable cooperation between immune cells, resident microbiota and gastro-intestinal (GI) epithelium (McCracken and Lorenz 2001). When this balance is altered, it may trigger a state of dysbiosis that can lead to systemic or chronic diseases, (Sekirov *et al.* 2010; Prakash *et al.* 2011; Abbasi *et al.* 2015, Federici *et al.* 2017). In this respect, the intestinal microbiota is considered a very metabolically active organ that can be affected by multiple factors such as host gene, age and delivery pattern, antibiotics and diet (Hasan

Received: 10 January 2020; **Accepted:** 2 March 2020

and Yang 2019). The latter relationship is still not completely understood, even though recent studies highlighted the contribution of particulars nutrients or chemicals components, and following the transfer of different microorganisms, in affecting the intestinal microbiota (Scott *et al*. 2013; David *et al*. 2014; Graf *et al*. 2015). Epidemiological and clinical-experimental evidences highlighted the crucial role played by the intestinal microbiota in the modulation of genotoxic and mutagenic risk in the GI tract (Burns and Rowland 2000; Davis and Milner 2009).

Therefore, the interest concerning dietary components (including food-borne microbes) that can modulate the intestinal microbiota and potentially confer benefits to the consumer is now growing (Gratz, Wallace and El-Nezami 2011; Claesson *et al*. 2012; Sharma and Shukla 2016). Probiotics and prebiotics represent the most used strategies for the production of functional foods and for the maintenance of the intestinal ecosystem equilibrium (Hill *et al*. 2014; Abuajah, Ogbonna and Osuji 2015; Gibson *et al*. 2017; Champagne, Gomes da Cruz and Daga 2018; Kerry *et al*. 2018). In particular, some microorganisms, mainly of the genera *Lactobacillus* and *Bifidobacterium*, that are present in food as probiotics, exert positive effects by protecting against intestinal pathogens, modulating the immune response, reducing the cholesterol levels and by improving the digestion of lactose (Shah 2007; Sekirov *et al*. 2010; Nagpal *et al*. 2012; Hill *et al*. 2014; Marco and Golomb 2016). The positive clinical outcomes are due to the ability of microorganisms to endure the harsh GI tract conditions and to persist stably herein. Recently, the microbial ability to make a protective role at GI level by counteracting the biological activity of genotoxic compounds is thought important for reducing gut pathologies and is considered a new tool for probiotics and functional foods (Trotta *et al*. 2012; Raman *et al*. 2013; Prete *et al*. 2017).

In this perspective, diet cannot be exclusively conceived as a source of nutrients for both humans and gut microbiota and a vehicle of potentially active microbes (O'Hara *et al*. 2006; Sekirov *et al*. 2010), but also as a supply of genotoxic compounds that can be commonly found in the gut. Recently, beside food-related mutagens, a group of pollutants commonly used in cosmetics, food packaging and/or in thousands of everyday products called EDs deserves great interest. EDs are exogenous substances or mixture that alter the functions of the endocrine system through estrogenic and anti-estrogenic activity, which are known to interfere with normal hormonal function in human and wildlife (Albert and Jégou 2014; Perugini *et al*. 2019; Zezza *et al*. 2019). Recently, the exposure of EDs as environmental stressors has been associated with metabolic disorders, such as diabetes and obesity (Le Magueresse-Battistoni *et al*. 2017), besides many others adverse effects in various organs including, in some cases, carcinogenic effects through DNA damage (Voss *et al*. 2005; Ventrice *et al*. 2013; Zarean *et al*. 2016). Up to date, the microbial antigenotoxic activity has been widely investigated against model genotoxins (i.e. 4-nitro-quinoline-1-oxide (4-NQO), methylnitronitrosoguanidine (MNNG)) and some food-related mutagens (i.e. aflatoxins, nitrosamines) by primary DNA damage *in vitro* assays, mainly SOS-Chromotest and Comet assay (Caldini *et al*. 2005; Caldini *et al*. 2008; Corsetti *et al*. 2008; Fang *et al*. 2008; Verdenelli *et al*. 2010; Walia, Keshani and Kanwar 2014; Bocci *et al*. 2015; Pithva *et al*. 2015; Prete *et al*. 2017; Chandel *et al*. 2019; Janosch *et al*. 2019). However, only few studies have been focused on evaluating the genotoxic effects of EDs (Cabaton *et al*. 2009; Umbuzeiro, Machala and Weiss 2011; Martinez-Paz *et al*. 2013; Erkekoglu and Kocer-Gumusel 2014) and their impact on gut homeostasis and immune response modulation and this field of research is now an emerging area of interest.

Furthermore, several studies showed validation in *in vivo* using murine models (Dominici *et al*. 2014) or by evaluating the antigenotoxicity using faecal water (FW) (Nowak, Slizewska and Otlewska 2015; Federici *et al*. 2017). About that, the use of zebrafish (*Danio rerio*) as *in vivo* model to validate different probiotic effects as well as to evaluate toxicity and genotoxicity of several compounds is an emerging tool (Avella *et al*. 2012; Zhang *et al*. 2012; Zhou *et al*. 2012; Caballero-Gallardo, Olivero-Verbel and Freeman 2016; Gioacchini, Rossi and Carnevali 2017; Dal Santo, Grotto and Boligon 2018). Several studies reported that administration of probiotic containing *Lactobacillus rhamnosus* modulates the composition of the gut microbiota, which subsequently changes host lipid and glucose metabolism, activates the endocannabinoid system, controls immune response and inflammation and restructures intestinal epithelial organization, affecting fish growth (Falcinelli *et al*. 2017; Gioacchini, Rossi and Carnevali 2017).

In particular, zebrafish is a well-characterized *in vivo* model, with a relatively short life cycle (few months) that allows full life-cycle tests (Ankley and Johnson 2004). In addition to that, the zebrafish genome can be considered approximately 70% equivalent of human orthologs, providing a further value as a model species for biomedical research (Howe *et al*. 2013). Zebrafish has emerged as an ideal *in vivo* model to study the aquatic toxicity of environmental chemicals including EDs (Chen *et al*. 2018a; Perugini *et al*. 2019).

Thus, this paper reviews the main applications of probiotics and food-associated microbes, mainly lactobacilli, as a bioprotective tool to counteract genotoxic and mutagen agents, by biologically inhibiting the related DNA damage in the gut. Starting with an overview of the main EDs, an emerging group of mutagens, and their adverse effects at intestinal level, the most widely applied *in vitro* models to investigate the microbial antigenotoxic activity are described. The final part of the review highlights the innovative perspective to investigate the microbial bio-protective role at intestinal level, by using *Danio rerio* as a novel *in vivo* model to investigate probiotic effects.

EMERGING ENVIRONMENTAL MUTAGENS: ENDOCRINE DISRUPTORS (EDs)

In 2002, the WHO defined EDs as 'an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations' (WHO/UNEP 2012). Humans and animals may be exposed to a wide range of endocrine active substances through the diet as well as other sources. Endocrine disruption includes a variety of different mechanisms of toxicity that might affect different individual endpoints (Table 1). The molecular mechanisms by which EDs can exert their effects are various and include their ability: (i) to bind to estrogen receptors by triggering a cascade of molecular effects that ultimately modify gene expression; (ii) to alter the levels of endogenous hormones by changing the rate of their production; (iii) to mimic or inhibit the activity of endogenous hormones (Alofe, Kisanga and Inayat-Hussain 2019). A key characteristic of EDs action is a non-monotonic dose-response curve causing adverse effects at low doses but not necessarily at higher doses (Beausoleil *et al*. 2013). The majority of EDs are synthetic compounds released into the environment by anthropogenic activities, either alone or in combination, although some of them are naturally present as plant-derived phytoestrogens or fungus-derived mycoestrogens (Tavares *et al*. 2016; Darbre 2019).

Table 1. Principal EDs and their side effects on human health.

EDs classes	Representative compounds[a]	Adverse side effects	References
Pesticides	Chlorpyrifos, Atrazine, Glyphosate	Genotoxicity obesity, insulin resistance, gut microbiota dysbiosis	Jacobson-Pereira *et al.* 2018 Yuan *et al.* 2019
Polybrominated Diphenyl Ethers (PBDEs)	PBDE-47, PBDE-99, PBDE-153	Carcinogenicity	Dunnick *et al.* 2018
Polychlorinated Biphenyls (PCBs)	PCB-153, PCB-126, PCB-173	Mutagenicity, obesity, metabolic syndrome, non-alcoholic fatty liver disease	Liu *et al.* 2017 Wahlang *et al.* 2013
Heavy metals	Cadmium, Lead, Arsenic	Mutagenicity, oxidative stress, immunosuppression	Li *et al.* 2019 Zhang *et al.* 2017
Engineered nanomaterials (ENMs)	Graphene oxide, Titanium dioxide	Oxidative stress, gut microbiota dysbiosis, immunotoxicity	Zheng *et al.* 2019 Chen *et al.* 2016
Personal care products (PCPs)	Methylparaben, Propylparaben, Butylparaben	Estrogenic, anti-androgenic properties, obesity	Nowak *et al.* 2018
Phenolic derivates	BPA, BPS, BPF	Obesity, genotoxicity, oxidative stress	Legeay and Faure 2017 Hercog *et al.* 2019
Phthalates	DEHP, DnHP	Hormones disruption, genotoxicity	Karabulut and Barlas 2018
Nitrosamines	NDMA, NDPA, NMOR	Hormones disruption, obesity	Somade *et al.* 2016 Zhu *et al.* 2019

[a]EDs noted as: 2,2′,4,4′-Tetrabromodiphenyl ether (PBDE-47), 2,2′,4,4′,5-Pentabromodiphenyl ether (PBDE-99), 2,2′,4,4′,5,5′ Hexabromodiphenyl ether (PBDE-153), 2,2′,4,4′,5,5′-Hexachlorobiphenyl (PCB-153), 3,3′,4,4′,5-Pentachlorobiphenyl (PCB-126), 2,2′,3,3′,4,5,6-Heptachlorobiphenyl (PCB-173), Bisphenol A (BPA), Bisphenol S (BPS), Bisphenol F (BPF), Di-(2-ethylhexyl) phthalate (DEHP), Di-n-hexyl phthalate (DnHP), N-nitrosodimethyamine (NDMA), N-nitroso-di-n-propylamine (NDPA), N-nitrosomorpholine (NMOR).

Emerging evidence indicates an association between exposure to EDs and modifications of gut bacterial communities leading to increase susceptibility to pathogens and/or the occurrence of metabolic diseases (Velmurugan *et al.* 2017). Indeed, gut bacteria were demonstrated to be efficient in the biotransformation of multiple classes of contaminants modulating the EDs toxicological potential toward host organism (Evariste *et al.* 2019). Moreover, recent studies report that some EDs also induce genotoxic effects (Güzel and Ayaz 2019; Hercog *et al.* 2019).

Among different type of chemicals known as EDs, only a small fraction of them, such as many pesticides, have shown to interfere also with the metabolism of target organism (MacBean 2012). In addition, the relationship between the occupational exposure to various pesticides and the presence of DNA damage and oxidative stress in humans has been demonstrated (Jacobsen-Pereira *et al.* 2018). Pesticides can also act on gut microbiota composition, affecting their metabolites and destroying intestinal mucosa (Yuan *et al.* 2019).

Other types of EDs that represent a great concern for their mutagenic and genotoxic activity are synthetic chemicals, such as polychlorinated biphenyl (PCBs) and polybrominated diphenyl ethers (PBDEs), both widespread anthropogenic compounds commonly found in dietary sources (Liu *et al.* 2017; Dunnick *et al.* 2018). Recently, prenatal exposure to PBDEs and PCBs was associated with changes in gut microbiota of infants and children (Iszatt *et al.* 2019; Laue *et al.* 2019).

Heavy metals are naturally occurring elements that have wide range of industrial, domestic, agricultural, medical and technological applications (Tchounwou *et al.* 2012). Their distribution in the environment is raising concerns over their potential effects on human health, since sub-lethal levels of heavy metals increase mutation rates and enrich *de novo* mutations (Li *et al.* 2019). Heavy metals reveal also a direct impact on the gut microbiota that, in turn, have an essential role in limiting heavy metal body burden (Breton *et al.* 2013). Moreover, probiotic-based diet containing *Lactobacillus plantarum* strains showed a protective effect on the intestinal microbiota following exposure to waterborne cadmium (Zhai *et al.* 2017).

On the other hand, engineered nanomaterials (ENMs) are increasingly used in many applications across military, industrial and consumer field (Agans *et al.* 2019). The cytotoxicity and genotoxicity properties of ENMs are not well established as well as their interaction with gut microbiota and intestinal barrier. However, available data suggest that nanoparticles may affect the microbiota, and clinical disorders such as colitis, obesity and immunological dysfunctions might follow (Pietroiusti, Magrini and Campagnolo 2016).

Pharmaceuticals and personal care products (PPCPs) are a group of emerging environmental pollutants able to induce physiological effects in humans at very low doses (Ebele, Abdallah and Harrad 2017). Among these PPCPs, antimicrobial agents used for human and veterinary medicines or included in consumer products as preservatives, are continuously released in the environment. Although the exposure to some PPCPs (e.g. parabens) has been associated with metabolic syndrome in humans, the long-term effects on the host microbiome remain still unclear (Kim and Chevrier 2019). The effects of triclosan, largely used as antimicrobial agent, are widely investigated because its high detection levels in human fluids and its impact on the metabolism and the gut microbiota in animal models (Ma *et al.* 2020). Recently, dietary administration of *L. plantarum* ST-III has shown to alleviate intestinal damages and behavioural symptoms resulting from triclosan exposure (Zang *et al.* 2019).

Microplastics, plastics that break down into pieces smaller than 5 mm, are sometime included in the group of EDs because they seem to lead dysbiosis through mechanical disruption or acting as a vector of chemicals and other EDs (Fackelmann and Sommer 2019). Bisphenol A (BPA) is widely used for manufacturing plastic products and has been classified as an environmental obesogenic compound acting through several mechanisms

(Legeay and Faure 2017). Recently, BPA and its analogues bisphenol S, bisphenol F and bisphenol AF, showed cytotoxic, genotoxic and oxidative potential activities (Maćczak *et al.* 2017; Hercog *et al.* 2019). Moreover, dietary BPA intake influences the gut microbiota composition and functions that could be an important factor to develop of metabolic syndrome (Lai *et al.* 2016).

Phthalates are another group of chemicals used to make plastics more flexible and harder to break. They are used in a wide variety of products, such as vinyl flooring, adhesives, detergents, lubricating oils, automotive plastics, plastic clothes and personal care products (Monneret 2017). Postnatal exposure to phthalates results in a transient gut microbial dysbiosis in human new-borns (Yang *et al.* 2019). Di-(2-ethylhexyl) phthalate (DEHP) induced genotoxic, morphometric and hormonal effects in pre-pubertal male rats (Karabulut and Barlas 2018). However, the genotoxic potential and toxicological effects of other phthalates such as di-n-hexyl phthalate (DnHP), are not yet clarified even though one of the probable mechanism could be the generation of reactive oxygen species and thus the induction of oxidative stress, strictly related to the inflammatory diseases and cancer (Erkekoglu and Kocer-Gumusel 2014).

Finally, nitrosamines (NAs) are well established carcinogenic, mutagen and hepatotoxic agents which can be formed mostly through the oxidation of dialkylhydrazine intermediates, in a variety of food stuffs such as smoked fish, dried malt, beer, milk products, meat products and preserved fruit juices (Somade *et al.* 2016). They are able to interfere with thyroid and reproductive functions as well as to induce changes in gut microbiota leading to obesogenic microbiota profile at environmentally relevant levels (Somade *et al.* 2016; Zhu *et al.* 2019).

In recent years, EDs have been linked to a wide range of pathologies and long-term effects as decreased fertility or infertility, alteration in the normal onset of puberty and puberty-related development as well as the promotion of breast, testicle and prostate cancer (Wallace 2015). Furthermore, some EDs have been considered obesogens because they might also interfere with regulatory processes in metabolism and in the control of adipocyte function, resulting in an imbalanced regulation of body weight, which can lead to obesity (Nappi *et al.* 2016) metabolic syndromes and diabetes (Fénichel and Chevalier 2017). At the end, it is important to highlight that EDs can be found in mixtures and a cumulative action can occur, thus their potential combined effects should be useful to understand their role in a wide range of pathologies.

IN VITRO INVESTIGATIONS OF MICROBIAL ANTIGENOTOXIC ACTIVITY

Experimental evidence for antigenotoxic activity of probiotics and/or food-borne microbes, mainly lactobacilli and bifidobacteria, has been primarily obtained by evaluating the microbial ability to inhibit DNA damage, the earliest event in carcinogenesis, in *in vitro* models (Hiramaya and Rafter 2000; Commane *et al.* 2005). To date, the most widely applied assays to evaluate bacterial genotoxic inhibition are represented by primary DNA damage *in vitro* assays, such as the SOS-chromotest with prokaryotic target and Comet assay by using eukaryotic cell lines as targets (Fig. 1). Among short-term mutagenic assays, the SOS-chromotest has been proposed as a screening tool to evaluate probiotic antigenotoxicity due to the practical advantages, such as sample preparation, rapid procedure, specificity in carcinogens recognition and concordance with other mutagenicity tests (e.g. Ames test). SOS-chromotest evaluates the DNA damage induced by genotoxic compounds on a prokaryotic tester organism (*Escherichia coli* PQ37). Briefly, *E. coli* PQ37 (*sfi*A::*lac*Z, *uvr*A, rfa, Pho^c) carries a *sfi*A::*lac*Z gene fusion, responsible for β-galactosidase (BG) induction in the presence of DNA damage, easily detectable through a colorimetric enzymatic assay (Quillardet and Hofnung 1993).

Several probiotics and/or food-associated bacterial strains, mainly lactic acid bacteria (LAB), have been investigated using SOS-Chromotest for their inhibition effects of model genotoxins and food-related mutagens of different nature in which a consistent decrease in biological activity was observed (Table 2). Overall, the results highlighted a widespread inhibition activity among various LAB strains, but with a clear strain-dependent specificity and a specific bacteria-genotoxin interaction as a putative bioconversion mechanism responsible for genotoxin detoxification (Corsetti *et al.* 2008; Prete *et al.* 2017), also confirmed by gas chromatography-mass spectrometry (GC-MS) and Infrared-Raman analyses (Verdenelli *et al.* 2010, Bocci *et al.* 2015). Other studies were conducted on probiotics *Bacillus* spp. (Caldini, Trotta and Cenci 2002; Cenci *et al.* 2008), *Streptococcus salivarius* var. *thermophilus* and *Enterococcus faecium* strains (Raipulis, Toma and Semjonovs 2005; Walia, Keshani and Kanwar 2014) and in yeasts strains (Trotta *et al.* 2012; Prete *et al.* 2017). Remarkably, most of the strains that are able to suppress the effects of genotoxic compounds have also displayed *in vitro* (Trotta *et al.* 2012) and *in vivo* (Deabes *et al.* 2012) antioxidant properties. Interestingly, most *in vitro* studies have underlined a strong antigenotoxic activity in many food-associated strains similar to that of probiotics, confirming that probiotic properties can be common features of food-borne microbes (Marco, Heeney and Binda 2017).

Similar results have also been reported by investigating primary DNA damage in *in vitro* intestinal cell lines (i.e. HT-29, Caco-2) or in *in vivo* test subjects by Comet assay, a single cell gel electrophoresis test (Table 2). This is an easy and highly sensitive cellular assay widely used in genotoxicity and DNA damage/repair studies for detecting single/double strand breaks, and a variety of DNA lesions (i.e. apyrimidinic/apurinic and excision repair sites) in individual mammalian cells (Singh *et al.* 1988). The comet assay has been used to investigate the antigenotoxic activity of some food-associated lactobacilli and bifidobacteria in the colon mucosa of rats treated with MNNG and or 1,2-dimethylhydrazine (DMH), in which a dose-dependent protective effect of microbial viable cells was reported (Pool-Zobel *et al.* 1996). Similar results were also found for different LAB species against different genotoxins in which concordance of *in vitro* results obtained with SOS-Chromotest and Comet assay was also confirmed (Caldini *et al.* 2005; Chandel *et al.* 2019). Comet assay was also used to investigate prebiotics and probiotics antigenotoxicity on human genotoxic FW by using adenocarcinoma cell lines with interesting findings on the prebiotics protective effect against early DNA damage (Burns and Rowland 2004; Nowak, Slizewska and Otlewska 2015). Antigenotoxic activity was also reported for Gram-negative probiotic strains, *E. coli* Nissle 1917 (Janosch *et al.* 2019).

Furthermore, the capacity of probiotics to degrade and/or adsorb environmental chemicals such as EDs is also emerging. Yasushi *et al.* (2007) reported that lyophilized cells of LAB could remove BPA from the medium by adsorption mechanism and a similar property was also found in yeast *Pichia pastoris* (Mergler, Wolf and Zimmermann 2004). Recently, a panel of commercial probiotic strains, belonging to multiple species (i.e. *Lactobacillus* spp., *Bifidobacterium* spp. and *Streptococcus thermophilus*) has been investigated for their ability of detoxifying and degrading

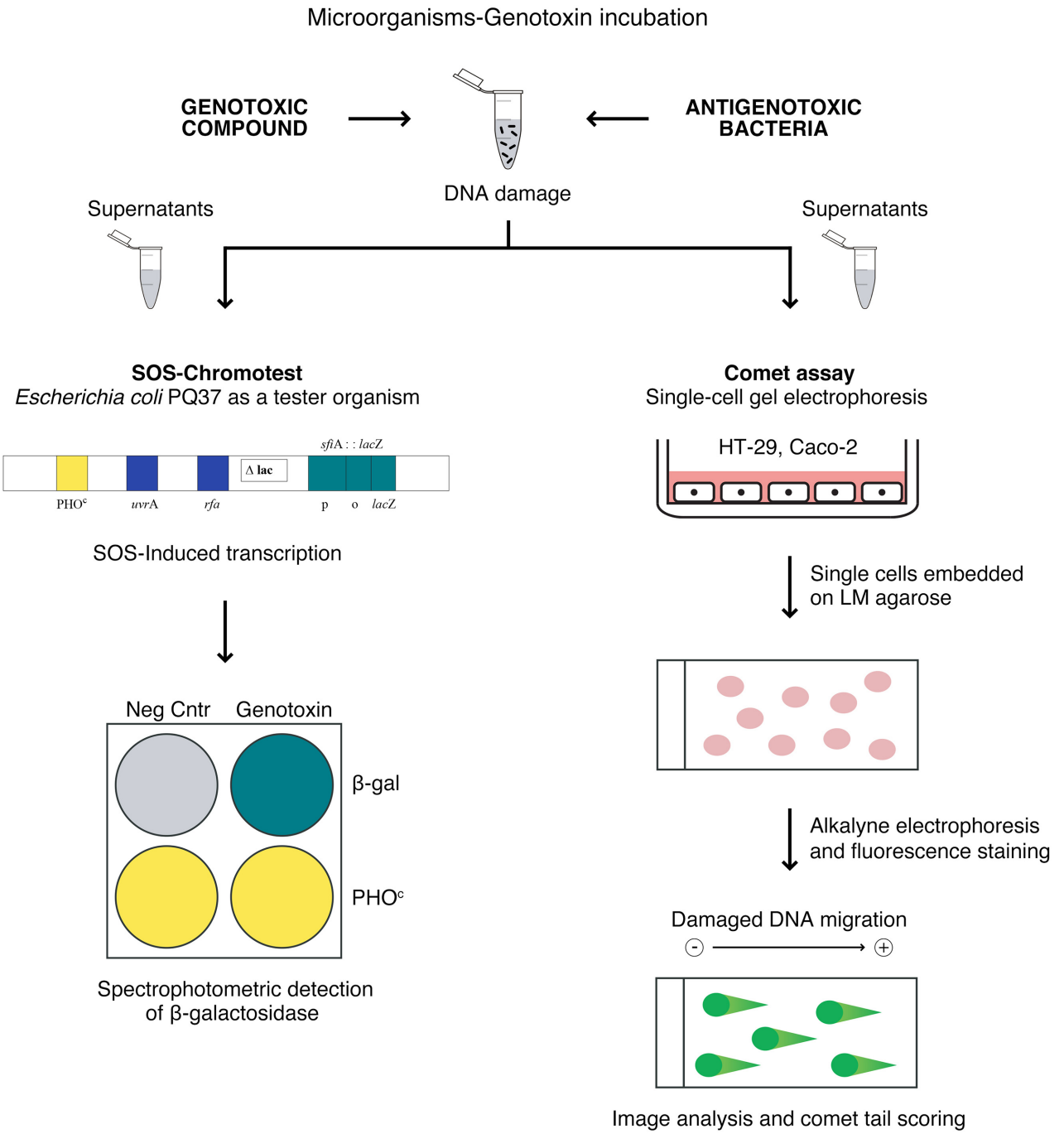

Figure 1. Schematic representation of *in vitro* primary DNA damage assays used to assess microbial antigenotoxic activity. SOS-Chromotest is a rapid colorimetric assay that evaluates the DNA damage induced by genotoxic compounds through the measure of the SOS-response of a prokaryotic tester organism *E. coli* PQ37 (*sfiA*::*lacZ*, *uvr*A, rfa, Pho^c), by quantifying β-galactosidase production under the induction of the SOS repair system. Comet assay is based on the encapsulation of eukaryotic cells in a low-melting (LM) point agarose suspension, followed by electrophoresis of lysed cell in neutral or alkaline conditions. Electrophoresis results in comets-like DNA migration, visualized by fluorescence microscopy. Undamaged DNA preserves a highly compact structure appearing as head of the comet, whereas DNA damaged strands migrates to resemble a comet tail, whose intensity reflects the amount of DNA damages.

environmental BPA (Solouki, Fazeli and Solouki 2018). Dairy lactobacilli also displayed *in vitro* binding activity towards pesticides (Trinder *et al.* 2015) and BPA (Zhu *et al.* 2017), confirming the bioremediation by using microbial cells as a low cost putative strategy to face increasing EDs exposure (Rai, Ganguli and Neogi 2017). Despite these efforts, the role of probiotics and food-borne microorganisms in degrading EDs and hence exhibiting protective effects remains unclear. Therefore, no investigations are available about the microbial antigenotoxicity activity against EDs, thus we performed a preliminary study that enlarged the investigation of the antigenotoxic activity of two documented probiotics *L. plantarum* strains IMC510 and IMC513 (Prete *et al.* 2017; Garcia-Gonzalez *et al.* 2018; Prete *et al.* 2020) against two different EDs: BPA and Di-n-hexyl phthalate (DnHP),

Table 2. Probiotics and food-associated bacteria evaluated for their antigenotoxic activity against different mutagenic compounds.

Antigenotoxic bacteria	Methods	Genotoxic agents[a]	References
Probiotics/human strains			
B. breve, *B. longum*	Comet assay	MNNG; DMH	Pool-Zobel *et al.* 1996
L. acidophilus, *Bifidobacterium* spp.	Ames test	4-NQO; MNNG; NF; NPD; AFB; MeIQ, PhIP, MeAαC	Lankaputhra and Shah 1998
Bacillus spp. (probiotics)	SOS-Chromotest	4-NQO	Caldini, Trotta and Cenci 2002
Bifidobacterium sp. 420, *Bifidobacterium lactis* Bb-12, *L. plantarum*, *Streptococcus thermophilus*, *L. bulgaricus*, *Enterococcus faecium*	Comet assay	FW	Burns and Rowland 2004
L. plantarum KL and 8014, *L. casei* Shirota, *L. acidophilus* T20, *Streptococcus salivarius* var. *termophilus*, *B. lactis* Bb-12	SOS-Chromotest	Furazolidone	Raipulis, Toma and Semjonovs 2005
Bacillus spp. (probiotics)	SOS-Chromotest	4-NQO, MNNG, MeIQ, AFB1	Cenci *et al.* 2008
L. acidophilus J76, *L. casei* 5H10, *L. bulgaricus*, *L. fermentum*, *L. plantarum* J25, *L. rhamnosus* J54, *B. longum*	SOS-Chromotest; Ames test	Heating cooking oils	Isidori and Parrella 2009
L. rhamnosus IMC501	GC-MS; SOS-Chromotest	4-NQO	Verdenelli *et al.* 2010; Bocci *et al.* 2015
L. casei LOCK 0900, *L. paracasei* LOCK 0919	Comet assay	PhIP	Nowak, Slizewska and Klewicka 2011
L. rhamnosus 0900 and 0908, *L. casei* 0919, *L. casei* Shirota, *L. casei* DN 114–001, *L. johnsonii* La1, *L. rhamnosus* GG, *B. animalis* ssp. *lactis* Bb-12, *L. delbrueckii* 0987, *L. mucosae* 0988	Comet assay	FW	Nowak, Slizewska and Otlewska 2015
L. rhamnosus Vc	SOS-Chromotest; Ames test	MNNG	Pithva *et al.* 2015
L. plantarum IMC510, IMC513, WCFS1	SOS-Chromotest	4-NQO	Prete *et al.* 2017
Escherichia coli Nissle 1917	Comet assay, Ames test	4-NQO	Janosch *et al.* 2019
L. rhamnosus MD14, *L. plantarum* GMD, *P. pentosaceus* GMD17A	SOS-Chromotest; Comet Assay	DMH, 4-NQO	Chandel *et al.* 2019
Food-associated strains			
L. acidophilus, *L. gasseri*, *L. confusus*, *S. thermophilus*	Comet assay	MNNG; DMH	Pool-Zobel *et al.* 1996
L. delbrueckii subsp. *bulgaricus*, *L. casei*, *L. rhamnosus*, *L. acidophilus*, *L. plantarum*	SOS-Chromotest	4-NQO	Cenci *et al.* 2002
L. casei, *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. rhamnosus*, *L. plantarum*	SOS-Chromotest; Comet Assay; Ames test	4-NQO, MNNG	Caldini *et al.* 2005
Lactobacillus spp.	SOS-Chromotest	4-NQO, MNNG	Caldini *et al.* 2008
L. casei	SOS-Chromotest	4-NQO, MNNG	Corsetti *et al.* 2008
L. salivarius FDB89	SOS-Chromotest	4-NQO	Fang *et al.* 2008
Lactobacillus spp.	Ames test	Sodium azide, NF	Darsanaki *et al.* 2012
E. faecium, *Bacillus coagulans*, *L. plantarum*	SOS-Chromotest, Ames test	4-NQO, Furazolidone	Walia, Keshani and Kanwar 2014
L. plantarum	Comet assay	AFB1	Kurhan and Cakir 2016
L. plantarum	SOS-Chromotest	4-NQO	Prete *et al.* 2017
LAB	SOS-Chromotest	DMH	Sharma, Chandel and Shukla 2020

[a]Genotoxic compounds noted as: methylnitronitrosoguanidine (MNNG), 1,2-dimethylhydrazine (DMH), 4-nitro-quinoline-1-oxide (4-NQO), 2-nitrofluorene (NF), 4-nitro-O-phenylenediamine (NPD), Aflatoxin-B (AFB), 2-amino-3-methyl-3H-imidazoquinoline (MeIQ), (2-amino-1-methyl-6-phenyl-1H-imidazo[4,5-b]pyridine (PhIP), 2-Amino-3-methyl-9H-pyrido[2,3-b]indole (MeAαC), genotoxic faecal water (FW), Aflatoxin-B_1 (AFB1).

commonly used in food packaging and/or in thousands of everyday products. As reported in Fig. 2, co-incubation of EDs with both *L. plantarum* probiotic strains showed appreciable genotoxicity inhibition (>75% BPA and > 50% DnHP), confirming the ability of *L. plantarum* strains to inhibit the biological activity of genotoxic compounds (Prete *et al.* 2019). Based on these preliminary results (data not shown), *L. plantarum* IMC 513 and DnHP, which induced the major genotoxicity on the tester strain, were selected as a combined treatment to use in an ongoing *in vivo* experimental trail with zebrafish.

IN VIVO STUDIES: ZEBRAFISH AS A MODEL FOR ANTIGENOTOXICITY

In vivo studies have shown that some probiotics (e.g. *Lactobacillus* spp.) could be capable of counteracting the DNA damage produced by potent genotoxins with consequent reduction of preneoplastic state and protection against neoplastic activities of these compounds (Dominici *et al.* 2011). Among mechanisms by which probiotics may exert their beneficial effects, significant differences exist between different probiotic strains and model genotoxins, including direct and indirect modes of

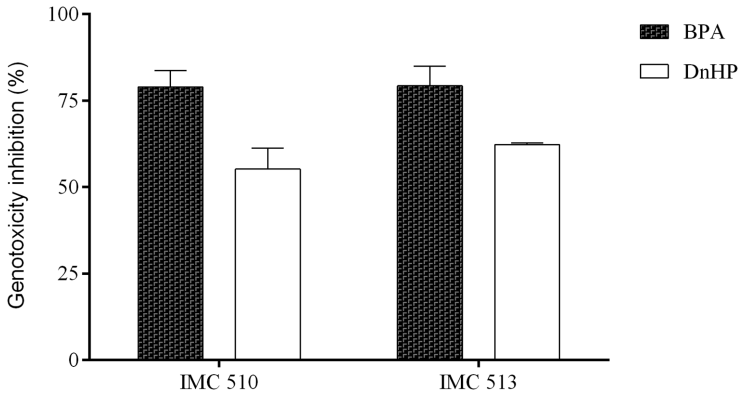

Figure 2. *In vitro* antigenotoxic activity of probiotics *L. plantarum* strains against bisphenol A (BPA) and di-n-hexyl phthalate (DnHP), evaluated by the SOS-Chromotest. Percent genotoxicity inhibition was calculated from residual activity (SOS induction factor) on supernatants in relation to that of a positive control. Data are shown as means ± SD from three replicates.

action. Indeed, probiotics can prevent the absorption of genotoxic xenobiotics in the intestine, reduce their bioavailability or could be responsible for the enzymatic inactivation of mutagens (e.g. scavenging). Moreover, Dominici and co-workers (2014) have demonstrated the capability of *L. rhamnosus* IMC501 to modulate faecal microbial ecosystem in mice treated with PhIP, one of the most frequent heterocyclic amines found in cooked meat, and to reduce the activity of β-glucuronidase and β-N-acetyl-glucosaminidase. β-glucuronidase has the ability to reverse phase II detoxifying reactions in which chemicals (e.g. dietary carcinogens) undergo conjugation in the liver; as a consequence, conjugated carcinogens are not excreted and parent genotoxic xenobiotics are released in the colon lumen (Dominici *et al.* 2014). The pre-treatment of male albino mice with *L. rhamnosus* GG before intoxication with aflatoxins has ameliorated the oxidative status by decreasing lipid peroxidation, increasing superoxide dismutase activity and reducing glutathione contents (Deabes *et al.* 2012). Other mechanisms potentially involved in the protective role of *Lactobacillus* strains (*L. salivarius* REN) in rats exposed to 4-NQO could be related to the suppression of cell proliferation, induction of cell apoptosis and/or downregulation of cyclooxygenase-2 expression (Zhang *et al.* 2013). Rodent models have been also used to investigate the effects of BPA and ethinyl estradiol exposure on gut microbiota (Javurek *et al.* 2016), and the protective effects of *L. casei* and *B. breve* against BPA (Oishi *et al.* 2008).

As previously stated, mice and rats have been used extensively to study antigenotoxicity properties of probiotics. However, *in vivo* models prove to be very expensive compared to *in vitro* approaches since a large number of animals is requested and a copious amount of test compounds have to be expended.

Zebrafish, on the other hand, has emerged as an attractive alternative model for studying probiotic properties and genotoxic activities of several EDs. Zebrafish has offered an alternative to animal experiments in mammals based on the 3Rs-replacement, reduction, and refinement. It has been extensively used for testing different types of toxicity, including developmental toxicity, cardiotoxicity, nephrotoxicity, and hepatotoxicity (Chakravarthy *et al.* 2014). The attributes that make zebrafish an useful *in vivo* model have been related to *ex vivo* fertilization and embryogenesis, optical transparency of embryos and larvae, rapid embryological development, cheap housing costs and genetic tractability (Lieschke and Currie 2007). Moreover, 71.4% of human genes have at least one zebrafish orthologue and, reciprocally, 69% of zebrafish genes have at least one human orthologue (Howe *et al.* 2013). More importantly, about 84% of human genes known to be associated with human diseases are also present in zebrafish (Howe *et al.* 2013). Zebrafish has also been used to study host-microbe interactions in the digestive system. Zebrafish colonization is characterized by a condition very similar to that of human, despite the relevant differences between human and zebrafish gut microbiota. The role of gut microbiota on host biology is similar between zebrafish and mammals and, in both species, intestinal microbiota participates in the education of the immune system, maturation of the gut, and promotion of nutrients metabolism in the host (Bates *et al.* 2007). The number of microbiota related studies in zebrafish are still limited but growing, in developmental and physiological microbiology, colitis models, effects of antibiotics and immune responses (Okazaki *et al.* 2019).

Moreover, the administration of probiotics seems to modulate the gut microbiome composition of zebrafish, leading to beneficial properties. *L. rhamnosus* has attenuated obese phenotype (Falcinelli *et al.* 2017), the induction of oocyte maturation, the increase of fecundity in female zebrafish as well as the acceleration of zebrafish backbone calcification (Avella *et al.* 2012; Gioacchini *et al.* 2012). A probiotic mixture of lactobacilli, bifidobacteria and *Streptococcus thermophilus* has activated the endocannabinoid system in zebrafish, leading to the modulation of immune cells function by inducing gene expression of toll-like receptors and other immune related molecules (Gioacchini, Rossi and Carnevali 2017).

Recently, the effects of several EDs on zebrafish gut microbiome have been reported. Gut microbiota dysbiosis in zebrafish has been associated to metals, pesticides, fungicides and microplastics exposure (Jin *et al.* 2017; Xia *et al.* 2018; Zhang *et al.* 2018; Qiao *et al.* 2019). Chronic co-exposure to titanium dioxide nanoparticles and bisphenol A have led to oxidative stress in zebrafish and this event has been correlated with higher abundance of gut pathogenic bacteria (Chen *et al.* 2018b). Moreover, it has been also proved the protective role of bioactive compounds, identified in a plant species from South America, against oxidative stress and genotoxic activities induced by glyphosate-based herbicide in adult zebrafish (Dal Santo, Grotto and Boligon 2018).

Based on that, the use of zebrafish as an *in vivo* experimental model to evaluate the antigenotoxic properties of probiotics and/or food-associated bacteria could be a promising tool to investigate the probiotics bio-protective role at GI level against different food and environmental genotoxic compounds.

CONCLUSION

Nowadays, the interest in the diets or dietary components able to modulate the risk of genotoxicity and GI diseases is relevant, considering that we are everyday exposed to high level of exogenous and endogenous risk factors. Within the huge variety of genotoxic compounds that can be found in the gut, mainly related to Western diets and lifestyle, EDs are emerging as novel genotoxic agents that can be associated to a wide range of pathologies and long-term effects. Experimental evidences confirm the magnitude of the diet-health relationship and probiotics and/or food-associated microbes assume an interesting role as potential bio-protective tool in the modulation of food-related genotoxic and mutagenic risk at GI level. Protective effects of probiotics, prebiotics and food-associated microbes over EDs have also been touch on, although further *in vivo* studies are needed to elucidate the mechanisms underlying these genotoxic-preventing effects. In line with this, the use of zebrafish as experimental model may open new perspectives in deciphering effective intervention for inflammatory and cancer prevention.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Synbiotec s.r.l. (Camerino, Italy) to kindly provide us *L. plantarum* probiotic strains (IMC510 and IMC513).

FUNDING

This work has received financial support from the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement 713714 ESR 07 (to NG-G).

Conflicts of interest. None declared.

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