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Antitumor bioactivity and gut microbiota modulation of polyhydroxybutyrate (PHB) in a rat animal model for colorectal cancer

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ABSTRACT

Polyhydroxybutyrate (PHB) is a non-toxic polyhydroxyalkanoate polymer produced by several microorganisms, widely used as a biological substitute for plastics derived from fossil hydrocarbons. In this work, PHB polymer has been tested in an animal model for colorectal cancer. In the animal model, PHB has been able to reduce the number of polyps by 48,1%, and the tumoral extension area by 58,1%. Also, PHB induces a selective increase in beneficial gut bacterial taxons in this animal model, and a selective reduction in pro-inflammatory taxons, demonstrating its value as a nutraceutical compound. This antitumor effect is caused by gut production of 3-hydroxybutyrate and butyrate. In this animal model, 3-hydroxybutyrate is also observed in plasma and in brain tissue, after PHB consumption, making PHB supplementation interesting as a bioactive compound in other extraintestinal conditions, as 3-hydroxybutyrate has been reported to enhance brain and cognitive function, cardiac performance, appetite suppression and diabetes. Therefore, PHB could be postulated as an interesting non-polysaccharide antitumor prebiotic, paving the way towards its future use in functional foods.

1. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide (after breast and lung cancers), with 1,931,590 new cases in 2020 and a rate of 24.8 cases per 100,000 habitants, which at global scale is higher in areas as North America, Europe, Russia, Japan, Australia and New Zealand [1–4]. CRC is increasing every year, especially in Western countries, due to environmental risk factors (tobacco, alcohol, chlorine in water) and dietary habits (saturated fats, salty food nitrosamines, benzopyrene from overcooked food, low consumption of fruit and vegetable fiber), which affect the colon mucosa healthy status [5].

Colon mucosa is structured as a monolayer epithelium of colonocytes, which increases its surface *via* the existence of millions of crypts (invaginations of this epithelium, distributed following a regular pattern). These crypts allow this tissue to increase the mucosal functional surface for absorption of nutrients and water. Also, these crypts contain, at their bottom, the stem cells in charge of renewal of the whole colon mucosa, which are cells with constant multiplication capabilities [6]. These stem cells at the bottom of each colon mucosa crypt may suffer DNA mutations in genes (such as *apc*, *k-ras*, *dcc* and *p53*), leading to their transformation in cancer cells with uncontrolled growth, which will proliferate towards aberrant crypt foci, then towards a microadenoma, a polyp (large adenoma), and finally will render a metastatic colon carcinoma [7–9].

This CRC development scenario can be altered, or even stopped, thanks to the presence in the colon lumen of some nutraceutical compounds, such as prebiotic fibers. Prebiotic fibers are plant polysaccharide polymers formed by D-fructose chains (or other sugars such as xylose, galactose, *etc.*), which are not digested by human mouth, pancreatic nor intestinal enzymes [10–13]. This is in contrast to other, more abundant D-glucose plant polymers, such as starch, which is fully digested towards free glucose and absorbed in the small intestine for

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energy purposes [14]. Regarding CRC prevention, these prebiotic fibers are very important, as once consumed, they are not digested nor absorbed in human digestive tract, arriving intact to the colon, where they selectively stimulate the growth or activity of some colon indigenous beneficial probiotic bacteria, such as *Bifidobacterium*, *Faecalibacterium*, *Lactobacillus*, *Roseburia* and others [15–19].

This colon microbiota fermentation of prebiotic fibers generates specific metabolites, short-chain fatty acids (SCFAs), which include for example acetate, butyrate, hexanoate, isobutyrate, lactate, propionate and valerate, among others [12,20,21]. From these SCFAs, the most important ones in colon homeostasis are butyrate and, to a lesser extent, propionate, as normal colonocytes metabolize them in order to generate energy. But, more importantly, butyrate exercises an inhibitory multiplication effect on tumor colonocytes, as it has inhibitory activity on histone deacetylases (HDACs), stimulating the expression of genes involved in differentiation and/or cell death in these colonocytes. Therefore, the presence of SCFAs in the colon lumen (especially butyrate) protects against the initial stages of colon tumorogenesis, the aberrant crypt foci. This has been proved in murine models treated with mutagens able to induce early stages of CRC (such as azoxymethane or hydrazine), where the number of these colon tumors decreased in rats fed with prebiotic fibers [22–29].

Some bacterial energy reserve polymers, such as polyhydroxybutyrate (PHB) are widely used at industrial level as substitutes for plastic polymers (for example in surgery for sutures, bone grafts, valves or cardiovascular patches, eye vitreous substitutes with enhanced transparency for vitreoretinal surgery, or in nanoparticles for CRC diagnosis or for antitumor compounds controlled delivery in cancer cells *in vitro* and *in vivo* studies (including better performance regarding drug solubility and reduced side effects, such as hemolysis at injection site in the animals models), neuroprotective substrates, *etc.*), given the fact that this polymer is non-toxic and biocompatible [30–37]. This polymer is composed of 3-hydroxybutyrate monomers, connected *via* an ester bond. Similar polymers are found in archaea, bacteria and eukaryotic cells (such as microalgae), such as polyhydroxypropionate or polyhydroxyvalerate, known as polyhydroxyalkanoates polymers (PHA). Producer cells use PHB (generated from acetyl-CoA as precursor) as carbon storage molecule, and also as protectant against some stresses (UV radiation, osmotic pressure, desiccation) [38].

In this work, PHB produced at the Bio-On's facilities has been tested in an animal model for CRC (chemically induced with azoxymethane (AOM) and dextran sodium sulfate (DSS)), as a potential antitumor compound of interest in the prevention of this common cancer in human populations. For this, PHB (molecular weight 300,000.00 Da) has been incorporated to the animals' feed at two different concentrations, 10% and 20%, and the evolution of the induced colorectal tumors has been

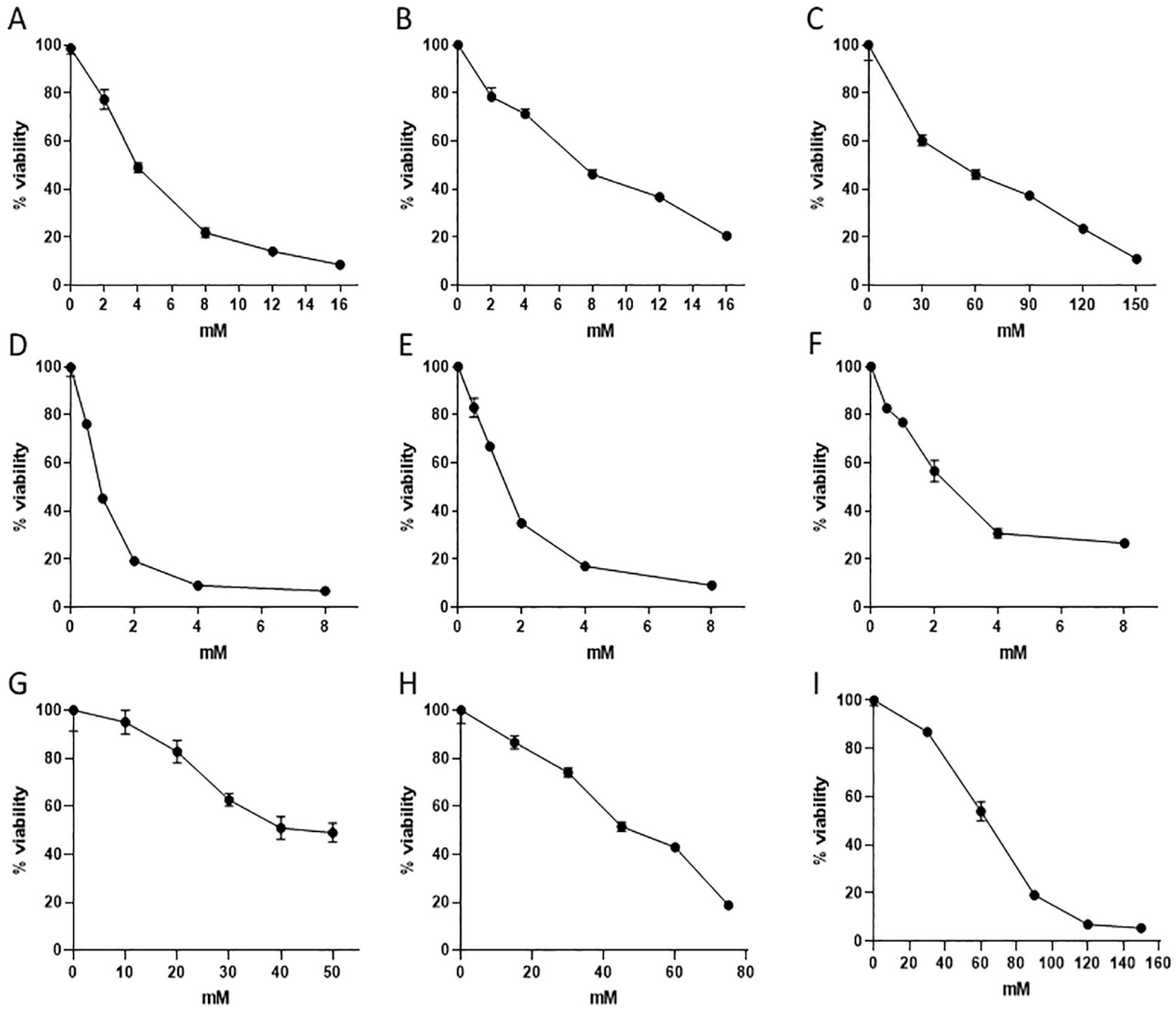

Fig. 1. Effects of propionate, butyrate and 3-hydroxybutyrate in the cellular viability of human CRC cell lines. The percentage of surviving cells is indicated for the different tested concentrations of short-chain fatty acids: Propionate concentrations in A: HCT116, B: HT-29, C: T84; Butyrate concentrations in C: HCT116, D: HT-29, E: T84; and 3-hydroxybutyrate concentrations in G: HCT116, H: HT-29, I: T84.

monitored after 20 weeks treatment, in comparison with control feed. The number of colon polyps in these animals, the intestinal microbiota changes associated to these treatments, as well as the production of SCFAs have been analyzed, shedding light on the potential use of PHB as an ingredient for functional foods, of interest in the prevention of CRC.

2. Results

2.1. In vitro cellular viability tests in human CRC cell lines

From the different SCFAs tested on the three human CRC cell lines, in the case of propionate, all three CRC cell lines showed inhibition with this antitumor SCFA, although its effect was stronger in HCT116 (IC_{50} 4.73 mM) than in HT-29 (IC_{50} 7.3 mM) and in T84 (IC_{50} 68.54 mM) (Fig. 1A–C). The antitumor effect of butyrate is the most successful one, in HCT116 (IC50 1.13 mM), HT-29 (IC50 1.52 mM) and T84 (IC50 5.93 mM) cell lines (Fig. 1D–F). Finally, in the case of 3-hydroxybutyrate, the concentrations corresponding to the IC50 values were much higher: 44.2 mM in HCT116, 51.21 mM in HT-29 and 68.8 mM in T84 (Fig. 1G–I).

2.2. In vivo rat model for CRC prevention studies

The rats' cohort number was selected based on being able to detect at least a difference of 2 tumors between the average numbers of tumors in each cohort, assuming a confidence interval of 95%, a potency of 80% and a maximum variance value of 1.59. In this way, tumor differences between cohorts will be obtained in a statistically significant way. For each of the 10 animals in each rat cohort, 8 animals were randomly selected for chemical induction of CRC, and 2 animals in each cohort were maintained as absolute controls (no induction of tumors, see Methods section). In order to enhance the effects on the colon mucosa of the tumor inducer AOM (administered twice intraperitoneally to those 8 animals per cohort), the proinflammatory compound DSS was included in this animal model (added to the drinking water for 7 days, in two separate weeks). DSS generates a transitional ulcerative colitis in the colon mucosa, reinforcing the tumorigenicity of AOM [22,39].

Monitoring of animals' body weight along the whole experiment demonstrated that the evolution of this parameter was similar among the three cohorts, indicating absence of toxicity of the PHB compositions present in these diets. This fact is reinforced by the absence of digestive symptoms in the 2 rats used in cohort 2 (10% PHB in feed) and 3 (20% PHB in feed) as absolute controls (no AOM, no DSS treatments). However, at the end of the experiment (weeks 12 to 18), the control feed cohort stopped gaining body weight in the 8 animals where CRC was induced, these differences were statistically significant between CRC feed animals and the other cohorts (Fig. 2B).

In total, two out of the eight rats in control cohort where CRC was induced were showing no diarrhea in this timeframe. This number was high in the 10% PHB cohort (3 rats with no diarrhea) and in the 20% PHB cohort (five animals). Mild diarrhea was observed only in two animals from the 20% PHB cohort. Diarrhea with blood crops was present in four animals from the control cohort, three animals from the 10% PHB cohort and one animal from the 20% PHB cohort. Finally, diarrhea with hemorrhage was observed in three animals from the control cohort (one of them died during the second DSS challenge) and two rats from the 10% PHB cohort.

After sacrifice, the caecum from each animal was weighted, in order to detect any possible prebiotic effect on the caecum microbiota. Statistically significant differences were observed among the three cohorts, with increasing caecum weight from control cohort animals (2.14 g ± 0.15) to 10% PHB (3.48 ± 0.18) and 20% PHB (4.25 g ± 0.4) cohorts (Fig. 2C).

This potential prebiotic effect was further studied, though the quantification of the different SCFAs that may have been produced because of this intestinal fermentation on PHB as the sole fiber ingredient in the feeds from cohorts 2 and 3. The only SCFA detected in GC–MS in statistically significant amounts in all animal caecum samples was butyrate (Fig. 3A). Its levels were higher in the 10% PHB cohort (0.994 mM ± 0.268) than in the feed cohort (0.0 mM), but the highest concentrations were detected in the 20% PHB cohort (1.709 mM ± 0.186).

In the case of propionic acid, the concentrations in feed, 10% PHB and 20% PHB cohorts were 0.436 mM, 0.646 mM and 0.596 mM respectively, with not statistically significance between these three cohorts (Fig. 3B). The concentrations of isobutyric acid, valeric acid, isovaleric acid and hexanoic acid were in all animals under the detection limits of the analytical method. In the case of these SCFAs quantifications in plasma samples, regarding propionate, butyrate, isobutyrate, valerate, isovalerate and hexanoate; only propionate was detected in significant amounts over detection limits. Propionate plasma concentrations were statistically significantly higher in the plasma from 10% PHB (average 0.92 mM) and 20% PHB (average 1.31 mM) animal cohorts than in the plasma from feed animal cohort (average 0.13 mM) (Fig. 3D). However, there were no statistically significant differences between both PHB cohorts regarding plasma propionate.

All other SCFAs were not detected in plasma samples, including butyrate, which only appears in 0.27 mM and 0.33 mM concentrations in the plasma of two of the animals (both belonging to the 20% PHB cohort).

Regarding brain tissue quantification of these SCFAs (propionate, butyrate, isobutyrate, valerate, isovalerate and hexanoate), all of them were below detection limits (0.1 mM) in all three cohorts, also including propionate and butyrate.

In the case of 3-hydroxybutyrate quantification, caecum samples from animals belonging to the control feed cohort did not show any

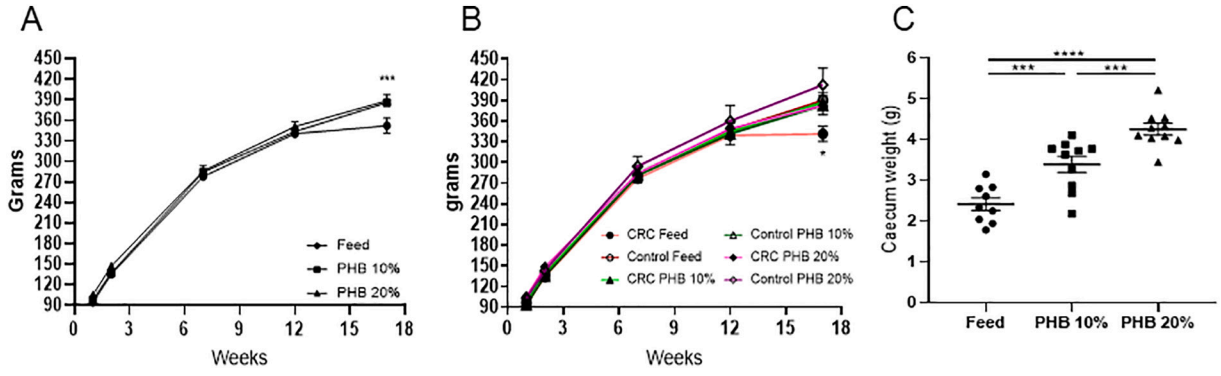

Fig. 2. Body weight gain in the three different rat cohorts along the nutritional intervention study. A: average weekly weight of the two absolute control animals in each cohort (those ones without CRC induction). B: average weekly weight of the 8 animals in each cohort where CRC was chemically induced with AOM. C: Caecum weight of each animal from the three different rat cohorts. Asterisks indicate statistical significance differences.

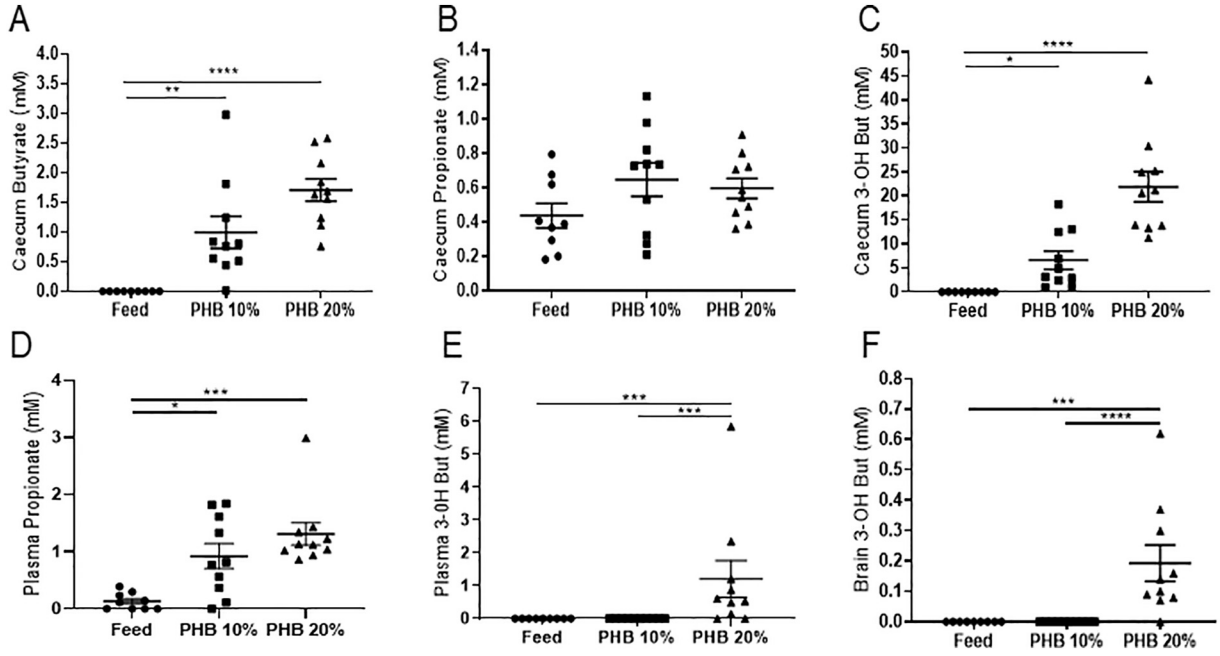

Fig. 3. Concentrations of selected SCFAs in different rat tissues (caecum, plasma, brain). Asterisks indicate statistically significant differences.

(Fig. 3C) However, the 10% PHB and 20% PHB cohorts showed increasing concentrations of 3-hydroxybutyrate, with mean values of 6.597 mM (±1.897) and 21.91 mM (±3.174) respectively (Fig. 3C). These differences were statistically significant.

In plasma samples, the detected concentrations of 3-hydroxybutyrate were much lower, with respect to caecum ones. Here, the control feed and 10% PHB cohorts did not show 3-hydroxbutyrate over the detection lower limit, but 20% PHB cohort showed 1.204 mM (±0.56) concentration of this SCFA (Fig. 3E). The concentration in 20% PHB cohort was statistically significant. Therefore, only a part of the 3-hydroxybutyrate generated in the caecum due to the microbiota degradation of the PHB polymer was transferred through the enterocyte to peripheral blood circulation. Accordingly, only in the 20% PHB cohort the initial caecum 3-hydroxybutyrate concentration was high enough in order to generate significant plasma concentrations of this SCFA.

Finally, in the brain tissue, no SCFAs are detected, except 3-hydroxybutyrate, which is not detected in the control feed and in the 10% PHB cohorts, but it is detected at a low concentration in the 20% PHB cohort (0.193 mM ± 0.05) (Fig. 3F). The differences between this 20% PHB cohort with the other two ones are statistically significant.

Regarding the number of colon tumors in the three rat cohorts, colon mucosas were analyzed after the sacrifices, and tumors bigger than 1 mm were quantified. Fig. 4 shows the average colon tumors number from each cohort (rats 1 to 8 in the three cases, except in control cohort, where one animal was dead during the second DSS challenge). Absolute control animals (rats 9 and 10 from each cohort) showed not tumors, as expected (data not shown). The obtained values are described hereafter. Control feed cohort: 51.14 tumors ± 5.57; 10% PHB cohort: 33.5 tumors ± 4.56; and 20% PHB cohort: 26.5 tumors ± 4.58 (Fig. 4A). A statistical highly significant difference ($p = 0.0058$) was particularly observed among control feed cohort and 20% PHB cohort, which showed a drastic 48.18% reduction in the number of colon tumors. The number of tumors was also reduced (34.49% reduction) in the case of 10% PHB cohort with respect to control feed cohort (Fig. 4A). Fig. 4B shows the total

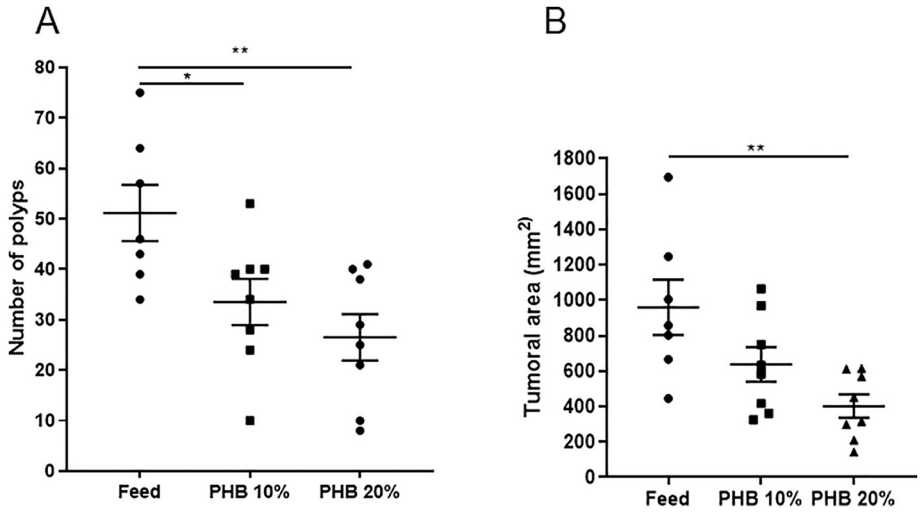

Fig. 4. Number of polyps in the colon mucosa of the experimental animals were CRC was induced with AOM/DSS treatment, and total tumoral area. Asterisks indicate statistically significant differences.

tumor area in the three rat cohorts, in mm^2. As it can be observed, control feed cohort showed an average of 960.1 $mm^2 \pm 155.1$; 10% PHB cohort an average of 638.2 $mm^2 \pm 97.17$; and 20% PHB cohort 402.2 $mm^2 \pm 65.67$. Therefore, 20% PHB cohort showed a 58.1% reduction in this parameter with respect to control feed cohort, and this reduction was statistically highly significant (0.0047) (Fig. 4B). The total tumor area was also reduced (35.52% reduction) in the case of 10% PHB cohort with respect to control feed cohort.

2.3. Intestinal microbiota studies

Metagenomics sequencing results at the family level showed increasing biodiversity indexes (Fig. 5A), as well as different phyla composition between control feed, 10% PHB and 20% PHB cohorts (Table 1). The two observed statistically significant differences at phylum level involved the phyla *Firmicutes* and *Proteobacteria*. Firmicutes phylum showed an important increase in the two cohorts with prebiotic PHB (Table 1), independently of the percentage of added fiber: 55.73% (±4.96) in control feed cohort, 72.92% (±1.77) and 71.89% (±3.35) in 10% PHB and 20% PHB cohorts respectively. Diverse families of this phylum are described as beneficial gut bacteria in animals, such as *Lactobacillaceae*, *Clostridiaceae*, *Eubacteriaceae*, *Peptococcaceae* or *Sutterellaceae*, some of them including genera specifically involved in fermentation of prebiotic fibers (such as *Ruminococcus* or *Parasutterella*). In the case of *Proteobacteria* phylum (Table 1), both PHB cohorts suffered a drastic reduction in its gut populations, from 25.86% (±5.90) in control cohort, to 5.51% (±0.56) in 10% PHB cohort and 8.65% (±0.97) in 20% PHB cohort. Intestinal microbiota diversity indexes showed higher taxonomical diversity in the two animal cohorts fed with PHB (Fig. 5A).

At the family level (Table 2), the major statistical differences were observed in the case of families *Desulfovibrionaceae* (phylum *Proteobacteria*); *Micrococcaceae* (phylum *Actinobacteria*); *Pasteurellaceae* (phylum *Proteobacteria*); *Sphingobacteriaceae* and *Flavobacteriaceae* (phylum *Bacteroidetes*); *Clostridiaceae*, *Lactobacillaceae*, *Erysipelotrichaceae*, *Eubacteriaceae*, *Peptococcaceae*, *Streptococcaceae*, *Sutterellaceae*, *Peptostreptococcaceae*, *Oscillospiraceae* and *Paenibacillaceae* (phylum *Firmicutes*), with almost all these family populations differences (increases or reductions) being proportional between control feed cohort, 10% PHB and 20% PHB cohorts (Fig. 5B).

Table 1
Phyla composition for intestinal microbiota and statistical differences between cohorts. Asterisks indicate statistically significant differences.

	Feed	10% PHB	20% PHB	F-10%	F-20%	10%–20%
Actinobacteria	0.13	0.17	0.30			
Bacteroidetes	16.12	17.61	17.77			
Deferribacteres	1.64	2.52	0.89			
Firmicutes	55.73	72.92	71.89	**	**	
Proteobacteria	25.86	5.51	8.66	****	*	
Verrucomicrobia	0.43	0.24	0.17			
Synergistetes	0.03	0.03	0.00			
Tenericutes	0.03	0.23	0.08	***		*
Spirochaetes	0.00	0.68	0.23	**	**	
unclassified bacteria	0.00	0.10	0.01	*		

All other families showing statistically significant differences showed an increase in their populations (Table 2). Major increases were produced in members of the phylum *Firmicutes*, as in the case of *Clostridiaceae* (8.72% in control feed cohort and 12.94% in 20% PHB cohort), *Lactobacillaceae* (0.23% in control feed cohort and 4.91% in 20% PHB cohort), *Erysipelotrichaceae* (5.66% in control feed cohort and 9.07% in 20% PHB cohort), *Eubacteriaceae* (2.56% in control feed cohort and 4.30% in 20% PHB cohort) and *Streptococcaceae* (0.53% in control feed cohort and 1.94% in 20% PHB cohort); but also in the case of the *Sutterellaceae* (0.41% in control feed cohort and 1.15% in 20% PHB cohort) (Table 2) (phylum *Proteobacteria*). Also, *Flavobacteriaceae* (a family from phylum *Bacteroidetes*) showed an increase in the prebiotic diet cohorts (0.37% in control feed cohort and 1.84% in 20% PHB cohort).

Lactobacillaceae family members, such as *Lactobacillus reuteri*, *L. vaginalis* and *L. murinus* (Fig. 5B) showed a big increase from control

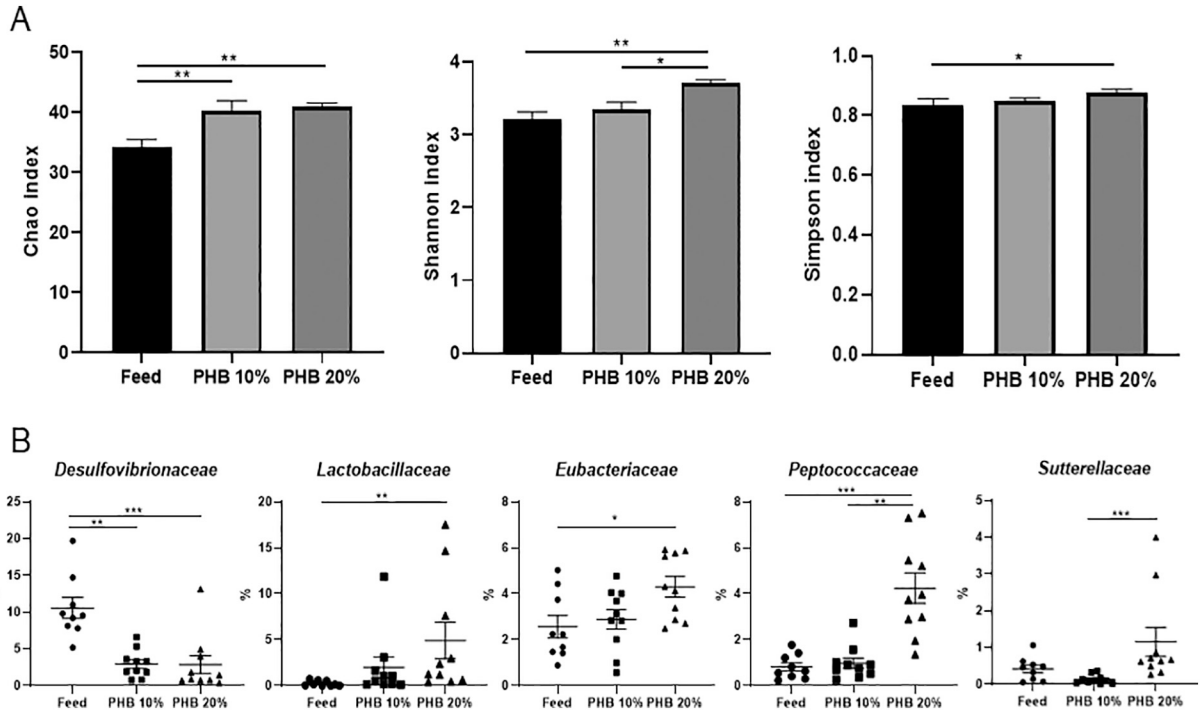

Fig. 5. A: Gut microbiota diversity indexes in the three animal cohorts. B: Percentages of selected microbiota families and statistical differences between the three cohorts. Asterisks indicate statistically significant differences.

Table 2
Families' composition for intestinal microbiota and statistical differences between cohorts. Asterisks indicate statistically significant differences.

	Feed	PHB 10%	PHB 20%	F-10%	F-20%	10%–20%
Rikenellaceae	0.601	1.095	0.219			*
Cytophagaceae	0.281	0.568	0.589	*		
Flavobacteriaceae	0.372	0.485	1.848		**	**
Sphingobacteriaceae	0.003	0.000	0.129			*
Paenibacillaceae	0.011	0.033	0.102		*	
Sporolactobacillaceae	0.000	0.147	0.025	***		
Enterococcaceae	0.971	0.046	0.743			*
Lactobacillaceae	0.236	1.975	4.911		**	
Streptococcaceae	0.532	0.444	1.945		*	*
Clostridiaceae	8.727	12.698	12.945	*	*	
Eubacteriaceae	2.567	2.886	4.306		*	
Oscillospiraceae	0.070	0.247	0.324		*	
Peptococcaceae	0.813	0.968	4.238		***	**
Peptostreptococcaceae	0.312	0.798	1.247		**	
Ruminococcaceae	12.011	21.454	6.962	**		***
unclassified Clostridiales	0.309	1.122	1.585		*	
Erysipelotrichaceae	5.669	3.618	9.077			**
Sutterellaceae	0.414	0.127	1.150			***
Desulfovibrionaceae	10.562	2.941	2.866	**	***	
Pasteurellaceae	0.049	0.267	0.743	*	***	
Brachyspiraceae	0.00	0.68	0.20	**	**	

feed cohort to 20% PHB cohort. In a similar way, PHB supplementation increased another beneficial family, *Streptococcaceae*, where the different species of the probiotic genus *Lactococcus* showed augmented populations (Table 3). Also, potentially pathogenic or pro-inflammatory *Proteobacteria* from the *Enterobacteriaceae* family, such as *Escherichia*, suffered a reduction associated to PHB supplementation, as well as *Enterococcus faecalis*, *E. gallinarum* and *Coprococcus* (Table 3). Principal component analysis at family level showed that the control feed and 10% PHB cohorts were more similar regarding microbiota composition than 20% PHB cohort (Fig. 6).

Finally, a statistically significant negative correlations were found between the concentration in the caecum of 3-hydroxybutyrate with the number of polyps (Spearman r: −0.53, Fig. 7A) and also with tumor area extension (Spearman r: −0.54, Fig. 7B). Furthermore, a statistically significant positive correlation was observed between the concentrations of 3-hydroxybutyrate and butyrate in the caecum (Spearman r: 0.87, Fig. 7C) as well as with the family *Flavobacteriaceae* (Spearman r: 0.59, Fig. 7D), the family *Streptoccocaceae* (Spearman r: 0.70, Fig. 7E), the family *Erysipelotrichaceae* (Spearman r: 0.61, Fig. 7F), the genus *Lactococcus* (Spearman r: 0.76, Fig. 7G), the genus *Dorea* (Spearman r: 0.64, Fig. 7H), the genus *Turicibacter* (Spearman r: 0.79, Fig. 7I), the species *Blautia producta* (Spearman r: 0.67, Fig. 7J), the genus *Enterococcus* (Spearman r: 0.71, Fig. 7K) and the species *Clostridium hiranonis* (Spearman r: 0.61, Fig. 7L).

3. Discussion

This study represents the first evidence of the potential prebiotic effect and antitumor action against CRC of PHB, a type of polyhydroxyalkanoate. This natural polymer is quite different from those ones which usually possess scientific evidence about their beneficial health effects on this cancer type (polysaccharides such as inulin, oligofructose, *etc.*), prebiotic fibers known to produce SCFAs in colon [12,13]. The use of PHB is safe for human health [40,41]. In addition, PHB is widely used in aquaculture such as in gibel carps, shrimps, or sea basses diet, where PHB produces structural changes in the composition of their gut microbiota, improving survival, animal growth, animal immunity and disease resistance [42–45].

In line with this, diverse cellular viability assays have been carried out in this work, using those SCFAs which were supposed to be eventually generated due to intestinal fermentation of PHB by gut microbiota taxons. These *in vitro* studies showed that the metastatic cell line T84 is more resistant to the antitumor effect of the different SCFAs tested, and the most sensitive cell line is HCT116. The fact that HT-29 and T84 cell lines have got extra chromosomal mutations (such as TP53) with respect to the initial adenoma HCT116 cell line, can explain their resistance to the apoptosis induction by these SCFAs. Also, butyrate showed the highest antitumor activity, followed by propionate, and with much higher IC_{50} values in the case of 3-hydroxybutyrate. In general, 3-hydroxybutyrate was about 37 times less potent as antitumor than butyrate in the case of HCT116 cells, 33 times less potent in the case of HT-29 cells, and 11 times less potent in the case of T84 cells (Fig. 1).

Once these *in vitro* cell lines data were available and based on the structural similarity between butyrate and 3-hydroxybutyrate, the objective of this work was to test if the PHB polymer was able to be metabolized by colon microbiota into SCFAs with antitumor activity, therefore rendering a protective effect against the development of CRC in this animal model. Structurally, PHB is a source of 3-hydroxybutyrate, and eventually of other SCFAs originated from its catabolism, like butyrate. For this, propionate, butyrate, isobutyrate, 3-OH-butyrate, valerate, isovalerate and hexanoate were analyzed and quantified in cecum content, plasma, and brain tissue in all the animals from the three cohorts in this work: control feed, 10% PHB in feed and 20% PHB in feed. Trying to simplify the interpretation of the intestinal fermentation of PHB, in this work, the based feed formula was designed in a way that its composition was lacking any potential source of prebiotic fiber (such as inulin), to be sure that the only SCFA-producing precursor in these experiments was PHB. By designing a control rat feed lacking any possible source of SCFAs, this plant prebiotic fibers masking effect is absent in this experimental animal model.

First, regarding physical symptoms, all rats from 10% PHB and 20% PHB cohorts were alive along the experiment, which indicates that PHB diet may help against the challenges with the chemical compounds AOM (CRC induction) and DSS (ulcerative colitis induction). Also, all digestive symptoms were less severe (or absent) in the two PHB cohorts, in comparison with the control one. Furthermore, the growth of the animals in the two PHB cohorts was better than in the feed cohort, where there was a slowdown in growth in the CCR-induced animals, due to the advanced stage of this injury (Fig. 2A, B).

Second, regarding caecum weight in the PHB cohorts animals (Fig. 2C), this was proportional to the PHB content in feed, indicating that this polymer is able to increase the bacterial populations in the digestive tract, and that probably some of those intestinal taxa are able to survive carrying out PHB fermentation, in a similar way to other better known prebiotic fibers [12,17,47].

Third, regarding SCFAs concentrations in caecum, plasma and brain, statistically significant differences were observed only in the case of caecum for butyrate (Fig. 3). This is consistent with the design of the special rat feed, as this feed was free of any type of prebiotic fiber, to make any prebiotic (and antitumor) effect of PHB clearly visible and without interferences from other potential prebiotic fibers in the *in vivo* analysis. Also, the detection of 3-hydroxybutyrate in caecum samples only in the cases of 10% PHB and 20% PHB cohorts means that PHB is effectively degraded in the digestive tract of the animals towards 3-hydroxybutyrate, its building block, probably due to the action of intestinal microbiota enzymes (Fig. 3C).

Butyrate was not detected in plasma samples, in agreement with literature, as it is the preferred energy source for colonocytes and therefore, butyrate is not transferred to blood stream from colon mucosa [46]. However, plasma propionate concentrations (higher in 10% PHB cohort, and even more in 20% PHB cohort) in accordance with the dogma of its transport from colonocytes to peripheral blood, where it is used as energy source in diverse tissues [46] (Fig. 3D). From all analyzed SCFAs, the only one achieving the brain was 3-hydroxybutyrate in the 20% PHB cohort, where a minor proportion of the plasma 3-hydroxybutyrate, 16.1%, can achieve this organ (Fig. 3E and F). Finding this metabolite in the brain, as well as in the bloodstream, as opposed to

Table 3
Genus and species composition for intestinal microbiota and statistical differences between cohorts. Asterisks indicate statistically significant differences.

Genus	Species	Feed	10% PHB	20% PHB	F-10%	F-20%	10%–20%
Bacteroides		7.573	3.228	8.120	**		
	massiliensis	0.098	0.286	0.550		*	
	oleiciplenus	0.264	0.086	0.000	*	****	
	stercorirosoris	0.190	0.065	0.000	*	****	
	uniformis	0.880	0.560	0.131		*	
Parabacteroides	*distasonis*	0.350	0.093	0.212	*		
Paraprevotella		0.024	0.030	0.070		*	
Alistipes		0.601	0.767	0.103		*	**
	finegoldii	0.129	0.125	0.016		*	**
Enterococcus		0.966	0.046	0.746			**
	faecalis	0.089	0.000	0.000	*	*	
	gallinarum	0.071	0.000	0.000	*	*	
Lactobacillus		0.219	1.962	4.869		**	
	reuteri	0.006	0.121	0.408		**	
	vaginalis	0.060	0.340	0.656		**	
	murinus	0.024	0.102	0.165		**	
Lactococcus		0.300	0.323	1.774		*	*
	lactis	0.159	0.161	0.653		*	*
Clostridium		4.278	9.038	10.265	*	****	
	celatum	0.112	0.095	0.011		*	
	hiranonis	0.010	0.090	0.239		***	
	perfringens	0.068	0.000	0.000	**	**	
	sp.	0.566	1.886	4.072		**	
Blautia		3.846	0.714	3.145	**		**
	hansenii	0.174	0.055	0.427			**
	producta	1.172	0.273	1.005	*		*
Coprococcus		0.917	0.552	0.003		*	*
Dorea		1.163	0.402	1.105	**		*
	Dorea	0.842	0.312	0.907	*		*
Eubacterium		0.004	0.054	0.355		****	**
Lachnoclostridium		0.324	0.201	0.070		*	*
Roseburia		0.013	0.212	0.011	**		**
	faecis	0.000	0.096	0.003	***		**
Tyzzerella	*propionicum*	0.319	0.035	0.000			**
[*Ruminococcus*]		2.902	4.480	6.089		**	
Faecalibacterium	*prausnitzii*	0.168	0.292	0.078			*
Ruminiclostridium		0.076	0.601	0.004	*		****
Ruminococcus		2.141	4.664	2.091			**
	flavefaciens	1.301	3.584	0.001		*	****
	sp.	0.056	0.088	0.328			*
Subdoligranulum	sp.	0.208	0.009	0.003	*	**	
Pseudoflavonifractor		0.029	0.923	0.549	**	**	
[*Eubacterium*]	*dolichum*	0.243	0.018	0.916	*		***
Holdemania	*filiformis*	0.052	0.001	0.137	*		***
Turicibacter		0.011	0.114	0.799		****	*
Parasutterella		0.414	0.127	1.143			***
	excrementihominis	0.356	0.115	1.008			***
Bilophila		10.266	0.116	2.480	****	*	
	wadsworthia	9.187	0.116	2.116	****	*	
Desulfovibrio		0.242	0.462	0.001		**	***
	C21_c20	0.000	0.338	0.000	**		**
	sp.	0.242	0.125	0.001		***	*
Escherichia		0.180	0.000	0.000	*	*	
Escherichia	*coli*	0.081	0.000	0.000	*	*	
Pasteurella		0.008	0.012	0.162		**	**

butyrate, which is limited to the caecum, could be interesting as a bioactive compound in other extraintestinal conditions, as 3-hydroxybutyrate has been reported to enhance, in mammals, brain and cognitive function (of interest among others in neurodegenerative disorders and epilepsy), cardiac performance (heart hydraulic efficiency), appetite suppression and diabetes, as this compound has shown *in vitro* neuroprotective effects [48–51].

The fact that, for example, in the 20% PHB cohort, caecum samples showed a mean value of 21.9 mM for 3-hydroxybutyrate and only 1.7 mM for butyrate, indicates that about 7.8% of the monomer 3-hydroxybutyrate present in the digestive tract (most probably *via* microbiota fermentation of PHB) is converted into butyrate, most probably due to the action of intestinal microbiota enzymes. Therefore, the remaining 3-hydroxybutyrate in the digestive tract may contribute as a secondary actor for the observed protection again CRC in this animal model, as its antitumor activity is much lower. Regarding this, the *in vitro* experiments showed that 3-hydroxybutyrate is between 37 and 11 times less potent as antitumor compound against CRC cell lines than butyrate (Fig. 1), but 3-hydroxybutyrate showed digestive tract concentrations 14 times higher than for butyrate (Fig. 3). 3-hydroxybutyryl-CoA (generated from PHB degradation) can be converted in crotonyl-CoA (a dehydrated product at carbons C2 and C3), due to the action of a 3-hydroxybutyryl-CoA dehydratase (such as EC 4.2.1.55), and finally in butyryl-CoA, due to the action of crotonyl-CoA reductase (such as EC 1.2.1.86) or a trans-2-enoyl-CoA reductase (such as EC 1.3.1.9 or EC 1.3.1.44) [52,53].

Therefore, most probably, the first microbial degradation product of PHB in the rat digestive tract is 3-hydroxybutyryl-CoA, which can render 3-hydroxybutyrate after hydrolysis of the CoA cofactor (by microbial enzymes), or butyryl-CoA (after the sequential action of the two

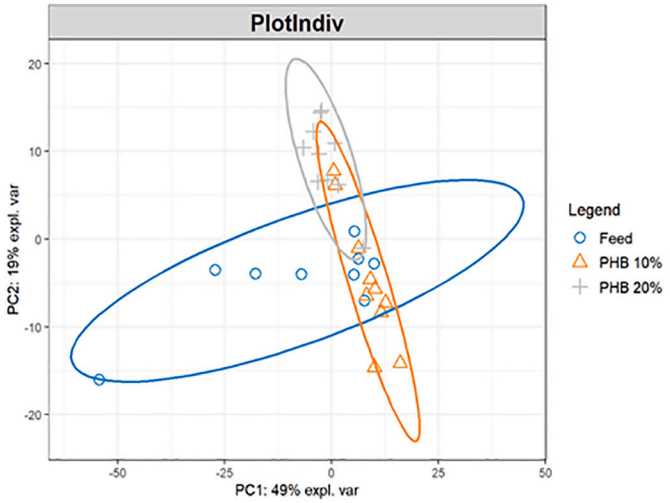

Fig. 6. Principal component analysis based on the gut microbiota composition of the 29 surviving animals.

enzymes described above). A similar hydrolysis of this butyryl-CoA would render the detected free butyrate in the rat digestive tract. Furthermore, in this animal model, a direct correlation has been found between the concentration of caecum 3-hydroxybutyrate and caecum butyrate (12 times lower concentration than 3-hydroxybutyrate), therefore suggesting that the presence of butyrate is derived from the transformation of this compound (Fig. 7C).

Fourth, regarding tumors in the colon mucosa, PHB, added to the animals diet, achieved a 48.1% reduction in the number of polyps and a 58.1% reduction in the tumoral area compared to the control cohort (Fig. 4). In addition, a direct correlation of a lower number and size of tumors was observed in those animals which contained a higher concentration of caecum 3-hydroxybutyrate (Fig. 7A, B). The main antitumor action mechanism described for 3-hydroxybutyrate and butyrate is by blocking the HDACs, allowing the DNA to acquire an open conformation that will enable tumor suppressor genes to be transcribed, eventually leading tumor cells to suffer apoptosis [50,54–56].

Finally, regarding gut microbiota analyses, the higher abundance of *Firmicutes* phylum (Table 1) in the two PHB rat cohorts is in accordance with the fact that diverse families of this phylum are described as beneficial gut bacteria in animals, such as *Lactobacillaceae*, *Clostridiaceae*, *Eubacteriaceae*, *Peptococcaceae* or *Sutterellaceae* (Fig. 5B), some of them including genera involved in fermentation of prebiotic fibers and SCFAs production (such as *Ruminococcus* or *Parasutterella*). Also, the important reduction observed in the phylum *Proteobacteria* in the case of the two PHB animal cohorts is interesting, as this phylum is associated with pro-inflammatory species, such as members of the *Enterobacteriaceae* (genus *Escherichia*) and *Desulfovibrionaceae* (genus *Desulfovibrio* and *Bilophila*) families.

The reduction in the case of the family *Desulfovibrionaceae* (Table 2, Fig. 5B) (phylum *Proteobacteria*) is of particular importance, as members of this family are important sulfite-reducing bacteria, generating H_2S, an important pro-inflammatory and genotoxic actor in the gut ecosystem. Therefore, its reduction from 10.5% in control feed cohort to 0.7% in 20% PHB cohort can be explained as a beneficial effect of this functional prebiotic diet. Inside this family, there are a major reduction in the levels of the disease associated *Bilophila wadsworthia* (9.2% in control feed cohort to 2.1% in 20% PHB cohort) (Table 3). This species, mainly associated to low vegetables diets (as those ones based on meat), generates H_2S, and it has been found in persons affected with appendicitis or inflammatory bowel disease [57–62].

The genus *Lactobacillus* (Table 3), with a statistically significant increase in both PHB animal cohorts, is considered highly beneficial for the gut ecosystem, as these non-pathogenic bacteria secrete immunomodulatory and anti-inflammatory compounds, together with peptides (bacteriocins) and other factors (H_2O_2, reuterin) showing antimicrobial properties against pathogenic species. Also, species of this genus have been described as able to ferment prebiotic compounds [63,64]. Therefore, the increase on the gut populations of this genus can be considered also a prebiotic effect of PHB supplementation in this animal model.

The observed increases in *Clostridiaceae*, *Lactobacillaceae* and *Streptococcaceae* families (Table 2), mainly associated to beneficial bacterial genera, are in agreement with recent studies where PHB dietary supplementation in the shrimp *Litopenaeus vannamei* also increased the gut population levels of *Lactobacillus*, *Lactococcus* and *Clostridium* genera [42]. There is a positive correlation of these and other families (*Flavobacteriaceae*, *Erysipelotrichaceae*) and genera (*Lactococcus*, *Dorea*, *Turicibacter*, *Blautia*, *Enterococcus* and *Clostridium*) with 3-hydroxybutyrate gut concentrations (Fig. 7), which could imply that those taxons are actively participating in the intestinal degradation of PHB.

The *Proteobacteria* family *Sutterellaceae* (Table 2, Fig. 5B), and particularly the species *Parasuterella excrementihominis* showed an increase associated to PHB supplementation (Table 3). This species has been described as a SCFAs producer, and together with other genera involved in SCFAs production, could be responsible in this animal model for the increased levels of these protective compounds derived from PHB fermentation [65].

At genus level, *Bacteroides* populations were increased in the PHB cohorts (Table 3). This genus contains species involved in prebiotics fermentation, and this increase could reflect the fermentation capability of these bacteria regarding the use of PHB as an energy source, as with conventional prebiotic fibers [66].

In general, PHB supplementation in this work, especially at 20% in animals feed, showed an association with a decrease in potential pathogenic or pro-inflammatory families, genera and species (such as those ones from *Desulfovibrionaceae* and *Enterobacteriaceae*), and an increase in beneficial taxons, especially in probiotic genera such as *Lactobacillus* or *Lactococcus*, and in prebiotic fermenters such as *Parasuterella excrementihominis*.

In conclusion, the addition of 10% PHB or 20% PHB to the diet of these animals showed an important prevention against the development of CRC tumors, probably due to intestinal generation of both butyrate (main protective antitumor agent), and its precursor 3-hydroxybutyrate, which is present in gut concentrations 14 times higher than butyrate, but is between 37 and 11 times less potent as antitumor. This biodegradation of PHB, by intestinal microbiota enzymes, will probably depend on the crystallinity and the molecular weight of the ingested polymer, as less compact and smaller granules would enhance the solubility and the enzymatic processing of the polymer, due to an increased surface available for enzyme interaction with the PHB complex [67]. This, together with the lack of toxicity in PHB, could allow its future use as a preventive agent for this type of digestive neoplasia in human populations. These data support the potential use of PHB as a nutraceutical compound in functional foods suitable for other extraintestinal conditions (neurodegenerative, epilepsy, cardiac disorders, diabetes), as in this work, 3-hydroxybutyrate has been also quantified in other tissues, such as plasma and brain, after its generation by gut microbiota populations from ingested PHB.

4. Materials and methods

4.1. Cellular viability tests

Three CRC cell lines were used in these experiments: HCT116 (epithelial, carcinoma, primary, mutated in KRAS and PIK3CA), HT-29 (epithelial, adenocarcinoma, primary, mutated in APC, BRAF, PIK3CA, SMAD4 and TP53) and T84 (epithelial, adenocarcinoma, metastasis in lung, mutated in APC, KRAS, PIK3CA and TP53). All of them were cultivated in DMEM/F12 1:1 medium, supplemented with 10% FBS, 2

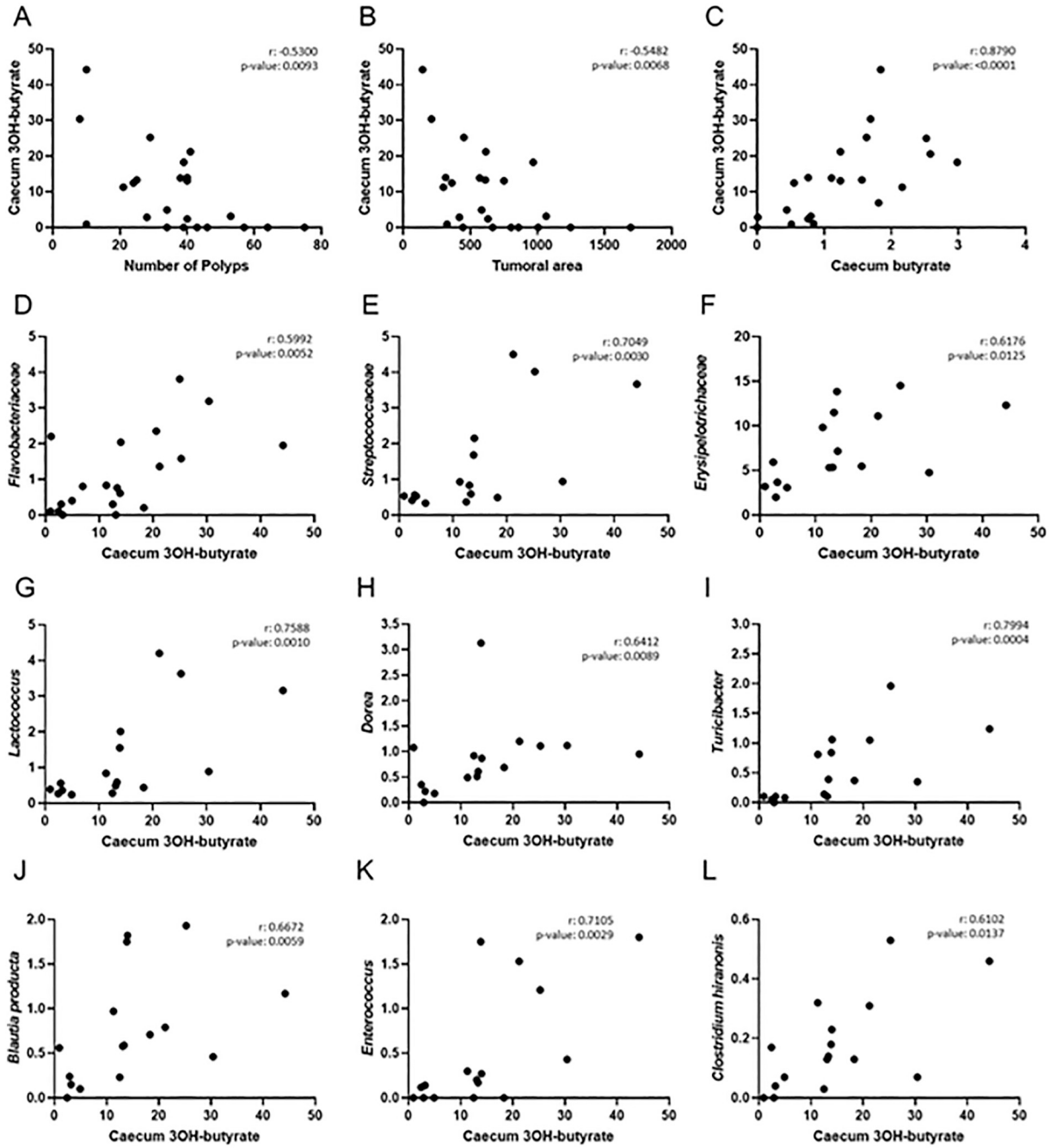

Fig. 7. Correlations between concentration of caecum 3-hidroxybutyrate and A: Number of polyps. B: Tumoral area. C: Caecum butyrate. D: Family *Flavobacteraceae*. E: family *Streptococcaceae*. F: Family *Erysipelotrichaceae*. G: Genus *Lactococcus*. H: Genus *Dorea*. I: Genus *Turicibacter*. J: Species *Blautia producta*. K: Genus *Enterococcus* and L: species *Clostridium hiranonis*. All these correlations are statistically significant, p-value and Spearman r indexes are shown in each graph.

mM L-glutamine, streptomycin (100 μg/mL) and penicillin (100 IU/mL). Cells were incubated at 37 °C with 5% CO_2 in a humidified CO_2 incubator (Thermo Scientific 8000DH), using 96-microtiter plates (90 μL cell suspensions per well): HCT116: 5×10^3 cells/well; HT-29: 7.5×10^3; T84: 1×10^4. These cell densities were incubated for 24 h to 75% confluence until complete adherence, and then 10 μL of the corresponding SCFAs concentrations in sterile distilled water were added (three replicates), except in the control wells (only solvent added). IC_{50} concentrations were calculated using Quest Graph™ IC_{50} Calculator. SCFAs tested concentrations were 0 to 8 mM for butyrate, 0 to 150 mM for propionate, and 0 to 150 mM for 3-hydroxybutyrate. Also, three blank control microtiter wells were used, without cells, to subtract the average interference background signal caused by the medium.

The Neutral Red cell uptake method was used in these cancer cell viability tests: 100 μL of Neutral Red (40 μg/mL, dissolved in culture medium) was added to each microtiter well after removing the culture medium, then the plate was incubated at 37 °C during 2 h. Then the dye was removed and 150 μL PBS were used twice for washing the cells. Finally, 150 μL of destain solution (1% acetic acid-50% ethanol) were added and mixed during 10 min (shaking), to extract the dye from the cells: absorbance was measured at 560 nm, using a microtiter plate reader spectrophotometer (GLOMAX, Promega).

4.2. Animal model and experimental design

30 male Fischer 344 rats (*Rattus norvegicus*) were maintained in the

installations of the Animal Facilities at the University of Oviedo (authorized facility No. ES330440003591). All experiments were performed in accordance with regulations from the Federation of European Laboratory Animal Science Associations (FELASA), and after the approval from the Ethics Committee of the Principality of Asturias (authorization code: PROAE 16/2015).

These five weeks old rats were divided into three cohorts of 10 individuals each one and fed *ad libitum* in individual cages. Cohort 1 was fed control feed (Research Diets Inc., New Jersey, USA). This feed contained 16.7% protein, 5.8 fat, 53.6% carbohydrates (of which cellulose content is 200.16 g/kg), and its caloric content was 3.33 kcal/g. Cohort 2 was fed a similar feed but replacing half of the cellulose content by PHB (produced by Bio-On S.p.a.), in a way that this feed contained 10% PHB. And finally, the feed used for cohort 3 contained 20% PHB (and no cellulose at all). Caloric content was the same for the three different formulas.

The molecular characterization of the PHB used in these experiments included analyses on the molecular weight and molecular weight distribution, which was determined by Gel Permeation Chromatography (GPC). An Agilent apparatus, equipped with a Tosoh Bioscience TSK gel G5000HHR column (5 μm particles size, 7.8 × 300 mm) and a refractive index detector, was employed for this. Samples were dissolved, injected and eluted with chloroform, at room temperature and at a concentration of 5 mg/mL, maintaining a flux of 1.00 mL/min. Retention time for PHB was comprised between 6 and 9 min. Calibration is carried out with 6 polystyrene standards (2000–900–600–300–120–30 kDa, Sigma Aldrich) diluted in chloroform at a concentration of 5 mg/mL. The chemical integrity was 99,5% as checked using ^{1}H NMR and comparison with commercial and literature-known samples.

4.3. CRC induction and monitoring

CRC induction with two intraperitoneal doses of AOM (weeks 2 and 3), reinforced *via* intestinal inflammation with the oral administration (in drinking water) of DSS, during two weeks (weeks 4 and 15), has been described previously [22]. In each cohort, two animals were kept as absolute controls, free of CRC induction (receiving intraperitoneal 0.9% NaCl solution, instead of AOM; and free of DSS treatments), and the other 8 animals were submitted to CRC induction as described (10 mg AOM per kg body weight, from a stock solution of 2 mg/mL in 0.9% NaCl; plus, the two DSS challenges). Along the 18 weeks, animals were monitored for stool consistency, body weight and rectal bleeding.

4.4. Histological analysis

After the 18 weeks monitoring (after the first administration of AOM), animals were anesthetized with isoflurane, before carrying out the sacrifices (using bilateral pneumothorax). During anesthesia, 2 mL of blood were obtained from the heart, in order to get plasma (after 15 min centrifugation at 3000 rpm), which was frozen at −20 °C until analysis for SCFAs.

After sacrifices, caecum was also extracted from each animal and weighted with a precision scale before frozen it at −20 °C until SCFAs analysis and metagenomics. Also, colon was extracted, opened longitudinally and its mucosa washed with phosphate buffer saline (PBS), finally it was stored in 4% formaldehyde at 4 °C, until its analysis for the presence of polyps (those ones larger than 1 mm). Shape (spherical, plane circular, plane irregular, pedunculated) and size of each polyp was recorded, in order to further calculate polyps' total area in this mucosa. Finally, brain was extracted and frozen at −20 °C until SCFAs analysis.

4.5. SCFAs analysis by GC–MS and HPLC-MS

Quantification of SCFAs (butyrate and propionate) in cecum, plasma and brain tissues was carried out as described before, using internal deuterated controls from commercial standards (Cambridge Isotope Laboratories, USA) to a final concentration of 0.4 mM each one [68]. In the case of 3-hydroxybutyrate, the method used was HPLC-MS (BRUKER Impact II, ESI-Q-TOF; coupled to a THERMO UPLC Dionex Ultimate 3000; Luna Omega Polar C18, 1.6 μm, 100 Å, 150 × 2.1 mm column), as due to its polarity, GC–MS was unsuccessful in this case. A program was developed to separate 3-hydroxybutyrate from its deuterated standard. For the UPLC (mobile phase A is water, mobile phase B is acetonitrile): 90% mobile phase A at time 0 min, 90% mobile phase A at time 1 min, 0% mobile phase A at 11 min, 0% mobile phase A at 12.5 min, 90% mobile phase A at 14 min, 90% mobile phase A at 16 min. For the MS (ESI): negative mode detection, capillarity 4500 V, nebulizer 2.4 Bar, heater 250 °C. Standards calibrations were made with 5 μM normal and deuterated 3-hydroxybutyric acid.

4.6. Intestinal microbiota studies and phylogenetic analysis

The method for genomic DNA extraction (from 200 mg cecum content) and PCR amplification of the 16S rRNA variable regions, and their metagenomics sequencing using the ION PGM™ system has been described [22,39]. Each metagenomics sequencing experiment (one per animal) generated a consensus spreadsheet with the percentages of each bacterial taxonomic level. This spreadsheet was downloaded from ION Reporter software (version 5.6, Life Technologies Holdings Pte Ltd, Singapore) and used for comparing frequencies between individual animals and their corresponding cohorts (QIIME-2 software). Microbiota metadata have been uploaded in SRA database (bioproject code PRJNA645062).

4.7. Statistical analysis

Shapiro–Wilk's test was used for analyzing the normality of the different variables, expressing then these data as the mean value ± standard error of mean (SEM). Levene's test was used for testing equality of variances, which was present in the following parameters: cecum weight, SCFAs concentrations, number of polyps, total polyps' area, number of hyperplastic Peyer's patches, as well as in the metagenomics analyses (phyla, families, genera and species). One-way ANOVA (analysis of variance Dunn's and Holm-Sidak's multiple comparisons test) was used for testing the differences among cohorts. Spearman's correlation coefficient was calculated to explore associations between variables. Graphic representation of the different data generated was carried out using GraphPad Prism software (version 9, GraphPad Software, San Diego, CA, USA), and considering a p value < 0.05 as statistically significant (*$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$; ****$p < 0.0001$).

Ethical approval

All experiments were performed in accordance with regulations from the Federation of European Laboratory Animal Science Associations (FELASA), and after the approval from the Ethics Committee of the Principality of Asturias (authorization code: PROAE 16/2015).

Availability of data and materials

Microbiota metadata have been uploaded in SRA-NCBI database (bioproject code PRJNA645062).

Funding disclosure

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CRediT authorship contribution statement

Conceptualization: MCF, PS, JF, FL
Formal analysis: JF, CJV
Funding acquisition: PS, MCF
Investigation: JF, FL
Methodology. JF, FL, MCF
Project administration: FL
Supervision: MCF, FL
Writing – original draft: FL
Writing – review & editing: JF, CJV, PS, MCF, FL.

Declaration of competing interest

This research work is part of the international patent WO2019175725A1.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2022.01.112.

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