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ORIGINAL ARTICLE

Preclinical Evidence of Probiotics in Colorectal Carcinogenesis: A Systematic Review

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Abstract

Background Colorectal cancer, the second major cause of cancer deaths, imposes a major health burden worldwide. There is growing evidence that supports that the use of probiotics is effective against various diseases, especially in gastrointestinal diseases, including the colorectal cancer, but the differences between the strains, dose, and frequency used are not yet clear.

Aims To perform a systematic review to compile the results of studies carried out in animal models and investigated the effect of probiotics on colorectal carcinogenesis.

Methods Studies were selected in PubMed/MEDLINE and Scopus according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines. Search filters were developed using three parameters: probiotics, colorectal cancer, and animal model.

Results From a structured search, we discovered 34 original articles and submitted them to a risk of bias analysis using SYRCLE's tool. The studies show a great diversity of models, most were conducted in rats (55.8%) and used 1,2 dimethylhydrazine as the drug to induce colorectal carcinogenesis (61.7%). The vast majority of trials investigated *Lactobacillus* (64%) and *Bifidobacterium* (29.4%) strains. Twenty-six (86.6%) studies found significant reduction in lesions or tumors in the animals that received probiotics. The main methodological limitation was the insufficient amount of information for the adequate reproducibility of the trials, which indicated a high risk of bias due to incomplete characterization of the experimental design.

Conclusions The different probiotics' strains showed anti-carcinogenic effect, reduced the development of lesions and intestinal tumors, antioxidant and immunomodulatory activity, and reduced fecal bacterial enzymes.

Keywords Colorectal neoplasms · Carcinogenesis · Probiotics · Animal model · Systematic review

Introduction

Chronic non-communicable diseases are responsible for the majority of global deaths, and cancer represents an important cause of morbidity and mortality worldwide. Cancer incidence is related to the westernization of lifestyle, and social and economic transition in countries [1]. The GLOBOCAN 2018, published by the International Agency for Research on Cancer (IARC), estimated the occurrence of 18.1 million new cases and 9.6 million cancer deaths worldwide in 2018. For colorectal cancer, 1.8 million new cases and 881,000 deaths are estimated to occur in 2018, representing the third neoplasia in incidence and second in cause of mortality, with average case fatality higher in countries with lower HDI (Human Development Index) [2, 3].

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With multifactorial etiology, cancer is associated with genetic factors, nutrition, and inflammatory processes. The increase in incidence of colorectal cancer is associated with changes of dietary patterns, obesity, and factors related to lifestyle [4]. There is convincing evidence that shows that processed meat, alcoholic drinks, and accumulation of body fat increase the risk of development of the disease. On the other hand, physical activity is a protective factor [4–6].

There is evidence that indicates the role of diet in the development of colorectal cancer. Dietary compounds may influence pathways by which carcinogens are metabolized and epigenetic changes that lead to cancer development [4, 7]. There is an indication that some probiotics' strains can affect the host's immunologic response, stimulating anti-inflammatory cytokines, antioxidants, and anti-carcinogenic compounds [8]. In this respect, probiotics are associated with anti-cancerous and anti-mutagenic activity [9, 10]. According to the Food and Agriculture Organization/World Health Organization (FAO/WHO), probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" [11]. However, the differences between the strains, doses, and frequency used, as well as the mechanisms by which they exert their effects, are not yet clear.

Given the difficulty in studying the effects of several treatments, including nutritional aspects, in colorectal carcinogenesis, preclinical models for colorectal carcinogenesis are used to induce lesions similar to colorectal cancer in humans, being widely used in experimental studies [12]. However, as the findings of preclinical studies often originate from relatively small experiments and are quite heterogeneous, they may not always be applicable in a translational context to enhance human health and well-being [13, 14]. Based on this, the objective of the present study was to systematically review the preclinical evidence in a qualitative manner (unlike the widely used narrative reviews). We believed that a study like this might provide us with reliable and solid new evidence on whether or not probiotic supplementation could be beneficial in the context of colorectal carcinogenesis. We performed a critical analysis of preclinical studies in order to improve the quality of the reports and to prevent the spreading of methodological failures, which could compromise the development of future clinical studies.

Methods

The systematic review was elaborated according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses—PRISMA [15], whose methods include data source and search, study selection, eligibility criteria, data extraction, analysis of results, and risk of bias. The protocol was registered at the International Prospective Register of Systematic Reviews—PROSPERO (registration number: CRD42018111201).

Search Strategy

The bibliographic search was performed using the electronic databases MEDLINE (PubMed platform—https://www.ncbi.nlm.nih.gov/pubmed) and Scopus (https://www.scopus.com/home.uri), admitting only studies in animal models. The keywords for the construction of the filters followed three criteria: probiotics AND colorectal cancer AND animal models (Supplementary file S1). The hierarchical distribution of the MESH terms was the strategy used to develop the filter on the PubMed platform. We applied a standardized filter in the Scopus platform for animal studies, and the same PubMed search strategy was adapted and used. Views, comments, notes, and unpublished studies were not included. No restrictions were imposed for language or date of publication. The bibliographies of the eligible studies were checked manually to find possible publications of interest.

Selection of Studies

We included all the original experimental studies that evaluated the administration of probiotics in an animal model (in vivo) of colorectal carcinogenesis. Prespecified eligibility and exclusion criteria were set using the PICOS (Population, Intervention, Comparison, Outcome and Study design) strategy. The following exclusion criteria were used: (1) studies that analyzed the associated effect of probiotics with prebiotics and/or nutritional supplements; (2) in vitro studies; (3) descriptive studies, such as annals of congresses, editorials, letters, case reports, and review works; (4) in vivo studies with humans were also excluded. Abstracts or unpublished reports have been disregarded. The evaluation of the eligibility of the studies was performed independently by two reviewers (P.G.A.B. and S.C.P.D.L). In the case of disagreements, another group of reviewers (R.V.G., M.C.G.P, and R.D.N.) decided whether the study met the inclusion and exclusion criteria. Inclusion or exclusion was verified by evaluating the full text of potentially relevant studies.

Extraction and Synthesis of Data

A detailed examination of the studies was carried out in order to evaluate the strength of the evidence and the validity of its inclusion in this review. Data extraction and compilation tables were developed according to the following information: (1) publication characteristics: authors, publication year, and country; (2) characteristics of the experimental model (animal model, sex, age, number of animals, control group, and carcinogenic model) and main characteristics of

the intervention (strain, dose, and duration); (3) effects of probiotics and its main outcomes. When essential information was absent, the authors were contacted in order for us to obtain it. The outcomes on the development of aberrant crypt foci (ACF), intestinal tumors, fecal enzymes activity, antioxidant activity, and immune markers were analyzed and presented. The data were subsequently compared, and conflicting information was identified and corrected after discussion among the reviewers.

Risk of Bias

The risk of bias was analyzed using the SYRCLE tool (Systematic Review Centre for Laboratory animal Experimentation), based on Cochrane Collaboration (RoB 2.0), which aims at evaluating the methodological quality of the studies. This instrument was adjusted for bias aspects that play a specific role in animal intervention studies. The objective was to establish consistency and to avoid discrepancies in the evaluation of methodological quality in the field of animal experimentation. In order to increase transparency and applicability, signaling issues have been formulated to facilitate judgment, based on the following areas: (1) random sequence generation, (2) baseline characteristics, (3) allocation concealment, (4) random housing, (5) investigator blinding, (6) blinding of outcome assessment, (7) blinding of outcome, (8) incomplete outcome data, (9) selective outcome reporting, and (10) ethical considerations [16].

Results

Study Selection

Initially, 778 references were found in the databases. By reviewing titles and abstracts, 738 citations were excluded for different reasons (human model, in vitro studies, intervention or outcome not pertinent, review articles, other diseases). Forty articles were selected for full-text examination; then, nine studies were excluded due to insufficient data ($n=3$), full text not available ($n=1$), other tumors ($n=1$), and other treatments ($n=4$). Three additional citations were included by manual search after research in the references of the articles that were initially included. Figure 1 shows the flow diagram of the study selection process.

Study Characteristics

Thirty-four studies with different probiotic preparations were selected and reviewed. The selected studies were performed in 12 different countries, most of them in the USA (23.5%) followed by India (17.6%), Argentina (11.7%), Canada (8.8%), and Japan (8.8%). All studies were published in English. The articles selected for this qualitative review show a great diversity of models, age of animals (21 days to 20 weeks old), and duration of treatment (7 days to 36 weeks). Most of the studies were conducted in rats (55.8%), and the remainder in mice (44.2%). The proportion of the animals' sex was 52.9% male ($n=18$), 26.4% female ($n=9$), 11.8% both ($n=4$), and three studies omitted the animals' sex (8.9%).

The majority of studies investigated the *Lactobacillus* (64%) and the *Bifidobacterium* (29.4%) genera (Table 1). For the dietary treatment, either as isolated strain, combined formulation, or probiotic food (e.g., kefir), the works mostly used commercial bacterial cultures and reported no microbiological counting. Out of the 34 studies included, 25 used only isolated strains (73.5%), three investigated the effect of two or more combined strains (8.8%), and six evaluated probiotic foods (17.7%).

The Effect of Probiotics in the Development of Lesions and Intestinal Tumors

Probiotics have been tested individually or in combination with different concentrations. Our results showed that the majority of studies offered the probiotic source daily (91%). Thirty studies (88.2%) presented the results of development of ACF, early precancerous lesions, and/or intestinal tumors after the subjects were exposed to the carcinogen. In 26 studies (86.6%), there was a significant reduction in lesions or tumors in the animals that received probiotics.

Of the articles included, 21 (61.7%) used 1,2 dimethylhydrazine (DMH) as the inducing drug of colorectal carcinogenesis, and the doses varied from 15 to 40 mg/kg. The second most used drug was azoxymethane (AOM) (17.6%). Out of the 34 studies, 27 (79.4%) utilized chemically induced models by DMH or AOM, and the usage duration of these chemicals carcinogens was of 14.3% for the administered for up to 2 weeks; 47.6% between 3 and 19 weeks, and 38.1% for 20 or more weeks. For AOM, 50% of the studies used the carcinogen for up to 2 weeks, the remainder between 3 and 19 weeks. Application frequency occurred once a week (77.7%) or twice a week (22.3%).

The studies that have evaluated the effects of the *Lactobacillus* genus (*acidophilus*, *casei*, *fermentum*, *delbrueckii*, *gasseri*, *rhamnosus*, *plantarum* species) observed reductions in lesions [17–30] in the intestinal tumors [31–42]. Table 2 presents the main effects of different probiotics' strains on the development of histopathological parameters in the studied models.

In the groups supplemented with milk fermented by *L. bulgaricus* and *S. thermophiles* or isolated *L. bulgaricus* [43, 44], the strains had no significant effect on the incidence of tumors. It was also shown that the effects depended on the dose, meaning that the protective effect of *Lactobacillus*

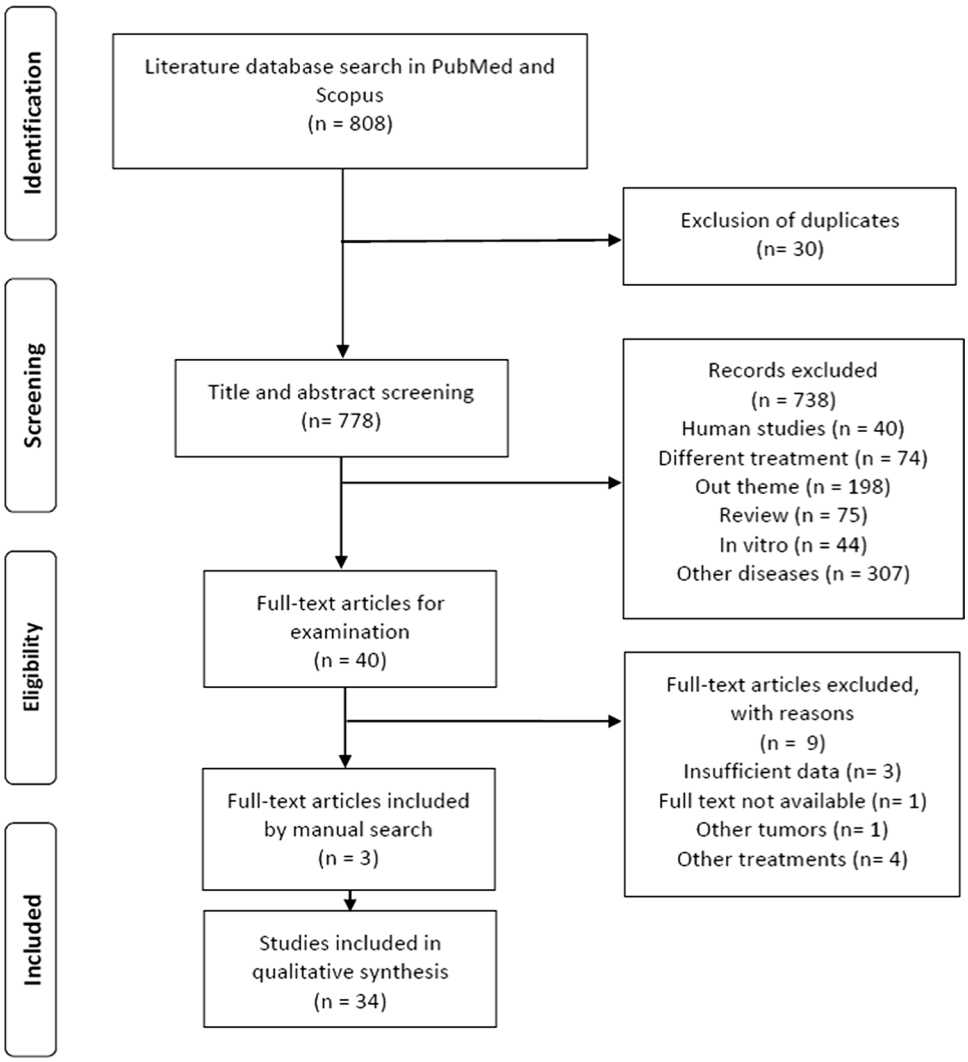

Fig. 1 Flow diagram of the search results of our systematic literature review. Based on "Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement." http://www.prisma-statement.org From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009)

bulgaricus was only detected in those animals treated with the higher dose [44]. When the administration of *Bifidobacterium* was evaluated, almost every study showed inhibition in the development of ACF compared to the control groups [17–19, 23, 45]. Using genetically modified strains (*Streptococcus thermophilus* and *Lactococcus lactis subsp. cremoris*), the analysis of histologic damages showed the highest scores in the samples obtained from the DMH and *L. lactis* groups. Mice that received genetically modified lactic acid bacteria showed decreased damage scores compared to the DMH group [46]. Seven studies worked with fermented foods [26, 34, 38, 39, 43, 47, 48], including kefir (2), yogurt (3), probiotic curd (1), and dahi (1), showing that the oral administration of milk and soy milk kefirs inhibited the tumor growth significantly [34]. When the use of yogurt was evaluated, only one study observed results in the development of intestinal tumors, signaling that the yogurt diet significantly reduced the number of colorectal tumors induced by DMH in male rasH2 mice [39].

Secondary Effects of Probiotics

Antioxidant Activity

Five studies evaluated the antioxidant activity exerted by probiotics [25, 35, 46, 48, 49]. Two studies of the same group assessed the effects of different genetically modified lactic acid bacteria. Mice that received a catalase-producing *L. lactis* strain (*L. lactis* KAT) presented an increase in catalase activity in samples taken from small and large intestines. H_2O_2 concentrations were slightly lower in samples from animals that were supplemented with *L. lactis* KAT as opposed to *L. lactis* NZ or no bacterial supplementation (DMH group) [35]. The same pattern was observed for *Streptococcus thermophilus* and *Lactococcus lactis subsp. cremoris*—which produced antioxidant enzymes (catalase or superoxide dismutase) [46]. The evaluation of the antioxidant capacity of the probiotic strains revealed that the malondialdehyde level was significantly lower in

Table 1 Characteristics of the experimental model and intervention of the studies regarding the use of probiotics in studies of colorectal carcinogenesis in animal models

Author, year	Country	Animal model	Sex	Age (weight)	N animals/group	Control group	Strain	Dose	Duration (probiotic)	Carcinogenesis model
Goldin and Gorbach [17]	USA	F344 rats	♂	6–8 weeks	11–22	Meat diet	*L. acidophilus*	10^{10}–10^{11} cells	20/36 weeks	DMH (20 mg/kg) s.c.
Shackelford et al. [18]	USA	F344 rats	♀	4 weeks	28	Commercial diet	*L. bulgaricus Streptococcus thermophilus*	?	20 weeks	DMH (20 mg/kg) s.c.
Kulkarni and Reddy. [19]	USA	F344 rats	♂	5 weeks	11	Semi-purified diet (AIN-76A)	*B. longum*	1.5 and 3% in diet (2×10^{10} cells/g)	13 weeks	AOM (20 mg/kg) s.c.
Abdelali et al. [20]	France	Sprague–Dawley rats	♂	26 days	6	Commercial diet	*B.*?	~6×10^{9} cells	4 weeks	DMH (25 mg/kg) i.p.
Goldin et al. [21]	USA	F344 rats	♂	?	8–21	Experimental diet (5%/20% corn oil)	*L. GG*	1% in diets ~2–4×10^{10} cells/day	24/27 weeks	DMH (20 mg/kg) s.c.
Challa et al. [22]	USA	F344 rats	♂	7 weeks?	15	Semi-purified diet (AIN-76A)	*B. longum*	0.5% in diet (1×10^{8} cells/g)	13 weeks	AOM (16 mg/kg) s.c.
Singh et al. [23]	USA	F344 rats	♂	5 weeks	12	Semi-purified diet (AIN-76A)	*B. longum*	2% in diet ~2×10^{10} cells/g	16 weeks	AOM (15 mg/kg) s.c.
Balansky et al. [24]	Bulgaria	BD6 rats	♂/♀	16–20 weeks (180–220 g)	30–32?	Commercial diet	*L. bulgaricus* (FFM.B144 or FFM.B5)	1.3 g/2.5 g/animal FFM.B144: 4×10^{7} cfu/g FFM.B5: 3×10^{6} cfu/g	8 months	DMH (21 mg/kg) s.c.
Rao et al. [25]	USA	F344 rats	♂	6 weeks	12	Semi-purified diet (modified AIN-76A)	*L. acidophilus*	2%/4% in diet 4.2×10^{9} cells/g	10 weeks	AOM (15 mg/kg) s.c.
Gallaher and Khil. [26]	USA	Wistar rats	♂	?	15–20	Semi-purified diet (modified AIN-76A) + skim milk	*B.*?	10^{8}–10^{9} cfu/animal	3.5–5 weeks	DMH (15 mg/kg) gavaged
Liu et al. [27]	Taiwan	ICR mice	♀	6–7 weeks (24 ± 0.8 g)	10	Commercial diet + water	Kefir?	?	30 days	S180 tumor cells in saline (1×10^{8} cells/ml) 0.2 ml s.c.
Tavan et al. [28]	France	F344 rats	♂	?	15	Commercial diet + 20% water	*B. animalis Streptococcus thermophilus*	$5.4\pm1\times10^{8}$ cfu/day	15 weeks	HAA (115 µl) in diet
De Moreno and Perdigón [29]	Argentina	BALB/c mice	?	? (25–30 g)	45–50	Commercial diet + skim milk	Yogurt (*L. delbrueckii* + *Streptococcus thermophilus*)	? 2×10^{8} cells/ml	10 days (cyclically)	DMH (20 mg/kg) s.c.

Table 1 (continued)

Author, year	Country	Animal model	Sex	Age (weight)	N animals/group	Control group	Strain	Dose	Duration (probiotic)	Carcinogenesis model
Lee et al. [30]	Korea	F344 rats	♂	5 weeks (185 ± 10 g)	9	Commercial diet	*Bacillus polyfermenticus*	3×10^6 cfu/day in diet	10 weeks	DMH (30 mg/kg) s.c.
De Moreno et al. [31]	Argentina	BALB/c mice	♂/♀	6 weeks (25–30 g)	30–35	Commercial diet	*Lactococcus lactis* NZ/KAT	1×10^9 cfu/day	6 months	DMH (20 mg/kg) s.c.
Takagi et al. [32]	Japan	BALB/cByJ mice	♀	6 weeks	12	Commercial diet	*L. casei* Shirota *L. fermentum* *L. acidophilus* *L. plantarum* *L. reuteri* *L. rhamnosus*	~ 10^9 cells/mg cells 0.005% (w/w)	12 weeks	3-Methylcholanthrene (1 mg/0.1 ml) s.c.
Cenesiz et al. [33]	Turkey	BALB/c mice	♂/♀	12 weeks (average 31.5 g)	5	Commercial diet	Kefir?	? 50% (w/v) ad libitum instead of water	13 weeks	AOM (5 mg/kg) s.c.
Urbanska et al. [34]	Canada	C57BL/6 J-*Apc* Min/+ mice	♂	7–8 weeks (20–25 g)	11	Commercial diet + saline	*L. acidophillus*	10^{10} cfu/ml	8, 10, and 12 weeks	*Apc* (Min/+)
Kumar et al. [35]	India	Rats?	?	10 weeks	25	Experimental basal diet	Probiotic curd Probiotic cultures: *L. acidophilus* + *L. casei*	?	15 weeks	DMH (20 mg/kg) s.c.
Narushima et al. [36]	Japan	rasH2 mice	♂/♀	8 weeks	?	Commercial diet + non-fermented milk	Yogurt (*L. delbrueckii* + *Streptococcus salivarius*)	?	3 weeks	DMH (20 mg/kg) s.c.
Foo et al. [37]	Taiwan	ICR mice	♂	6 weeks	5–18	Semi-purified diet (AIN-76A) + skim milk	*B. longum* *L. gasseri*	*B. longum* ~ 5×10^9 cfu/g *L. gasseri* ~ 1×10^{11} cfu/g	15/24 weeks	DMH (20 mg/kg) i.m.
Chang et al. [38]	Korea	F344 rats	♂	5 weeks (average 130 g)	15	High-fat diet (HF)	*L. acidophilus*	2×10^9 cfu/ml	10 weeks	DMH (20 mg/kg) i.m.
Verma and Shukla [39]	India	Sprague–Dawley rats	?	? (100–150 g)	6	Commercial diet + saline	*L. rhamnosus* *L. casei* *L. acidophilus* *L. plantarum* *B. bifidum*	1×10^9 cfu	7 weeks	DMH (20 mg/kg) i.p.
Urbanska et al. [40]	Canada	C57BL/6 J-Apc Min/+	♂	5–6 weeks	24	Commercial diet + saline	*L. acidophilus* + 2% yogurt	?	17 weeks	*Apc* (Min/+)
Mohania et al. [41]	India	Wistar rats	♂	3 weeks	24	Experimental basal diet + buffalo milk (BM)	Dahi culture *L. acidophilus* + *B. Bifidum*	Dahi culture 1% 2×10^9 cfu/g, *B. bifidum* and *L. acidophilus* each	8, 16, and 32 weeks	DMH (40 mg/kg) s.c.

Table 1 (continued)

Author, year	Country	Animal model	Sex	Age (weight)	N animals/group	Control group	Strain	Dose	Duration (probiotic)	Carcinogenesis model
Verma and Shukla. [42]	India	Sprague–Dawley rats	♂	? (100–200 g)	8	Commercial diet	*L. acidophilus* *L. rhamnosus*	1×10^9 lactobacilli	19 weeks	DMH (20 mg/kg) i.p.
Walia et al. [43]	India	Sprague–Dawley rats	♀	? (125–175 g)	6	Commercial diet	*L. plantarum* *L. rhamnosus*	10^{10} cells	8 and 16 weeks	DMH (30 mg/kg) s.c.
Shin et al. [44]	Japan	BALB/c mice	♀	5 weeks	6	Commercial diet	*L. plantarum*	10 mg	3 weeks	Meth-A tumor cells (1×10^6 cells) s.c.
Lenoir et al. [45]	Argentina	C57BL/6 mice	♀	6 weeks (22–25 g)	30–35	Commercial diet	*L. lactis* *L. casei*	1% in the drinking water (average $1\pm0.4\times10^9$ cfu/mouse)	3, 4, 5, and 6 months	DMH (20 mg/kg) s.c.
del Carmen et al. [46]	Argentina	BALB/c mice	♀	6 weeks (22–25 g)	10	Commercial diet	*Streptococcus thermophiles*[b] *Lactococcus lactis subsp. cremoris*[b]	1×10^{10} cfu/ml in the drinking water (average intake ~ 3 ml/animal/day	3–6 months	DMH (20 mg/kg) s.c.
Irecta-Nájera et al. [47]	Mexico	BALB/c mice	♀	14–16 weeks (25 ± 2 g)	10	Commercial diet	*L. casei*	10^6 cfu	31 weeks	DMH (20 mg/kg) s.c.
Kahouli et al. [48]	Canada	C57BL/6 J Apc Min/+	♂	4 weeks	5	Commercial diet + saline	*L. acidophilus* + *L. fermentum*	1×10^{10} cfu	12 weeks	*Apc* (Min/+)
Walia et al. [49]	India	Sprague–Dawley rats	♀	? (125–200 g)	6	Commercial diet	*L. plantarum* *L. rhamnosus*	2×10^{10} cells	16 weeks	DMH (30 mg/kg) s.c.
Agah et al. [50]	Iran	BALB/c mice	♂	6–8 weeks	9–10	Commercial diet	*L. acidophilus* *B. bifidum*	1×10^9 cfu/g (1.5 g probiotics in water)	5 months + 10 days	AOM (15 mg/kg) s.c.

wk, week; d day; ? absent or unclear information; USA, United States of America; ♂, male; ♀, female; *L. lactobacillus*; *B. bifidobacterium*; DMH, 1,2 dimethylhydrazine; AOM, azoxymethane; s.c., subcutaneous; i.m., intramuscular; i.p., intraperitoneal; (*), genetically modified strains; HAA, heterocyclic aromatic amines; yogurt culture: *L. delbrueckii subsp. bulgaricus* and *S. thermophilus*; *Lactococcus lactis;* NZ isogenic non-catalase-producing strain; *Lactococcus lactis* KAT catalase-producing *L. lactis* strain; *L. lactobacillus*; *B. bifidobacterium*; Apc (Min/+) germ line mutations in the APC gene that lead to spontaneously development of neoplasms; curd culture: *Lactococcus lactis biovar. diacetylactis*; dahi culture: *Lactococcus lactis* ssp. *cremoris* and *Lactococcus lactis* ssp. *lactis biovar diacetylactis*

Table 2 Effects of different probiotics on the development of histopathological parameters in animal models of colorectal carcinogenesis

Probiotic strain	Effect	Sample size	Outcomes	Percentage of inhibition (*p* value)*
Lactobacillus acidophilus	Reduction/without alteration	$n=9$	Incidences of colonic lesions [50]	57% ($p<0.05$)
		$n=20$		37% ($p<0.02$)
		$n=12$	Colon cancer [17]	29–39% ($p<0.01$–0.001)
		$n=15$	Total number of ACF in colon [25]	41.1% ($p<0.05$)
		$n=6$		96% ($p<0.05$)
		$n=11$	Total number of ACF in colon [38]	12.5–50% ($p>0.05$)
		$n=8$		0% (p?)
			Percentage of ACF [39]	
			Adenomas in large intestine [34]	
			Incidence of tumor [42]	
Lactobacillus rhamnosus	Reduction	$n=21$	Small intestinal tumors [21]	28.5% ($p<0.02$)
		$n=6$	Percentage of ACF [39]	98% ($p<0.05$)
		$n=8$	Incidence of tumor [42]	11.12% (p?)
		$n=6$	Tumor incidence [43]	33.4% (p?)
Lactobacillus bulgaricus	Without alteration	$n=28$	Colon tumor [18]	0% ($p>0.05$)
Lactobacillus casei	Reduction	$n=6$	Percentage of ACF [39]	45% ($p<0.05$)
		$n=5$	Number of damage score [45]	45% ($p<0.01$)
		$n=10$	Number of ACF [47]	68.1% ($p<0.01$)
Lactobacillus plantarum	Reduction	$n=6$	Percentage ACF [39]	89% ($p<0.05$)
		$n=6$	Tumor incidence [43]	50% (p?)
Bifidobacterium longum	Reduction	$n=11$	Number of ACF [19]	43–53% ($p<0.01$–0.001)
		$n=10$	Total number of ACF in colon [22]	23.3% ($p<0.05$)
		$n=12$		31.2% ($p<0.05$)
		$n=9$	Intestinal tumor incidence [23]	35–43% ($p<0.05$)
			Number of microadenomas and adenomas [37]	
Bifidobacterium bifidum	Reduction	$n=6$	Percentage ACF [39]	74% ($p<0.05$)
		$n=9$	Incidences of colonic lesions [50]	27% ($p>0.05$)
Bifidobacterium longum + *Lactobacillus gasseri*	Reduction	$n=9$	Number of ACF [37]	25–30% ($p<0.05$)
Lactobacillus acidophilus + *fermentum*	Reduction	$n=5$	Intestinal polyp [48]	40% ($p<0.05$)
Mix[a]	Reduction	$n=10$	Number of tumors [46]	100% (p?)

ACF, aberrant crypt foci; n, number of animals in the treatment groups; ? absent or unclear information

[a]Mix: genetically modified *S. thermophilus* strain that produces the antioxidant enzymes catalase and superoxide dismutase, combined with *L. lactis* IL-10

[b]Results extracted from the original studies

animals that received probiotics when compared to those that received DMH alone. The DMH treatment of animals significantly decreased the amount of glutathione and the activities of the enzymes glutathione-S-transferase, superoxide dismutase, catalase, and glutathione peroxidase. These changes appear to be reversed by probiotic supplementation [25, 49].

Fecal Bacterial Enzymes

The activity of fecal bacterial enzymes was evaluated in six studies, demonstrating a decrease within all included studies. Fecal bacterial activity of β-glucuronidase declined significantly in animals that received probiotics [17–19, 24, 25]. Whole yoghurt maintained the enzyme levels lower or similar to control [47]. Bacterial β-glucosidase activity was reduced by the administration of different probiotics, including *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, and *Bifidobacterium bifidum* [24, 25]. Was also observed significant decrease in nitroreductase activity in groups that received *Lactobacillus casei* and *plantarum* [25]. In large intestine fluid, whole yoghurt feeding decreased or maintained enzyme levels, similar to the non-treatment control. These mice were fed with yoghurt cyclically again after 8 weeks. The animals from yoghurt–DMH–yoghurt group showed lower nitroreductase activity compared to the group that received only DMH [47].

Immune System

Cytokines and immune cells were evaluated in six studies, presenting different results, but in general the probiotics seem to modulate immune response. In model *Apc* (Min/+) mice, results show that the average levels of inflammatory cytokine IL-6 reduced after the mice received *L. acidophilus* [37]. In the same group, mice receiving a probiotic yogurt formulation containing microencapsulated live *Lactobacillus acidophilus* showed higher concentrations of CD8 cells than the tissue of animals in the control group. Relatively similar Mac-1 expressions were found in animal tissues from both control and treatment groups, 6.02 and 5.43% similarity, respectively. Results suggest that oral administration of the probiotic formulation may lower the expression of markers directly related to intestinal inflammation [40]. Mice receiving *L. casei* had significantly decreased MCP-1 and TNF-α pro-inflammatory cytokine levels in the intestinal samples and had increased levels of the anti-inflammatory cytokine IL-10, compared to other groups [27, 30]. The mucosal IgA was verified by Liu et al. [27], indicating that feeding milk kefir and soy milk kefir significantly increased the total IgA level in the tissue from the small intestine.

Protein Expression

Four studies have evaluated the expression of proteins involved in carcinogenesis, demonstrating that *B. longum* significantly suppressed the expression ras-p21 in colonic mucosa [33]. In another paper, the authors observed that the expression of cleaved caspase-3 was reduced, 11.36 in the control group versus 6.09% in the treatment group (*L. acidophilus*) [40]. Probiotic supplementation was able to restore the normal expression of both p53 and Bcl-2 after DMH administration [49]. The administration of *L. acidophilus* + *L. fermentum* (46.2 ± 3.4%) led to reduced aberrant β-catenin signaling and nuclear staining of β-catenin when compared to the saline group (54.7 ± 0.9%), meaning that the aberrant β-catenin signaling in the tumors was suppressed by probiotic strains' administration [29].

Study Quality and Risk of Bias

The results of our risk of bias assessment of the 34 studies included in this systematic review are shown in Fig. 2. None of the studies presented a low risk of bias in all the methodological criteria and reached the desired quality. Considering each criterion analyzed individually, none of the studies reported information as investigator blinding and declared the sample calculation for the number of animals used. However, most of the studies displayed food availability information during the experiment, use of standardized diets, management conditions, details of animal allocation and experimental groups, food consumption, and body weight. Mortality information and comments on study limitations were poorly addressed.

Discussion

Our results indicated that a large variety of probiotics strain were effective in reduction in development of lesions and intestinal tumors in animal models. The vast majority of the studies evaluated the administration of *Lactobacillus*

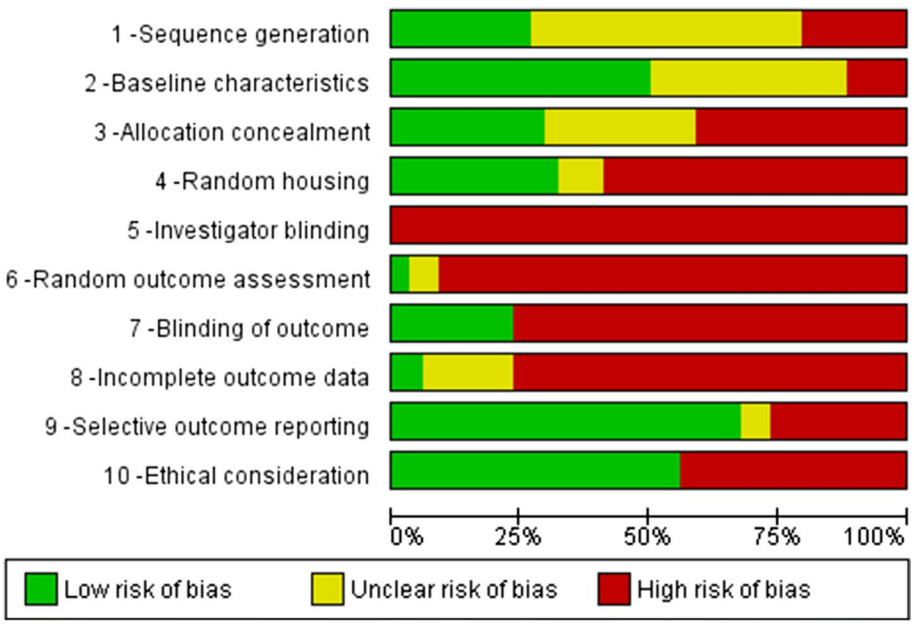

Fig. 2 Evaluation of the animal studies using the SYRCLE's risk of bias tool for animal studies

and/or *Bifidobacterium* genera, which is supported by the safety evidence of these strains [50, 51]. Furthermore, the probiotics presented other benefits, such as modulation of the immune system, antioxidant activity, and decrease in fecal bacterial enzymes, all associated with the main result. These data thus provide evidence that probiotics can act as an effective strategy on prevention of colorectal cancer. The results in Table 2 are consistent with this.

Despite the fact that the studies included in this review show a wide methodological variability, some common ground was observed. Murine models were the main animal model used for the study of carcinogenesis. The use of animals provides lower costs, allowing more controlled and careful analysis of the outcome measures, which is particularly important because it presents itself as a viable tool for research regarding colorectal cancer, a disease that has great relevance in the world morbidity and mortality [52]. Male animals were often chosen, which may have occurred due to sex-specific mechanisms. However, the relationship between sex and the development of colorectal cancer is not completely clear [53, 54, 55].

A wide variation was found for the age of the animals used in the experimental models. The vast majority of studies used animals of 5 or more weeks of age. However, some studies did not report the age of the animals. It is interesting to note the relevance of the preclinical model for age-dependent carcinogenesis, since the cancer is predominantly a disease of elderly people [56, 57]. The studies included in this review evaluated animals between 3 and 20 weeks of age, but only two studies evaluated the consumption of probiotic in the early stages of life, as in post-weaning [18, 26]. This is particularly relevant, as it indicates the lack of information on the use of probiotics in critical periods of development and their long-term effect, since it is known that early exposure to different substances can program the individual's in adulthood, beneficially or not [58].

The studies analyzed presented a great variability in relation to carcinogens, mainly the type, dose, and frequency in which these compounds are administered to animals. There is evidence that high doses can affect the number and growth features of ACF and of tumor outcome. The ACF are the earliest visible lesions in the colon and rectum and are considered potential precursors of colorectal cancer, being also identified in patients at a high risk of colorectal cancer [12]. The two chemical substances with carcinogenic potential that were the most used were DMH and AOM, an active metabolite of DMH, which enables the chemopreventive and chemotherapeutic study of other compounds, such as probiotics [59–61]. The intestinal mucosal injury DMH-induced involves a sequential process with gradual increase in the number of ACF, which may lead to the development of colorectal cancer. The majority of these tumors develop mutations in the β-catenin gene, which is similar to hereditary non-polyposis colorectal cancer, with inactivation of the β-catenin destruction complex, generally by *APC* (adenomatous polyposis coli) mutations [59, 62]. The use of genetically modified animals in the study of colorectal carcinogenesis was also observed, especially of *Apc* (Min/+) mice, in which the *Apc* gene is the homolog of human *APC* gene. Due to the fact that its standard molecular and pathologic structure is similar to human familial adenomatous polyposis, it is widely used to study the development, treatment, and prevention of colorectal cancers that contain somatic *APC* mutations [52, 63, 64].

In this review, the most common investigated genera were *Lactobacillus* followed by *Bifidobacterium*, once lactic acid bacteria represent the main microorganisms added to probiotic products [65]. The two lactic acid bacteria employed in the production of yogurt from milk are the *Streptococcus thermophilus* and *Lactobacillus delbrueckii subsp. bulgaricus*. These bacteria appear to be involved in the prevention of carcinogenesis and in immune stimulation, decreasing colorectal cancer risk [65–67]. Included in the classification of fermented foods, the kefir, a probiotic fermented milk with a complex composition of bacteria and yeasts in a polysaccharides matrix, has demonstrated anti-proliferative, anti-inflammatory, and anti-mutagenic activity [68, 69]. These effects are consistent with the results found in the studies included in this systematic review [34, 48].

Our findings indicate that several of the studies did not submit or could not establish the dose administered [17–20, 22, 27, 32–34, 39, 40, 43, 46–48, 70]. This lack of information was caused by not counting the colony-forming units (cfu) or by ad libitum offer of probiotic supplementation in water or in experimental diets, that led to estimations of consumption, but not the exactly values. The use of probiotic microorganisms must grant health benefits to the host in the studied dosage and duration of use. It is not possible to establish a general minimum dose because each strain is effective at a specific dose [71]. The different probiotics differ in depending on the way the complex interactions between food, microbiota, microorganism, and intestinal mucosa takes place [72]. Surprisingly, this important information was often underreported in this review, hindering the studies reproducibility and representing an important indicator of heterogeneity among the preclinical models.

Some of the studies included analyzed the expression of markers involved in carcinogenesis, as ras-p21, cleaved caspase-3, Mac-1, Ki-67, β-catenin, E-cadherin, p53, Bcl-2, Bax, caspase-9, and caspase-3 [29, 33, 40, 49]. The identification of these markers plays an important role in the early diagnosis and identification of colorectal cancer and in the development of prevention strategies. With the exception of a few specific cases, colon and rectum cancers have remarkably similar patterns of genomic alteration. In almost all tumors, there are diverse alterations involving the TGF-β and

p53 pathways [62], reinforcing the results found in studies included in this review.

In this sense, some protective effects are attributed to probiotics, including the maintenance or enhancement of intestinal barrier function, explained in part by the increase in the expression of the genes involved in tight junction signaling in intestinal epithelial cells [73]. Other mechanisms that relate to probiotic action involve the fecal bacterial enzymes, including β-glucuronidase, β-glucosidase, and nitroreductase, which catalyze the release of procarcinogenic substances in the intestine. The alteration of the intestinal metabolism by modulating the activity of these bacterial enzymes may be one of the possible mechanisms by which probiotics may reduce the risk of the onset of colorectal cancer [74]. According to the results presented, consumption of probiotics reduced fecal bacterial enzymes in all studies that evaluated this effect [17, 18, 20, 24, 25, 47].

Another important mechanism is in the relation of the intestinal mucosa and the host–microbiota. Although the intestinal microbiota were not the focus of the included studies [75, 76], three studies evaluated fecal bacteriology and observed that the probiotic strains were recovered in the feces of all the rats that were given probiotic supplementation [32]. In another study, the *L. acidophilus* group had fecal pH, aerobic bacteria, and *E. coli* count reduced [24], and when comparing the concentration of probiotics strain in feces before and after treatment, a significant increase was found (*L. acidophilus* 4–74% and *B. bifidum* 1–36%) [30]. These effects may be mediated by adherence to enterocytes, intestinal pH reduction, and mechanisms of competition with bacterial pathogens [24, 32, 74].

Probiotic bacteria also show immunomodulatory activity, stimulating production of IL-10 and IgA in intestinal epithelial cells and decreasing pro-inflammatory pathways (via reduction in IL-1β, IL-6, and TNF-α) [75, 77, 78]. Several mechanisms have already been related to the modulation of intestinal barrier function and include the innate and adaptive defense responses, such as of IgA, Toll-like receptors, cytokines, gut-associated lymphoid tissues, and signaling pathways [75, 77, 79, 80]. Some of the mechanisms were verified in the studies we reviewed, such as the reduction in levels of IL-6 and TNF-α and the increase in IL-10 and IgA [27, 30, 34, 37].

Complementarily, a few probiotics show antioxidant activity, inhibiting the generation of reactive oxygen species (ROS), such as superoxide ions, free radicals, and peroxides. These reactive species in excess result in oxidative stress, and they can lead to damage in the cellular structure and in its constituents (DNA, RNA, proteins, and lipids) [81, 82]. Therefore, oxidative stress has an important role in diseases of the gastrointestinal tract, including inflammatory bowel diseases and colon cancers. Thus, reduction in its levels may represent an effective strategy against the development of tumors.

As a result of the high variability of experimental designs and of the finding of methodological bias, the preclinical evidence for the probiotics is still limited. The data heterogeneity, with different strains, doses, and duration of treatment, as well as the extremely diverse induction model of colorectal carcinogenesis represent the main limitations in the evidence availability. The risk of bias analysis was performed individually as a way to ensure the validity of the findings and assessing the methodological quality of the studies, demonstrating that the application of standard protocols is essential to the reproducibility and synthesis of results.

Conclusions

The probiotics were effective in preventing colorectal cancer and the development of preneoplastic lesions, demonstrating that their effects and the metabolic pathways involved are diverse and depend on the probiotic strain administered, on the dose, and on the duration. The limited methodological description and incomplete characterization of protocols and outcomes were the limitations we found, as well as the methodological heterogeneity in studies. We believe that our critical analysis can promote new preclinical research with lower methodological bias, enable researchers to determine the exact mechanisms by which probiotics act, along with their long-term effects, and more importantly guide public health policies that may have an impact on the reduction in colorectal cancer worldwide.

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Authors' contribution PGAB, SCPDL, MCGP, RDN, and RVG conceived the study. PGAB and RVG collected all data and analyzed and interpreted the data. PGAB drafted the manuscript. All authors commented on drafts of the paper. All authors have approved the final draft of the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

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