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Original Research

A systemic review of the role of enterotoxic *Bacteroides fragilis* in colorectal cancer

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Abstract

Enterotoxigenic *Bacteroides fragilis* (ETBF) has received significant attention for a possible association with, or causal role in, colorectal cancer (CRC). The goal of this review was to assess the status of the published evidence supporting (i) the association between ETBF and CRC and (ii) the causal role of ETBF in CRC. PubMed and Scopus searches were performed in August 2021 to identify human, animal, and cell studies pertaining to the role of ETBF in CRC. Inclusion criteria included the use of cell lines, mice, exposure to BFT or ETBF, and detection of *bft*. Review studies were excluded, and studies were limited to the English language. Quality of study design and risk of bias analysis was performed on the cell, animal, and human studies using ToxRTools, SYRCLE, and NOS, respectively. Ninety-five eligible studies were identified, this included 22 human studies, 24 animal studies, 43 cell studies, and 6 studies that included both cells and mice studies. We found that a large majority of studies supported an association or causal role of ETBF in CRC, as well as high levels of study bias was detected in the *in vitro* and *in vivo* studies. The high-level heterogeneity in study design and reporting made it difficult to synthesize these findings into a unified conclusion, suggesting that the need for future studies that include improved mechanistic models, longitudinal *in vitro* and *in vivo* evidence, and appropriate control of confounding factors will be required to confirm whether ETBF has a direct role in CRC etiopathogenesis.

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Keywords: Colorectal cancer (CRC), Enterotoxigenic *Bacteroides fragilis* (ETBF), *B. fragilis toxin* (BFT), Etiology

Introduction

Colorectal cancer (CRC) is the third most diagnosed cancer in men and women around the world [1]. The vast majority of the 1.9 million annual CRC cases are sporadic and can be attributed to a variety of environmental factors [2]. The environmental influence of the gastrointestinal microbiome has become an important research consideration in the etiology of CRC, including the role of microbes and microbially-produced metabolites and toxins as causal agent in the initiation and progression of CRC. Numerous studies of CRC in humans, animal models, and cell models have provided data supporting the role of microbes as a causative agent of CRC. This review focuses on one area generating strong interest, the role of enterotoxigenic *Bacteroides fragilis* (ETBF) as a causal agent of CRC [3–5].

A brief overview of the historical evidence for the role of ETBF in the etiology of CRC is given by Fig. 1. Briefly, the *B. fragilis* toxin (BFT) is a metalloprotease located on a pathogenicity island, BfPAI, which encodes both the *bft* gene and *mpII*, a second metalloprotease [6,7]. The association between certain strains of *B. fragilis* and secretory diarrhea in farm animals and humans was initially reported in the 1980s and BFT was subsequently isolated and characterized [8–10]. A review of the literature shows that ETBF has been associated with both colitis and CRC [11,12]. For example, Toprak et al. report a 38% carriage rate of *bft* in CRC patients using stool samples [12] and Boleij et al. report an ETBF colonization rate of 85.5% in mucosal tissue samples taken from CRC patients [60]. Mechanistic studies carried out using

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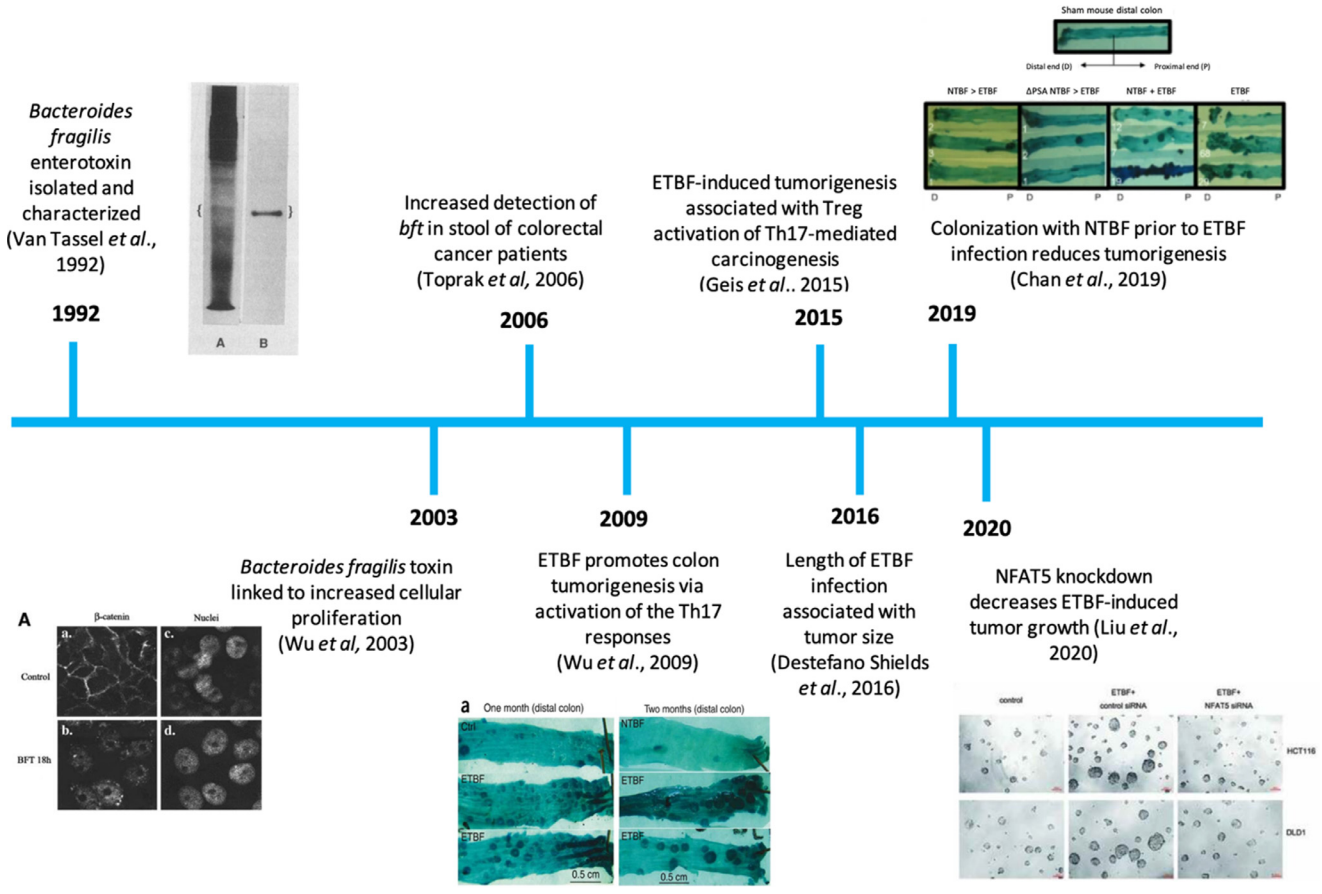

Fig. 1. A timeline of some of the key discoveries concerning the potential causal relationship between ETBF colonization and colorectal cancer.

human cell lines and animal models also provide evidence for the ability of ETBF to enhance tumorigenesis, including immune-mediated inflammation. This review summarizes existing evidence for association of ETBF and CRC as well as the current state of knowledge regarding molecular mechanisms by which BFT influences the etiology of CRC.

Methods

Search strategy and paper selection

A systematic literature search was performed in August 2021 using SCOPUS and PubMed to identify human observational studies that investigated an association between ETBF colonization and a CRC diagnosis, and *in vitro* cell studies and *in vivo* mouse studies that explored a causal relationship between BFT/ ETBF colonization and CRC pathology.

Potential human observational studies were identified using search terms "colo* cancer", "*Bacteroides fragilis*", "bft", "enterotoxigenic *Bacteroides*", "enterotoxigenic", "ETBF", "fragilysin", "metagenome*", "microbiota*; *in vitro* studies were identified using search terms "*Bacteroides fragilis* enterotoxin", "ETBF", "BFT", "enterotoxigenic *Bacteroides*", "fragilysin", "*in vitro*", and "cell"; and *in vivo* studies were identified using search terms "*Bacteroides fragilis*", "ETBF", "BFT", "enterotoxigenic *Bacteroides*", "fragilysin", "mouse", and studies were limited to the English language.

Following an initial screen of titles and abstracts, full text papers were screened for eligibility using pre-defined criteria (Fig. 2, Supplementary Materials S1 Fig. 1). Inclusion criteria for the human observational studies included the detection of ETBF carriage and/ or *bft* (gene or transcript) in CRC patients using colonic washings, stool samples, mucosal samples, and tissue biopsies of colorectal neoplasms (polyps, adenomas, carcinomas). Inclusion criteria for the *in vivo* studies included use of mouse models and exposure to ETBF and/ or purified or recombinant BFT, and inclusion criteria for the *in vitro* studies involved use of cell lines, primary cells, or tissues biopsies and exposure to ETBF and/ or BFT. Studies were excluded if they were review papers, did not present original data, presented protocol development only, and/or performed toxin characterization only. Additionally, 3 studies were removed following concerns regarding how the qPCR analysis was performed, and 1 study was removed for relying on aerobic culture only.

Assessment of methodology quality and risk of bias

The Newcastle-Ottawa Quality Assessment (NOS) tool adapted to human cross-sectional studies, as described by Modesti et al. [13], was performed to assess study design and bias in the human observational studies, the Systematic Review Center for Laboratory animal Experimentation (SYRCLE) tool was used to assess the *in vivo* mouse studies [14], and ToxRTools used to analyze the *in vitro* cell studies [15]. Difference assessment tools were used to account for differences in study design across the three study groups.

The NOS and SYRCLE tools involved a set of criteria designed to assess potential bias with regards to selection of study subjects, comparability between case and control subjects, and assessment of outcome. Additionally, SYRCLE also included a list of criteria designed to detect potential bias as a consequence of flaws in the experimental design (see Supplementary Material

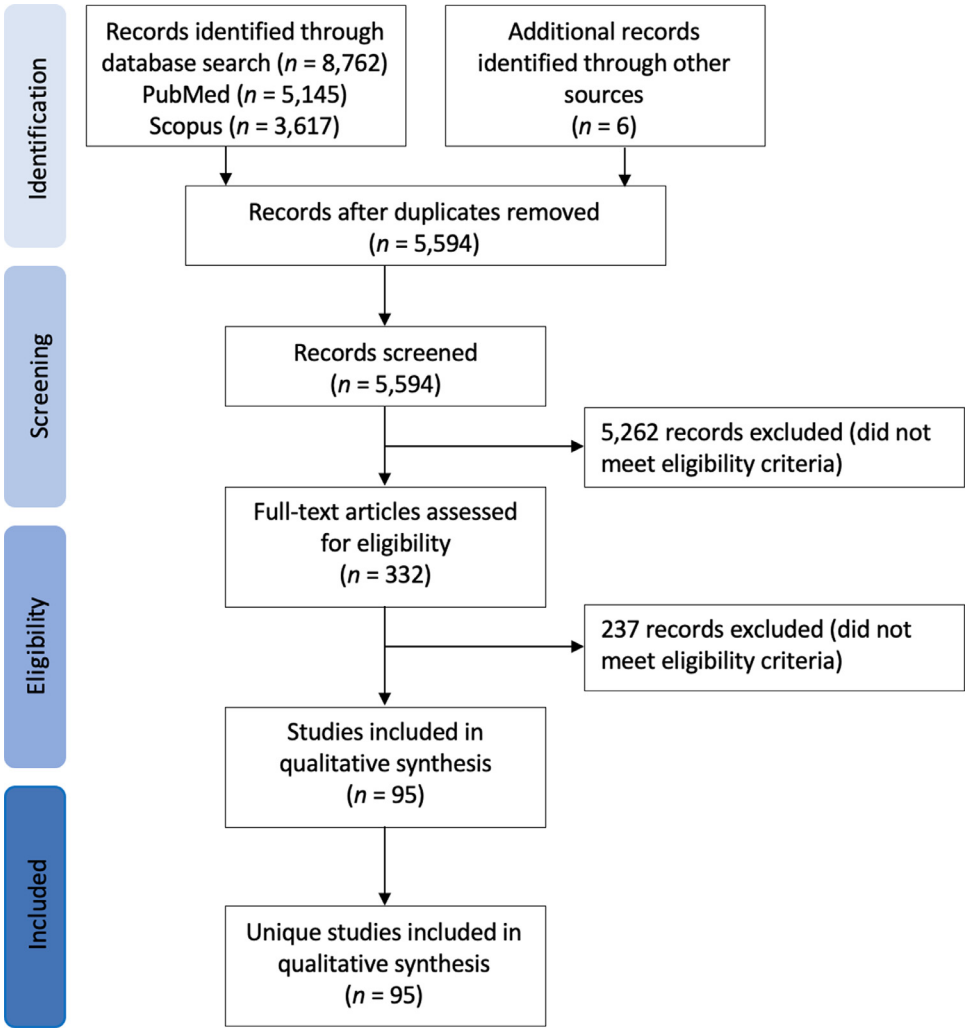

Fig. 2. PRISMA flow diagram for identification, screening, eligibility, and inclusion of human observational studies, mouse *in vitro* studies and cell *in vivo* studies included in this systematic review.

S2 for a full list of criteria). A 'yes' score indicates that the criteria has been achieved and that there is low risk of bias, a 'no' score indicates that the criteria was not achieved and that there is a high risk of bias, and an 'unclear' score is recorded when the paper has not provided sufficient information to determine whether the criteria has been achieved or not.

The ToxRTool differed in that it involved five criteria groups designed to assess the reliability of toxicological data by evaluating how the test substance was identified (total possible score of 4), how the test system was characterized (score $\leq$ 3), the description of the study design (score $\leq$ 6), how the results were documented and presented (score $\leq$ 3), and the plausibility of the study design and data (score $\leq$ 2). For each criteria a score of one is achieved when the criteria is met and a score of zero is achieved when the criteria has not been met. The total score is quantified and used to assess how reliable the study is. For *in vitro* studies, a total score of 15–18 indicates the study is reliable without restrictions (reliability category 1), a score of 11—14 indicates the study is reliable with restrictions (reliability category 2), and a score $<$11 indicates that the study is not reliable (reliability category 3). Additionally, six criteria, one in Group I, four in Group 2, and one in Group V, are deemed essential. A failure to meet all six criteria results in the study being categorized as not reliable (see Supplementary Material S2 for the full list of ToxRTool criteria).

Results

The goal of this review was to provide a systematic overview of the current state of evidence for the causal role of ETBF in CRC using standardized guidelines. A meta-analysis of guidelines for meta-analyses show broad agreement on the need to carefully access a few key characteristics across studies, including eligibility criteria (100%), method of data extraction (100%), risk of bias (100%), and heterogeneity in study design (100%) [16]. We highlight the study design, methodologies, risk of bias, and provide quantitative and qualitative descriptions of heterogeneity in our discussion of the study results to highlight the difficulty of synthesizing the existing evidence.

Observational human studies

Study designs

Twenty-two human observational studies were included in the analysis [12,17–37] (Table 1). Study formats were cross-sectional, case-control or meta-analysis. Nine studies examined samples taken from CRC patients [19,20,23,28–30,34,37] and 13 studies compared sample results from CRC patients to healthy controls [12,17,21,22,24–27,31–33,35,36].

Table 1

Observational studies ($n = 22$) included in review. CRC = colorectal cancer.

Author [reference]	Number of Subjects	Method of detection	Major findings
Toprak et al. 2006 [12]	132 (73 CRC, 59 control)	qPCR of stool culture isolates	*bft* detected at higher rates in CRC compared to controls (38% v 12%, P = 0.009)
Van et al. 2012 [17]	99 (49 polyps, 50 without polyps)	Cytotoxicity assay, qPCR of colonic washing culture isolates	ETBF carriage did not positively correlate to polyp incidence.
Dutilh et al. 2013 [28]	12 (12 CRC)	Metatranscriptomics of DNA extracted from tissue biopsy	No significant *bft* expression in tumor or matched normal sections
Zeller et al. 2014 [31]	491 (114 CRC, 41 adenoma, 335 control)	Metagenomics of DNA extracted from stool samples	*B. fragilis* was not detected
Boleij et al. 2015 [32]	98* (49* CRC/adenoma, 49** control) *23 antibiotic-treated cases excluded from comparison to controls due to poor culture recovery **including 11 adenomas	qPCR of bacterial colonies isolated anaerobically from mucosal colon tissue	The *bft* gene was associated with colorectal neoplasia, especially in late-stage CRC. Detection of *bft* occurred more often in the right tumor
Nakatsu et al. 2015 [33]	276 (102 CRC, 88 adenoma, 86 control)	Characterized the colorectal mucosal microbiome using 16S rRNA sequencing	*B. fragilis* was enriched in the adenoma-carcinoma sequence
Viljoen et al. 2015 [34]	73 (73 CRC*) *55 fresh-frozen, 18 FFPE	Quantified ETBF in paired tumor and normal tissue samples from 55 CRC patients using qPCR	ETBF was enriched in late stage (III/V) colorectal cancers
Keenan et al. 2016 [35]	142 (71 CRC, 71 control)	Screened stool samples for *bft* gene using PCR or qPCR of DNA or cultured colonies isolated from stool.	qPCR was more sensitive than standard PCR for *bft* detection. *bft* was detected at an increased rate in CRC patients
Lennard et al. 2016 [37]	19 (19 CRC)	Transcriptomics (microarray) of DNA extracted from tissue biopsy	Found no differential expression between ETBF-positive and negative tumor, and no differential expression between ETBF-positive and negative adjacent normal
Purcell et al.2016 [18]	19 (19 CRC)	Standard PCR, qPCR, digital PCR to detect *bft* gene from matched luminal and stool samples from 19 CRC patients	SYBR qPCR under-detected *bft* in clinical samples
Snezhkina et al. 2016 [19]	36 (36 CRC)	qRT-PCR was used to quantify *SMOX* gene and ETBF colonization in 50 paired specimens of stages I-IV CRC tumors and adjacent morphologically normal tissues from CRC patients	Found no association between ETBF colonization and SMOX expression
Zhou et al. 2016 [36]	135 (87 CRC, 48 control)	Quantified ETBF present in resected tumors and adjacent normal tissues from 97 CRC patients using qPCR	ETBF was detected significantly higher in the tumor tissues compared to normal tissue and healthy controls
Purcell et al. 2017 [30]	150 (77 CRC/adenoma/dysplasia, 73 without lesions)	qPCR was used to quantified *bft* gene present in mucosal tissue from up to four different colonic sites obtained from a consecutive series from 150 patients referred for colonoscopy	ETBF positivity was associated with the presence of low-grade dysplasia, tubular adenomas, and serrated polyps. Increased ETBF and abundance was also associated with left-sided biopsies.
Hale et al. 2018 [20]	83 (83 CRC)	16S rRNA sequencing was performed on paired colon tumor and normal-adjacent tissue and mucosa samples from patients who underwent partial or total colectomies for CRC	*B. fragilis* was enriched in deficient MMR CRC but not proficient MMR CRC
Bao et al. 2019 [23]	96 (96 CRC)	qPCR was used to quantify ETBF, mRNA, and microRNAs present in CRC tissue samples	Increased expression of *BFAL1* and high abundance of ETBF in CRC tissues predicted poor outcome in CRC patients

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Table 1 (*continued*)

Author [reference]	Number of Subjects	Method of detection	Major findings
Haghi et al. 2019 [21]	120 (60 CRC, 60 control)	Stool samples were screened for *B. fragilis* using PCR targeting the marker genes *neu* and *bft*	*B. fragilis* was detected at a higher frequency in the CRC patients. Detection of *bft* was greater in stage III samples compared to stages I and II.
Saffarian et al. 2019 [22]	67 (58 CRC, 9 control)	Characterized the microbiome from crypts and associated adjacent mucosal surfaces from CRC patients and controls using 16S rRNA gene sequencing, qPCR, and FISH analysis	*B. fragilis* was more abundant in the right-side tumors. *B. fragilis* abundance was increased in tumor samples compared to the controls
Wirbel et al. 2019 [24]	768 (386 CRC, 382 control)	Metagenomic meta-analysis of DNA extracted from stool samples	No significant difference in *bft* between cases and controls
Jasemi et al. 2020 [25]	62 (31 CRC, 31 control)	Phenotypic tests and PCR were performed on bacterial isolates cultured from colorectal tissue	*bft* gene was detected with a greater frequency in the CRC samples compared to the controls. ETBF had an increased ability to form biofilms
Zamani et al. 2020 [26]	120 (68 CRC, 52 control)	qPCR was used to detect *B. fragilis* and *bft* gene from bacterial isolates cultured from mucosal biopsies from patients with precancerous and cancerous legions and healthy controls	*B. fragilis* and *bft* was detected with increased frequency in the patient samples compared to the controls. ETBF was associated with serrated lesions and adenoma with low-grade dysplasia
Shen et al. 2021 [27]	24 [8 colorectal adenoma, 11 laterally spreading tumor (LST), 5 control] – mucosal 475 (113 CRC, 208 adenoma, 109 LST, 113 control) - stool	16S rRNA sequencing was performed in mucosal samples and qPCR was performed on fecal samples to characterize microbial signature	High abundance of ETBF was associated with LST and CRC groups. ETBF also had strong diagnostic power and was associated with malignant LST and IL-6.
Shariati et al. 2021 [29]	30 (30 CRC)	qPCR was used to quantify *B. fragilis* present in paired tumors and normal tissue specimens from CRC patients	*B. fragilis* was detected in equal levels in the tumor and control samples. 15% of *B. fragilis* patients were infected with ETBF in both adenocarcinoma and matched adjacent normal samples

Method of ETBF assessment

Methods of detection included direct PCR or qPCR of ETBF marker genes (*bft, neu*) (13 studies) [18–23,26,27,29,30,33,34,36], selective culture followed by PCR (four studies) [12,17,25,32], comparison of direct PCR to culture with PCR (one study) [35], transcriptomic analysis (one study) [37], or detection via metagenomic or metatranscriptomic data (three studies) [24,28,31]. Nine studies examined tissue biopsy samples for *bft* and/ or ETBF colonization [19,20,23,25,28,29,34,36,37], six examined stool samples [12,18,21,24,31,35], five looked at mucosal samples [22,26,30,32,33], one study used colonic washings [17], and one study examined both stool and mucosal samples [27] (Table 1).

Study results

In nine studies that examined only CRC subjects, the prevalence of ETBF ranged from 0 to 100% [18–20,22,23,28,29,34,37] (Fig. 3). Most studies used qPCR for detection of *bft*, but specific methods, including primers, vary between studies. In patients positive for *bft* in tissue by qPCR, Bao et al. [23] found greater ETBF in tumor samples, but two other studies found no difference between tumor and adjacent normal [29,34]. In addition, Hale et al. [20] identified *bft* in only six of 75 subjects by qPCR. Dutilh et al. [28] used metatranscriptomic data to search for bacterial toxin gene expression. While they identified many reads mapping to the *B. fragilis* genome, there was no significant expression of *bft*. Lennard et al. [37] found no difference in host gene expression between ETBF positive and negative samples, for either tumor or normal samples.

In studies examining CRC and controls, the association between ETBF and CRC was unclear (Fig. 3). Six of eight case-control studies found differences in ETBF prevalence between cases and controls [12,21,25,26,31,32,35,36]. Boleij et al. [32] performed selective *Bacteroides* culture followed by touch-down PCR of selected *Bacteroides* colonies and identified a statistically significant difference between cases and controls, but only after excluding almost half of the cases and not controls for poor culture recovery, potentially biasing the results. Three cross-sectional studies identified an association between ETBF and CRC, but involved different or unreported inclusion criteria, or explicitly did not address irritable bowel disease (IBD) as a potential confounder [27,30,33]. Unfortunately, systematic comparison across studies with so much heterogeneity is not straightforward. An attempt to perform a meta-analysis of fecal metagenomics data across 768 subjects found clearly detectable *bft* in deeply sequenced fecal metagenomes but no significant difference between cases and controls [24]. It was reported that *bft* levels differed broadly with respect to abundance, significant and cross-study consistency of enrichment [24], and thus lack of significance may be due to variability in sequencing depth across the different studies included in the analysis, and thus further metagenomic studies will be required to confirm these findings. Conflicting results may be a consequence of study design. It was observed that studies that reported a significant difference

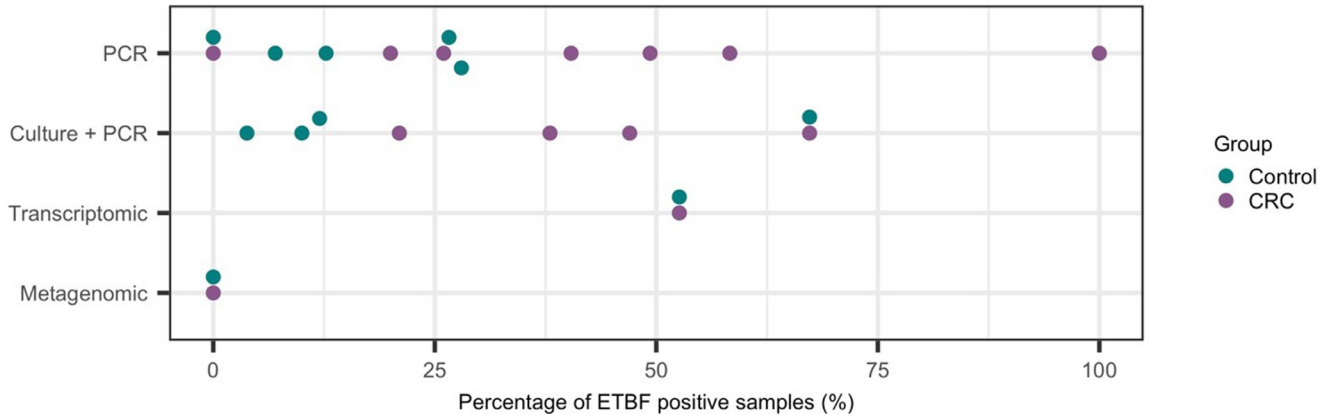

Fig. 3. Comparison of enterotoxin *Bacteroides fragilis* (ETBF) prevalence using different methods of detection. The percentage of colorectal cancer (CRC) and healthy control subjects that tested positive for the *bft* gene are plotted. Each data point represents the results reported from a different study, and only studies that clearly reported the abundance of *bft* are plotted.

in *bft*/ ETBF often involved the use of selective bacterial culturing prior to performing *bft* PCR, while studies that found no significant difference were typically studies that performed PCR/ sequencing directly on DNA samples obtained from the clinical samples. It may be that in samples with low levels of *B. fragilis*, performing PCR and/ or sequencing directly on DNA extracted from the clinical samples may not detect as significant difference as a result of low levels of *B. fragilis* DNA, and that improved *B. fragilis* DNA concentrations as a consequence of selective culturing may improve statistical power. However, it should be noted that the metagenomics studies included significantly higher numbers of subjects, and thus future studies should aim to increase total number of subjects included in order to determine if DNA concentrations and/ or number of included subjects influences the overall findings.

The ability to identify association between ETBF and precursor lesions would be consistent with the hypothesis that ETBF plays a causal role in CRC. Conflicting results between the seven studies and heterogeneity in reporting [17,26,27,30–33] has made it difficult to meaningfully compare the results from different studies. We note some examples to highlight some of the difficulties in making broad assessments. In a cross-sectional study of colonoscopy patients using qPCR Van et al. [17] did not find a difference in ETBF prevalence between patients with polyps or without polyps. Boleij et al. [32] defined subjects with tubular adenomas (TA) as either cases or controls based on the type of medical procedure they underwent (surgical resection or colonoscopy). Nakatsu et al. [33] identified a significant percentage of *bft* positive cases in neoplasms relative to controls in a discovery cohort but not validation cohort. We refer the interested reader to the studies listed in Table 1.

Study limitations and bias analysis

Observational studies were analyzed for bias using the NOS bias tool adapted to cross-sectional studies and the criteria of selection, comparability and outcome. The included observational human studies displayed a relatively low risk of bias (Fig. 4). All studies, however, failed to perform a power analysis to determine the minimum number of subjects and/ or samples required to successfully demonstrate an association between ETBF colonization and CRC. This, therefore, makes it difficult to determine whether the results presented are complete. Observational studies without the engineered use of a toxin/microbial exposure are relatively simple protocols with little risk presented to the individuals, and it may be this simplicity in design that enabled the studies to score a low risk of bias.

In addition to the examples already discussed, it is worth noting some of the limitations in the remaining studies included in Table 1. For example, while Shen et al. [27] found a difference between lateral spreading tumors compared to controls or adenomas, results were only provided as part of a figure, and explicit values were not reported. Purcell et al. [30] reported a significant association of ETBF colonization with the left side of the colon, however, they also reported a within-subject concordance rate of 86% (e.g., a subject's samples were all negative or all positive for ETBF for 129 of 150 subjects). Presumably the reported association is then limited to the 21 cases with discordant results between anatomic sites, raising questions regarding the significance of this result. Additionally, the study identified an association with dysplasia, but did not differentiate between dysplasia arising in adenoma or dysplasia within the setting of IBD. Given that there is a well-established association between IBD and CRC, and that ETBF has also been associated with IBD, this is an important potential confounder and limitation of the study. Nakatsu et al. [33] examined two geographically distinct cohorts and results were reported as percentages only, making it difficult to compare results even within their one study.

In vivo *studies*

Study designs

Thirty *in vivo* mouse studies were identified and included in the analysis [38–68]. 87% of these studies used the C57BL6 mouse strain (26 studies [38,40–49,51–53,57–69]), 10% used the BALB/c mouse strain (3 studies [54–56]), and 3% used a germ free (GF) NIH mouse strain (1 study [39]) to investigate the role of ETBF in colitis and tumorigenesis (Table 2). The mouse strains used had varying genetic backgrounds (see Supplementary Materials S1, Table 1) and 47% of the studies involved knockout (KO) mouse strains, this included 2 studies that used just the C57BL/6J-Apc^{Min} (Min) mouse strain [41,51], six studies that compared wild type (WT) C5BL6 mice to Min mice [49,53,62,65,67,68], two studies that compared WT C5BL6 mice to various KO mouse strains [45,60], and four studies that compared WT C5BL6, MIN, and KO mouse strains [47,49,64,66] (for additional details on KO strains used, see Supplementary Materials Table 1).

Study methods

Study methods include inoculation of specific pathogen free (SPF) mice following antibiotic treatment, mono-colonization of GF mice, colonization in conjunction with chemical instigation of colitis, and exposure to purified or recombinant toxin. The two most commonly used murine models of CRC was the azoxymethane/dextran sodium sulfate (AOM/DSS) model of colitis-associated carcinoma [70] and the Min model of multiple intestinal neoplasia [71]. The AOM/DSS model used a pro-carcinogen (AOM) as initiator followed by repeated cycles of DSS-induced colitis, while the Min model involved the use of an engineered *APC* mutation in the

Table 2

Mouse studies ($n = 30$) included in review.

Author [reference]	Study design	Method of analysis	Major findings
Kim et al. 2005 [38]	SPF C57BL6Cr were injected with BFT	Histopathological examination	Inhibition of p38 prevented BFT-induced enteritis
Kim et al. 2006 [50]	SPF C57BL6Cr mice were treated with either buffer or a COX-2 inhibitor and injected with BFT	cAMP assay, Histopathological examination, ELISA	Suppression of COX-2 activity prevented BFT-induced fluid secretion
Nakano et al. 2006 [39]	GF NIH mice were treated with ETBF or NTBF	Histopathological examination, multiplex-PCR	ETBF induced ulceration, edema, and inflammatory infiltration in the intestine. NTBF was not associated with histological alterations.
Rabizadeh et al. 2007 [61]	SPF C57BL/6 mice were inoculated with buffer, NTBF, or ETBF	PCR, hematoxylin and eosin staining	ETBF alone stimulated colitis and significantly enhanced colonic inflammation
Rhee et al. 2009 [63]	SPF C57BL/6J or GF 129S6/SvEv mice were orally inoculated with WT ETBF, WT NTBF, WT NTBF overexpressing *bft* (rNTBF), or WT NTBF overexpressing a biologically inactive mutated *bft*	Colonic histopathology, Western blot, *ex vivo* E-cadherin cleavage	ETBF and rNTBF caused colitis in both SPF and GF mice but was lethal only in GF mice. Colonic neoplasms were not observed in mice persistently colonized with ETBF or rNTBF (up to 16 months)
Wu et al. 2009 [64]	SPF multiple intestinal neoplasmia (Min)$^{Apc716+/-}$ mice, C57BL/6 mice, and conditional CD4 Stat3–KO mice were colonized with ETBF or NTBF	Histopathology, flow cytometry, depletion of T lymphocytes, cytokine blockade, RT-PCR, Western blotting	Only ETBF triggered colitis and strongly induced colonic tumors. This was associated with Stat3 activation and a selected Th17 response
Goodwin et al. 2011 [65]	SPF C57BL/6 and Min mice were treated with ETBF	Immunohistochemical staining, Western blotting, qRT-PCR	ETBF treatment induced colitis that was associated with increased SMO expression. Treatment with MDL 72527 reduced ETBF-induced chronic intestinal inflammation and proliferation, and reduced ETBF-induced colon tumorigenesis in the Min mouse mode
Wick et al. 2014 [66]	C57BL/6 WT, C57BL/6$^{Stat3\Delta IEC}$, and Rag-1 mice were inoculated with NTBF or ETBF	Immunohistochemistry (hematoxylin and eosin staining), Western blot, EMSA, mucosal permeability, flow cytometry	ETBF increased mucosal permeability and induced rapid-onset colitis that persisted for up to a year. Stat3 activity was increased.
Geis et al. 2015 [67]	C57BL/6 and Min mice were inoculated with ETBF	Flow cytometry, quantitative RT-PCR, histology and microadenoma counts	Tregs initiate IL17-mediated carcinogenesis. Depletion of Tregs in ETBF-colonized C57BL/6 FOXP3DTR mice enhanced colitis but diminished tumorigenesis
Destefano Shields et al. 2016 [68]	SPF Min mice and SPF C57BL/6 mice were colonized with ETBF	Mucosal colonization, TaqMan qPCR analysis	Median colon tumor numbers increased with duration of ETBF colonization. ETBF clearance associated with decreased IL-17 expression
Hecht et al. 2016 [40]	SPF C57BL/6J mice were co-colonized with NTBF and ETBF	Histological staining, ELISA, quantitative reverse transcriptase PCR, sequential colonization	Competitive exclusion of ETBF by NTBF limited toxin exposure and protected against ETBF-induced colitis.
Housseau et al. 2016 [41]	Min$^{Apc+/-}$ mice were colonized with ETBF	Tumor counting, histopathology, flow cytometry, RT-PCR,	Ablation of Th17 cells delayed but did not eliminate ETBF-induced tumorigenesis. IL17 blockade significantly attenuated tumorigenesis

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Table 2 (*continued*)

Author [reference]	Study design	Method of analysis	Major findings
Wagner et al. 2016 [42]	GF C57BL/6 mice were colonized with human fecal microbiota containing NTBF or ETBF	*bft* PCR assay, histological analysis, mass spectrometry, immune cell isolation and characterization, microbial RNA-seq, cytokine quantification	ETBF caused weight loss and NTBF reduced BFT expression
Casterline et al. 2017 [44]	SPF C57BL/6 mice were inoculated with NTBF and then challenged with ETBF	Sequential colonization, Western blot, quantitative reverse transcriptase PCR,	In sequential *B. fragilis* colonization, secondary colonization in SPF mice was strain-specific. Bfpai is neither necessary nor sufficient for secondary colonization but was demonstrated to provide an advantage to one strain of ETBF in successful secondary colonization.
Hecht et al. 2017 [45]	SPF C57BL/6 $Muc2^{+/+}$ and SPF C57BL/6 $Muc2^{-/-}$ mice were inoculated with various ETBF clones	Protein overexpression, bacterial mutants, Western blot, qRT-PCR, EMSA	Muc2-deficient mice succumbed to lethal disease from ETBF colonization in a BFT- dependent manner. BFT expression was suppressed by RprXY. Overexpression of RprXY was sufficient to prevent lethal disease in Muc2-deficient mice.
Lv et al. 2017 [46]	SPF C57BL/6J mice were treated with AOM/DSS and BFT	Histopathological examination, immunohistochemical examination, tumor examination	BFT blocked formation of adenocarcinoma and size of tumors. BFT treatment was associated with increased adenoma counts.
Thiele Orberg et al. 2017 [47]	C57BL/6 (WT), CD45.1 C57BL/6, $Min^{Apc716/+}$ (Min), and OT-1 T cell receptor transgenic $RAG^{-/-}$ mice were colonized with ETBF	Flow cytometry, cell sorting.	ETBF-triggered colon tumorigenesis was associated with an IL-17 driven myeloid signature characterized by subversion of steady-state myelopoiesis in favor of the generation of pro-tumoral monocytic-MDSCs (MO-MDSCs)
Chung et al. 2018 [48]	Mice with a C57BL/6 background were colonized with ETBF	Tumor and microadenoma counts, flow cytometry and cell sorting, gene expression, immunohistochemistry, immunofluorescence, Western blot, immunoblotting	ETBF-induced tumorigenesis requires BFT, epithelial IL-17 and Stat3 signaling.
Dejea et al. 2018 [49]	*Apc+/Δ716* Min mice and SPF C57BL/6J mice treated with AOM were colonized with ETBF	Flow cytometry, qRT-PCR, ELISA, immunohistochemistry	Tumor-prone mice co-colonized with *E. coli* and ETBF showed increased IL-17 in the colon and DNA damage in colonic epithelium with faster tumor onset and greater mortality, compared mono-colonized mice.
Chan et al. 2019 [53]	SPF C57BL/6 WT and $Min^{Apc716+/-}$ mice were treated with NTBF and ETBF	qRT-PCR, histology, microadenoma & macroadenoma counts, immunofluorescence and FISH staining, flow cytometry	Sequential treatment with NTBF followed by ETBF diminished ETBF-induced colitis and tumorigenesis
Gu et al. 2019 [51]	SPF C57BL/6J-Apc^{Min} mice were colonized with ETBF	Flow cytometry, cytospin analysis, IFN β neutralization, qRT-PCR	Expansion of Treg in the colon of ETBF-colonized mice was driven by $CX3CR1^{+}$ tissue-resident macrophages in a IFN β-dependent manner. Knockout or suppression of CX3CR1+ myeloid cells reduced tumors
Hwang et al. 2019 [52]	SPF C57BL/6 mice was colonized with ETBF and treated with zerumbone	Hematoxylin and eosin staining, Western blot, qRT-PCR, ELISA, nitric oxide assay, immunohistochemistry	Zerumbone did not affect ETBF colonization or BFT-mediated E-cadherin cleavage. Zerumbone did prevent weight loss, splenomegaly, decrease macrophage infiltration, and suppress BFT-induced NF-kB signaling and aIL-8 secretion

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Table 2 (*continued*)

Author [reference]	Study design	Method of analysis	Major findings
Cho et al. 2020 [54]	SPF ETBF-colonized BALB/c mice were treated with AOM/DSS and zerumbone	V3-V4 16S MiSeq sequencing, microbiome taxonomic profiling	*B. fragilis* could be activated by zerumbone. ETBF significantly decreased microbial diversity
Hwang et al. 2020 [55]	ETBF-colonized BALB/c mice with AOM/DSS-induced tumorigenesis were treated with zerumbone	Tumor enumeration, histopathology	Oral treatment with zerumbone inhibited colonic polyp numbers and macroadenoma progression
Hwang et al. 2020 [56]	SPF BALC/c mice were colonized with ETBF or NTBF	Histology, quantitative reverse transcriptase PCR, ELISA,	ETBF colonization resulted in formation of numerous, larger-sized polyps in the colon. Polyp formation was associated with *bft* expression
Hwang et al. 2020 [57]	SPF C57BL/6 mice were colonized with ETBF and fed a normal salt diet (NSD) or high salt diet (HSD)	qPCR, nitric oxide assay, histology, ELISA	HSD decreased ETBF-induced tumorigenesis through suppression of IL-17A and iNOS expression
Liu et al. 2020 [58]	SPF C57BL/6 mice were treated with AOM and colonized with ETBF or ETBF. SPF BALB/c nude mice were injected with ETBF-treated or untreated cancer stem cells	RT-PCR, Western blotting, RNA interference, ChIP assays, immunohistology	ETBF increased the number and volume of intestinal tumors and enhanced expression of NANOG and SOX2. NFAT5 and TLR4 knockdowns decreased tumor growth
Patterson et al. 2020 [59]	SPF C57BL/6 mice were colonized with ETBF	Lipidomic analysis, confocal microscopy, qRT-PCR, Western blot, flow cytometry	BFT increases glucosylceramide levels
Boleij et al. 2021 [60]	SPF WT C57BL6 and GPR35$^{-/-}$ (KO) mice were colonized with ETBF	qPCR, Histopathology,	Choice of antibiotic pre-treatment influenced severity of ETBF-colitis. GPR35 knockdown resulted in reduced ETBF-induced weight loss, less severe colitis, increased survival rate, and reduced expression of *IL-22, Cxcl5*, and *Mt2*
Destefano Shields et al. 2021 [62]	C57BL/6J and BRAF^{V600E}Lgr5^{Cre}Min (BLM) mice were colonized with ETBF	Flow cytometry, histology and immunohistology, immunohistochemistry, MBD-Seq, RNA-seq, anti-PD-L1 therapy	*BRAF* mutation drove right-sided ETBF-induced colon tumorigenesis and resulted in disruption of the mucus layer and significant changes in myeloid populations in ETBF-colonized mice

murine genome that results in the formation of multiple small intestine adenomas. Investigation of the potential causal association between ETBF colonization and CRC was achieved by colonizing mice with ETBF in 53% of studies [41,45,47–49,51,52,54,55,57,59,60,62,65,67,68], colonizing mice with ETBF or NTBF in 23% of studies [39,56,58,61,63,64,66], colonizing mice with both ETBF and NTBF (either concurrently or subsequential) in 10% of studies [40,44,53], and inoculating mice with BFT in 10% of studies [38,46,50]. Additionally, mice were inoculated with human fecal microbiota (either ETBF positive of NTBF positive) in one study [42], and in another study the mice were inoculated with ETBF-treated or untreated stem cells [58]. Studies that involved colonizing the mice with ETBF and/ or NTBF typically utilized antibiotics to disrupt the gastrointestinal microbiota prior to bacterial inoculation in order to encourage successful colonization. In total 10% of studies utilized a GF mouse model [39,42,63], 67% of studies utilized antibiotics to generate SPF mouse models [38,40,45,46,49–54,56–61,64,65,68,72], 3% of studies utilized both GF and SPF mice [63], and 23% of studies involved WT mice with no disruptions to the gastrointestinal microbiota [41,47,48,55,62,66,67].

The most commonly used methods of characterizing the effects of ETBF/ BFT exposure in the mouse studies included histopathological examination (utilized by 73%) to assess changes to intestinal inflammation, the formation of tumors, polyps and neoplasms, and the development of ulceration, edema, colitis; PCR (70% of studies) to confirm the presence of ETBF/ BFT and to quantify mRNA levels for inflammatory genes of interest; flow cytometry (40% of studies) for cell quantification; and Western blot (30% of studies) and ELISA (23% of studies) for quantification of BFT, inflammatory proteins (chemokines, cytokines) and other proteins of interest (antibodies, Stat3, Casp3, E-Cadherin) (Table 2).

Study results

Of the 26 studies that colonized mice with ETBF and/ or NTBF, 24 studies reported induced pathogenic traits associated with

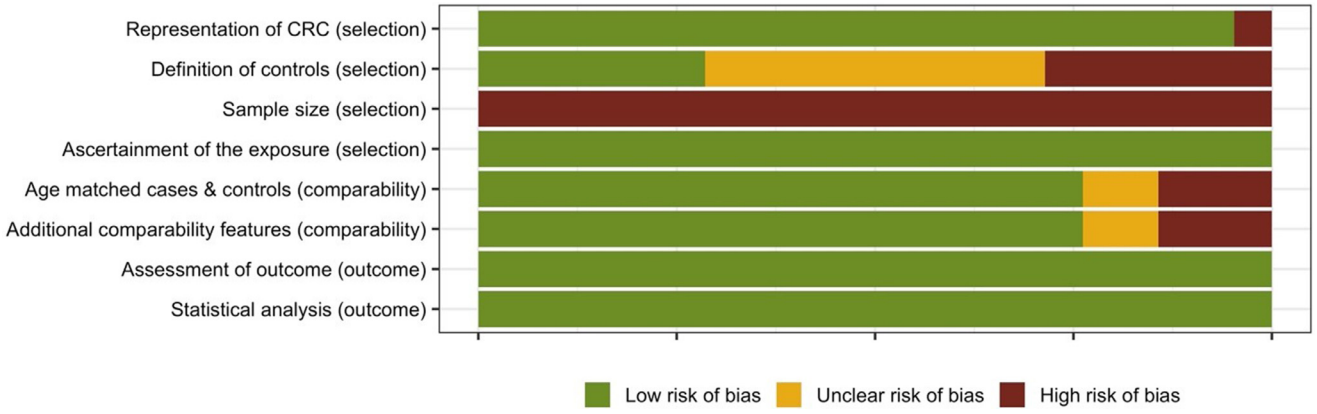

Fig. 4. Quality of reporting and risk of bias assessment using the NOS bias tool adapted to cross-sectional studies. Assessment of the selection, comparability, detection, and outcome is presented as a percentage across all included observational human studies.

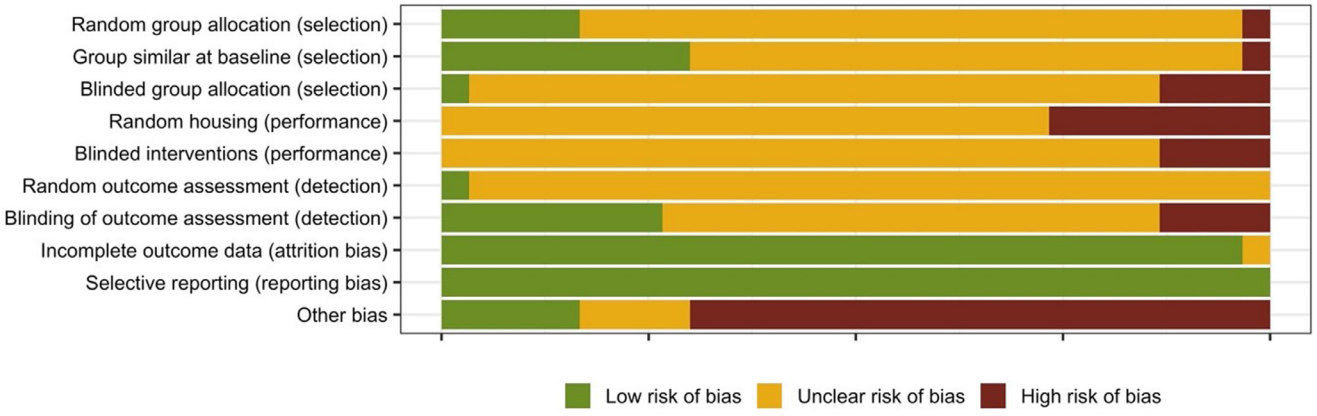

Fig. 5. Quality of reporting and risk of bias assessment using SYRCLE'S risk of bias tool. Assessment of the selection, performance, detection, attrition, reporting, and other bias is presented as a percentage across all included *in vivo* mouse studies.

CRC, this included tumorigenesis (reported in 11 studies) [41,46–49,51,53,57,58,62,64,65,67,68], intestinal inflammation (reported in six studies) [39,47,51,52,61,65], colitis (reported in six studies) [40,53,60,61,63–67], polyp formation (reported in two studies) [55,56], and ulceration [39], edema [39], splenomegaly [52], and macroademona progression [55] (all reported in one study each). Of the studies that colonized mice with NTBF, colonization was found to be non-pathogenic [39,56,61,63,64], and colonization with NTBF prior to colonization with ETBF was observed to reduce the toxic effects of ETBF colonization [40,42,53] (Table 2). Inoculation with just BFT, however, produced more variable results, Kim et al. [38] and Kim et al. [50] reported BFT-induced enteritis and fluid secretion, respectively, while Ly et al. reported that inoculation with BFT blocked the formation of CRC and reduce the number and size of tumors [46], indicating that BFT was in fact protective against CRC.

Study limitations and bias analysis

In the majority of the *in vivo* studies, poorly reported methodology led to an unclear risk of bias for the selection, performance, and detection sections of the bias assessment tool. The authors frequently failed to discuss how the mice were assigned to the treatment group, whether the caregivers, intervenors, or assessors were blinded to which treatment the mice received, whether mice for each treatment group were housed separately or together, and whether results from all the mice were included in the outcome assessment (Fig. 5). Additionally, a significantly number of the *in vivo* studies used pre-treatment antibiotics but failed to address how this might impact outcome, resulting in a high risk of other bias (Fig. 5).

Analysis of the study design revealed a number of limitations associated with the *in vivo* studies. Firstly, despite widespread use of SPF mouse models, excluded pathogens vary by vendor and institution and were not reported by investigators [73]. Extremely limited data was available about gastrointestinal microbiota community composition prior to antibiotic treatment or following ETBF colonization This was of significant concern given that Boleij et al. reported that the choice of antibiotic used to generate SPF mice prior to ETBF colonization significantly influences the severity of ETBF-induced colitis [60]. Gut microbiome dysbiosis has been associated with colitis [74–77], and thus the use of SPF mice makes it difficult to determine whether the development of colitis is a consequence of microbial dysbiosis as a result of non-specific antibiotic targeting of gut commensals or if its caused by ETBF colonization. Moreover, the failure of most studies to determine whether *B. fragilis* strains were present in the gastrointestinal microbiota prior to treatment means that it is impossible to determine whether treatment response was due to ETBF, NTBF, or BFT inoculation or if it was the results of native species of *B, fragilis* or other microbial species undergoing expansion due to reduced competition as a result of antibiotic treatment [40,72].

Another concern was the lack of consistent results within the same model system. ETBF inoculation was found to enhance tumorigenesis in AOM/DSS-induced tumorigenesis model when reported by Hwang et al. [56], yet Lv et al. reported that BFT treatment reduced adenocarcinoma, as

evidenced by the reduced number and size of tumors in AOM/DSS mice treated with recombinant BFT compared to AOM/DSS mice not exposed to BFT [46]. However, BFT treatment was associated with increased adenoma counts and when the total number of adenocarcinomas and adenomas were combined the number of neoplasms detected were similar across the BFT-treated and non-treated groups [46].

The interaction between NTBF and ETBF has been shown to be important, but the lack of follow-up studies on these questions make the role of ETBF in carcinogenesis more uncertain. Hecht et al. [40] reported that co-colonization of NTBF and ETBF prevented the exacerbation of DSS-induced colitis caused by ETBF colonization alone. Wagner et al. [42] reported that NTBF reduced expression of ETBF and prevented weight loss in a human microbiome associated (HMA) mouse model of childhood undernourishment . Inoculation of the Min (APC $^{+/-}$) mouse model with ETBF promotes rapid development of colonic tumors [64]. However, wild-type strains have not demonstrated tumor development in response to ETBF colonization alone, despite development of chronic colitis [63]. Results of co-colonization of ETBF and NTBF in Min (APC $^{+/-}$) mice have not been reported, but this is an important question in light of the Wagner and Hecht studies.

In vitro *studies*

Study characteristics

Forty-nine *in vitro* studies were included in this analysis [6,7,10,38,50,58–60,65,78–117] (Table 3, see also Supplementary Materials S1, Table 2). At least thirty cell lines have been studied (Table 3, Supplementary Materials S1, Table 2), of which the colon carcinoma HT-29 cell line was the most frequently utilized (74% of studies), followed by the human intestinal epithelial cell lines T84 and Caco-2, which were utilized in 10% of the studies each (Table 3). Additionally, several of the studies used human primary colon cells [38,50,86,88,91,102], rat primary cells [83,100], and mouse primary cells [58,59,105] (Table 3, Supplementary Materials S1, Table 2).

Study methods

The most common method of investigation was direct exposure to the BFT protein (80% of studies) [6,7,10,38,50,59,60,65,80–108,110,111,114,115,118], followed by co-culture with both ETBF and NTBF (4% of studies, includes studies where identification of ETBF/ NTBF was determination after co-culture) [112,113,116,117], co-culture with ETBF (2% of studies) [58], co-culture with either ETBF or NTBF (2% of studies) [78] (Table 3). The effect of BFT/ ETBF exposure on the cultured cells was determined by a variety of methods (see Table 3), but the most common included Western blotting, utilized by 57% of studies to detect BFT [59,60,94], assess the ability of BFT to bind and / or degrade to proteins of interest [85,91], to cleave E-cadherin [60,87,96,104,116], determine protein expression/ levels [50,58,59,97–99,104–107,111,114], assess electrophoretic mobility [116] and protein activation [60,108]; PCR (reverse transcription, real-time) was used in 49% of studies to detect and quantify expression of *bft* [6,65,94,112,116], E-cadherin [87], Cox-2 [50,98], beta-defensin, ICAM-1 [100] cytokines [60,89,90,93,97,98,107,113], chemokines [38,91], Muc-2 [110], heme oxygenase [105,111], and transcription factors [58,92]; microscopy (bright-field, fluorescence, electron, confocal, immunofluorescence) was used in 33% of the studies to assess changes in morphology [59,60,79,82–89,92,103,117], proliferation [92], and permeability [59,82–84,88] in the exposed cells; and 12% of studies performed cytotoxicity assays to cellular sensitivity to BFT [10,78,81,86,115] (Table 3). The cytotoxicity were typically semi-quantitative and relied on morphologic changes such as cell rounding or detachment following incubation with purified toxin [16]. Quantification of purified toxin was reported as a concentration such as picomolar or ng/mL, or as titers of cytotoxic activity (highest dilution causing at least 50% cell rounding after 4-hour incubation).

Study results

Exposure to BFT was found to induce a number of cellular changes that are associated with CRC pathogenesis. This included morphological changes [79,80,84,85,89,117], cell permeability [83,84,88,89], cytotoxicity response [10,78,81,86,115], tissue damage [78,88], gene expression (cytokines, transcriptional factors, Cox-2, ICAM-1, B-catenin, Heme oxygenase) [38,50,65,89–93,97,99–101,105,107,108,110,111], cell proliferation [92,114], tumorigenesis [65], and reduced apoptosis [108]. Co-culture with ETBF also resulted in changes to morphology [117], CRC cell proliferation [113] and tumorigenesis [65], increased tissues destruction, increased expression of core stemness transcriptional factors [58], and activation of the TLR4 pathway [58].

Study limitations and bias analysis

The reliability of the toxicological data generated by the *in vitro* studies was assessed by the ToxR Tool (Fig. 6). The average score for Group criteria 1: test substance identified was 1.8 (range = 1–3, total possible score = 3), the average score for Group II: test system characterization was 1.8 (range = 0–3, total possible score = 3), the average score for Group III: description of study design was 4.1 (range = 2–6, total possible score = 6), the average score for Group IV: presentation of study results was 1.9 (range = 1–3, total possible score = 3, and the average score for Group V: plausibility of study design was 2.0 (range = 2–2, total possible score = 2) (Fig. 6). Overall, the average total score was 11.6, resulting in an average reliability score of 2, reliable but with restrictions (Fig. 6). However, 38 studies (78% of the studies) failed to meet the six critical criteria, resulting in 13 studies scoring 3 on the reliability scale (data is not reliable) and 25 studies being downgraded from a reliability score of 2 to a reliability score of 3. This led to an average adjusted reliability score of 2.7 (Fig. 6) (Supplementary Materials S2).

Of the six essential criteria, it was the essential criteria included in Group III: study design description where the *in vitro* studies analyzed failed to meet all essential criteria. The included studies frequently failed to disclose the concentration of BFT used, how the cells were exposed to BFT/ ETBF, the frequency and time points of exposure, and if a positive control had been included (Supplementary Materials S2). Studies which referred the reader to previous papers were score 'no' for these criteria, and often the studies the reader is recommended to refer to also failed to achieve these essential criteria. This meant that the *in vitro* studies were highly unreliable as the studies could not be replicated and potential influencing factors that might bias the results could not be determined.

The *in vitro* studies were found to have several flaws in methodology and presented with conflicting evidence. An early diagnostic test for the presence of BFT took advantage of the "exquisitely sensitive" response of the HT-29/C1 colon adenocarcinoma cell line [84,121] and since the development of this diagnostic tool the vast majority of *in vitro* studies investigating a potential causal relationship between ETBF and CRC have utilized the HT-29 cell line. However, Van Tassell et al. [10] also exposed 14 mammalian cell lines in addition to HT-29 to BFT, including the CCD-3CO cell line (human colon fibroblasts), NCI-H508 (human cecal adenocarcinoma), LS174T (human colon adenoma), Caco-2 (human colon carcinoma), and T-84 (human colon carcinoma). The authors found that BFT only induced a cytotoxic response on the HT-29 cells [10], suggesting that only the HT-29 cell line is sensitive to the toxin, and that this unique property may mean that that the HT-29 model may not be the most appropriate cell line to investigate the relationship between ETBF colonization and CRC. However, it should be noted that other groups have subsequently demonstrated T84 responsiveness to BFT [84].

There is also conflicting evidence for the effects of BFT on barrier function. While Chambers et al. [84] reported decreased monolayer resistance

Table 3

***In vitro* studies ($n = 49$) included in review.**

Reference	Study design	Method of analysis	Major findings
Van Tassell et al. 1992 [10]	Colon carcinoma HT-29 cells were treated with BFT	Cytotoxicity assay	BFT induced cytotoxic response (cell rounding)
Weikel et al. 1992 [117]	Human intestinal epithelial cells T84, Caco-2, HT-29 cell lines were co-cultured with ETBF and NTBF cultures	Cell morphology (bright-field light microscopy)	Exposure to ETBF induced morphological changes
Pantosti et al. 1994 [120]	146 *B fragilis* strains and 64 *Bacteroides* isolates were tested for ability to produce BFT	Anti-serum testing, Cytotoxicity assay	16 strains of ETBF were identified (11% of *B. fragilis* strains examined) Clinical isolates were associated with tissue destruction
Moncrief et al. 1995 [115]	HT29 cells were treated with BFT	Cytotoxicity, SSP-PCT, protein assays, ELISA, PAGE, Western blot	BFT exhibited cytotoxic activity that was inhibited by pretreatment with a metal chelator
Donelli et al. 1996 [79]	HT-29 cells were treated with BFT	Fluorescence and electron microscopy	BFT induces morphological cell changes by reversibly modifying the actin cytoskeleton
Koshy et al. 1996 [80]	Cloned human colonic epithelial cells (HT29/C1) were treated with BFT	Fluorescent phallicidin staining. Cell volume	BFT exposure resulted in distribution of F-actin with loss of stress fibers and cellular membrane blebbing
Saidi and Sears 1996 [81]	HT29/C1 cells were treated with BFT	Cytotoxicity assay	BFT rapidly and irreversibly intoxicates HT29/C1 cells in a concentration- and temperature-dependent manner
Wells et al. 1996 [82]	HT-29 enterocytes were treated with BFT and then co-cultured with enteric bacteria	Viability, transepithelial electrical resistance (TEER), Light and electron microscopy, bacterial internalization	BFT treatment decreased transepithelial electrical resistance, decreased *Listeria monocytogenes* internalization, increased internalization of other enteric species
Obiso Jr. et al. 1997 [83]	HT-29, rat lung type II, and canine kidney epithelium cells were treated with BFT	Mannitol flux assay, Tight junction resistance recovery assay, epifluorescence microscopy	BFT increased permeability of the paracellular barrier of epithelial cells
Chambers et al. 1997 [84]	T84 cells were treated with BFT	Light and electron microscopy, Cell viability, F-actin staining, Ussing chambers	BFT treated induced morphological changes, loss of cellular microvilli, and complete dissolution of some tight junctions
Saidi et al. 1997 [85]	HT29/C1 cells were treated with BFT	Spectrofluorimetry, Confocal microscopy, Western blot	BFT alters the F and G-actin cytoskeletal architecture of HT29/C1 cells without direct proteolysis of actin or decrease in F-actin content
Sanfilippo et al. 1998 [86]	Human primary colon cells were treated with BFT	Cytotoxicity, Electron microscopy	BFT treatment induced morphological changes (cell rounding, separation from adjacent cells, detachment from basement membrane) and cell cytotoxicity
Wu et al. 1998 [87]	HT29/C1 cells were treated with BFT	Western blot, Immunofluorescent, confocal microscopy, Northern blot, Reverse transcription PCR	BFT cleaves the extracellular domain of E-cadherin
Chung et al. 1999 [116]	89 *B. fragilis* strains were tested for BFT production HT29/C1 cells were co-cultured with NTBF and ETBF	Colony blot hybridization, PCR, Western blot	38% of *B. fragilis* strains examined were ETBF, BFT cleaved E-cadherin
Riegler et al. 1999 [88]	Treated colonic mucosa with BFT	Ussing chambers, confocal microscopy	BFT treatment increased cell permeability and damaged crypt and surface colonocytes

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Table 3 (*continued*)

Reference	Study design	Method of analysis	Major findings
Sanfilippo et al. 2000 [89]	Intestinal epithelial cell lines HT29, T84, Caco-2, and IEC-6 were treated with BFT	Transmission electron microscopy, reverse transcription PCR, sandwich ELISA	BFT exposure increased expression of IL-8 and secretion of TGF-β (T84), induced morphology changes (HT29), loss of tight junctions (T84), and detachment (T84)
Kim et al. 2001 [90]	HT29 and Caco-2 cells were treated with BFT	Quantitative real-time (qRT)-PCR, ELISA	BFT exposure increased expression of neutrophil chemoattractant and activators (ENA-78, GRO-α, IL-8)
Kim et al. 2002 [91]	HT29, T84, and primary human colon epithelial cells were treated with BFT	Supershift EMSA, Western blot, qRT-PCR, ELISA	BFT induced NF-κB activation and IκB degradation
Franco et al. 2002 [6]	HT29/C1 cells were treated with BFT	Reverse transcription PCR	The *B. fragilis* pathogenicity island and its flanking regions modulate *bft* expression
Wu et al. 2003 [92]	HT29/C1 cells were treated with BFT	Western blot, Immunofluorescent confocal microscopy, Reverse transcription PCR	BFT activates T-cell factor-dependent transcriptional activation and promotes cell proliferation
Wu et al. 2004 [93]	HT29/C1 cell were treated with BFT	Western blot, ELISA, reverse transcription PCR	BFT stimulates IL-8 secretion
Kim et al. 2005 [38]	HT29 cells were treated with BFT	qRT-PCR, ELISA, EMSA, Western blot	BFT activated three major MAPK cascades (p38, JNK, ERK1/2) and AP-1 signals composed of c-Jun/c-Fos heterodimers
Kim et al. 2006 [50]	HT29 cells were treated with BFT	qRT-PCR, Western blot, Luciferase assay	BFT exposure increased expression of COX-2 and prostaglandin E2
Sears et al. 2006 [94]	HT29/C1 cells were treated with BFT	Western blot, reverse transcription PCR	The deletion of 2 amino acids in the C terminus of BFT reduced biological activity
Wu et al. 2006 [95]	HT29/C1 cells were treated with BFT	Confocal microscopy, flow cytometry, acid wash	BFT binds irreversibly to intestinal epithelial cells in a polarized, metalloprotease-dependent manner
Wu et al. 2007 [96]	HT29/C1 cells were treated with BFT	Western blot, RNA interference, immunostaining	BFT mediated shedding of cell membrane proteins. Cleavage of E-cadherin was dependent on toxin metalloprotease and γ-secretase.
Kim et al. 2008 [97]	HT29 cells were treated with BFT	Cell Death detection ELISA, flow cytometry, qRT-PCR, Western blot, luciferase assay	BFT induced apoptosis and activated the phosphorylation of ERK1/2, p38, and JNK
Kim et al. 2009 [98]	HT29 cells were treated with BFT	Quantitative reverse transcription PCR, RT-PCR, ELISA, Western blot	BFT-induced phosphorylation of both IκBα and IκB kinase (IKK) signals was prevented in eupatilin-pretreated HT29 cells
Yoon et al. 2010 [99]	HT-29 and Caco-2 cells were treated with BFT	qRT-PCR, ELISA, EMSA, Western blot	BFT induced human ß-defensin 2 in a dose- and time-dependent manner that could be regulated by a MAPK, IKK-, and NF-kB-dependent signaling pathway. BFT also activated ERK1/2, p38, and JNK
Goodwin et al. 2011 [65]	HT29/C1 and T84 cells were treated with BFT	qRT-PCR, Western blot, enzyme activity assays,	BFT upregulates spermine oxidase (SMO), resulting in SMO-dependent generation of ROS and induction of a DNA damage marker (γ-H2A.x)

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Table 3 (*continued*)

Reference	Study design	Method of analysis	Major findings
Roh et al. 2011 [100]	HUVECs and rat aortic endothelial cells were treated with BFT	qRT-PCR, flow cytometry, immunofluorescence assay, EMSA, ELISA	BFT induced ICAM-1 expression. Upregulation of ICAM-1 was dependent on the activation of IkB and NF-kB signaling pathways.
Hwang et al.2013 [101]	HT29/C1 wells were treated with BFT	ELSA and Western blot	BFT induced E-cadherin degradation and IL-8 secretion
Yoo et al. 2013 [102]	HT29 cells were treated with BFT	Quantitative reverse transcriptase PCR, ELISA, EMSA, luciferase assay, Western blot	BFT induced upregulation of lipocalin 2 in an AP-1 signaling dependent manner that was regulated by MAPKs (ERK, p38)
Remacle et al. 2014 [103]	Human colorectal carcinoma cell lines (HTC116, HT29, HT29/C1) were treated with BFT	Immunofluorescence microscopy, immunoprecipitation of E-cadherin	BFT cleaved E-cadherin,
Shiryaev et al. 2014 [7]	HT29 cells were treated with BFT	Immunoprecipitation of E-cadherin, cell aggregation assay	BFT repressed cell aggregation
Kharlampieva et al. 2015 [104]	HT29 cells were treated with BFT	Site-directed mutagenesis, recombination, Western blot	BFT induced endogenous E-cadherin cleavage. Cleavage activity required the native structure of zinc-binding motif
Ko et al. 2016 [105]	Murine intestinal epithelial cells were treated with BFT	Quantitative reverse transcriptase PCR, EMSA, transfection assay, Western blot, ELISA immunofluorescence, apoptosis assay	BFT upregulated expression of heme oxygenase-1 (HO-1) in a p38 and IKK-NF-xB dependent manner
Ko et al. 2017 [106]	HUVECs were treated with BFT	Western blot, ELISA, immunofluorescence assay, EMSAs, transfection assay	BFT increased light chain 3 protein II (LC3-II) conversion from LC3-I and protein expression of p62, Atg5, and Atg12. BFT increased indices of autophagosomal fusion with lysosomes, activated ATP-1, and upregulated expression of C/EBP
Jeon et al. 2019 [107]	Human colon epithelial cells (HCT 116) were treated with BFT	Quantitative reverse transcriptase PCR, ELISA, Western blot	BFT reduced expression of β-catenin. Suppression of β-catenin resulted in increased NF-kB activity and IL-8 expression.
Metz et al. 2019 [109]	Ht29/C1 cells were treated with BFT	Morphological assay, thermal shift assay	Chenodeoxycholic acid inhibits BFT
Allen et al. 2019 [110]	HT29/C1 cells were treated with BFT	Quantitative PCR, RNA-seq assay	BFT induced differential expression of genes related to bacterial interactions with colon epithelial cells. *Ceacam1* was increased and *Muc2* was decreased
Jeon et al. 2020 [108]	HCT 116 cells were treated with BFT	Western blot, ELISA, EMSA, Cell death detection ELISA	BFT increased expression of sulfiredoxin 1 (Srx-1) in a time-dependent manner. BFT also activated transcriptional signals (Nrf2, AP-1, and NF-kB). Srx-1 induction was dependent on the activation of Nrf2 signals. Overexpression of Srx-1 attenuated apoptosis
Ko et al. 2020 [111]	Murine dendritic cells were exposed to BFT	Quantitative reverse transcriptase-PCR, EMSA, transfection assays, Western blot, ELISA, ROS assay	BFT upregulated HO-1expression and activated transcription factors (NF-kB, AP-1, Nrf2). Upregulation of HO-1 was dependent on Nrf2 activation and regulated by ERK and p38. BFT also increased production of ROS.

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Table 3 (*continued*)

Reference	Study design	Method of analysis	Major findings
Liu et al. 2020 [58]	Murine colonoids were co-cultured with ETBF	qRT- PCR, sphere forming assay, Western blot, immunohistochemistry	ETBF increased cell stemness and enhanced expression of core stemness transcription factors (NANOG, SOX2). ETBF also activated the Toll-Like 4 pathway
Patterson et al. 2020 [59]	Colon organoids and HT29/C1 cells were treated with BFT	Lipidomic analysis, confocal microscopy, q RT-PCR, Western blot, flow cytometry	BFT increased glucosylceramide levels and decreased colonoid permeability and bursting.
Becker et al. 2021 [112]	Caco-2 cells were exposed to *bft*-positive and -negative strains of *B. fragilis*	TEER, Real-time qPCR, whole genome sequencing, NMR spectroscopy.	BFT increased intestinal barrier function
Cao et al. 2021 [113]	Human CRC cell lines (HCT116, SW480) were co-cultured with NTBF and ETBF	microRNA sequencing, semiquantitative reverse-transcription PCR, RT-PCR	ETBF promoted CRC cell proliferation by down-regulating miR-149-3p
Xie et al. 2021 [114]	Human CRC cell line SW620 and normal colon cell line NCM460 were cultured/ treated with Recombinant BFT (rBFT)	ELISA, Western blot, cell proliferation assays,	rBFT promoted CRC cell proliferation and accelerated tumor growth. This was associated with upregulation of CCL3, CCR5, NF-kB, and TRAF-6
Boleij et al. 2021 [60]	HT29/C1 cells were treated with BFT	CRISPRcas GPR35-knockout, Western blot, ELISA, immunofluorescence, confocal imaging, RT-PCR	GPR35 identified as a signaling molecule for BFT

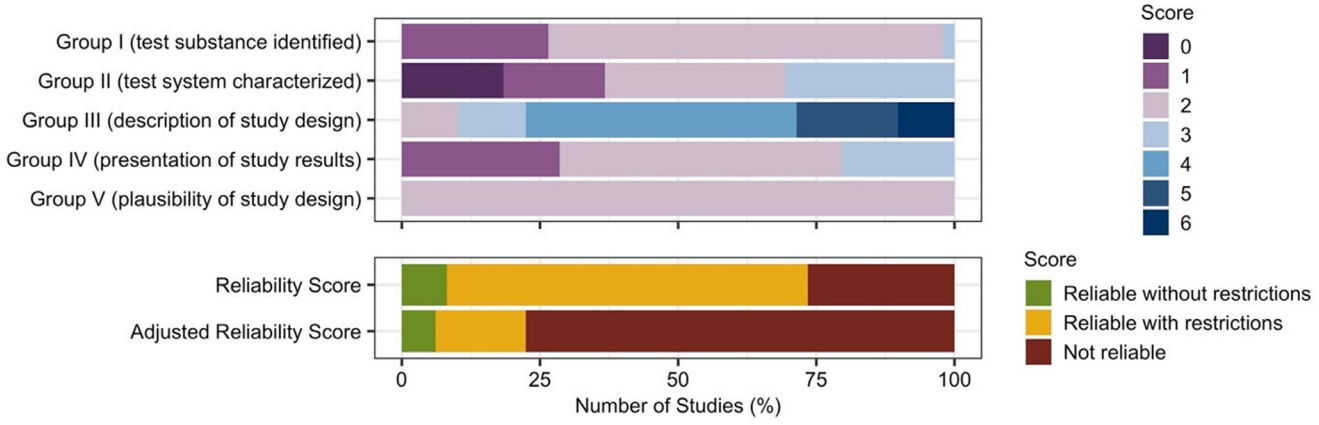

Fig. 6. Quality of reporting and the risk of bias assessment using ToxRTool. The reliability of the *in vitro* cell studies was determined by scoring the test substance identified, test system characterized, description of study design, presentation of study results, and plausibility of the study design. Total score is quantified and both reliability score and adjusted reliability score were quantified. Bias score is presented as a percentage across all included *in vitro* cell studies.

in T84 cells and Riegler et al. [88] identified increased permeability in primary human colonic mucosal strips, Becker et al. [112] reported increased barrier function in Caco-2 cells and human colonic organoids [84,88]. Differences in results across the three studies is likely due to the cell lines used in the different studies having different responses to BFT. Additionally, Becker et al. used live cultures while Chambers and Riegler exposed the cells to purified BFT. It has been previously reported that inoculation of *B. fragilis* corrects gut permeability in a maternal immune activation (MIA) mouse model [122], suggesting that the improved barrier function reported by Becker et al may be due to additional activities of *B. fragilis*. Further investigations will be required to determine whether differences in findings is a consequence of different concentrations of BFT used, different cell lines used, or the use of BFT compared to co-culture with ETBF.

Discussion

This systemic review lays out the evidence for the association between ETBF and human CRC as well as ETBF's role in causing CRC. Though the initial reports have been promising, important biological questions remain as part of future studies. One of the key challenges for any broader attempt at synthesizing evidence is the heterogeneity of the studies and the potential for bias. Future epidemiological studies of CRC could improve the status of our knowledge through stratification of normal, adenomas and carcinomas, and matched controls to assess known confounding risk factors such as colitis. On a technical level, newer sequencing technology can potentially reduce the variation in detection methods and the variability apparent in PCR and culture. There is opportunity to move from exploratory studies to more concrete assessments that more consistently report power calculations and detect ETBF using multiple testing methodologies to enhance reproducibility

and reduce bias. This would help us better understand the nature of any geographic variation in ETBF prevalence.

Mechanistic studies have been extremely promising but reducing the risk of bias by expanding the animal models used for testing will be a key additional piece of evidence in support of the causal role of ETBF in CRC. It is worth noting that many of the i*n vitro* studies do not provide key details essential for reproducibility including identification of the cell line being used, source of material for cell lines, and concentration of toxin being used in experiments. Reproducing these results would be a key first step to understanding the robustness of the overall findings from *in vitro* testing. Reporting concentrations of toxin exposures would be particularly helpful for understanding the physiological applicability to the human colon. Additional future areas of interest for understanding the broader relationship between ETBF and human CRC include assessing the amount of toxin production in an asymptomatic human carriers; what drives heterogeneity of colonocyte response to toxin; and how the context of the gastrointestinal microbial community, including the presence of other *B. fragilis* strains, modulates ETBF behavior.

Conclusion

The role of individual microbes and the gastrointestinal microbiome as a whole in the initiation and progression of CRC is an important area of active research. While the initial studies have brought to light an intriguing potential relationship between ETBF and CRC, a combination of multiple lines of high-quality evidence will be important to further this hypothesis. Future studies should seek to reduce heterogeneity and bias by employing appropriate controls for key confounding factors. In addition, reducing risk of bias in experimental testing by diversifying the models used as well as reporting key data such as cell line or toxin concentrations used would greatly improve the ability of synthesis findings into a broader understanding of the role of ETBF in CRC. This review of the literature supports the International Cancer Microbiome Consortia's 2019 statement that "there is currently no direct evidence that the human commensal microbiome is a key determinant in the etiopathogenesis of cancer" [123], at least with regards to ETBF, and identifies specific areas where additional evidence is needed.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2022.100797.

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