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Role of colonic microbiota in colorectal carcinogenesis: A systematic review

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ABSTRACT

Background and aim: The human colonic mucosa is populated by a wide range of microorganisms, usually in a symbiotic relation with the host. Sometimes this balance is lost and a state of dysbiosis arises, exposing the colon to different metabolic and inflammatory stimuli (according to the microbiota's changing profile). Recent findings lead to hypothesize that this unbalance may create a subclinical pro-inflammatory state that increases DNA mutations and, therefore, colorectal carcinogenesis. In this article we aim to systematically review the scientific evidence regarding colonic microbiota and its role in colorectal carcinogenesis.

Methods: Systematic review of PubMed searching results for original articles studying microbiota and colorectal cancer until November 2014.

Results: Thirty-one original articles studied the role of colon microbiota in colorectal carcinoma including both human and animal studies. Different and heterogeneous methods were used and different bacteria were considered. Nevertheless, some bacteria are consistently augmented (such as *Fusobacteria*, *Alistipes*, *Porphyromonadaceae*, *Coriobacteridae*, *Staphylococcaceae*, *Akkermansia spp.* and *Methanobacteriales*), while other are constantly diminished in colorectal cancer (such as *Bifidobacterium*, *Lactobacillus, Ruminococcus*, *Faecalibacterium spp.*, *Roseburia*, and *Treponema*). Moreover, bacteria metabolites amino acids are increased and butyrate is decreased throughout colonic carcinogenesis.

Conclusion: Conclusive evidence shows that colorectal carcinogenesis is associated with microbial dysbiosis. This information may be used to create new prophylactic, diagnostic and therapeutic strategies for colorectal cancer.

Key words: Colon microbiota. Microbiome. Colorectal cancer. Innate immunity.

INTRODUCTION

The human large bowel is known for its wide microbiota composition. In fact, there are as many as 100 trillion organisms that interfere with the host, usually in symbiotic relation. These microorganisms take the undigested nutrients that reach the colon as its substrates to live. Usually, those are innocuous commensals or are relevant to final product degradation, as well as vitamin formation, among other functions (1-3).

However, this balance is not always maintained and the chronic inflammation and immune evasion caused by inappropriate interactions may promote colorectal carcinogenesis. Indeed, there is growing evidence on microbial dysbiosis in colorectal cancer patients, although the mechanism is not fully understood and yet to be investigated (4,5).

Colorectal cancer (CRC) is a troublesome issue because it is a major cause of cancer deaths around the world, mostly in developed countries where its incidence is increasing. It is a multifactorial disease, associated with lifestyle (pointing out dietary habits and sedentary behaviuors), DNA mutations, inflammation and, most recently,

microbiota changes (6-8).

Although advances are still minimal, recent researches have attempted to identify the type of microorganisms' changes that may enhance carcinogenesis (namely, sequencing advances are being crucial to understand how it happens) (9,10).

Therefore, the aim of this systematic review is to discuss and deepen possible changes in microbiota in adenoma-carcinoma cascade and its interaction with immune response.

METHODS

Specific criteria were defined in order to guide this review. Firstly, a *PubMed* query to gather the articles related to the subject on was built: ("microbiota"[All Fields] OR "microbiome"[All Fields]) AND ("colorectal cancer"[All Fields] OR "colorectal carcinogenesis"[All Fields] OR "colon cancer"[All Fields] OR "rectal cancer"[All Fields]). With this query we intended to collect a wide range of articles, which then would be judiciously selected (total of 250 in November 2014).

A total amount of 254 articles were screened after the referred search (250 articles), cross-referencing (4 articles) and discarding the duplicates. The following inclusion criteria were used: a) Studies that were published until the end November 2014; b) the article should be written in English; and c) studies relevant to the subject (presenting original data). As exclusion criteria we defined: a) Studies considered by the authors as unrelated to the subject; and b) non-original studies. These criteria were applied by reading the title and abstract. After this step, 45 studies were selected for full-text reading. On a second level of eligibility, 14 more studies were excluded and 31 studies were selected, analysed and included in this revision (Table I and Fig. 1).

RESULTS

Colorectal carcinogenesis

It is now believed there are two major pathways for colorectal carcinogenesis; the APC/β-catenin pathway (chromosomal instability) and the microsatellite instability pathway (associated with DNA mismatch repair genes).

The first pathway referred commonly appears as a consequence of mutations on oncogenes and tumor suppressor genes, and represents about 85% of colonic sporadic tumors. Firstly, APC tumor suppressor gene is lost; secondly, the other and intact allele of APC gene is also lost. Other mutations also occur, as in K-RAS, SMAD2, SMAD4 and p-53. Referring to the phenotype, colonic mucosa originates adenomas that become more dysplastic and may drive to carcinomas (11-13).

Further, in the second pathway (about 15% of sporadic colonic tumours) a precursor lesion might not be apparent. However, a serrated adenoma is believed to precede carcinoma. The inactivation of mismatch repair genes (mostly MLH1 and MSH2) is the main event in this cascade, and probably the first, conferring microsatellite instability. Microsatellites are mainly in noncoding regions, originating silent mutations; nevertheless, some are in the coding region or in promoting region of genes that regulate cell cycle and apoptosis (as TGF-β and BAX) (13-15).

Initial events leading to these mutations are still unknown. Subclinical inflammatory stimuli are potential initiators. For instance, our group has shown that colorectal carcinogenesis sequence is accompanied by an increase of Toll-like receptors (TLR) as well as its inhibitors lowering (like Toll-interacting protein, TOLLIP) in colonic lesions and that TLR2 and 4 polymorphisms strongly change colorectal carcinogenesis risk. Taking into account that animal models suggest that bacteria appear to be crucial to the development of colorectal cancer, it is thus hypothesized that the activation of the innate immunity receptors by bacteria leading to a chronic pro-inflammatory status may favor carcinogenesis (16-18).

Microbiota, inflammation and cancer

Microbiota in CRC patients: Microbial dysbiosis?

In order to correctly understand and study microbiota's change in colorectal carcinogenesis it is important to classify each bacterium. In general, organisms are categorized into hierarchic levels (19) (Fig. 2). In this review, microbiota is considered generally by phyla and when appropriated by genus and species.

Some of the phyla analysed below comprise gram-negative bacteria. In first place *Fusobacteria* is a phylum of anaerobic bacilli that may both be commensals or pathogens. Also, *Bacteroidetes* are anaerobic bacteria and are fully distributed in gastrointestinal tract. On its turn, *Proteobacteria* is a big phylum that houses more than 200 genera of gram-negative bacteria, including a wide variety of pathogens as *E. coli*, *Salmonella* and *H. pylori* (20).

On the other hand, other phyla include gram-positive bacteria. For example, *Actinobacteria* usually are aerobic and are frequently mistaken for fungi. Likewise, *Firmicutes* comprises mostly gram-positive bacteria (20).

It is also important to estimate the normal proportions of these bacteria. Besides the high variability found, the proportions of the most predominant bacteria are somehow consensual. The most prevalent bacteria are *Firmicutes* and *Bacteroidetes* (57.2% and 32.0% of colonic microbiota, respectively), according to Wang et al. These authors also stated that the second most predominant phyla seem to be *Proteobacteria, Actinobacteria* and *Fusobacteria* (representing, respectively, 2.81%, 2.22% and 2.20% of normal colonic microbiota) (Fig. 2) (21). In table II, we can see how these bacteria may change during colorectal carcinogenesis.

Fusobacteria

Fusobacterium, genus of this phylum, was shown to be more prevalent in colorectal cancer individuals than in healthy rats and humans (p = 0.001) (22,23). In addition, Kostic et al. found *Fusobacterium* in 48% of adenomas and, in those patients, it was augmented in adenomatous tissue *vs.* surrounding tissue – p < 0.004 (24). Furthermore, comparing individuals itself, the ones with higher abundance of *Fusobacterium* were apparently more likely to have adenomas (OR 3.66, 95% CI 1.37-9.74, p = 0.005) (25). This suggests these bacteria may start to accumulate early in the colorectal carcinogenesis sequence.

Moreover, *Fusobacterium nucleatum* (human's oral cavity colonizer), was found in a higher prevalence in CRC patient's faeces than in healthy individuals (60% *vs.* 22.2% respectively; p = 0.07) (26). It is still unknown whether this is a cause or a consequence (24,26).

Animal studies have shown that introducing human isolates of *F. nucleatum* in Apc [Min/+] mouse model of intestinal tumorigenesis accelerates the onset of colonic tumours - the ones fed *F. nucleatum* developed a significantly higher number of colonic tumours compared to mice fed *Streptococcus spp*. (p < 0.001) (24).

Bacteroidetes

Referring to this phylum in general, a tendency to be augmented in the tumour, rather in adjacent mucosa, has been identified (27).

Bacteroides belong to this phylum and it is worth to mention that Wu et al. observed not only an increase of these bacteria in CRC but also a positive relation between the density of these bacteria and the disease status (R = 0.462, p = 0.046). Sobhani et al. that observed an increase from CRC patients stool and biopsy samples comparing with healthy individuals (5,22). This increase was also stated by animal-model based studies – for example, Zhu et al. shown that *Bacteroides* exhibited higher abundance in CRC rats compared with control animals (14.92% *vs.* 9.22%, p = 0.001). Also, Baxter et al., using transplanted faecal microbiota from both CRC patients and healthy individuals into germ-free mice, stated that *Bacteroides* where strongly correlated with increased tumor burden (p < 0.005). Finally, Zackular et al. found an enrichment of members of the *Bacteroides* in tumour-bearing mice (p < 0.001) (23,28,29).

Furthermore, Boleij et al. studied *Enterotoxigenic Bacteroides fragilis* (known for its role in acute diarrheal disease, inflammatory bowel disease, and colorectal cancer and which produce *B. fragilis* toxin) and concluded that the expression of the gene that encodes the toxin is strongly related with CRC (75% from CRC cases *vs.* 67% from healthy controls) and, particularly, with advanced CRC (100% from advanced stages *vs.* 72.7% from early stages; p = 0.093) (30).

Prevotella, genus which also belongs to *Bacterioidetes* phylum, was also shown to be overrepresented in CRC patients (p = 0.009) (5). Despite this, a mice-based study showed that family *Prevotellaceae* and, namely, members of the genus *Prevotella* were underrepresented in CRC animals (p < 0.05) (28).

Moreover, *Alistipes* (genus that belonging to the same phylum) were also in higher levels in tumour-bearing mice (relative abundance of 0.05) (29).

On the other hand, studies comparing *Bacterioidetes* levels between adenoma patients and controls, cases showed lower abundance of these bacteria (namely, showing lower proportions of *Bacterioides spp.*) - 29.14% *vs.* 34.14%, p < 0.05 (4). This result was also exhibited by Brim et al., in a study with pre-neoplastic lesions from African-Americans, particularly at a sub-genus level, and by animal studies in rats, comparing CRC animals with healthy ones (63.95% *vs.* 79.26%, respectively) (23,31). These results lead to hypothesise that *Bacteroidetes* may be fundamental at a cancer stage, rather than in adenoma-carcinoma sequence.

⸺ *Porphyromonadaceae:* This is a bacteria family composed by genera as *Porphyromonas* and *Dysgonomonas*, which appear to be less prevalent in healthy individuals (p = 0.001) (22). In animal studies, *Odoribacter* (other genus of this family) was increased in CRC-mice supporting the previous statement; though, different results were found for other members of this family, which appeared to be underrepresented in CRC-mice (28). Additionally, *Parabacteroides* (another genus of this family) were also shown to be positively related to tumorigenesis rate in mice transplanted with faecal microbiota from CRC patients, comparing with the ones transplanted with healthy individuals microbiota (29).

Actinobacteria

Bifidobacterium represents a ubiquitous, gastrointestinal, vaginal and oral cavity colonizer gram-positive bacterium, belonging to *Actinobacteria* phylum; it seems to be higher in control individuals than in CRC-patients (26). Also, when comparing colon cancer patients with diverticulitis patients, the first have lower counts of *Bifidobacterium* (*Bifidobacteria* were found in 100% of patients with diverticulitis

and in 76% of those suffering colon cancer) (26,32).

Coriobacteridae is a subclass which has been demonstrated to be increased in CRC tissue, namely the genera *Slackia* and *Collinsella*, regarded as gastrointestinal commensals (27).

Firmicutes

This is one of the predominant phyla both in health and disease. Although, when looking to the whole phylum, there is no difference between adenoma cases and healthy controls, specific subgroups show differences (4).

At a family level, *Staphylococcaceae* was shown to be higher in CRC patients than in healthy controls ($p = 0.011$) (22).

On the other hand, *Clostridiales* are a class of gram-positive bacteria that were negatively correlated with CRC formation in a mice-based study, which used faecal transplantation (29).

More specific studies on species from this class have been done; namely intraindividual temporal stability of *Clostridium coccoides*, is significantly ($p < 0.05$) different comparing CRC patients (65%) with both healthy controls (76%) and polypectomized individuals (77%). Furthermore, *C. leptum*'s temporal stability was also lower (although not significant) in CRC patients (33).

Eubacteriaceae, on its turn, is a family which belongs to *Clostridiales* order and that is statistically significant augmented in CRC patients ($p = 0.037$) (22). Despite this, *Eubacterium,* a butyrate-producing genus of this family, was reduced in CRC rats, comparing with the control group (23).

Lactobacillus, is a probiotic specie which also belongs to this phylum, seems to be diminished (although not statistically significant) in faecal samples of tumour patients rather than in healthy controls ($p = 0.064$). This decrease in CRC patients (comparing with healthy controls) was also observed in animal studies with rats (2.32% *vs.* 3.71%; $p < 0.001$). Furthermore, *Ruminococcus*, another probiotic specie, was similarly decreased (23,26).

⸺ *Faecalibacterium prausnitzii:* Bacteria generally present as a commensal in gastrointestinal tract and which was demonstrated to be diminished in CRC

patients (13.3% *vs.* 40%; p = 0.06) (22,26). Even though, different results are found comparing patients with adenomas with control individuals; Shen et al. found that *Faecalibacterium spp.* were increased in case subjects (21.7% *vs.* 10.3%, p < 0.05) (4).

Roseburia, which is a butyrate-producing genus, as well as *Faecalibacterium*, were also diminished in CRC patients (3.59% in healthy controls and 1.56% in CRC patients; p < 0.05); the same tendency was also seen in rats by Zhu et al. (21-23).

Proteobacteria

Analysing at a phylum level, components of this one were shown to be overrepresented in adenoma cases, comparing with controls (12.9% *vs.* 4.85%, respectively; p < 0.05) (4).

Animal studies in rats (animal model of 1,2-dimethylhydrazine - induced colon cancer) show no difference in the species of *Proteobacteria* between CRC animals and healthy controls, when comparing bacterial communities (p = 0.175), although its abundance was higher in CRC animals (1.06% in healthy rats, comparing with 2.95% in CRC rats) (23).

Enterobacteriaceae is a family from this phylum. It includes both harmless microorganisms and pathogens, and it seems to be augmented in cancer patients (46.6% in CRC patients *vs.* 0% in control group). In fact, when splitting tubular adenoma and adenocarcinoma patient's results, the last one has statistically significant higher levels of these bacteria (p = 0.035), which may lead to the hypothesis that there may be a positive correlation between bacteria level and tumour's stage (26). Nonetheless, controversial results were found; actually, members of this family, as *Citrobacter, Shigella, Cronobacter, Kluyvera, Serratia and Salmonella spp.*, have been shown to be lessened in cancer tissue, comparing with adjacent mucosa (27).

Further, *Campylobacteraceae,* other family from this phylum, has been shown to be less prevalent in healthy controls (p = 0.014) (22).

⎯ *E. coli: E. coli* (which belongs to *Proteobacteria* phylum) represents gram-negative commensal bacterium from human gut, although some strains have been recognized as pathogens, associated with an inflammation status and toxin production (as cyclomodulin). Studies have demonstrated that cyclomodulin-positive pathogenic strains are more prevalent in most advanced cancer stages (using TNM staging, TNM I - 45%; TNM II - 64% and TNM III/IV - 67%); furthermore, there is evidence of an increase of mucosa-associated and internalized *E. coli* in tumour's tissue, comparing with normal mucosa (from diverticulitis patients' controls). These two were also significantly correlated with proliferative index of the mucosa (p < 0.02 and p < 0.04, respectively) (34).

Still, experiments with IL-10$^{-/-}$ mice showed that host's inflammation is needed for E. coli cancer-promoting activity: In the absence of inflammation, high abundance of *E. coli* was not sufficient for tumorigenesis (35).

Other bacteria

- Treponema. Zhu et al. showed that, in rats, these gram-negative bacteria, belonging to *Spirochaetes* phylum, were reduced among CRC-rats group (2.43% in CRC group *vs.* 3.04% in control group, p < 0.001) (23).

- Methanobacteriales. This represents a group from *Euryarchaeota* phylum. *Methanobacteriales* were higher in polyps and tumours, rather than in healthy controls. Actually, a positive correlation was seen between CRC and *Methanobacteriales* presence (r = 0.537, p = 0.007). To be more accurate, significantly higher levels of faecal bacteria were observed when comparing the control group with the CRC group (p = 0.0033); however, no differences between controls and adenoma cases were observed (p = 0.48). Moreover, no significant differences between tumour and adenoma samples were found (p = 0.189) (26).

- Akkermansia muciniphila. Is an anaerobic, gram-negative, mucin-degrading bacterium (*Verrucomibrobia* phylum), which appears to be in higher levels in tumour patients (33.3% *vs.* 0%; p = 0.136 according to Mira-Pascual et al.;

and 12.8% *vs.* 3.54%, according to Weir et al.) (26,36). *Akkermansia* genus was also elevated in tumour-induced mice, rather than in healthy ones (28,29).

Bacterial metabolism

Butyrate

Butyrate is thought to protect against colonic inflammation and, therefore, CRC (36,37). Butyrate-producing bacteria seem to be underrepresented in CRC patients. Studies reveal that faeces of healthy individuals show higher butyrate levels, comparing with acetate rich CRC-patients stool (29,36).

GPR109a, coded by Niacr1, is a receptor for this metabolite and for niacin (metabolite that prevents inflammation). It's signalling stimulates anti-inflammatory response, allowing the differentiation of anti-inflammatory cells (namely regulatory T cells and IL-10 producing cells). Experiments with Niacr1$^{-/-}$ mice have shown that those were more susceptible to colonic cancer. This may be a good starting point for new prevention and therapeutic measures using, for example, a receptor agonist (37).

Other fatty acids

Healthy controls presented higher levels of poly and monounsaturated fatty acids in stool than CRC-cases. Healthy controls also presented higher ursodeoxycholic acid and glycerol (glycerol might be taken up by tumour cells) levels (36).

Amino acids

Faeces from CRC-patients show higher concentrations of amino acids; this might be due to a great variety of reasons as differences in protein consumption by the different microorganisms, reduction in nutrient's absorption because of the inflammation status and/or augmented autophagy, among others (36,38).

Cyclomodulin

Cyclomodulin, toxin produced by some (pathogenic) strains of *E. coli*, may participate in the carcinogenesis pathway. It is genotoxic and has great impact in several cell functions (cell-cycle progression, proliferation, differentiation, apoptosis, etc.). A particular experiment with *E. coli* strain 11G5, that encodes colibactin (which acts as a cyclomodulin), revealed that mice colonized with it showed greater tumor's size and number (34).

Actually, this toxin was higher in adjacent normal mucosa of colonic cancer patients, rather than in the diverticulitis ones (the colonic mucosa of CRC patients was mostly colonized by B2 cyclomodulin-producing phylogroup); moreover, this strains were also augmented in higher tumour's stages, comparing with the lower ones (34,39).

This information suggests that the association between the microbiota and CRC may be due to metabolic activity (Table III), overlapping bacterial phylogeny.

Biomarkers

Latest studies have emphasized the possibility of creating a new, non-invasive diagnostic test of CRC, using biomarkers (in fecal material).

Zackular et al. combined the characterization of microbiome in healthy, adenoma and carcinoma patients with recognized risk factors for CRC, and concluded that the discrimination between these groups strongly ameliorated, rather than the risk factors alone (40).

Analysing and investigating the presence of bacterial metabolites, new CRC biomarkers may arise, using a non-invasive diagnostic test (feces samples).

Further research is needed to test and consolidate these hypotheses and also to test the use of bacteria and various metabolites in order to create a new, non-invasive and trustable diagnostic test which can benefit clinical practise.

Immune signalling and inflammation

The impact of microbiota changes in immune signalling is undeniable and so it is the role of chronic inflammation in colorectal carcinogenesis. There are many published results that brace this idea.

McCoy et al. showed a significant positive correlation in CRC cases between *Fusobacterium* prevalence and local inflammatory cytokines gene expression, explicitly TNF-α (r = 0.33, p = 0.06) - suggesting that *Fusobacterium* may increase mucosal inflammation- and, remarkably, IL-10 (r = 0.44, p = 0.01), highlighting the complexity of the relationship host/intestinal microbiota (25).

When comparing healthy individuals' mucosa with adjacent mucosa of tumour lesion from CRC patients, the latter showed overrepresentation of IL-17 immunoreactive cells (mostly CD3 marking cells). Interestingly, not only IL-17A, but also IL-17C have been proved to be up regulated in CRC, both in human and mouse models, although being differentially regulated (5,41).

Further investigations on IL-17C production have led to new knowledge on this subject; even though it is up regulated in CRC, IL-17C signalling is crucial for and promotes tumorigenesis (by the induction of Bcl-2 and Bcl-x$_L$ expression). Moreover, throughout tumorigenesis microbiota drives its production leading, therefore, to CRC promotion (41).

Notwithstanding, a different investigation on IL-10 showed that its deficiency in mice worsened epithelial mitosis and polyp's growth; also IL-10$^{-/-}$ mice had more, more penetrant and multiple tumours. In addition, the expression of IL-12p40 and TNF-α mRNA was shown to be strongly augmented in IL-10$^{-/-}$ mice (18,42).

TLR/MyoD88 was also shown to be crucial in bacterial-induced carcinogenesis (inflammation-related CRC); in fact, IL-10$^{-/-}$; MyoD88$^{-/-}$ mice lacked neoplastic signs, unlike IL-10$^{-/-}$ mice. On the other hand, IRAK-M (IL-1 receptor associated kinase-M) deficient mice presented invasive and antibiotic resistant cancer (18,43).

Further human research showed changes on TLR's profile in normal mucosa adjacent to the lesion (either adenoma or carcinoma), comparing with normal, healthy subjects' mucosa. This supports TLR's involvement in CRC. Namely, a persistently positive TLRs expression (as TLR2) and lower expression of TLRs inhibitors (as TOLLIP) were associated with higher TLRs protein levels throughout all the spectrum of lesions of colon carcinogenesis (16).

TGF-beta signalling pathway is also important in gut's inflammatory and microbiota microenvironment. It is crucial down-regulating the inflammatory response a

maintaining the normal gut environment. Actually, SMAD4's (mediator of this pathway) haploinsufficiency was reported in two case reports and, then, tested in mice. The resultant inflammatory environment may be connected with CRC development (44).

Supplementary information shows that c-Jun/JNK and STAT3 signalling pathways are generated by colonic microbiota and anaemia, respectively and that both are thought to act synergistically in tumour's growth in APC$^{Min/+}$ mice. Supporting this statement it was seen that germ-free APC$^{Min/+}$ practically lacked colonic tumours (45). Nonetheless, some investigations have been made on inflamossomes, cytoplasmic protein complexes (Nod-like receptors- NLR's, pro-caspase-1 and eventually the adaptor protein apoptosis-associated speck-like protein containing a CARD- ASC). ASC$^{-/-}$ mice showed greater predisposition for CRC tumorigenesis, as well as NLRP6$^{-/-}$ mice. Besides, intriguingly, wild-type mice cohoused with ASC$^{-/-}$ and NLRP6$^{-/-}$ mice had a great increase in the propensity to develop an inflammation-induced CRC, comparing with singly housed ones- can this represent a transmissible cancer? (46).

DISCUSSION

The information resulting from the revised studies suggest that several different bacteria, isolated or acting concurrently, may play an important role in colorectal carcinogenesis. Evidence was gathered that this may happen by proinflammatory and metabolic stimulus. It looks clear that investigation of colon microbiota and its cellular pathways is an area of great potential.

However, several limitations must be assigned to this review and to the studies herein included. Firstly, it both includes human and animal studies, giving similar importance to both. Furthermore, different authors used different methodology (namely the usage of fecal samples/biopsy samples; the usage of biopsies from normal adjacent mucosa compared with tumors' mucosa versus normal healthy mucosa compared with tumors' mucosa; number of patients/animals used, etc.), which may affect the achieved results and can contribute to the observed differences. Despite the inherent limitations, the present review allowed us to reach valid and important results.

It seems clear that colorectal carcinogenesis is associated with a microbial dysbiosis. Actually, although with some controversial results, in carcinoma patients (either humans or animals) some bacteria were consistently found augmented (such as *Fusobacteria*, *Alistipes*, *Porphyromonadaceae*, *Coriobacteridae*, *Staphylococcaceae*, *Akkermansia spp.* and *Methanobacteriales*), while other were constantly diminished (such as *Bifidobacterium*, probiotic species- namely *Lactobacillus* and *Ruminococcus*, butyrate-producing bacteria- explicitly *Faecalibacterium spp.*, *Roseburia*-, and *Treponema*).

Concerning to bacterial metabolites, it looks sharply defined that butyrate-producing bacteria are lessened in CRC patients, as well as poly and monounsaturated fatty acids and ursodeoxycholic acid (29,36). Also, higher concentrations of amino acids are found in CRC individuals (36,38). Notwithstanding, cyclomodulin-producing *E. coli* strains were augmented in higher tumour's stages, comparing with the lower ones (34,39). All this evidence hints that metabolic environment might be deeply involved in colorectal carcinogenesis.

Moreover, the gathered information leads to the conclusion that different microbiota may have different effects in immune signalling and that this may contribute to a chronic proinflammatory stimulus in colorectal carcinogenesis. The results suggest that dysbiosis may be the missing link between the several studies that show immunologic changes in the colon mucosa (cytokine profile, TLR's expression) and colorectal cancer. Definitely, microbiota changes are closely related to inflammatory environment and to CRC's development. Other reviews on the subject also support this statement (47-50).

In view of this information, it seems appropriate to hypothesize that different bacterial profiles exposes colonic mucosa to different metabolic (given that different bacteria originates different metabolites) and inflammatory stimuli (namely, by activation of the innate immune response), and that this seems to create a subclinical pro-inflammatory state that enables DNA mutations and, therefore, colorectal carcinogenesis (Fig. 3).

Taking altogether, and even though from the analysis of the studies included in this review it is not possible to firmly conclude that dysbiosis is a cause or a consequence

in CRC, it looks clear that future research in this area is needed. Moreover, methods to study colonic microbiota should be homogenised in order to compare studies and to reach valid conclusions. The precise analysis of CRC microenvironment (composition, immunologic pathways and metabolite production) seems to deserve to be seen as a major point of interest in scientific research nowadays. Dietary and drug (possibly using antibiotics, probiotics and anti-inflammatory drugs) approaches might well be tried and further adopted in clinical practise. Furthermore, the usage of metabolites appears to be very useful as a non-invasive way of diagnosing this pathology.

In conclusion, microbiota appears to have an important role in colorectal carcinogenesis. However, more studies applying validated techniques are needed. It is predictable that in the future new prophylactic, diagnostic and therapeutic strategies can arise from microbiome research.

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Table I. Main characteristics of studies included in the systematic revision (including "Authors", "Year of publication", "Type of study", "Methods", "Limitations" and "Main conclusions").

Authors	Year	Type of study	Methods	Limitations	Conclusions
Gueimonde M, et al.	2007	Cross-sectional	- Distal colonic mucosa sampling; - DNA extraction; - PCR	- Samples from healthy controls were not available for comparison	- Aberrancies in mucosa associated microbiota are present in different intestinal diseases
Sato K, et al.	2007	Experimental study	- Cell culture; - Tissue sampling; - Western blotting; - Fluorescence assay; - Cell death assay; - Immunohistochemistry - Transmission electron microscopy	- Only usage of tissue sampling	- Autophagy is activated in colorectal cancers *in vitro* and *in vivo*, and it may contribute to the survival of the cancer cells in their microenvironment
Scanlan PD, et al.	2008	Cross-sectional	- DNA extraction; - PCR; - Gel electrophoresis and ribosomal intergenic spacer analysis	- Only usage of faecal material	- The intestinal microbiota and their metabolites are significantly altered in both colorectal cancer and polypectomized subjects compared with controls
Uronis JM, et al.	2009	Experimental study	- Model of CAC- azoxymethane-exposed, conventionalized-Il10$^{-/-}$ mice; - Tumour histology; immunohistochemistry; - Real-time PCR	- The involvement of MyD88 signalling in the development of CAC was not addressed in this study	- Colonic microbiota is essential to the development of colitis-associated cancer and that chronic colitis promotes the oncogenic potential; - Carcinogenesis is dependent on microbial recognition by the TLR/MyD88 system
Shen XJ, et al.	2010	Cross sectional	- Terminal restriction fragment length polymorphism;	-Inability to detect *Bifidobacterium spp.;* -Mucosal biopsies from the rectum (not from	-Adherent bacteria may be crucial in the development of adenomas and colorectal cancer

		study	- Clone sequencing; - Fluorescent *in-situ* hybridization	adenoma tissue or from the surrounding mucosa); -Potential differences in biopsy location	-Changes in the adherent bacterial in adenomas, namely increased *Proteobacteria* and decreased *Bacteroidetes*
Sobhani I	2011	Cross sectional study	DNA extraction; pyrosequencing	-Controls were not healthy volunteers; -Cases and controls differed in age and cases of polyps or cancer in relatives; -Possible missed differences (pyrosequencing)	- Distribution of colonic bacteria varied with the disease status; significant elevation of the *Bacteroides*/*Prevotella* population in CRC patients (this appeared to be linked with elevated IL-17 producing cells)
Li Y, et al.	2012	Experimental study	- Mice model; -Immunohistochemistry - Western blot; - Real-time qPCR; - Fluorescein isothiocyanate-dextran assay; - Flow cytometry; - Cell culture, *in vitro* assays	- Limitations in obtaining sufficient numbers of CD11bþ cells from the tumour directly	- The microbiota is implicated in the acceleration of tumour growth in APCMin/1 mice, through triggering the c-Jun/JNK and STAT3 signalling pathways in combination with anaemia
Ohigashi S. et al.	2012	Cross-sectional	- Faecal sampling; - Real-Time quantitative PCR; - Measurements of fecal organic acid concentrations and pH	- Small number of patients; - Regional and racial differences not taken in account; - Only usage of faecal material	- CRC patients showed significant differences in the intestinal environment, including alterations of microbiota, decreased short chain fatty acids, and elevated pH. These changes are not a result of CRC progression but are involved in CRC onset
Szigeti R, et al.	2012	Case-report; Experimental study	- Mice model (Smad4+/−mice; chronic colitis induction); - Pyosequencing	- Few samples studied	- Smad4 haploinsufficiency can be associated with higher susceptibility to large bowel inflammation, in association with microbiota. This may be implicated in colorectal cancer as well
Wang T, et al.	2012	Cross-	- DNA extraction;	- Only usage of faecal material	- Reduction of butyrate producers and increase of opportunistic

Author	Year	Study type	Methods	Limitations	Conclusions
		sectional	- Pyrosequencing; - Real-Time quantitative PCR		pathogens may constitute a major structural imbalance of gut microbiota in CRC patients
Arthur JC, et al.	2013	Experimental study	- AOM/Il10$^{-/-}$ mice model; - DNA extraction; - Quantitative PCR; - Illumina V6 16S library construction and sequencing; - RNA isolation; - Illumina RNA-sequencing	- The mono-association studies may not fully reflect *E. coli* behaviour in a human host harbouring a naturally acquired complex microbial community	- Host's inflammation is necessary for *E. coli* cancer-promoting activity
Brim H, et al.	2013	Cross-sectional, case-control	- Stool sampling; - microarray; - Pyrosequencing	- Only usage of faecal samples	- At the pre-neoplastic stages, there is a trend showing microbiota changes between healthy and colon polyp patients at the sub-genus level
Buc E, et al.	2013	Cross-sectional	- Biopsy samples; - DNA hybridization, PCR; - Single-cell gel electrophoresis; - Cytopathic and adhesion assays	- Data may be underestimated due to experimental procedure of preparing colonic specimens	- The study showed high prevalence of cyclomodulins-producing B2 *E. coli* in biopsies of CRC, particularly at the distal part
Dennis KL, et al.	2013	Experimental study	- Mice models; - Histology; - Bone marrow reconstitution; - DNA extraction; - 16SrRNA sequencing	- The mono-association studies may not fully reflect microbiota's behaviour	- Colon polyps are driven by microbes that accumulate and trigger local inflammatory responses. These are suppressed by IL-10 derived specifically from T cells and Tregs
Hu B, et al.	2013	Experimental study	- Mice model; - Histopathology; - Immunohistochemistry;	- Dysbiosis in IL-6-dependent models should have be taken into consideration	- Aberrant inflammasome-induced microbiota plays a critical role in CRC development. -The results also suggest that, under some conditions,

			- Cell culture; - ELISA		microbiota components may transfer CRC susceptibility between individuals
Klimesova K, et al.	2013	Experimental study	- Colitis-associated cancer mice model; - multiplex cytokine assay/ELISA; - Flow cytometry; - Pyrosequencing; - Fluorescence assay	- Further research is needed to reveal the exact role of IRAK-M in different types of tissues	- Microbiota promotes tumourigenesis by increasing the exposure of the epithelium to carcinogens and that IRAK-M negative regulation is crucial for CRC resistance, even in conditions of altered microbiota
Kostic AD, et al.	2013	Cross-sectional study	- Faecal and biopsy samples; - quantitative PCR; - FISH analysis; - Sequencing; - Flow cytometry	- Complex environment in which *Fusobacterium* exists	- *Fusobacterium* spp. are found at an increased abundance in the stool of patients with adenomas and colorectal cancer and are enriched in adenomas and adenocarcinomas relative to noninvolved colonic tissues
Le Chatelier E, et al.	2013	Cross-sectional.	- Faecal sampling; - DNA extraction; - Illumina sequencing; - Quantitative metagenomics; - Microarray analysis	- Only usage of faecal material	- The author's classifications based on changes in the microbiota, identify subsets of individuals in the general white adult population
Marchesi JR, et al.	2013	Cross-sectional.	- resections (colon adenocarcinoma); - rRNA sequencing	- Intra-patient variability in microbiome alterations; - Absence of a large reference database of mucosal microbiomes from healthy individuals	- There are striking differences in microbial colonization patterns between colon tumour tissue and adjacent non-malignant mucosa
McCoy AN, et	2013	Case-	- Biopsies;	- Complex environment in which	- It appears to exist a link between the abundance of

al.		control, cross-sectional study	- DNA extraction; - PCR; - Pyrosequencing	*Fusobacterium* exists	*Fusobacterium* in colonic mucosa and adenomas, further suggesting a possible role for mucosal inflammation in this process
Weir TL, et al.	2013	Cross-sectional	- Stool sampling; - DNA extraction; - Pyrosequencing	- Only stool samples and not tissue mucosa were analysed	- There were no significant differences in the overall microbiota structure associated with the disease state, but several bacterial genera, particularly butyrate-producing species, were under-represented in the CRC samples, while a mucin-degrading species, was about 4-fold higher in CRC
Wu N, et al.	2013	Cross-sectional case-control study	- Faecal collection; - RT-qPCR - Pyrosequencing	- Biases associated with different regions of the 16S rRNA gene	- Significant differences exist between CRC patients and normal controls; namely, a positive correlation was observed between the abundance of *Bacteroides* species and CRC status
Zackular JP, et al.	2013	Experimental study	- Mice model of inflammation-induced colon tumourigenesis. - Histological analysis; - RNA isolation and DNA extraction; - Sequencing and qPCR	- Large variation in the structure of the microbiome across individuals	- Changes in the gut microbiota directly contribute to tumourigenesis; interventions affecting it's composition may be a strategy to prevent the development of colon cancer
Baxter NT, et al.	2014	Experimental study	- Fecal transplantation to germ-free mouses; - DNA extraction; - Sequencing	- Assessment of fecal communities only; - Unable to recapitulate the structures of the human microbiota donors	- The initial structure of the microbiome determines susceptibility to colonic tumourigenesis
Boleij A, et al.	2014	Cross-sectional	- Sample collection (colonoscopy biopsies);	- Significantly higher number of colonies analyzed per tissue area from controls, and	- The bft gene is associated with colorectal neoplasia, especially in late-stage CRC

			- PCR; - bft isotype identification	surgical specimens exposed to air for longer than control biopsies	
Bonnet M, et al.	2014	- Cross-sectional; experimental study	- *E. coli* colonization of Min mice.	- Further studies are needed to understand the role of *E. coli* on CRC	- Pathogenic *E. coli* could be a cofactor in pathogenesis of colorectal cancer
Mira-Pascual L, et al.	2014	Case-control, cross-sectional study	- Mucosal biopsies and faecal samples; - Pyrosequencing; - qPCR	- Further studies are needed to understand the role of methanogens on CRC	- CRC risk is influenced by microbiota, showing differences according to disease progression and tumour's stage
Singh N, et al.	2014	Experimental study	- Mice models; - Cell cultures; - Quantitative real-time PCR; - ELISA		- Gpr10a has an essential role in mediating the beneficial effects of gut microbiota and dietary fibre in colon
Song X, et al.	2014	Experimental study	- Induction of CAC and Spontaneous Intestinal Cancer in mice; - Histology staining; - Real-time PCR; - Etc.		- IL-17C is a critical early cytokine that promotes intestinal tumour development. - This paper provides a mechanism for microbiota-mediated tumour development through IL-17C regulation
Zackular JP, et al.	2014	Cross-sectional	- Stool samples; - DNA extraction; - rRNA gene sequencing	- A larger, more diverse sample of individuals should be used; - Inability to assess the bacterial role and mechanisms in the development and progression of CRC	- Information on gut microbiota improved the ability to differentiate healthy, adenoma and carcinoma groups, combined with other clinical risk factors (comparing with risk factors alone)

| Zhu Q, et al. | 2014 | Experimental study | - Establishment of an animal model of 1, 2-dimethylhydrazine (DMH)-induced colon cancer
- Pyrosequencing | - Few detailed information concerning mucosa-associated microbiota | - A significant difference in intestinal bacterial flora exists between healthy rats and CRC rats (higher abundance of *Firmicutes*, *Proteobacteria* and *Actinobacteria* in CRC rats; lower abundance of *Bacteroidetes*) |
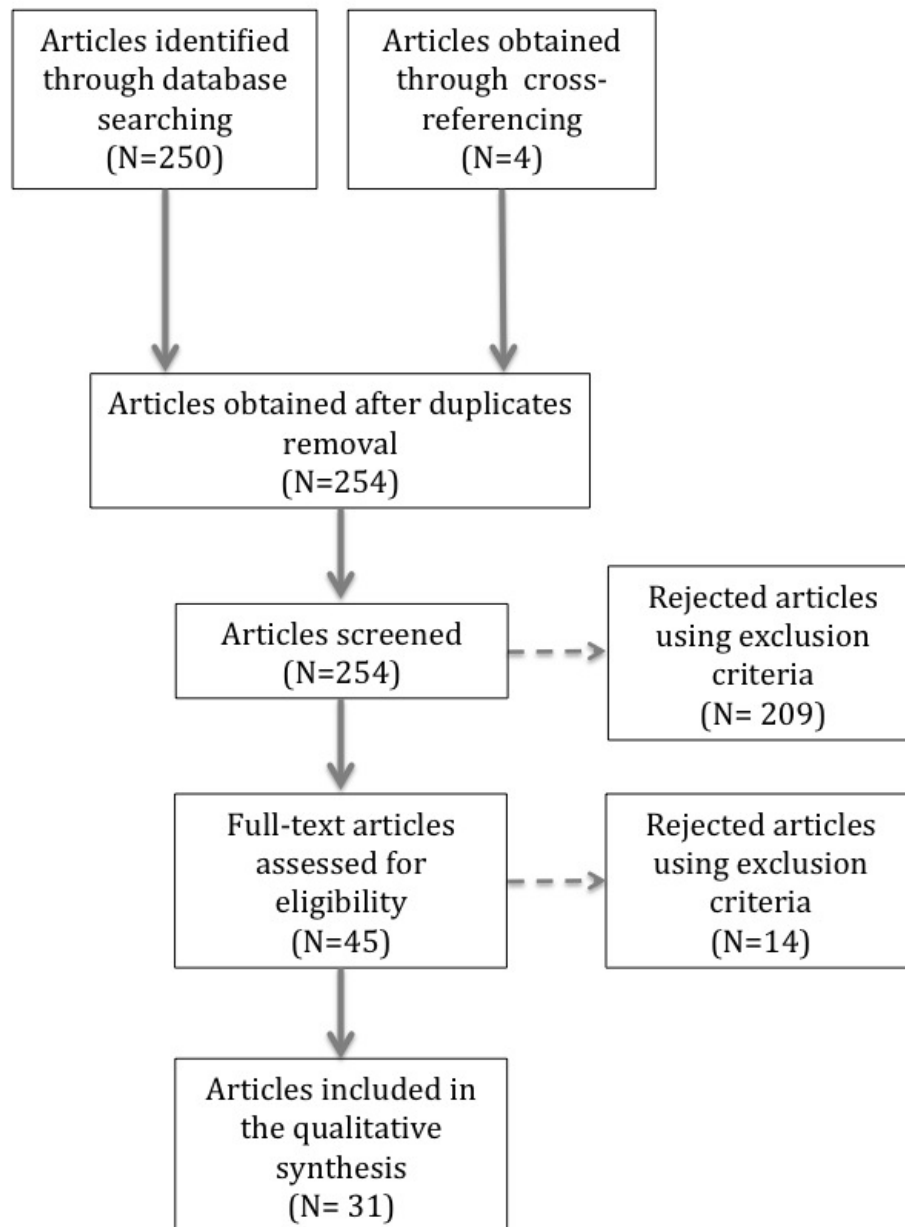
Fig. 1. Fluxogram.

Table II. Microbiota changes from normal individuals (either from biopsy or faecal samples, from studies based on humans, animals or both) to adenoma/carcinoma stages

		Adenoma	Carcinoma	Study subjects	References
	Fusobacteria				
	Fusobacterium	↑	↑	H, A	Wu et al. (22), Zhu et al. (23), Kostic et al. (24), McCoy et al. (25)
	F. nucleatum		↑	H, A	Kostic et al. (24), Mira-Pascual et al. (26)
	Bacteroidetes				
	Bacteroides	↑/↓ (?)	↑	H, A	Shen et al. (4), Sobhani et al. (5), Wu et al. (22), Zhu et al. (23), Zackular et al. (28), Baxter et al. (29), Brim et al. (31)
	Prevotellaceae		↓	A	Zackular et al. (28)
	Prevotella		↑	H	Sobhani et al. (5)
	Alistipes		↑	A	Baxter et al. (29)
	Porphyromonadaceae				
	Porphyromonas		↑	H	Wu et al. (22)

	Dysgonomonas		↑	H	Wu et al. (22)
	Odoribacter		↑	A	Zackular et al. (28)
	Parabacterioides		↑	A	Baxter et al. (29)
Actinobacteria					
	Bifidobacterium		↓	H	Mira-Pascual et al. (26), Gueimonde et al. (32)
	Coriobacteridae		↑	H	Marchesi et al. (27)
	Slackia		↑	H	Marchesi et al. (27)
	Collinsella		↑	H	Marchesi et al. (27)
Firmicutes					
	Clostridiales		↓	A	Baxter et al. (29)
	Eubacteriaceae		↑	H	Wu et al. (22)
	Eubacterium		↓	A	Zhu et al. (23)
	Staphylococcaceae		↑	H	Wu et al. (22)
	Lactobacillus		↓	H, A	Zhu et al. (23), Mira-Pascual et al. (26)
	Ruminococcus		↓	A	Zhu et al. (23)
	Faecalibacterium spp.	↑		H	Shen et al. (4)
	F. prausnitzii		↓	H	Wu et al. (22), Mira-Pascual et al. (26)
	Roseburia		↓	H, A	Wang et al. (21), Wu et al. (22), Zu el al. (23)

|				
Proteobacteria	↑	↑	H, A	Shen et al. (4)
Enterobacteriaceae	↑	↑↑/↓ (?)	H	Mira-Pascual et al. (26)
Campylobacteraceae		↑	H	Wu et al. (22)
E. coli (Cyclomodulin-positive)		↑	H	Bonnet et al. (34)
Other bacteria				
Treponema		↓	A	Zu et al. (23)
Methanobacteriales	↑	↑↑	H	Mira-Pascual et al. (26)
Akkermansia		↑	A	Zackular et al. (28), Baxter et al. (29)
A. muciniphila		↑	H	Mira-Pascual et al. (26); Weir et al. (36)

Here, ↑ represents an augment and ↓ a decrease; H: Study based on humans; A: Study based on animals.

Table III. Metabolites changes from normal individuals (either from biopsy or faecal samples, from studies based on human, animals or both) to adenoma/carcinoma stages

	Carcinoma	*Study subjects*	*References*
Butyrate	↓	H	Baxter et al. (29), Weir et al. (36)
Other fatty acids (poly and monosaturated fatty acids; ursodeoxycholic acid)	↓	H	Weir et al. (36)

Amino acids	↑	H	Zackular et al. (28), Sato et al. (38)
Cyclomodulin	↑	H	Bonnet et al. (34), Buc et al. (39)

Here, ↑ represents an augment and ↓ a decrease.
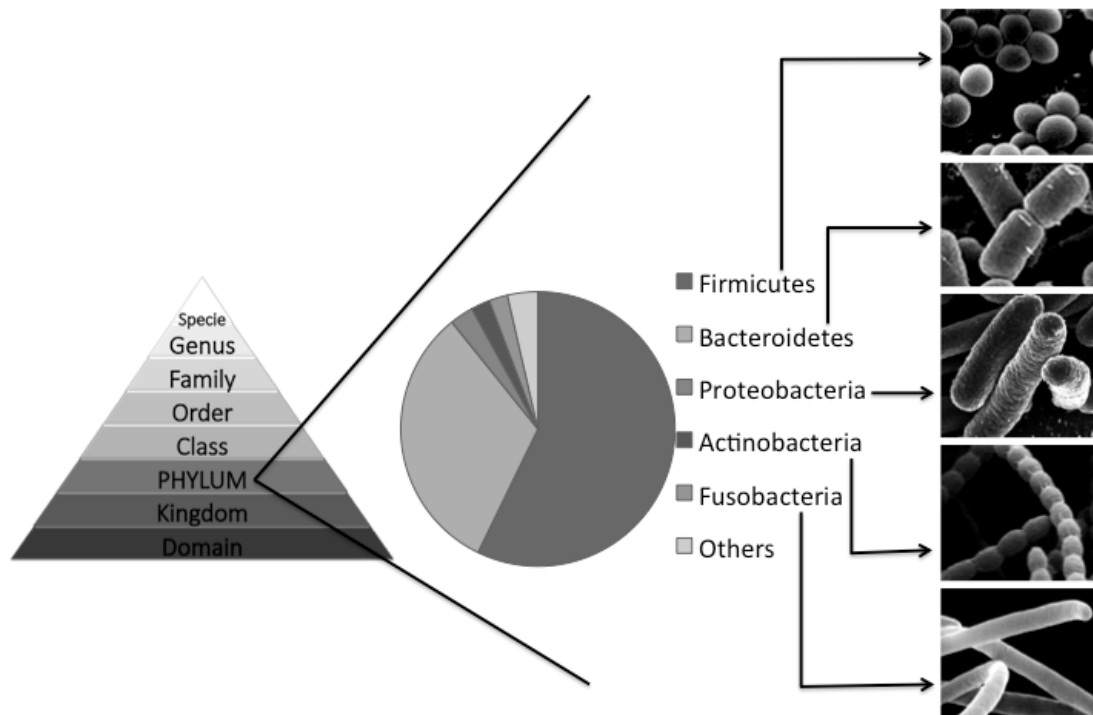
Fig. 2. Hierarchical organization of the taxonomy used to categorize organisms and comensal microbiota and normal microbiota proportions (based on Wang et al. results).
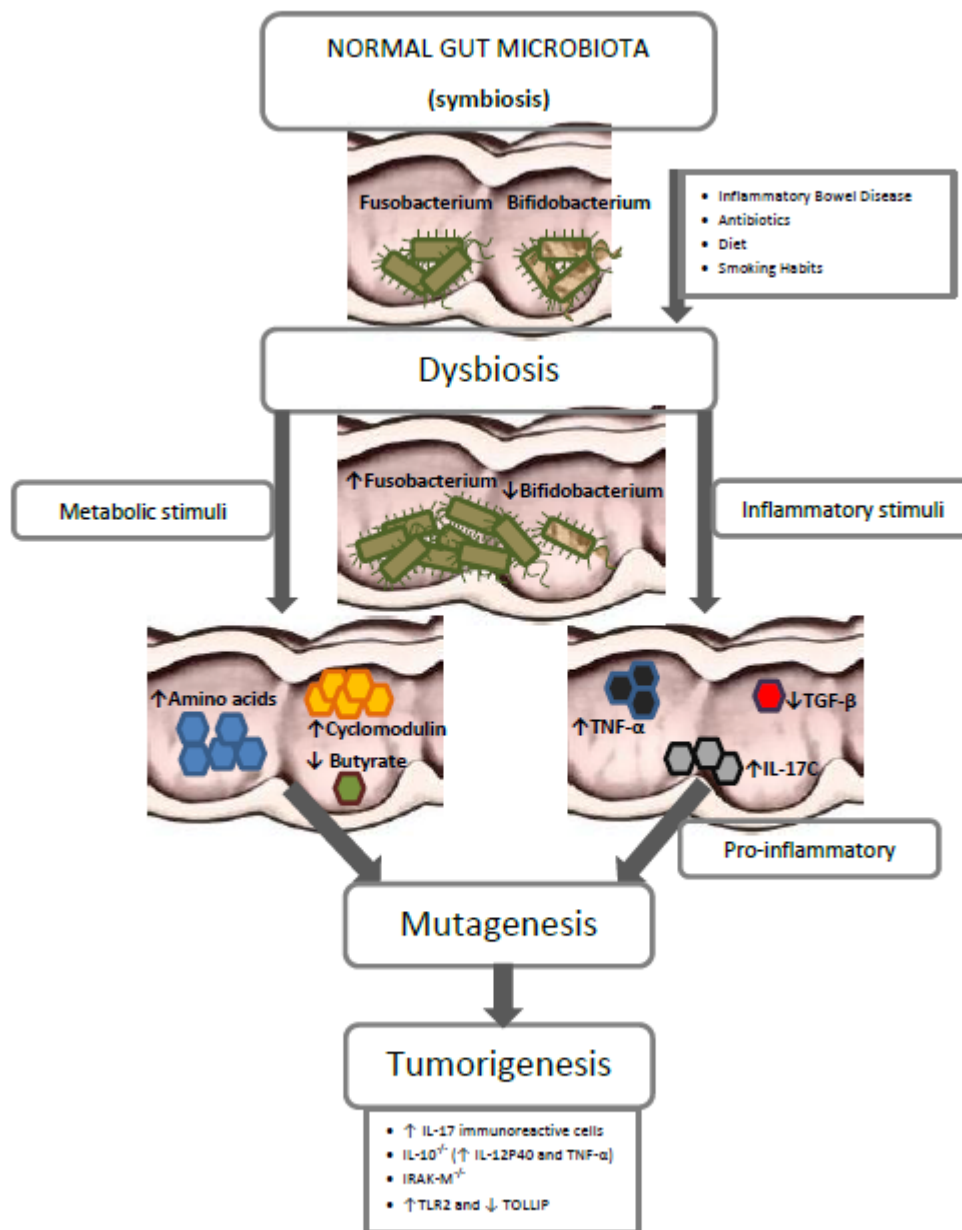
Fig. 3. Possible altered gut microbiota mechanisms of inducing colorectal carcinogenesis. Different bacterial profile exposes colonic mucosa to different metabolic and inflammatory stimuli, creating a subclinical pro-inflammatory state that enables DNA mutations and, therefore, colorectal carcinogenesis.