The controversial role of *Enterococcus faecalis* in colorectal cancer

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Abstract: Colorectal cancer (CRC) is a complex and widespread disease, currently ranked as the third most frequent cancer worldwide. It is well known that the gut microbiota has an essential role in the initiation and promotion of different cancer types, particularly gastrointestinal tumors. In fact, bacteria can trigger chronic inflammation of the gastric mucosal, which can induce irreversible changes to intestinal epithelial cells, thus predisposing individuals to cancer. Some bacterial strains, such as *Helicobacter pylori, Streptococcus bovis, Bacteroides fragilis, Clostridium septicum* and *Fusobacterium* spp. have a well established role in CRC development. However, the role of *Enterococcus faecalis* still remains controversial. While part of the literature suggests a harmful role, other papers reported *E. faecalis* as an important probiotic microorganism, with great applicability in food products. In this review we have examined the vast majority of published data about *E. faecalis* either in CRC development or concerning its protective role. Our analysis should provide some answers regarding the controversial role of *E. faecalis* in CRC.

Keywords: chronic inflammation, colorectal cancer, *Enterococcus faecalis*, gastrointestinal cancer, gut microbiota

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Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers among both men and women worldwide, being the third most frequent in many developed countries, with an estimated 135,430 new cases expected in 2017 in the United States.[1] The increasing incidence in developing countries seems to be closely related to changes in lifestyle.[2,3] In fact, only 15% of patients with this disease present a genetic background of familiarity, whereas 85% of cases are represented by sporadic forms.[4] Known environmental factors involved in CRC development include smoking, alcoholism, obesity, sedentary lifestyle, diabetes mellitus, consumption of red meat, a high-fat diet and inadequate intake of fibers.[5] The gut microbial composition has also been reported as another important factor associated with CRC progress.

Recent findings show that gut microorganisms could modulate the mucosal immune system and change the expression of some host genes associated with important functions, such as nutrient uptake, metabolism, angiogenesis and mucosal barrier function.[6,7] Accordingly, the imbalance of the symbiotic relationship that exists between the gut and its microbiota[8] may disturb the intestinal epithelial integrity, resulting in multiple downstream consequences, including inflammation, oncogenesis and the progression of primary tumors into metastasis.[9,10] However, even if some microorganisms constitute a fundamental part of natural gut composition, playing protective roles against cancer,[11] the role of other strains, such as *Enterococcus faecalis*, still remains unclear.

E. faecalis is a *Firmicutes* member, sometimes used as a probiotic product.[12,13] However, in some specific situations, *E. faecalis* can result in pathogenic and, as reported by some authors, a harmful microorganism on CRC development, due to its ability to damage colonic epithelial cell DNA.[14] Because of these conflicting roles, in this review we examine the most relevant published data that correlate *E. faecalis* with CRC either in a harmful or a protective way.

Features of CRC

CRC is a multifactorial disease that occurs in a multistep process involving accumulating mutations in tumor suppressor genes and oncogenes. This means that the colorectal tumorigenesis includes several genetic and epigenetic changes required for tumor initiation and progression.[15] CRC is one of the most genetically complex cancers that have been investigated, and its underlying genetic basis is described by the 'adenoma–carcinoma sequence' model (Figure 1), which posits that the genomic instability drives epithelial dysplasia and hyperplasia in the colon, resulting, eventually, in CRC.[16]

Differently from the CRC molecular phenotype originating from genetic familiarity, that is characterized by high-frequency microsatellite instability phenotypes, and by germline mutations in the mismatch repair genes[17] or the adenomatous polyposis coli (APC) gene,[18] the sporadic cases of CRC phenotypes present chromosomal instability and allelic imbalance at several chromosomal *loci* (reviewed by Cunningham and colleagues).[19] Besides the occurrence of genetic and epigenetic abnormalities, many aspects of CRC malignancy are affected by cancer-associated inflammation, such as proliferation and survival of malignant cells, angiogenesis and tumor metastasis. Finally, the presence of an inflammatory microenvironment also plays a crucial role in CRC development.[20,21] In this scenario, cancer could be described not just as a concentration of malignant cells, but it is also composed of the stromal and infiltrating immunological or inflammatory cells.

It is well known that the diet is definitely the most important (and previously identified) exogenous factor in CRC etiology,[22] since components, ingested through the diet, are the major source of mutagenic compounds that may promote both cancer initiation and progression.[23] In addition, epidemiological studies show that diet–gene interactions are one of the leading causes explaining the wide variation in CRC developing risk among different individuals.[24] For example, the excessive consumption of fats, animal proteins, processed meat, and heterocyclic amines (HCAs) has shown strong correlations with CRC incidence.[25,26] However, a vegetarian diet helps to prevent CRC, since fruits and vegetables invariably contain antioxidants, which scavenge free radicals, inhibiting the DNA damage responsible for mutations and eventually cancer.[27,28] The diet also influences the features of gut microbiota, as well as other factors, such as the host's age, sex, geography and ethnicity.[29]

General aspects of gut microbiota

The gut microbiota, now fully recognized as a natural defensive barrier against infections, is involved in several physiological functions and plays a large role in maintaining the gut homeostasis.[30] Almost immediately after birth, the human gastrointestinal tract (GI) is colonized by a large and diverse community of microorganisms which will compose the GI microbiota.[31] The colonization pattern is influenced by the type of delivery (vaginal delivery or caesarean section)[32] and the type of baby diet (breast or formula feeding).[33] These pioneer microorganisms modulate the expression of some genes from host epithelial cells, creating a flattering habitat for themselves, and also preventing the growth of other microorganisms.[34]

The adult phylogenetic composition of gut microbiota could be influenced by a lot of factors like diet, antibiotic consumption, external environmental microorganisms, geographic/cultural traditions and age.[35,36] Gut microbiota is normally compounded by autochthonous members, which occupy specific niches constituting the most stable populations over long periods, and by allochthonous members that may be found in any given habitat in significant numbers, but do not influence the gut ecosystem balance in the same way.[37] Humans have a close relationship with these microorganisms, given that their health and well-being are closely interconnected with this complex mutualism.[38] The microbiota plays a fundamental role in displaying some essential organismal functions such as helping food digestion, contributing to nutrition,[39] modulating the immune system[40,41] and defending against a lot of diseases caused by other pathological microbes.[42]

Over the gastric tract, there are variations in the concentration of microorganisms, species and specific functions. For example, in the proximal GI tract, the relatively low (10^8 cells/ml) microbial biomass is associated with the host amino acid requirements,[43] and is composed of *Bacteroidetes*, members of the *Clostridiales* clusters (clusters XIV and I), and specifically in lumen by *Enterobacteriaceae* V. This restricted bacterial composition is determined by several factors such

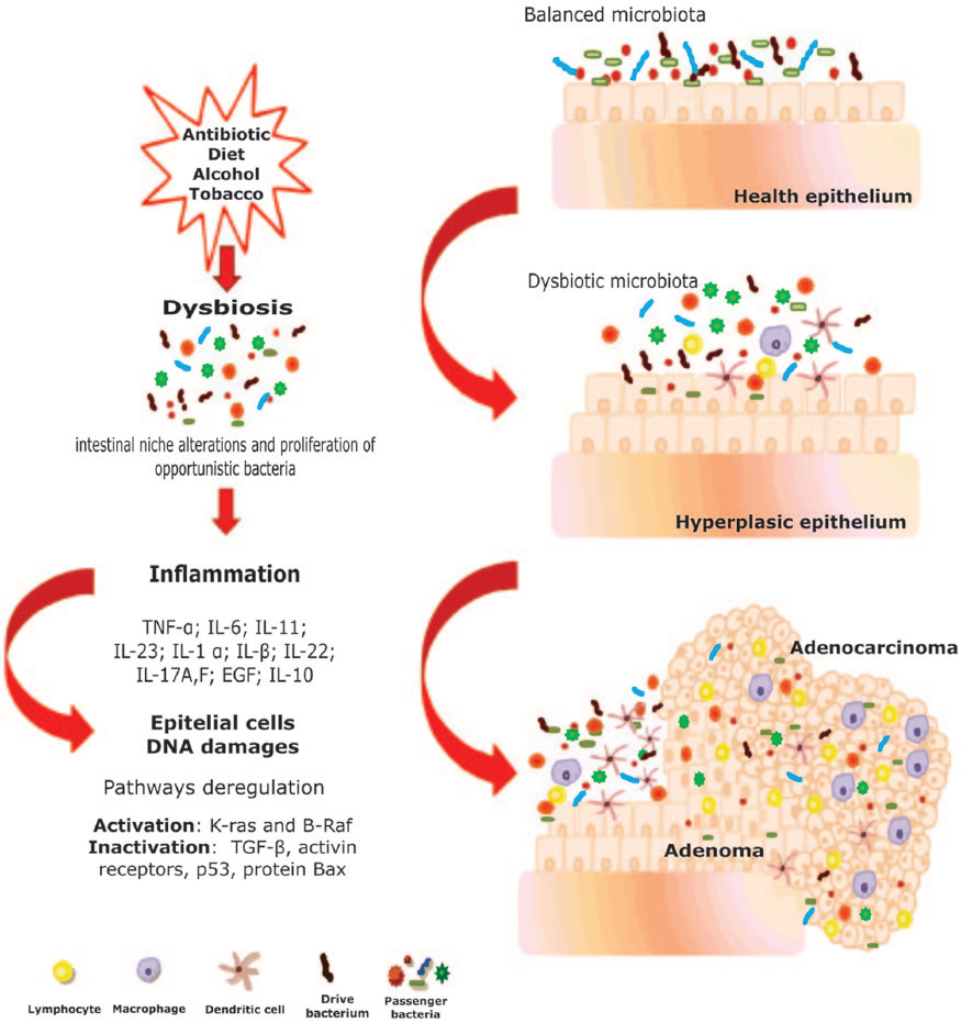

Figure 1. 'Adenoma–carcinoma' progression following the model proposed by Fearon and Vogelstein[17] incorporating the 'bacterial driver-passenger' model of Tjalsma and colleagues. In this situation, the adenoma–carcinoma progression occurs because of the genomic instability (accumulating genetic and epigenetic mutations), caused by changes in gut microbiota. These changes initiate with the presence of a 'drive bacteria' which drives the epithelial DNA damage and contributes to colorectal cancer (CRC) promotion. Then the tumorigenesis induces intestinal niche alterations, which favor the proliferation of opportunistic bacteria (bacterial passengers). EGF, epidermal growth factor; IL, interleukin; K-ras, Kirsten rat sarcoma viral oncogene homolog; TGF, transforming growth factor; TNF, tumor necrosis factor.

as acid pH, rapid lumen flow tendency, bile salts and the presence of immunoglobulin A (IgA). In contrast, the large intestine, which is associated, for example, with the production of short-chain fatty acids (SCFAs) by fermentation of dietary compounds (that escape from the digestion in the small intestine), is colonized by higher concentrations (10^{11} cells/g) of microorganisms, mainly by *Firmicutes* (clusters IX, XIV, and XVI), *Bacteroidetes*, *Actinobacteria*, *Verrucomicrobia*, *Proteobacteria* and *Fusobacteria* (reviewed by Walter and Ley).[44]

Even if these data are well known, there is no consensus about what is a 'healthy' or 'average' intestinal microbiota. For example, studies with rodents have demonstrated that a given bacterial species can have opposite effects in disease induction in one susceptible host, but can protect another rodent strain. Furthermore, different bacterial

species can induce variable clinical phenotypes in a single genetically susceptible host.[45] Nevertheless, it seems that beyond the organization of this complex and diverse bacterial ecosystem, the two most common phyla present in the gut, are the *Firmicutes* and the *Bacteroidetes*,[46] with a documented hierarchy of dominant anaerobic bacteria, like *Bacteroides, Eubacterium, Bifidobacterium, Peptostreptococcus, Ruminococcus, Clostridium* and *Propionibacterium*, and a subdominant bacteria represented by the *Enterobacteriaceae* family, especially *Escherichia coli* and the genera *Streptococcus, Enterococcus, Lactobacillus, Fusobacterium, Desulfovibrio* and *Methanobrevibacter*.[47] In fact, one of the most studied parameters regarding the 'normal composition' of gut microbiota is the *Firmicutes*/*Bacteroidetes* ratio, as well as its variations between individuals,[42] and it seems to be closely linked to health problems such as obesity and metabolic disorders.[48,49]

Gut microbiota and CRC

As well as in different types of tumors such as skin, liver, lungs and breast cancers, CRC can be associated with host microbiome dysbiosis.[50,51] In fact, microorganisms are suspected to be involved in approximately 20% of cancers,[52] especially CRC,[53] since the dynamic crosstalk between intestinal epithelial cells (IECs), the microbes that colonize their apical surface and the surrounding local immune cells, is necessary to maintain intestinal homeostasis.[54] Changes in the *Firmicutes*/*Bacteroidetes* ratio[55] as well as other microbiota imbalances[56,57] are associated with the beginning, maintenance, and determination of the phenotype of human inflammatory bowel diseases (IBDs), especially Crohn's disease and ulcerative colitis. These diseases, characterized by chronic inflammation of the GI tract (as a result of inappropriate activation of intestinal mucosal immunity), affect more than 0.4% of Europeans and North Americans,[58] and are associated with an increased risk of CRC development.[59,60]

The relationship between the commensal microbiota and IBD could occur in different forms (reviewed by Sartor and Mazmanian),[61] but all of them seem to be related to the increase in bacterial antigens' exposure to effector T cells and innate immune cells (resident in the intestinal mucosa) or alteration of the host immune response to commensal bacteria.[62] Nevertheless, different studies have also demonstrated that patients with IBD present a reduction of *Firmicutes* members, specifically *Faecalibacterium* and *Roseburia*, which play a role in protecting the intestine by producing SCFAs.[63]

However, an interesting way to explain the role of microorganisms in CRC development was proposed by Tjalsma and colleagues in the 'bacterial driver-passenger' model.[64] According to this model, the DNA damage caused by distinct indigenous intestinal bacteria (*driver bacteria*) can drive genome instability, which starts the first phase of cancer progression. These microorganisms may be able to induce alterations in mucosal permeability, which favors the translocation of bacteria and bacterial toxins, causing a gut inflammatory response that contributes to the development and progression of cancer. The intestinal inflammation can result from an aberrant ratio of protective (tolerogenic) to aggressive (proinflammatory, damage-inducing, protumorigenic) microbiota, since GI bacteria are able to trigger production of both interleukin (IL)-10 (tolerogenic) and IL-17 (proinflammatory) cytokines.[65] This CRC microenvironment could impact the microbial regulation, alter microbiota composition by selective pressure on the microbial community, and thus could support the outgrowth of specific opportunistic bacteria (*passengers bacteria*) that potentially have carcinogenic effects[63,66] (Figure 1).

Furthermore, this inflammatory scenario has an important role in tumor development and maintenance. Once activated, inflammatory cells produce reactive oxygen species (ROS) and reactive nitrogen that can promote the accumulation of additional mutations and epigenetic changes. These mutations can activate oncogenes or inactive tumor suppressor genes, thus increasing the risk of cancer development.[23,67,68] One of the important bacteria cited as an 'inducer' of genetic instability in colonic epithelial cells' DNA, through the oxidative process, is *E. faecalis*.[61,69]

Properties of *E. faecalis*

Among the enterococci that colonize the GI tract, the most prevalent cultured strain found in human feces is *E. faecalis* (10^5–10^7 colony-forming units (CFU)/g) followed by *E. faecium* (10^4–10^5 CFU/g). However these numbers can change with the host's geographical location, and especially, diet. For instance, Hill and colleagues demonstrated that feces samples of individuals

from England, Scotland and the USA (who have a Western diet) present lower concentrations of *Enterococcus* spp. compared with subjects from India, Japan and Uganda, who adopt a mainly vegetarian diet.[70] This Gram-positive commensal bacterium belongs to the lactic acid bacteria (LAB), it is a facultative anaerobic, resistant to extreme environmental challenges and is usually found in the human oral cavity, GI and vagina mucosa.[71] However, it can emerge as a human pathogen of significant concern[72] and so can be associated with various pathologies including urinary tract infections,[73] endocarditis,[74] persistent endodontic diseases,[75] blood stream infections[76] and chronic periodontitis.[77]

E. faecalis is the first colonizer of the human GI tract and has a major impact on intestinal immune development in the very early stages of life.[78] In newborn babies, it plays a protective role regulating the colonic homeostasis during development by suppressing pathogen-mediated inflammatory responses in human IECs, inducing IL-10 expression,[79] and attenuating the secretion of proinflammatory cytokines, especially IL-8.[80] Moreover, because of this anti-inflammatory potential, *E. faecalis* is commonly adopted as a probiotic in the treatment of some diseases such as recurrent chronic sinusitis, bronchitis or infant acute diarrhea.[13,14] In fact, it has been demonstrated that, compared with 70 different LABs isolated from healthy adults, *E. faecalis* showed the highest probiotic activity.[81] Furthermore, due to its thermophilic fermentation potential (it can ferment different types of sugars, grows at 10°C, and survives at 60°C for 30 min), *E. faecalis* is commonly used in the production of some cheeses and fermented sausages.[82,83]

The 'Gianus' role of *E. faecalis* in cancer development

Data about the role of *E. faecalis* in CRC are discordant: some authors suggest a protective role or no role at all in CRC while others demonstrated harmful activity. For example, Viljoen and colleagues did not find any significant clinical association between this bacterium and colon adenocarcinoma.[84] In another study, when cocultivated with HCT-116 (an aggressive CRC linage), *E. faecalis* was able to downregulate the expression of the FIAF gene (angiopoietin-like protein 4), normally associated with the development of some cancer types.[85] In a mouse model of ulcerative colitis, the increase in *E. faecalis* colonization after a treatment with vinegar was associated with the inhibition of inflammation by suppressing T helper (Th)-1 and Th17 responses.[86] On human peripheral blood mononuclear cells, the heat-killed *E. faecalis* YM-73 strain shows immunomodulatory ability, increasing Th1- and reducing Th2-associated cytokines.[87]

In a recent study, when murine primary colon epithelial cells were cocultured with M1 macrophages polarized by *E. faecalis*, their Wnt/β-catenin signaling was activated and pluripotent transcription factors associated with dedifferentiation were induced. Consequently, these cells were reprogrammed and transformed the primary colon epithelial cells, thus suggesting a role of the microbiome in inducing CRC.[88] In contrast, Miyamoto and colleagues demonstrated that in Min mice (APC-mutant mice) which develop many intestinal polyps through activation of β-catenin signaling, administration of the heat-killed *E. faecalis* EC-12 strain tends to reduce polyp development in the proximal to middle portion of the small intestine, by suppressing β-catenin signaling.[89] Nevertheless, the same probiotic strain EC-12 has been demonstrated to protect the host against pathogens by inducing B-cell activation in the gut.[90] The other probiotic strain, CECT7121, can protect animals from lymphoma challenge and rechallenge by downregulating LBC cell – a poorly immunogenic cell line derived from a spontaneous murine T-cell lymphoma – proliferation, inducing apoptosis in these cells and enhancing the immune response.[91] This strain is also able to enhance and skew the cytokine profile to the Th1 phenotype in different conditions such as vaccination, antitumor immunity, and allergic reactions.[92,93] Furthermore, Hassan and colleagues suggested that *E. faecalis* strains, isolated from human breast milk, could be candidates for breast cancer prevention and treatment once they are able to inhibit the proliferative activity of breast cancer cells.[94]

The anticarcinogenic role of some LABs has already been described and related to their immunomodulatory activities, inducing changes in the cytokine profile.[95] These changes are orchestrated by the activation of dendritic cells (DCs), which recognize and respond to microbial structures *via* PRRs (pattern-recognition receptors) such as toll-like receptors (TLRs).[96] In addition, different stimuli can induce the production of specific cytokines that are responsible for the fine tuning

of an adequate immune response in each pathogen.[97] For example, in the case of the CECT7121 strain, it has been demonstrated that, whereas cell wall and soluble lysates are capable of activating DCs and induce dose-dependent secretion of IL-6 and IL-12, only the cell wall, but not the soluble lysates, can induce tumor necrosis factor α and IL-10 secretion.[93]

Despite the fact that commensal and clinical strains share the same evolutionary origin, behavioral differences may occur when harmless ones acquire antibiotic resistant or putative virulent genes from other bacteria, *via* horizontal gene transfer (HGT). HGT favors rapid changes occurring in bacterial structure, generating resistance and pathogenicity island (a dynamic component of their genome), which can affect or influence their virulence.[98,99] Many putative virulence factors of *E. faecalis* have been described. Most of them are associated with aggregation substance, surface adhesins, sex pheromones, lipoteichoic acid, extracellular superoxide, gelatinase, hyaluronidase and cytolysin (hemolysin) (reviewed by Kayaoglu and Ørstavik).[100]

The harmful role of *E. faecalis* has been suggested to be mainly associated with its ability to generate ROS and extracellular superoxide that can cause genomic instability, damaging colonic DNA, and because of that, predisposing the host to mutations and thus cancer.[16] Moreover, *E. faecalis* has been demonstrated to produce metalloprotease that can directly compromise the intestinal epithelial barrier and induce inflammation.[101] It can also activate the macrophage matrix metalloprotease MMP-9[102] and lead to disruption of monolayer integrity, which could be responsible for morphological transformation of progenitor cells that get a migrating phenotype, contributing to epithelial mesenchymal transition.[103] Furthermore, some authors have observed that, compared with healthy controls, the fecal *E. faecalis* population was increased in Indian patients with CRC,[11,104] which is also noted in oral cancerous lesions.[105,106]

These contrasting data suggest that the origin of the isolated strain is an important variable that should be considered when its role is analysed.[107] This is also motivated by the rapid and continuous changes in the DNA of *E. faecalis* that could modify genes associated with important features of enterococcal virulence, including hemolytic, gelatinase activities,[108] antibiotic resistance and biofilm production.[109] Different dysbiotic scenarios, such as those promoted by the use of antibiotics, which perturb the normal commensal microbiota and set the stage for intestinal domination, could favor these gene changes between the strains, thus suggesting possible explanations for the different roles of this bacterium in IBD, dysplasia and carcinoma.[110]

Discussion

In recent years, crucial discoveries and findings about gut microbiota and its impact on host functions have increased the interest evaluating the microbiome role in human health. Finding microorganisms that can be used as probiotics, either to contrast the infections, or for antitumor treatments, has become a fundamental area of investigation in translational research, generating high expectations for science and medicine.

In this review, we report that *E. faecalis* has been associated with human IEC injury by ROS production. However, it has also been described as showing a protective role in the same cells by inducing IL-10 expression, attenuating the secretion of the proinflammatory cytokines (e.g. IL-8).

In order to provide an explanation for this scenario, we suggest that this conflicting role could be attributed to the different isolated and investigated strains of *E. faecalis*. In fact, this bacterium could come up against various mutations due to gene transfer. These mutations have shown the potential to make it more or less virulent,[111] especially by changing the cytokine/functional profile of the respondents of APCs, like DCs and macrophages.

Furthermore, when we analyzed the studies associating *E. faecalis* with cancer development, we noted that data demonstrated only an increase in its concentration, but not the functional roles of its bacterium on CRC development. In the case of documented cell or tissue injuries, the conditions were extreme, such as immunosuppressed animals. Therefore, we propose that in CRC, *E. faecalis* could act as a 'passenger' bacterium, rather than a 'driver', as suggested by Tjalsma and colleagues. We also suggest that, due to its intrinsic resistance and ability to acquire it (by mutations or HGT), *E. faecalis* can emerge as pathogenic only when the major environment undergoes alterations, such as during the

appearance of cytokines and mucins or during changes in oxygen tension (typically observed on CRC onset and progression conditions).[112] By considering all environmental changes caused by tumorigenesis, *E. faecalis* can grow uncontrolled, thus increasing the possibility of new mutations that can modify its virulence and also change the final product of its metabolism, becoming potentially harmful to the epithelial tissue. This could be an explanation for the increased concentrations of *E. faecalis* that were found in some studies on CRC patients' feces, as well as its harmful role in immunodeficient mice.

Conclusion

By considering all these contrasting data, we suggest that the role of *E. faecalis* should be investigated in more detail and under different experimental conditions since its adoption not only as a fermentative bacterium in food manufacturing, but also as a probiotic product. Our group is currently studying the role of this bacterium in Italian patients with CRC. Our recent data are showing interesting results about the presence and incidence of *E. faecalis* in patients with CRC, as well as the effects of different strains on tumor cells and inflammatory response (data forthcoming).

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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