Montanari *et al. Molecular Cancer* (2025) 24:148
https://doi.org/10.1186/s12943-025-02348-0

Molecular Cancer

REVIEW Open Access

Biofilm formation by the host microbiota: a protective shield against immunity and its implication in cancer

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Abstract

Human-resident microbes typically cluster into biofilms - structurally organized communities embedded within a matrix of self-produced extracellular polymeric substance (EPS) that serves as a protective shield. These biofilms enhance microbial survival and functional adaptability, favoring a symbiotic relationship with the host under physiological conditions. However, biofilms exhibit a dual role in modulating the immune response. If their ability to promote tolerance is key to safeguarding homeostasis, by contrast, their persistence can overcome the cutting-edge balance resulting in immune evasion, chronic inflammation and development of numerous diseases such as cancer. Recent evidence highlights the significance of cancer-associated microbiota in shaping the tumor microenvironment (TME). These microbial inhabitants often exhibit biofilm-like structures, which may protect them from host immune responses and therapeutic interventions. The presence of biofilm-forming microbiota within the TME may promote chronic inflammation, and release of bioactive molecules that interfere with immune surveillance mechanisms, thereby enabling cancer cells to evade immune destruction. This review delves into the complex interplay between biofilms and cancer, with particular focus on the tumor-associated microbiota and the implications of biofilm involvement in modulating the immune landscape of the TME. Addressing this intricate relationship holds promises for innovative therapeutic approaches aimed at reprogramming the microbiota-cancer axis for better clinical outcomes.

Keywords Biofilm, Microbiota, Cancer, Immune modulation

Background

A biofilm is a community of microorganisms, including bacteria, fungi, protozoa, and algae, that attaches to surfaces, typically at solid-liquid interfaces [1]. Unlike the planktonic state, where free-floating microorganisms are highly vulnerable to environmental pressures, these microorganisms are encased and protected by a matrix of highly hydrated extracellular polymeric substances (EPS), which consist primarily of polysaccharides, proteins, extracellular DNA (eDNA), lipids and water. The EPS provides structural integrity, keeping cells in close proximity, fostering interaction, and creating a cooperative

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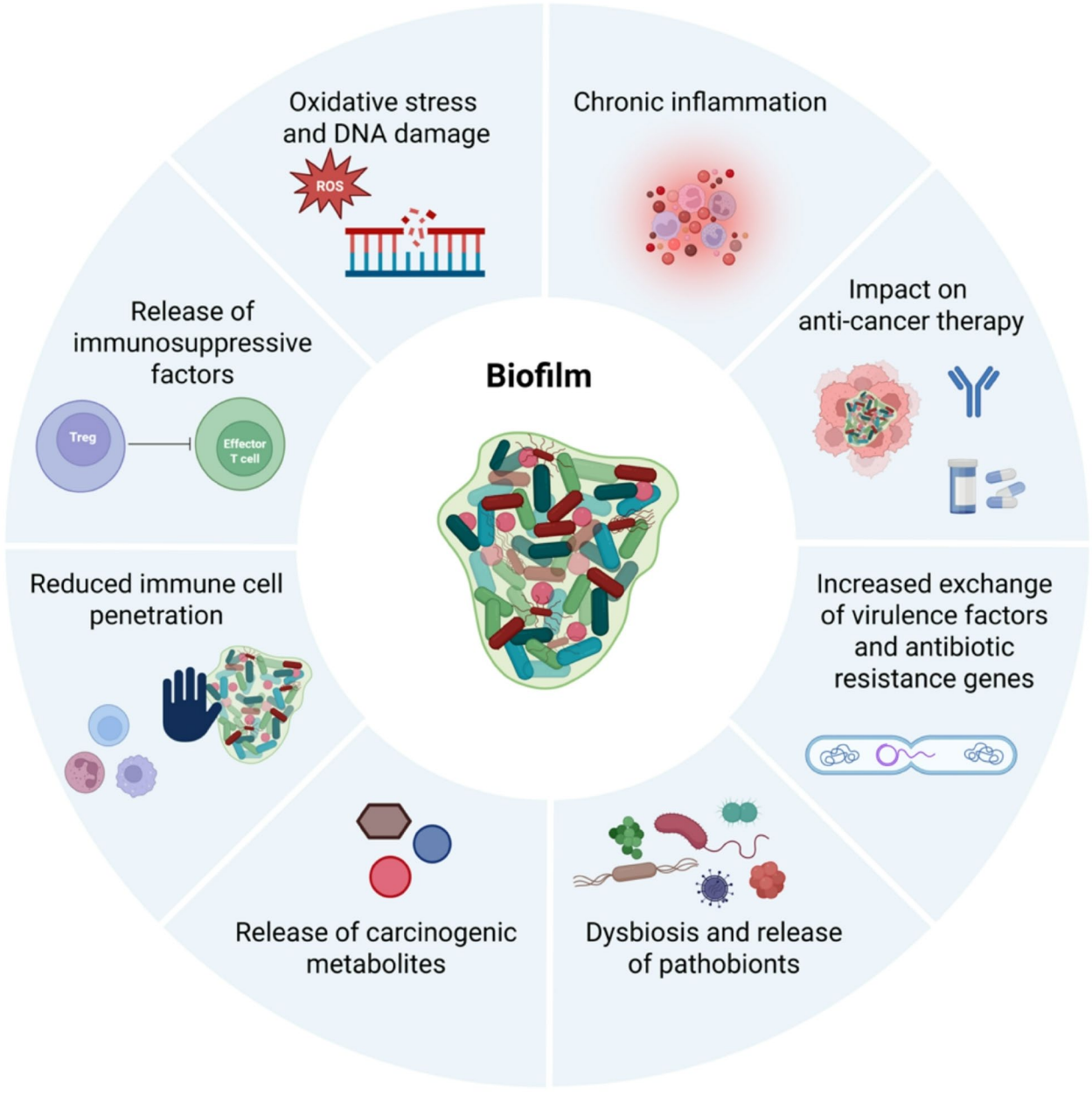
Graphical abstract



microenvironment [2]. Indeed, the biofilm represents the predominant mode of bacterial growth and offers significant survival advantages to bacterial cells [3]. The human body harbors a multikingdom community of microorganism comprising both prokaryotic and eukaryotic organisms - collectively known as the microbiota - that are widespread along almost all the mucosal surfaces and cavities, including sites thought to be sterile (e.g. lung, breast, liver and pancreas) [4]. Microbes in the human body can be broadly categorized as commensals, opportunists, or pathogens [5]. Commensal bacteria coexist peacefully with the host and support vital functions, such as *Lactobacillus* that helps maintaining vaginal health [6]. Under certain conditions, such as immune suppression or dysbiosis - an imbalance in the microbiota linked to numerous human diseases - some commensals may become opportunistic pathogens, capable of causing infections. For instance, *Staphylococcus aureus* (*S. aureus*) can shift from harmless skin resident to pathogenic if it enters wounds [7]. In contrast, true pathogens can cause disease even in healthy individuals and typically originate from external sources, such

as *Helicobacter pylori* (*H. pylori*), responsible for gastritis and ulcers [8]. Consequently, the impact of biofilms within the human body can vary significantly depending on the composition of the microbiota. In a balanced microbial environment and under normal immune function, biofilms formed by commensal bacteria play a protective role, acting as a physical barrier that prevents colonization by pathogenic organisms and contributing to the maintenance of tissue homeostasis [9]. However, when the microbial equilibrium is disrupted due to factors such as antibiotic use, immune suppression, or epithelial damage, biofilms can become pathogenic adopting more virulent behaviours, resisting clearance by the host immune system, and contributing to chronic or recurrent infections [10]. Therefore, biofilms are not inherently harmful or beneficial; their effects are highly context-dependent, shaped by the host environment and the dynamic interactions within the microbial community.

Being an active compartment of our body, the human microbiota contributes to vital functions that the host organism alone cannot perform, thereby supporting the maintenance of homeostasis. In return, the host immune system has evolved multiple means to maintain a symbiotic relationship with the microbiota. Perturbations of this mutually beneficial interplay have been extensively studied for their detrimental effects on human health, with dysbiosis recognized as key factor in various pathogenetic conditions, including chronic inflammatory diseases of the digestive system, such as Inflammatory Bowel Disease (IBD) and Irritable Bowel Syndrome (IBS), metabolic disorders, cardiovascular diseases, asthma and Chronic Obstructive Pulmonary Disease (COPD), atherosclerosis, immune-related conditions, such as allergies and autoimmune diseases, and cancer [11]. The overgrowth of potentially pathogenic commensals (pathobionts), the loss of commensal bacteria, or the reduced microbial diversity can drive harmful changes in the microbiota composition. This altered microbiota can trigger tumorigenesis and play a role in tumor progression by producing genotoxins or carcinogenic metabolites, and by interfering with immune system activity, inducing both pro- and anti- inflammatory phenotypes [12]. Given these crucial effects, the study of the bacterial community associated with tumor has become a major focus of the scientific research in recent years.

Emerging findings suggest that the biofilm structure, by creating a specialized microenvironment that directly influences microbial growth and metabolism, can contribute to pathogenic mechanisms in cancer [13]. In this review, we present novel evidence on host-microbe interactions, mainly focusing on the impact of the biofilm phenotype on immune system responses during cancer initiation and progression.

Biofilm: a survival strategy of human-associated bacteria

Biofilm formation and adaptation

In the human body, resident bacteria typically adopt a three-dimensional organization, forming complex multicellular communities, termed biofilms, that occupy specific sites across various mucosal surfaces including the gut, oral cavity, skin and respiratory tract. Current estimates suggest that up to 80% of bacterial and archaeal cells reside in biofilms. Within biofilms, microbes create microenvironments with distinct gradients of nutrients, oxygen, and other factors, allowing different species to colonize tailored ecological niches [14]. Biofilm formation is highly regulated through a multi-step process initiated by the reversible adherence of free-swimming planktonic microorganisms to a surface. In this stage, various mechanisms, including Brownian motion, active motility (e.g., flagella or pili-driven movement), as well as passive transport such as convection and sedimentation, allow microbial cells to reach the surface. The initial adhesion is weak and transient, governed by non-specific physical forces, like van der Waals interactions, hydrophobic effects and electrostatic forces. Once microorganisms sense favourable environmental conditions, intracellular signalling pathways are activated, inducing the accumulation of a small, cyclic nucleotide signalling, the bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP), which triggers a phenotypic switch from a motile to a sessile lifestyle in microbial cells. This shift involves the downregulation of flagella-mediated motility and the upregulation of bacterial surface organelles, such as adhesins, pili/fimbriae and surface-associated proteins that are projected towards the surrounding environment, promoting a strong, specific binding to the surface. Upon the transition from weak-to-covalent attachment is achieved, cells start to proliferate, leading to microcolony formation [15, 16]. This event is coupled to in situ self-production of EPS that forms multilayered matrix scaffolds in which a plethora of densely packed microbes are embedded. The EPS matrix production is crucial for biofilm maturation, as it provides structural integrity, stabilizes the microbial biomass, and facilitates bacterial adhesion to both tissue surfaces in the body and neighbouring cells. As biofilm matures, it develops a sophisticated architecture that includes nutrient gradients, water channels and distinct regions of metabolic activity, allowing bacterial cells to exhibit various interactive behaviours. The final stage of biofilm lifecycle involves the dispersal of cells from the mature community, allowing them to colonize new locations and initiate new biofilms (Fig. 1). This highly organized process confers survival advantages to bacterial communities, enhancing their resilience and persistence in both natural environments and pathologic condition [17].

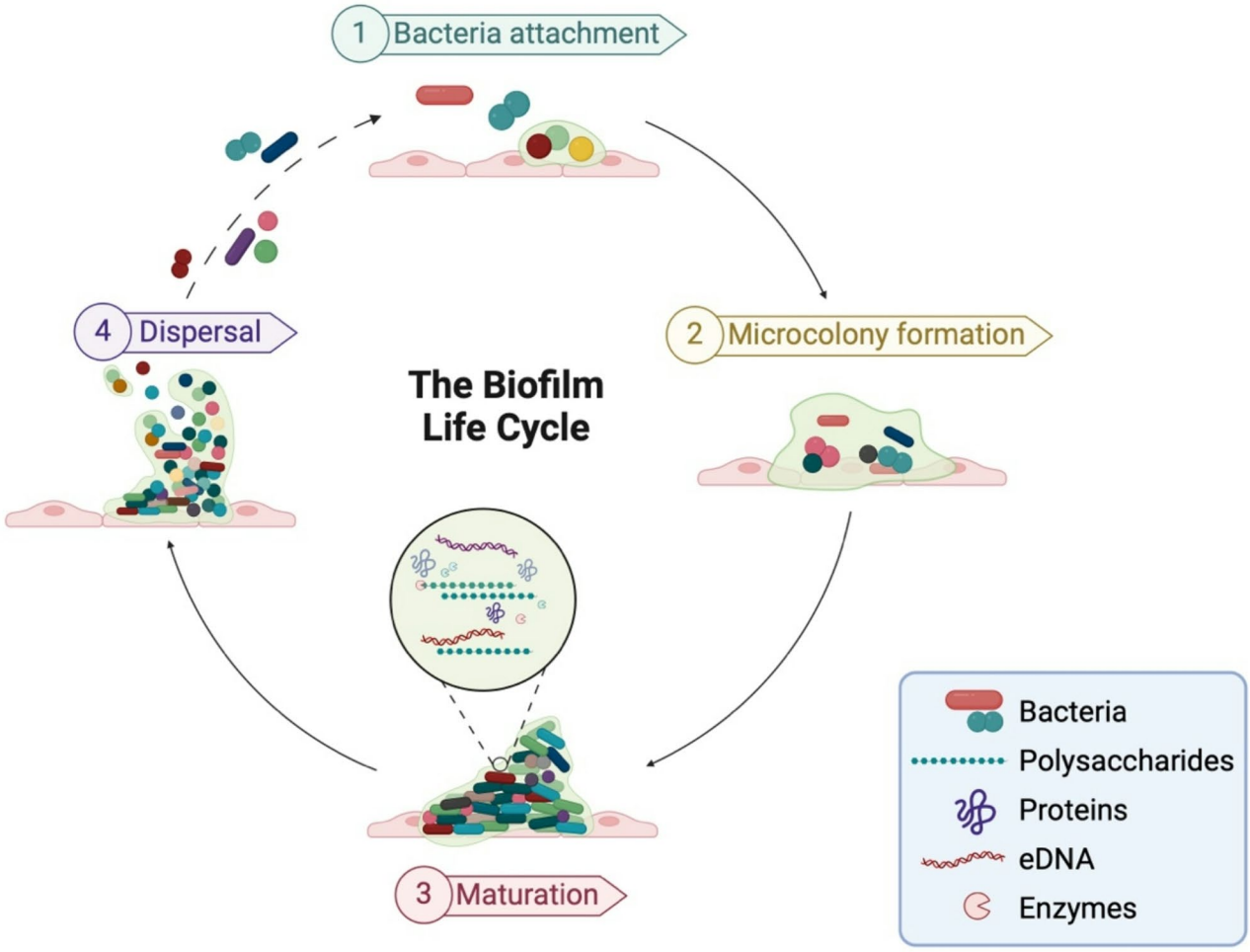


Fig. 1 The Biofilm Life Cycle. The biofilm formation is a dynamic and adaptive process involving four key stages that enables microbial communities to develop and propagate on various surfaces. In the initial phase, bacteria shift from a planktonic to a sessile lifestyle by adhering to a surface (1, "bacteria attachment"). Once adhered, microbes start to multiply and aggregate, forming microcolonies encased in a self-produced extracellular polymeric substance (EPS) matrix (2, "microcolony formation"). As the biofilm grows, it develops a mature three-dimensional structure with distinct microenvironments. The EPS matrix components (polysaccharides, proteins, extracellular DNA and enzymes) play critical roles in the biofilm architecture, stability, resistance to antimicrobials, and establishment of cooperative behaviours among the microbial community (3, "maturation"). In the final stage, bacteria are released in the surrounding environment, facilitating the spread of biofilm-forming cells and their colonization of new locations (4, "dispersal")

Microbial dynamics in biofilms: balancing cooperation, competition and communication

The spatial stratification of biofilm-forming bacteria enables diverse populations to coexist and operate as a coordinated consortium, performing complementary metabolic functions. This intricate organization is primarily supported by various communication forms between microbial cells, with *quorum-sensing* (QS) being one of the most critical mechanisms [18]. QS allows bacteria to sense the population density through the release and detection of signaling molecules called autoinducers (such as acylated homoserine lactones (AHL) for Gram-negative and processed oligopeptides for Gram-positive bacteria) that tune biofilm behaviors and regulate the community composition. QS also enables microbes to gather information from the environment to govern other essential activities, such as resource sharing and cooperation in defense mechanisms that help biofilm-embedded microorganisms to thrive in diverse and often hostile milieu [18]. It has been observed that *S. aureus* produces 8-amino acid cyclic short peptides as a QS signaling molecules which accumulate around the bacterial cells, activate membrane-bound receptor proteins finally inducing the expression of virulence genes [19]; *Pseudomonas aeruginosa* (*P. aeruginosa*) communicates through the QS to change the state of free-living plankton cells to form biofilms and enable them to become resistant to applied antibiotics [20]. Bacteria within biofilms establish synergistic relationships that enhance their survival and functional efficiency. One of the most prominent forms of cooperation is metabolic cross-feeding, where different bacterial species exchange metabolic byproducts

to optimize resource usage, thereby creating a network of metabolic interdependencies. For example, a study showed that *Enterococcus faecalis* (*E. faecalis*) growth is sustained by *S. aureus*-derived heme which is essential to activate the aerobic respiration of *Enterococcus* species which cannot synthetize heme themselves [21]. Moreover, spatial proximity enhances defense strategies within biofilms. The EPS matrix facilitates the diffusion of nutrients and signals while acting as a physical barrier that shields the community from external attacks such as antibiotics or host immune responses [22]. Even bacteria lacking intrinsic resistance can become less susceptible to antimicrobials when grown in biofilms. This biofilm-mediated resistance has been recognized as an adaptive mechanism, as the antibiotic efficacy is restored when the matrix protection is lost. Several factors account for biofilm resistance to antibiotics, generally including restricted agent penetration through the EPS barrier, reduced antibiotic uptake due to lower microbial growth rates, and increased horizontal gene transfer which promotes the proliferation and survival of persister cells, which are dormant and highly tolerant to antibiotics, but repopulate the biofilm once the antibiotic is removed [23].

In addition to cooperative behaviors, microbial interaction modes also involve antagonism and competition that frequently arise when space or resources become limited. In such cases, microorganisms within biofilms can alter the fitness of neighboring cells and facilitate their detachment by manipulating the local environment, such as pH or oxygen levels, to create unfavorable conditions for competitors. Alternative mechanisms of interference also include the production of toxic byproducts and antimicrobial substances that inhibit the growth of competing bacteria [24]. These processes are fundamental to maintain eubiosis, as seen in the oral cavity where commensal streptococci regulate the composition of the tissue microbiota by antagonizing and preventing colonization of cariogenic and periodontal pathogens [25]. Collectively, the ability to dynamically shift between cooperation and competition reflects the remarkable adaptability of biofilm communities to changing environments, underscoring their role in human health. However, the same traits can become harmful, contributing to dysbiosis and driving the pathogenicity of biofilms, which are increasingly recognized as a global health threat in clinical settings.

The dual role of biofilms in the host: from beneficial to pathogenic effects

As discussed, biofilms possess unique properties that offer significant advantages to resident microbial communities, allowing them to survive and thrive in diverse environments. Biofilm formation primarily acts as a natural barrier, creating inhospitable niches that prevent pathogen colonization, reduce infection risk, and limit the growth of invasive species, thereby helping to maintain community equilibrium. These mechanisms are crucial for supporting population balance and preserving long-term stability of a healthy microbiota, fostering a symbiotic relationship with the host [9]. Nevertheless, biofilms exhibit a dual nature: they can both safeguard favorable human-microbe interactions, and disrupt this harmony, depending on specific circumstances. As such, they can act as gatekeepers to homeostasis or, conversely, become sources of disease. Different mechanisms have been proposed to explain the pathogenicity of biofilms [10], one of which is the increased bacterial density resulting from the aggregation of FadA within the EPS matrix. This dense clustering often leads to a reduction in microbial diversity which contributes to dysbiosis. For instance, *Gardnerella vaginalis* (*G. vaginalis*), a member of the healthy vaginal microbiota, is thought to trigger bacterial vaginosis by initiating a polymicrobial biofilm that colonizes the vaginal epithelium. In this environment, beneficial lactobacilli are outnumbered by other virulent micro-aerophilic and anaerobic organisms that promote biofilm maturation and contribute to infection [26]. Therefore, biofilms can harbor pathogenic or opportunistic bacteria that overgrow and persist chronically on body surfaces, causing local infections. At the same time, the periodic release of bacterial cells from the mature niche during the biofilm lifecycle, enables microbes' migration and colonization of distant anatomic locations [27]. This dispersal mechanism, a key pathogenic feature of polymicrobial populations, allows potentially harmful pathogens to establish new biofilms elsewhere in the host, facilitating infection dissemination in previously unaffected tissues and making biofilm-associated diseases more challenging to treat. Many studies have shown that the oral microbiota is a major endogenous source of pathogenic species involved in the development of extra-oral conditions, with specific oral biofilm strains such as *Fusobacterium nucleatum (F. nucleatum)*, *Porphyromonas gingivalis (P. gingivalis)*, and *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) being able to translocate to the gut, leading to intestinal dysbiosis and systemic inflammatory responses [28]. Notably, it has been documented that *F. nucleatum* exerts its pathogenic activity in the colon through the production of Fusobacterium adhesion A (FadA), a protein with amyloid properties [29]. FadA plays a crucial role in the bacterium's ability to adhere to host tissues, facilitating the spread of infections and influencing various cellular processes. A well-characterized mechanism involves the FadA binding to E-cadherin, a transmembrane protein found on epithelial cells that mediates cell-cell adhesion. This interaction not only facilitates the initial attachment

of *F. nucleatum* to the epithelium but also promotes the disruption of epithelial cell-cell junction, leading to bacterial persistence and invasion in the human colon. Furthermore, FadA contributes to tissue inflammation through the activation of the NF-kB intracellular signaling pathway and is implicated in cancer cell transformation and proliferation processes by triggering the Wnt/β-catenin pathway [30]. In addition to mediating direct interactions with host cells, FadA also contributes to biofilm formation, particularly in the gastrointestinal tract. This biofilm helps *F. nucleatum* persist in the colon while altering the local microbiota in favor of pathogenic species. Although evidence of a direct correlation between biofilm formation and increased FadA secretion is still limited, it is well understood that biofilm-associated bacteria typically exhibit enhanced virulence by locally accumulating microbial toxins at higher concentrations as part of their survival and defense mechanism [30]. From the human perspective, one of the most alarming consequences of biofilm formation is the development of tolerance to existing antibiotics and immune system attacks. The EPS architecture provides protection and enhances microbial resistance to exogeneous stressors at multiple levels. The self-generated matrix limits drug penetration at bactericidal concentrations, particularly into deeper biofilm layers, both by physically slowing the diffusion of antimicrobial agents through the multicellular surface and by enabling enzymatic inactivation or degradation of drugs [31]. As a result, only a limited amount of killing agents reaches inner bacterial cells, which often lie in a slow-growing or dormant state, making them less susceptible to external insults [2].

Furthermore, the proximity of biofilm-embedded bacteria fosters horizontal gene transfer, accelerating exchanges of genetic material among microbial communities. The high frequency of this process significantly contributes to the pathogenicity of biofilms in clinical settings, as it allows the rapid acquisition of survival traits like antibiotic resistance which further complicates eradication efforts [32]. This is particularly relevant in cystic fibrosis patients, where *P. aeruginosa*, a common opportunistic pathogen, typically produces biofilms on lung epithelial cells. Within the biofilm environment, this bacterium can acquire several antibiotic resistance genes, including those encoding drug-neutralizing enzymes or efflux pumps that actively expel antibiotics from bacterial cells. The emergence of these withstands mechanisms coupled with the co-occurrence of multidrug-tolerant persister cells, usually found in biofilms, frequently leads to treatment failures and recurrent infections [33].

The role of biofilms in disease is gaining increasing recognition. The spread of persistent and drug-resistant bacteria is posing a major challenge in clinical settings, calling for deeper exploration of underlying mechanisms and highlighting the urgent need for innovative therapeutic strategies that can effectively disrupt and target biofilm-associated pathogens to mitigate their adverse effects on human health.

Microbial biofilms: a complex relationship with host immune system

Biofilm formation represents a protected mode of growth employed by microorganisms not only to reduce their susceptibility to antimicrobials but also to evade host immune responses. The relationship between host immunity and microbiota biofilms is complex and inherently contradictory, embodying both shielding and pathogenic roles [34]. During homeostasis, biofilm-forming commensals play a crucial role in maintaining a balanced interaction between host immunity and the microbiota by (i) supporting immune tolerance towards commensal organisms while ensuring that the immune system can still mount an effective response against invading pathogens; (ii) protecting epithelial tissues from pathogen colonization by forming a physical barrier on mucosal surfaces; (iii) preventing excessive immune activation through the secretion of signaling molecules or metabolites with anti-inflammatory effects [35].

From birth to death the human body is continuously exposed to beneficial biofilms which the immune system can recognize and distinguish from opportunistic pathogens through specialized pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs) (Fig. 2). These receptors detect microbial-associated molecular pattern (MAMPs), aiding to maintain a state of immune tolerance toward beneficial biofilms while enhancing immune activation when potential threats arise, ensuring a balanced host-microbiota relationship [36]. A well-known commensal species with health-promoting effects able to form biofilm is *Lactobacillus*, which plays a crucial role in maintaining vaginal homeostasis. It is involved in the generation of T regulatory cells (Tregs), creating a local environment that improves the fitness of beneficial microbes while preventing colonization of pathogens [37]. Biofilm matrix components are also involved in host-immunity effects of biofilm. For example, exopolysaccharides produced by *Lactobacillus plantarum (L. plantarum)* reduced the production of pro-inflammatory cytokines IL-6, IL-8, and MCP-1 (monocyte chemotactic protein-1, also known as CCL2) by increasing negative regulators of Toll-like receptor-4 (TLR4), such as cell wall-associated high molecular mass polysaccharides [38]. A similar behaviour is embraced by *Bacteroides fragilis* (*B. fragilis*) in the gut, where biofilm-produced polysaccharide A (PSA) modulates host immune reactions *via* stimulating Treg development contributing to homeostasis and limiting excessive inflammation [39] (Table 1). Raffatellu et

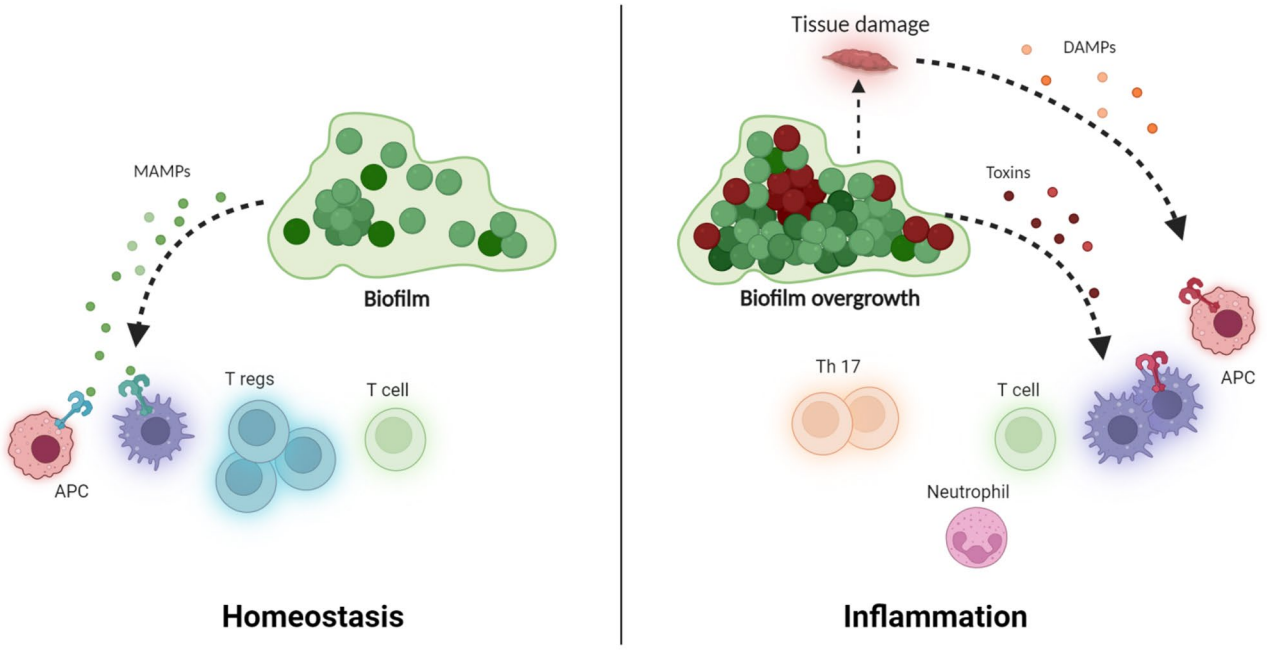


Fig. 2 The conflicting role of biofilm in host immunity-microbiota interaction. The immune system continuously interacts with beneficial biofilms, recognizing them through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs). These receptors detect microbial-associated molecular patterns (MAMPs), promoting immune tolerance toward biofilm-forming bacteria while ensuring immune activation against potential pathogens (left side). Biofilm overgrowth can lead to pathogenic states, characterized by the release of microbial toxins and overstimulation of the immune system. This process is driven by danger-associated molecular patterns (DAMPs) released from damaged host cells, leading to enhanced inflammation through macrophages, neutrophils, T and Th17 cells' activation (right side)

al. showed that capsular exopolysaccharides from *Salmonella typhi (S. typhi)* biofilm can also reduce IL-8 expression by human intestinal epithelial cells [40]. Additionally, exopolysaccharides from a clinical strain of *Burkholderia cepacia (B. cepacia)* inhibited neutrophil chemotaxis and production of reactive oxygen species (ROS) [41]. Biofilm growth can also become pathogenic, often due to species composition changes that favor enrichment of harmful strains, loss of gut barrier function, and release of microbial toxins. This transition entails an overstimulation of the immune system mediated by the release of danger-associated molecular patterns (DAMPs) from damaged host cells, leading to the activation of dendritic cells, macrophages and adaptive immune responses [22] (Fig. 2). For instance, in the gut the loss of *B. fragilis* and its PSA, coupled with the simultaneous overgrowth of opportunistic pathogens like *F. nucleatum* or *Enterobacteriaceae* species, shifts the immune system from a state of tolerance to activation. Indeed, this event reduces Treg induction while promoting enhanced inflammation by triggering macrophages, neutrophils, and Th17 cells activity [42] (Table 1).

Acetate residues of the alginate, the exopolysaccharide forming the EPS of *P.aeruginosa*, are bound to hydroxyl groups that can become covalently linked to the complement opsonins C3b and C4b to the bacterial surface, escaping the macrophage killing by inhibiting complement activation [43]. Additionally, *P. aeruginosa* EPS can bind to calcium ions, which are essential secondary messengers in various pattern recognition receptor (PRR) pathways, impairing the host immune cells' ability to effectively respond to the establishment of *P. aeruginosa* biofilm [44]. eDNA component of *P.aeruginosa* biofilm has also been found to influence immune cells' function. Its degradation by DNAse1 greatly reduced the release of neutrophil pro-inflammatory cytokines IL-8 and IL-1β, suggesting the role of eDNA as an immunomodulatory component of the EPS matrix [45]. Additionally, signaling molecules that are part of the QS of bacteria, such as 3-oxododecanoyl homoserine lactone (3OC12-HSL) autoinducer used by *P. aeruginosa*, can regulate the expression of virulence genes, in turn modulating the host immunity. It was reported that this molecule increased mRNA levels of a common set of pro-inflammatory genes (interleukin (IL)-1α, IL-6, IL-8/KC, and COX-2) in both murine fibroblasts and human lung epithelial cells [46]. Additionally, the rhamnolipid, a QS regulated virulence factor of *P. aeruginosa*, has been shown to induce lysis of various components of the human immune system, including macrophages and

Table 1 Table showing the main mechanisms of interactions between biofilm-inducing bacteria and host-immunity

Biofilm-inducing bacteria	Effects on host immunity	References
Lactobacillus plantarum	Induction of T regs *via* IL-10 and TGFβ production by DCs Reduction of pro-inflammatory cytokines, IL-6, IL-8, MCP-1	[37, 38]
Bacteroides fragilis	Polysaccharide A (PSA) inducing IL-10 expression from T regs	[39]
Salmonella typhi	Reduction of IL-8 expression by human intestinal epithelial cells	[40]
Burkholderia cepacia	Inhibition of neutrophil chemotaxis and production of ROS	[41]
Fusobacterium nucleatum	Pro-inflammatory cytokines production	[52]
Pseudomonas aeruginosa	Escaping the macrophage killing by inhibition of the complement activation Induction of pro-inflammatory cytokines by neutrophils, murine fibroblasts and human lung epithelial cells Lysis of immune cells as macrophages and PMN	[43, 45, 46]
Staphylococcus aureus	Cleavage of the complement system components through metalloprotease secretion	[48]
Streptococcus pneumoniae	Cytokine release modulation *via* pneumolysin production in the lung	[49]
Clostridium difficile	Production of toxins (TcdA and TcdB) leading to inflammation	[50]
Escherichia coli	Production of toxins leading to urinary tract infections (UTI)	[51]

polymorphonuclear leukocytes (PMNs) [47]. *S. aureus*, commonly associated with biofilm-mediated infections on medical devices, secrete aureolysin, a metalloprotease which cleaves components of the complement system, aiding in immune evasion [48]; *Streptococcus pneumoniae (S. pneumoniae)* which forms biofilms in the respiratory tract can secrete pneumolysin that, in turn, modulates cytokine production and reduces immune cell function [49] (Table 1). These mechanisms allow pathogens to persist in a protected environment without triggering a robust immune reaction. In addition, pathogenic biofilms can alter the local microenvironment, creating conditions that favor bacterial survival and resistance to immune attack. Pathogens such as *Clostridium difficile* (*C. difficile*) and *Escherichia coli* (*E. coli)* exemplify this ability to evade immune detection and suppress host defenses through biofilm formation. *C. difficile* can produce toxins that damage intestinal epithelial cells and disrupt tight junctions, facilitating its survival within biofilms while evading immune recognition [50]. Instead, *E. coli* can form biofilms on urinary tract surfaces, resisting to immune cells clearance and leading to persistent urinary tract infections (UTIs) [51] (Table 1). The ability of certain pathogens to exploit biofilm formation as a strategy for immune evasion raises critical implications for developing therapies aimed at restoring balance in the microbiota while combating infection. Addressing biofilm-related pathogenicity will be essential for improving treatment outcomes and reducing the burden of chronic infections.

Dissecting the role of biofilms in cancer

In recent years, an expanding body of research has highlighted the influence of microbiota on cancer susceptibility and progression, particularly emphasizing the impact of dysregulated tissue-associated bacterial populations on host pathophysiology and immune function. Consequently, dysbiosis has emerged as a critical hallmark of cancer, further revolutionizing our understanding of microbiota's role in human diseases [53]. Despite initial thoughts that individual bacterial strains could contribute to tumor promotion, it is becoming more evident that tumorigenesis can stem not only from single pathogen infections, but largely from widespread shifts in microbiota composition, suggesting a broader role for microbial dysbiosis in cancer development and progression. These findings underscore how tumor-promoting properties of microbes not only rely on their individual virulent capabilities but may be significantly strengthened through complex interactions among opportunistic bacteria that collectively influence the host environment. *H. pylori* has been demonstrated to be directly involved in neoplastic transformation events of gastric epithelial cells by delivering its virulence factor, the cytotoxin-associated gene A (CagA), into the gastric epithelium [54]. However, it was subsequently realized that *H. pylori* does not induce gastric cancer by itself; instead, its infection leads to the disruption of the gastric microbiome's balance, by inhibiting the colonization of other bacterial and contributing to the formation of a microenvironment conducive to carcinogenesis [55].

To date, most studies in literature have concentrated on pro-tumorigenic effects of individual bacterial species in their planktonic form. However, it is important to acknowledge that many of these cancer-associated species possess the ability to form biofilms, a feature that may significantly enhance their tumor-promoting potential. The pro-tumorigenic activity of biofilms can be manifested through various mechanisms including (i) the induction of a persistent inflammation that can exacerbate in immunosuppression and (ii) the ability to release toxins and/or metabolites, finally fostering a tumor-friendly environment [56] (Fig. 3).

Biofilm-driven inflammation in cancer

Chronic inflammation is a hallmark of cancer [57], contributing to oxidative stress, DNA damage, and cellular transformation, which support cancer initiation [58].

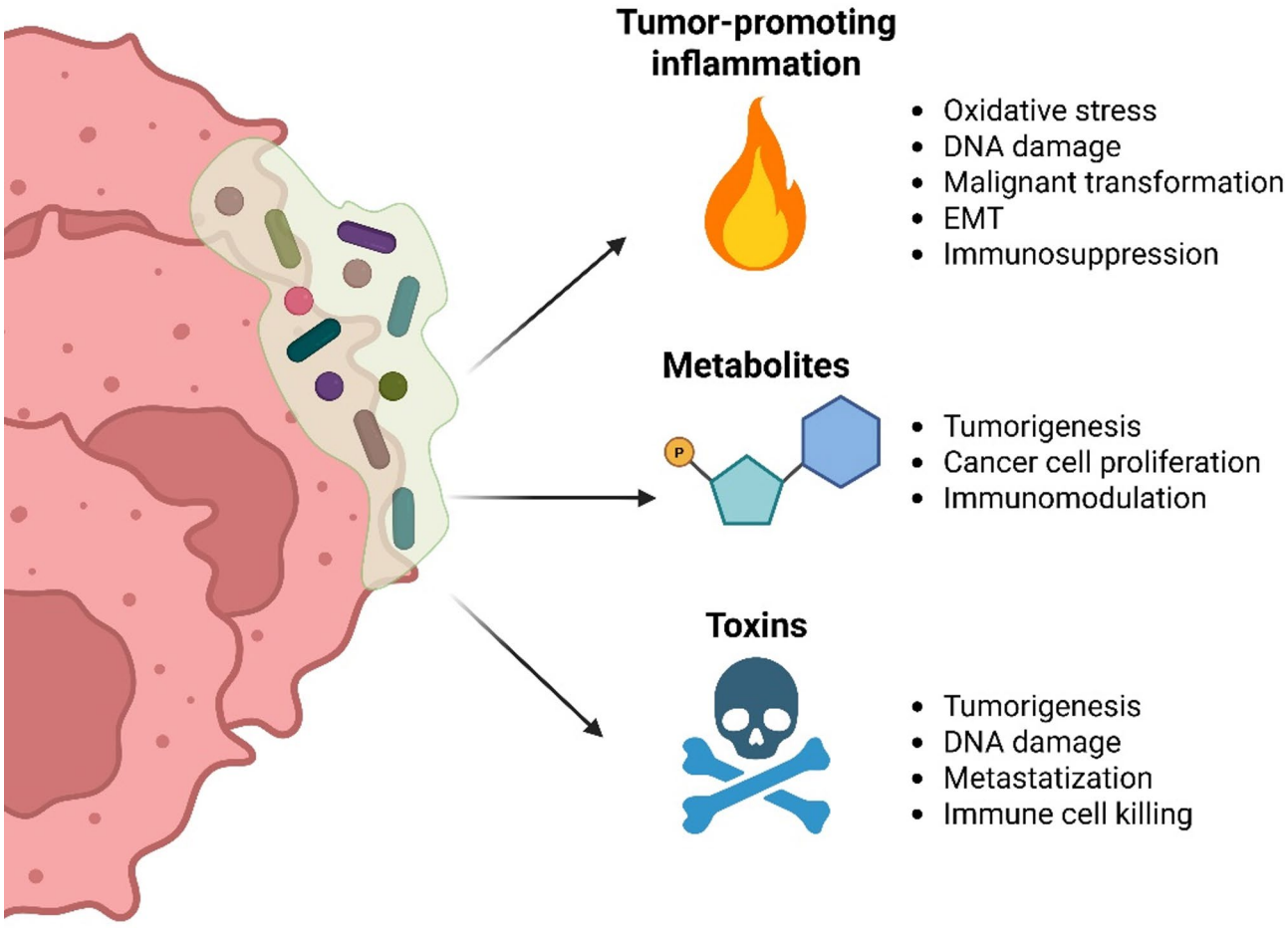


Fig. 3 Tumor-promoting effects of biofilms. The figure illustrates key mechanisms by which bacterial biofilms contribute to tumor initiation and progression. These include: tumor-promoting inflammation, which leads to oxidative stress, DNA damage, epithelial-to-mesenchymal transition (EMT), malignant transformation, and immunosuppression; the release of biofilm-derived metabolites, which can promote tumorigenesis, support cancer cell proliferation, and modulate immune responses; and biofilm-associated toxins, which contribute to tumor development, induce DNA damage, facilitate metastasis, and may directly kill immune cells

Several insights suggest that bacterial biofilm can play a significant role in supporting tumorigenesis by triggering chronic inflammation. Tomkovich et al. demonstrated that tumor-associated biofilms promote colon cancer development in genetically engineered mouse models prone to develop tumor [59]. The carcinogenic potential of these biofilms was attributed to their capacity to recruit myeloid cells and induce IL-17–mediated inflammation, thereby facilitating CRC development [59]. Furthermore, the presence of bacterial biofilms on the colonic epithelium has been linked to elevated levels of IL-6 in epithelial cells and enhanced STAT3 activation, accompanied by a reduction in crypt cell E-cadherin expression and increased proliferation [60]. Given the role of the IL-6/STAT3 signaling pathway in promoting the epithelial-to-mesenchymal transition (EMT) in the colon [61], these findings support the link among biofilms, inflammation and carcinogenesis. The pro-inflammatory action of biofilms is not limited to the early stages of tumor development but is also evident in established neoplastic disease. For instance, high levels of bacterial biofilm, that the authors defined as large patches of aggregated bacteria, have been detected in colon cancer samples compared to normal tissue counterparts. This biofilm, primarily composed of *F. nucleatum* and *B. fragilis*, has been associated with increased expression of pro-inflammatory cytokines, such as IL-8, IL-1β and IL-6, and immunomodulatory factors in the tumor specimens but not with tumor stage and lymph node or distant metastases [62]. Nonetheless, additional studies suggest that *F. nucleatum*, an anaerobic opportunistic pathogen frequently detected in colorectal cancer (CRC) samples, may contribute to tumor invasion and the development of distant metastases [63]. Mechanistically, *F. nucleatum* downregulates miR-5692a, resulting in the upregulation of IL-8 in CRC cells. This, in turn, enhances ZEB1 expression, thereby facilitating CRC metastasis to the liver [64].

Although many studies investigating the relationship between biofilm-induced chronic inflammation and tumor occurrence and progression have primarily focused on the gastrointestinal tract, this interplay has also been observed in other tumor types. *F. nucleatum* and *P. gingivalis*, an anaerobic bacterium known for forming biofilms in the oral cavity [65], have been shown to enhance the growth and aggressiveness of oral squamous cell carcinoma (OSCC). This effect is mediated through the activation of TLR signaling in the oral cavity, which stimulates IL-6 production and subsequently activates the STAT3 pathway, ultimately inducing OSCC proliferation and invasiveness [66]. Additionally, a 2014 case report described a patient who developed esophageal adenocarcinoma five months after a *Streptococcus suis* (*S. suis*) infection [67]. Although there is no direct evidence linking this bacterium to tumor development, it has been reported that the bi- and tri-acylated carbohydrates of this bacterium biofilm can be detected by monocytes through TLR2 involvement, triggering the release of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, TNFα and MCP-1, that can sustain cancer development [67, 68].

In the context of breast cancer, an increased infiltration of *S. epidermidis*, a commensal skin bacterium, has been detected in both human and murine tumor mammary glands [69, 70]. This microbe, known to form biofilms, can exert immunoregulatory functions in the breast [71]. We recently reported that the presence of a tumor orthotopically implanted in the mouse mammary gland modifies the local microbiota composition compared to healthy tissue and that peritumoral antibiotic treatment reduced breast cancer growth. The anti-tumor effect exerted by antibiotics was found to be linked to a reduction in the abundance of *S. epidermidis* in tumor lesions. In vitro we observed that this bacterial strain polarizes macrophages toward a pro-tumor phenotype characterized by the augmented release of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-18 and TNFα [70]. Therefore, the presence of intratumoral *S. epidermidis* encapsulated in biofilm could foster a highly pro-inflammatory microenvironment conducive to tumor progression. Overall, these findings emphasize the connection between biofilm-forming bacteria and cancer development and progression, with increasing evidence suggesting that biofilms not only protect bacterial communities from host immune responses but also contribute to a pro-carcinogenic environment through persistent inflammation and dysregulation of host immune cell signaling.

Biofilm-derived metabolites in cancer

Recent evidence highlights the additional role of biofilm-associated metabolites in both cancer progression and immune response regulation [72]. A significant upregulation of N^1, N^{12}-diacetylspermine, a polyamine metabolite associated with tumor-resident bacteria was observed in both biofilm-positive CRC tissues and their matched normal counterparts. Oral antibiotic treatment in patients undergoing colon cancer therapy led to a reduction in N^1, N^{12}-diacetylspermine levels, aligning them with those observed in biofilm-negative tissues [73]. This metabolite has been shown to enhance proliferation and ATP production in CRC cell lines by downregulating miR-559, which leads to the upregulation of the enzyme cystathionine β-synthase (CBS) [74]. CBS, overexpressed in CRC, contributes to tumorigenesis by promoting a shift toward anabolic metabolism, thereby increasing cellular energy production [75, 76]. It has also emerged that metabolites derived from biofilms not only have the potential to directly impact tumor cell biology, but also profoundly influence immune cells, thereby modulating the antitumor immune response. Much of current knowledge regarding biofilm's capacity to shape immune cell phenotypes stem from studies conducted in the context of infection. For instance, macrophages and myeloid-derived suppressor cells (MDSCs) co-cultured with biofilm from *S. aureus* mutant for the alpha subunit of ATP synthase were characterized by an increased release of pro-inflammatory cytokines, such as IL-12p70, TNFα and IL-6, highlighting the immunomodulatory role of ATP [77, 78]. Furthermore, D- and L-lactate produced by *S. aureus* biofilm induced the expression of IL-10 by MDSCs and macrophages thus creating an immunosuppressive microenvironment and hindering bacterial clearance [79]. Since the presence of biofilm has also been detected within tumors [13], it is therefore plausible to hypothesize that immunomodulatory effects of biofilm-associated bacterial metabolites may also occur in the TME, although direct evidence supporting this remains limited. In this regard, a recent study described that *Clostridium scindes (C. scindes)*, belonging to the *Clostridium* species reported to be biofilm-producing bacteria [80], is able to negatively impact CD8 + T cell effector function by producing deoxycholic acid (DCA). By targeting plasma membrane Ca^{2+}-ATPase (PMCA), DCA inhibitis Ca^{2+}-dependent activation of the nuclear factor of activated T cells 2 (NFAT2) signaling pathway, thus attenuating $CD8^+$ T cell responses in both human cancer patients and mouse models [81]. Even when encapsulated within a biofilm, bacteria are capable of maintaining a dynamic dialogue with their surrounding environment, in part through the release of metabolites. Notably, the effects of individual bacterial metabolites originate from studies on single planktonic bacterial species. Moving forward, it is crucial to expand our investigations to encompass more complex bacterial communities, particularly those residing within biofilm structures.

Biofilm-derived toxins in cancer

As already reported for free-floating bacteria [82], biofilm-enveloped microbial communities can also release toxins with cancerogenic properties. Most of the available data originates from studies pertaining to digestive system tumors. In the gallbladder, the presence of bile and gallstones facilitates the formation of biofilm by *S. typhi*, leading to chronic infection resistant to antibiotic treatment. Upon colonization of the gallbladder mucosa, this bacterium starts to release several carcinogenic and DNA damaging agents, including cytolethal distending toxins (CDT), that promote gallbladder cancer onset [83]. Furthermore, by analyzing surgical specimens from patients with familial adenomatous polyposis (FAP), Dejea et al. identified the frequent presence of bacterial biofilms within the majority of the samples. Further characterization of these biofilms revealed a predominant colonization by two bacterial strains implicated in colorectal tumorigenesis: enterotoxigenic *B. fragilis* (ETBF) and *E. coli* harboring the polyketide synthase (pks) genotoxic islands (pks$^+$ *E. coli*), known for its ability to produce the DNA-damaging toxin colibactin [84, 85]. In vivo studies using mouse models of CRC demonstrated that robust tumor formation occurred only upon co-colonization with both ETBF and pks$^+$ *E. coli*. This synergistic effect appears critical, as ETBF degrades the colonic mucus layer, thereby facilitating the adherence of pks$^+$ *E. coli* to the epithelial surface. This close interaction enhances the delivery of colibactin to epithelial cells, significantly increasing the possibility of DNA damage and subsequent tumor development [86].

The procarcinogenic activity of these bacteria does not appear to be limited to the development of tumors in the gastrointestinal tract, but may also extend to neoplastic diseases in other body regions. Parida et al. demonstrated that ETBF colonization of the breast gland induces mammary hyperplasia, tumor growth and metastatization through the release of its toxin (BFT) that triggers the β-catenin–NOTCH1 axis in cancer cells [87]. Moreover, this bacterium can also promote stemness features and chemoresistance in breast cancer cells, thus contributing to tumor progression [88]. Despite the absence of data on the presence of ETBF biofilm in the breast, but considering the ability of this bacterium to form biofilms in CRC [86], it is plausible that a comparable situation may also take place within the mammary TME, thereby facilitating tumor development and modulating immune signaling pathways to support oncogenesis.

It is important to recall that several biofilm toxins not only exhibit carcinogenic activity, as previously discussed, but also directly impact on the immune system. *S. aureus* biofilms release α-hemolysin (α-toxin) and leukocidin AB, both of which suppress phagocytosis and induce cellular dysfunction in macrophages [89]. *Vibrio cholerae (V. cholerae)* can form biofilms around different types of immune cells, a strategy that promotes the localized delivery of hemolysin toxin and significantly enhances immune cell killing [90]. Biofilms also produce detergent-like molecules with potent cytotoxic effects on immune cells, such as rhamnolipids from *Pseudomonas* biofilms that are cytotoxic for neutrophils [91, 92]. Although direct evidence of immune cell-killing effects by biofilm-associated toxins within the TME is currently lacking, the presence of bacterial biofilms in tumors suggests that these toxins could undermine the anti-tumor immune response, contributing to the establishment of an immunosuppressive microenvironment.

Impact of biofilm on anticancer therapies

The tumor is supported by a complex and dynamic system, the TME, which not only influences its growth and metastatic potential but also modulates its susceptibility to various forms of anticancer therapy [93]. It is now widely recognized that tumor-associated bacteria constitute an integral component of the TME, and that their biological activity plays a critical role in shaping the outcome of neoplastic disease [94], likely influencing the response to different anticancer treatments.

Focus on chemotherapy

In colon cancer, *F. nucleatum* has been implicated in the development of resistance to oxaliplatin and 5-fluorouracil (5-FU). Specifically, this bacterium downregulates miR-18a* and miR-4802 through TLR4 and MYD88 signaling pathways, promoting autophagy activation in tumor cells and contributing to chemotherapy resistance [95]. In breast cancer, Ma et al. identified a correlation between the presence of ETBF within tumors and reduced chemotherapy efficacy in breast cancer patients [88]. Consistently, 4T1 tumor-bearing mice pre-treated with ETBF *via* intragastric gavage exhibited diminished responsiveness to docetaxel, a chemotherapeutic agent commonly employed in breast cancer treatment [96]. Mechanistically, the ETBF-secreted virulence factor BFT-1 engages the receptor NOD1, particularly expressed by breast cancer stem cells (CSCs). Subsequently, NOD1 triggers a series of events ultimately leading to the activation of the NOTCH1–HEY1 signaling pathway. This cascade resulted in the enrichment of the breast CSCs population by enhancing their self-renewal and chemoresistant properties, explaining the initial observations in cancer patients and mice [88]. Furthermore, we previously demonstrated that in the 4T1 mouse mammary tumor model, the microtubule-targeting agent paclitaxel was ineffective when administered as a monotherapy, whereas its therapeutic efficacy was significantly enhanced when combined with oral ampicillin. This antibiotic intervention profoundly reshaped the composition

of the tumor-associated microbiota, most notably leading to a marked reduction of *S. epidermidis* within the TME [70]. These findings underscore the dual role of this Gram-positive bacterium, which not only modulates the phenotype and function of tumor-infiltrating immune cells but also actively influences the response to chemotherapy. It is worth emphasizing that these studies have focused on the activity of individual planktonic bacterial species. To date, only limited evidence exists concerning the role of bacterial biofilms in modulating responses to anticancer therapies, although preliminary insights are progressively emerging. Using an integrated microfluidic platform to investigate the impact of intracellular and extracellular bacteria on bladder cancer progression, Deng et al. demonstrated that extratumoral bacteria promoted the formation of a biofilm localized at the periphery of cancer cell clusters. Notably, the presence of the biofilm enriched a subpopulation of cancer cells expressing stemness markers. Given that such cancer cell populations are known to exhibit resistance to chemotherapeutic agents [97], it was hypothesized that the bacterial biofilm sustains tumor cells from chemotherapy. Accordingly, the combination of antibacterial agents capable of disrupting the biofilm with doxorubicin, a chemotherapeutic drug commonly used in the treatment of bladder cancer [97], resulted in an increased tumor cell killing compared to doxorubicin treatment alone in this tumor type [98]. Furthermore, extracellular lipids (ECLs) secreted by *Candida albicans* (*C. albicans)* biofilm resulted in the reduction of the cytotoxic activity of the topoisomerase I inhibitor camptothecin against two dysplastic and neoplastic oral cell lines. Although ECLs of *C. albicans* biofilms did not interfere with the cellular internalization of camptothecin, they promoted the formation of lipid droplets (LDs) capable of sequestering the drug within the cytoplasm, thus preventing its perinuclear localization and the possibility to exert its anti-tumor activity [99]. While the presence of bacterial biofilms can alter the efficacy of chemotherapy, it is also true that many chemotherapeutic agents possess antibiotic properties and can, in turn, influence biofilm development. Groizeleau et al. reported that doxorubicin enhanced *P. aeruginosa* biofilm formation, despite a decrease in intracellular levels of bis-(3′–5′)-cyclic dimeric guanosine monophosphate (c-di-GMP), a secondary messenger crucial for biofilm development [100], and the downregulation of several biofilm-associated genes. The authors proposed that biofilm formation was driven by the release of extracellular DNA (eDNA) from a population of lysed bacteria [101]. Therefore, the interaction between chemotherapy and bacterial biofilms is bidirectional, and this relationship must be carefully considered in the design of future therapeutic strategies aimed at targeting both biofilms and tumor cells.

Focus on immunotherapy

Given the immunomodulatory properties of biofilms, it is plausible that they could influence the response to immunotherapy. To date, the most widely employed form of immunotherapy in clinical oncology remains the immune checkpoint inhibitor-based treatment. It is now well established that both gut-resident bacteria and, more recently, tumor-infiltrating bacteria represent critical factors in determining the outcome of this type of therapy. A metagenomics study conducted on melanoma cancer patients undergoing anti PD-1 immunotherapy revealed differences in gut microbiota composition between responders and nonresponders [102]. In particular, the authors found that the presence of certain biofilm-forming bacteria in the gut, such as Bacteroides species, was associated with reduced responsiveness to PD-1 blockade. On the contrary, they could enhance its therapeutic efficacy. Indeed, Gao et al. found that *F. nucleatum* levels correlate with improved therapeutic responses to PD-1 blockade in patients with CRC. *F. nucleatum* enhanced the antitumor effects of PD-L1 blockade on CRC in mice and prolonged survival [103]. Additionally, it was found that a "normal" relative abundance of intestinal *Akkermansia* in NSCLC patients was associated to better response to anti-PD1 immunotherapy, while its overabundance was linked with a worse overall survival [104]. These examples highlight the need to consider the bacterial component in the context of immunotherapy. However, it is important to emphasize that, although many of the bacteria investigated as modulators of the response to immune checkpoint inhibitors are capable of producing biofilms, a direct link between bacterial biofilms and the outcome of immune checkpoint therapy has yet to be fully elucidated. Emerging data are beginning to shed light on this relationship. For instance, oral administration of smectite, a silicate clay, was found to promote *Lactobacillus* expansion and biofilm formation in the colon [105]. In melanoma-bearing mice, treatment with smectite resulted in significantly reduced tumor growth compared to control animals or those receiving clays lacking biofilm-promoting properties. Consistently, the combined administration of smectite and *Lactobacillus* biofilm exhibited superior anti-tumor efficacy relative to either agent alone. Analysis of the TME revealed a marked activation of the T-cell response. Mechanistically, the enhanced *Lactobacillus* biofilm formation driven by smectite facilitated the differentiation, maturation, and migration of dendritic cells *via* TLR2 engagement, ultimately leading to T-cell activation. Furthermore, in the same melanoma preclinical model, the smectite–*Lactobacillus* biofilm combination potentiated the anti-tumor effects of both doxorubicin and anti-PD-1 antibody therapies [105]. Therefore, a critical next step will be to refine the current knowledge regarding the impact of individual

tumor-associated bacteria on therapeutic outcomes, by integrating these findings within a more complex biological system, such as bacterial biofilm.

Conclusions

Our understanding of tumor-associated microbiota should evolve beyond focusing on single or few microbial species to encompass the complex, cooperative bacterial communities that interact synergistically to influence several biological processes. Bacteria form structures such as biofilms, composed of EPS, that act as a protective barrier that shields bacteria from immune responses and antimicrobial agents. It is becoming evident that, in some circumstances, the biofilm matrix can represent the primary driver of the biological effects exerted by the tumor-associated microbiota. Biofilm-producing bacteria have emerged as significant players in the context of cancer, as their persistent colonization of tissues and resistance to eradication create a microenvironment conducive to carcinogenesis and tumor progression (Fig. 3). These conditions, indeed, are sustained by the induction of a chronic inflammatory state and the release of metabolites or toxins that not only contribute to DNA damage and genetic instability, but also foster conditions that promote tumor initiation, growth, and metastasis. Moreover, emerging evidence suggests that bacterial biofilm is implicated in the resistance to chemotherapy and immunotherapy effectiveness *via* the formation of a protective shield and the shaping of the local immune microenvironment. The intricate interactions between biofilm-producing bacteria, the host immune system, and cancer cells highlight a complex but crucial connection, paving the way for innovative therapeutic approaches aimed at disrupting these pathological processes. By developing strategies to dismantle or inhibit the formation of this matrix, it may be possible to mitigate the pro-inflammatory and tumor-promoting effects of biofilm-associated bacteria. Such interventions could reduce cancer risk in susceptible individuals and enhance the efficacy of existing treatments, including chemotherapy and immunotherapy, thereby expanding the arsenal of therapeutic options available for diverse cancer types. However, while targeting biofilm-associated bacteria could hold promising therapeutic advantages, potential limitations and risks need to be taken into consideration, such as the disruption of microbiota homeostasis, off-target effects and the alteration of the mucosal integrity. Balancing the benefits of biofilm-targeting therapies with these potential risks will require a nuanced approach to ensure effective and safe interventions.

Acknowledgements
Not applicable.

Author contributions
EM and GB contributed equally to this work as first authors.MS and LS contributed equally to this work as co-last authors.LS and MS had the idea for the article and supervised the entire work; EM and GB performed the literature search; EM, GB, SM and LS drafted the manuscript; VLN, MA, SMP, ET, MS and LS revised the manuscript. All authors read and approved the final manuscript.

Funding
The research leading to this manuscript has received funding from: AIRC under IG 2020 – ID. 24718 project - PI Sfondrini Lucia; Ricerca Strategica Istituzionale Fondazione IRCCS INT- 5xmille funds for healthcare research (Ministry of Health); PRIN: PROGETTI DI RICERCA DI RILEVANTE INTERESSE NAZIONALE – Bando 2022 PNRR, Prot. P2022R5TCA – PI Sfondrini Lucia.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 26 February 2025 / Accepted: 5 May 2025
Published online: 21 May 2025

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