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Positive biofilms to guide surface microbial ecology in livestock buildings[☆]

Virgile Guéneau[a,b], Julia Plateau-Gonthier[b], Ludovic Arnaud[b], Jean-Christophe Piard[a], Mathieu Castex[b], Romain Briandet[a,*]

[a] Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, 78350, Jouy-en-Josas, France
[b] Lallemand SAS, 31702, Blagnac, France

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ABSTRACT

The increase in human consumption of animal proteins implies changes in the management of meat production. This is followed by increasingly restrictive regulations on antimicrobial products such as chemical biocides and antibiotics, used in particular to control pathogens that can spread zoonotic diseases. Aligned with the One Health concept, alternative biological solutions are under development and are starting to be used in animal production. Beneficial bacteria able to form positive biofilms and guide surface microbial ecology to limit microbial pathogen settlement are promising tools that could complement existing biosecurity practices to maintain the hygiene of livestock buildings. Although the benefits of positive biofilms have already been documented, the associated fundamental mechanisms and the rationale of the microbial composition of these new products are still sparce. This review provides an overview of the envisioned modes of action of positive biofilms used on livestock building surfaces and the resulting criteria for the selection of the appropriate microorganisms for this specific application. Limits and advantages of this biosecurity approach are discussed as well as the impact of such practices along the food chain, from farm to fork.

1. Introduction

Nowadays, a significant increase in the production of meat and fish is observed around the world [1]. This is linked to the growing consumer demand associated with the demographic and consumption increase in developing countries. To cope with this societal demand, an increase in the number and size of farms and a densification of animals in the buildings are envisioned. High animal density, particularly in confined buildings, can lead to the emergence of diseases, such as zoonosis. According to the World Organization for Animal Health, 60% of the 1400 human pathogens have an animal origin and 75% of emerging animal diseases can infect humans. Microbial pathogens in farms can trigger human diseases by direct contact with animals, but can also affect the whole food chain up to processed products such as meat or dairy products [2]. Characterizing pathogenic agents in livestock buildings and finding appropriate means to reduce their establishment and their propagation are therefore important challenges in the agrifood domain.

Prophylactic means of control such as vaccines to prevent the onset, spread and worsening of diseases or curative methods such as antibiotic therapy are mostly used to control these infectious diseases in animals. To limit animal contamination in farms, the surfaces of livestock buildings are cleaned and disinfected according to defined “biosecurity schemes” between each batch of animals. These protocols are aimed at limiting the microbial surface load that can be a reservoir of pathogens in livestock buildings before animals enter. However, these protocols may lack efficiency [3]. This variable effectiveness could be associated with the formation of spatially organized biofilms by the surface microbial communities. Biofilms are three-dimensional microbial structures adhering to a surface and buried in self-produced extracellular polymeric substances (EPS) [4]. They colonize all biotopes and are the most prevalent mode of life of microorganisms in nature [5]. The presence of an EPS matrix, the heterogeneity of cell types and the existence of specific intercellular communication phenomena such as the density-dependent intercellular communication system called *quorum sensing* give rise to emerging biofilm properties, including an extraordinary adaptation to environmental fluctuations. Microorganisms within a biofilm tolerate much more intense stresses than their planktonic counterparts, such as dehydration or the action of disinfectants

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[*] Corresponding author.
E-mail address: romain.briandet@inrae.fr (R. Briandet).

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[6]. Besides, these complex communities can harbor bacteria, viruses, yeasts, other fungi, microalgae, archaea, protists and can constitute reservoirs of pathogenic microorganisms [7,8]. The National Institutes of Health (NIH) have estimated that approximately 80% of human infections are associated with microbial biofilms [9]. These biostructures generate dramatic health issues and intensive efforts are used in the medical field to find new control strategies [10–13]. In farms, biofilms are found on walls, floors as well as in drinkers, feeders and even on the animals themselves. Biofilms are frequently associated with disinfection failure and pathogen persistency on surfaces [14–16].

Currently, there is strong pressure from consumers and legislators to reduce chemical disinfectant inputs in livestock farming to limit environmental impact and improve animal welfare and human health [17,18]. To ensure both farming sustainability and biosafety management, innovative solutions based on guided microbial ecology approaches have emerged recently in livestock production. The use of beneficial microorganisms to protect surfaces is applied under different usage names depending on the sector [7]: *e.g.* biocontrol for plants [19], biopreservation for food [20,21] or bioremediation for environmental issues [22]. In livestock buildings the term “positive biofilms” is now widespread for biosafety issues [23]. In this review, “positive biofilms” refers to the application of defined mixtures of bacteria in livestock buildings, selected for their capacity to colonize this environment and generate biofilms able to outcompete undesirable microorganisms. This review describes the concept, functioning and current use of positive biofilms and how they can complement conventional antimicrobial interventions. Because of their action to limit pathogens in animal production systems, the use of positive biofilms fits well in the One Health concept [17]. This is intended to limit the use of antimicrobials in farms and to propose additional solutions to prevent zoonoses.

2. Biosafety tools for the livestock sector

2.1. Direct actions on animals to limit diseases

2.1.1. Vaccines: successes and limitations

Vaccines are considered one of the greatest public health successes, along with the discovery of antibiotics. Vaccination aims to induce protective immunity to a targeted pathogen, thereby limiting the risk of developing the disease and reducing its potential impact on health. The principle is to inject a live attenuated pathogen, inactivated pathogen or part of a pathogen into a host [24]. These can be cellular debris, surface proteins or other molecules specific to the presence of the pathogen such as RNA molecules that will be used by the host cell to produce a pathogenic protein. Vaccines allow the body to obtain a long-lasting immune memory against the injected product. Typically, the active agent of a vaccine is recognized by cells of the immune system, which will produce large quantities of antibodies specifically directed against the pathogen. When the virulent pathogen infects the host, it will be immediately taken care of by a large number of cells secreting antibodies against it. Vaccines can have beneficial effects against pathogens other than the one initially targeted [25]. The use of vaccines helps to prevent the proliferation of pathogens in farms. It is a prophylactic action performed under the prescription of veterinarians. Because a vaccination plan is preventive, a strategy linked to a global prevention and control plan must be undertaken. The latter comprises local and global health recommendations [26]. The price of global vaccination in one farm is high, but the cost of diseases is higher [27,28]. One limit of the utilization of vaccines is silent infection [29]. Administration protocols may also be a limitation for some species and vaccines are not available for all infectious diseases in farms.

2.1.2. Antibiotic use for animals

Antibiotics are molecules that kill or inhibit the growth of susceptible bacteria. They are naturally secreted by fungi or other microorganisms and can be produced synthetically or by large-scale fermentation. The use of such molecules has saved countless lives, both human and animal, but their use is subject to worrying abuses [17]. Antibiotic sales increase in proportion to population growth. In some countries, they can be purchased without a medical prescription and in others they are used as growth promoters for livestock and aquaculture productions [30]. Antibiotic consumption may increase by 67% between 2010 and 2030 worldwide [31].

Careless use of antibiotics leads to the emergence of resistance in bacteria that may be transmitted to human microbiota. Antibiotic resistance is a genetically encoded mechanism in bacteria that allows a change in the target of the antibiotic or a reduction in the concentration of the antibiotic in the cell, preventing destruction [32,33]. The World Health Organization characterizes antimicrobial resistance (AMR) as a global public health crisis that must be managed with the utmost urgency [34]. Due to poor absorption by the body, 30–90% of antibiotics used in the animal food-producing industry are released into the environment [35,36]. The intensive exposure of environmental microbial communities to antibiotics can promote the emergence of intrinsic resistance and the transfer of resistance genes between bacteria (acquired resistance genes). Hence, animal carcasses can be contaminated in slaughterhouses with antibiotic-resistant pathogens [37]. As a consequence, food can contain bacteria harboring acquired antibiotic resistance genes that can be transmitted horizontally to bacteria from our gut microbiota [32]. The major health issue related to AMR is linked to the acquisition of resistance genes by bacteria capable of zoonosis. Some pathogenic bacteria such as *S. aureus* and *Escherichia coli* can become resistant to a very large spectrum of antibiotics [38,39].

According to the O'Neill report, more than 10 million deaths could have been caused by resistance to anti-infective drugs in 2016 which may become the leading cause of death in the world by 2050 if the situation does not change [40]. The economic cost could reach US $ 100 billion in this case [40]. To address this risk, the European Union implemented a regulation to ban antibiotics as growth promoters in animal feed in 2003 (Regulation 1831/2003/EC) and in June 2022 a new regulation will ban the use of therapeutic dose of zinc oxide, which is known to contribute to the emergence of bacterial resistance. The goal is not to ban their curative usage but to foster more reasonable use to limit the spread of antibiotic-resistant bacteria in the food chain. This is of particular interest as antibiotics used in veterinary medicine are often the same molecules as in human medicine. In the European Union, legislation will oblige member states to transmit sales and usage data of antimicrobials by species, before January 28, 2024, for cattle (including cattle of less than a year in age), pigs, poultry (chickens and turkeys).

2.1.3. Probiotics to maintain animals in good health

Probiotics are defined by the World Health Organization and the Food and Agriculture Organization of the United Nations as live strains of strictly selected microorganisms which, when administered in adequate amounts, confer a health benefit on the host [41,42]. Probiotics are given to animals or humans in aqueous solution, feed or in lyophilized form. They can be used to confer benefits such as promoting a beneficial microbiota and result in growth-promoting effects and morbidity reduction. The main microorganisms used as probiotics are *Saccharomyces* spp., *Bifidobacterium* spp., *Pediococcus* spp., *Lactobacillus* spp., *Lactococcus* spp., *Bacillus* spp., *Streptococcus* spp. *Enterococcus* spp. and *Escherichia coli* [43,44]. Research is under way to find new candidates as live biotherapeutics with specific probiotic properties such as *Faecalibacterium prausnitzii*, which has shown anti-inflammatory activity in the gut [45,46]. Probiotics can have positive effects on animal welfare, for instance by alleviating the stress of farming animals transitioning to a different production stage (such as the weaning phase in piglets). Probiotics can also stabilize animal intestinal flora and reduce the need for antibiotic treatments and associated propagation of AMR strains [47]. Probiotics are usually administrated to the host in “planktonic” form and are typically freeze-dried or spray dried. Recent reports have explored the possibility of formulating probiotics in a

Table 1
Major microbial pathogens isolated from biofilms in animal farms.

Zoonosis rank[a]	Pathogens	Place	Surface of development	Sources
1	*Campylobacter* spp.	poultry, slaughterhouse	stainless steel, surface-water isolates, human epithelial cells	[68–70]
2	*Salmonella* spp.	pig farm, poultry	eggshells, glass, broiler bedding material, polystyrene	[15, 71–74]
3	*Escherichia coli*	broiler material	broiler bedding material, air-handling system, water	[75]
4	*Yersinia enterocolitica*	mammals	flea intestine (vector of disease), polystyrene	[76,77]
5	*Listeria monocytogenes*	pork processing industry, floor drain and drain water, poultry meat	stainless steel, glass	[78–82]
	Staphylococcus aureus	bovines	bovine magpie	[83]
	Enterococcus faecalis	cattle farm	intestine	[84]
	Mycoplasma hyopneumoniae	pig farm	tracheal epithelium from pigs, glass	[85]
	Pseudomonas aeruginosa	drinking water of broiler houses, floor drain	stainless steel, glass	[86]
	Clostridium perfringens	poultry	livestock building (water supply lines, wall, feed)	[87,88]
	Pasteurella multocida	pigs and poultry	glass, calf trachea	[89]
	Streptococcus suis	pigs	endothelial cells	[90,91]
	Mycobacterium spp.	cattle	lung, liquid/air interface	[92,93]
	Vibrio spp.	aquaculture	glass, surface of the digestive tract of shrimp	[94,95]

[a] The European Union One Health 2019 Zoonoses Report [67].

biofilm form, allowing better tolerance to stressors encountered in the digestive tract and to boost the beneficial effect [48–50].

2.2. Management of undesirable microbes in the holobiont environment

2.2.1. Microbial flows in the farm influence the equilibrium of the holobiont

The holobiont is a biological organization composed of the host and the microbial communities associated with it, including viruses, and prokaryotic and eukaryotic organisms [51]. The whole genome of the holobiont is called a hologenome. If there is a disorder in the organization of the hologenome, it can affect the phenotype of the host and its microbiota. Comprehension of the relation between the host, its microbiota, and the environment is essential to understand how the addition of positive bacteria on farms can have beneficial effects on the host [52]. To safeguard animal health and holobiont balance, a set of biosecurity measures can be taken. Biosecurity is defined by the Food and Agriculture Organization as a strategic and integrated approach to the analysis and management of risks to the life and health of people, animals, plants and associated risks to the environment [53]. Good farming practices and effective biosecurity measures are essential, as they are the first barrier to the entry of pathogens into farms. External biosecurity measures designate strategies used to reduce disease introduction like fencing, quarantine, movement restriction, cleaning and disinfection procedures, and transport. Internal biosecurity measures are strategies used to reduce disease spread with, for example, the isolation of sick animals, and the control of rodents and insects. Biosecurity management is complex but first involves daily animal monitoring. Humans and animals present in the farm (*e.g.* cats, dogs, insects, rodents) can be vectors of contamination and may be responsible for transmitting them [54]. For example, flies are carriers of Enterobacteria, which are possibly pathogenic *e.g. Salmonella* spp. [55]. For 20 flies caught in pig farms, 10^4 to 10^6 *E. coli* were quantified [56]. To control these vectors, semi-containment measures are used in certain farms, along with airlocks that limit the risk of external contamination. Next comes hygienic practices such as the use of overshoes, hand cleaning, taking a shower, or the management of inputs including water, feed, litter and effluents such as manure. Indeed, water and feed can also transmit diseases to animals, which can lead to zoonoses [57,58]. Biosecurity management measures must be taken until the end of the chain and not only during breeding. Organic waste from livestock can be used as fertilizer for crops and thus disseminate pathogens in the environment [59].

Among internal biosecurity measures to limit the persistence and proliferation of pathogenic bacteria in livestock buildings, cleaning and disinfection (C&D) protocols are applied between each breeding cycle. In addition to environmental and safety concerns associated with their use, several studies have shown that these chemical biocides are poorly effective on biofilms and only allow the elimination of a small fraction of the microbiota present on surfaces [3,60,61]. Hence, it has been shown in avian farms that C&D protocols are not totally effective in eradicating residual pathogens responsible for cross-contamination between different batches of animals such as *Salmonella* spp.*, Campylobacter jejuni, Enterococcus* spp. and *Escherichia coli* [62–66]. This decrease in efficiency of C&D is directly associated with the formation of biofilms by these surface-associated communities. As shown in Table 1, microbial pathogens of major public health interest that can trigger zoonoses are able to form biofilms on surfaces typically encountered in livestock buildings [67].

2.2.2. Towards innovative biosecurity approaches in livestock buildings

For more than two decades, the international scientific community has been searching for alternative strategies to chemical biocides to control unwanted microorganisms. While physical processes (*e.g.* pulsed-light or plasma gas decontamination devices) are promising for specific industrial applications, their high demand in energy and cost limit large-scale use [96]. Most of the alternative processes considered nowadays for livestock building applications are based on biological systems. For instance, enzyme-based detergents improve the cleaning and disinfection process [97]. Different enzymes can be used such as proteases, cellulases, polysaccharide depolymerases, alginate lyase, dispersin B, or DNAse [98,99]. In industrial environments, numerous microbial species can grow within the same biofilm, thus increasing the biochemical heterogeneity of the matrix composition. Therefore, formulations used to destroy biofilm organization are generally composed of mixtures of enzymes with different substrate spectra. Novel anti-biofilm approaches targeting *quorum sensing* systems are emerging [100]. Several *quorum sensing* inhibitors, such as brominated furanones, interfering with biofilm formation in lab conditions [101,102]. Similarly, cyclic-di-GMP pathways that are involved in many biofilm formation processes could be promising drug targets [102–104]. Antimicrobial molecules extracted from natural compounds are also considered for use in livestock buildings. These are screened for having a high antagonistic effect against undesirable microorganisms while having a very low environmental impact. Honey bee products (bee venom, propolis or honey), plant essential oils and microorganism metabolites have shown antibacterial and antibiofilm activities [105–107]. A wide variety of organisms such as insects and amphibians can also secrete antimicrobial peptides with anti-biofilm activities against pathogenic bacteria [108–110]. The use of phages (viruses that infect

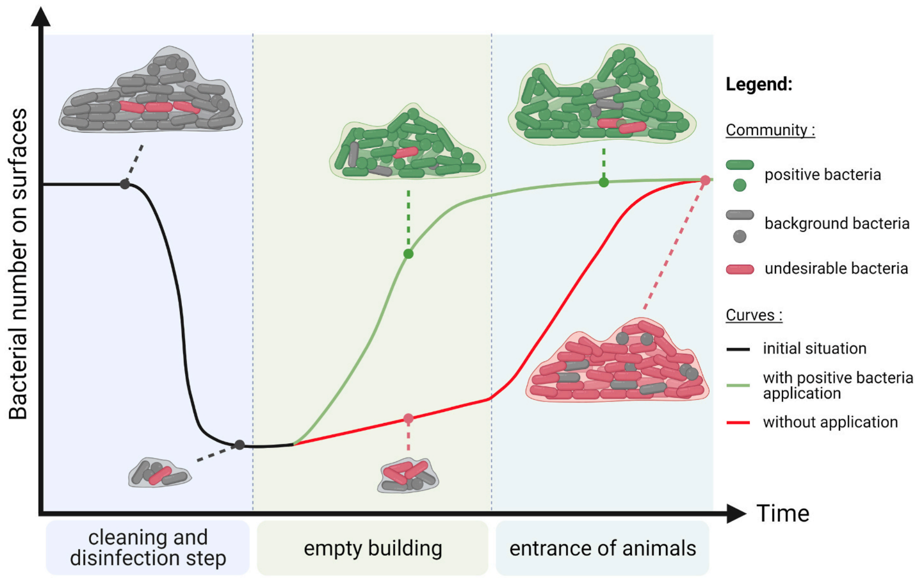

Fig. 1. Schematic representation of the concept of positive biofilms to guide the microbial ecology on the surface of livestock buildings. Biofilm communities that colonize livestock buildings are composed of background microorganisms that may contain undesirable bacteria. When animals leave the building, the microbial density on the surface is at its highest level. C&D protocols are used to reduce microbial load on surfaces (black curve). After the C&D procedures, two situations are possible: *i)* a positive biofilm is applied to the surface (green curve), *ii)* no application (red curve). In the first situation, the bacteria that are sprayed in large quantity adhere to surface to initiate a positive biofilm. This biofilm has an antagonistic effect on pathogens and prevents their proliferation. The objective is to have a mature positive biofilm before the entrance of animals in the building. In the second situation symbolized by the red curve, only the residual community that persisted after C&D is initiating a biofilm from a very low contamination level. Organic matter including feces or food projection is brought into surfaces when animals come into the livestock building. At that time, some undesirable bacteria that have survived the C&D protocols and that are competitive can proliferate with low competitive pressure. Figure created with https://biorender.com/. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

bacteria) to attack target pathogens is a promising solution that is already used in different sectors in some countries [111]. Phages can diffuse through the biofilm matrix [112] and are active on established biofilms [113]. They are already used in livestock buildings in poultry farms [114,115], and increasing research is being carried out to find new candidates [116]. Bacteria themselves can be used to sensitize unwanted biofilms to antimicrobial action. In a proof of concept study, Houry et al. [117] demonstrated that selected bacilli were able to swim inside exogenic biofilm matrix of pathogens such as *S. aureus*. Their infiltration generated a network of transient pores vascularizing the biofilm and increasing the efficacy of biocides. The authors also demonstrated that swimming bacilli that produce antimicrobial compounds could eradicate unwanted target biofilm [117].

None of these agents proved to be universal anti-biofilm molecules and combined approaches appear of value in limiting the emergence of resistance mechanisms [118]. Another way to use microorganisms in biosecurity applications is to guide the ecology of a surface by settling positive biofilms that will colonize and protect the surface from pathogen multiplication.

3. Positive biofilms to protect surfaces

3.1. Lessons from nature

Multispecies biofilms colonize most ecological niches. Antagonistic but also synergistic interactions can take place between species in these natural communities. A disorder of host microbial diversity (dysbiosis) can lead to the emergence of pathogens and associated diseases. This phenomenon is described with *Clostridium difficile* gastrointestinal infection which occurs essentially after antibiotic treatment alters competitive microbiota [119]. Similar situations can occur on the surface of livestock buildings after C&D protocols that leave free habitats on the surface for microbial pathogen settlement.

Many examples of natural positive biofilms illustrate competition with microbial pathogens. Indeed, selected bacteria, such as some strains of *Bacillus*, can act as plant growth-promoting rhizobacteria with the capacity to form a biofilm on the root [120–122]. In this case, bacteria can actively migrate by chemotaxis directed by root exudate. There it will form a biofilm preventing pathogen settlement by a set of complementary mechanisms of competition and interference. Similarly, some lactic acid bacteria (LAB) can naturally colonize amphibian skin and form biofilms that exclude the fungal pathogen *Batrachochytrium dendrobatidis* [123]. *Lactobacillus* spp. and *Bifidobacterium* spp. can form biofilms on the wall of honey crops with beneficial effects on beehive health through antimicrobial secretion [124]. Bacterial biofilms are also able to colonize coastal reefs and attract or repulse opportunistic algae [125]. These few examples of complex synergic and antagonist interactions inside a microbial community and its associated hosts are the subject of numerous mechanistic studies designed to decipher the mode of action of these positive biofilms against undesirable microorganisms.

3.2. Practical use of positive biofilms

3.2.1. Positive biofilms are already used in many sectors

Natural properties of positive biofilms have been used for centuries for many applications, particularly in fermented food production [126–129]. Biopreservation of food can use positive microorganisms, fermentation processes, metabolites or purified molecules to preserve food against pathogens. For example, *Lactococcus piscium*, a bacterium isolated from rainbow trout, has shown anti-*Listeria monocytogenes* activity in diverse foods [130]. Several products are commercialized as biocontrol agents for plants [131,132]. Bacteria from the rhizosphere used as biocontrol agents in plant culture have shown the capability to combat pathogens [133]. *Pseudomonas* spp. has shown anti-*Phytophthora infestans* activity in a potato model [134]. With the same principle, *Bacillus velezensis* QST713 can form biofilm on *Agaricus bisporus* compost excluding the green mold pathogen *Trichoderma aggressivum* [135,136]. Using microbial solutions to maintain the microbiological quality of inert surfaces is also envisioned in specific sectors. Some *Bacillus* spp. strains used as cleaning products on hard surfaces in hospitals can reduce by 50–89% healthcare-associated infections in comparison with conventional cleaning protocols [137]. The application of positive bacteria on building surfaces is an innovative solution already used in several sectors to control the establishment of microbial pathogens and limit the spread of AMR while reducing the use of toxic chemicals [138,139].

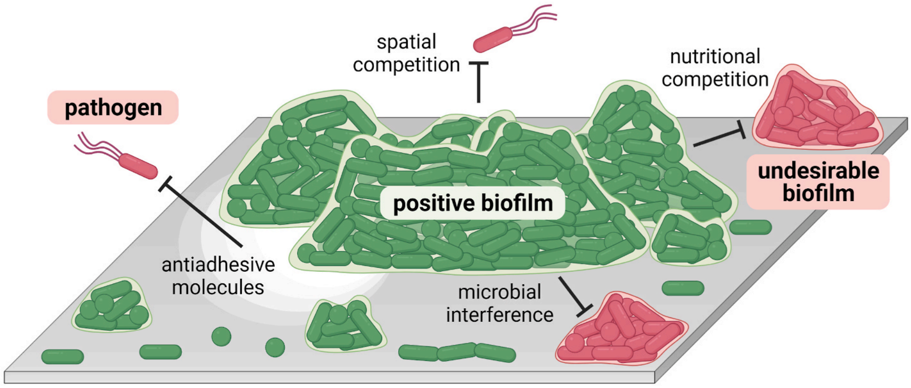

Fig. 2. Schematic representation of the mechanisms triggering exclusion of undesirable microorganisms by the settlement of positive biofilms. A positive biofilm can reduce the implantation of undesirable bacteria by several complementary mechanisms. *i)* An antiadhesive effect: the presence of the positive biofilm itself, or surface-active molecules it produces, can limit the initial adhesion of free planktonic pathogens on the surface. *ii)* A spatial and nutritional competition; by occupying the space and consuming available nutritional resources, the positive biofilm can limit the proliferation of pathogens recruited on the surface. *iii)* Microbial interference: through the secretion of specific effectors such as organic acids or antagonistic molecules, positive biofilm can reduce the presence of undesirable microorganisms on the surface. Figure created with https://biorender.com/.

3.2.2. *The case of positive biofilms in livestock buildings*

Selected bacteria can be applied to building surfaces to guide the microbial ecology of biofilm after cleaning and disinfection procedures. As described previously, biosecurity is crucial for livestock production. Current C&D protocols have shown their limits due to the development of negative biofilms on surfaces *e.g.* biofilm with microbial pathogens. These biofilms can persist between two production batches despite the application of biosecurity measures [3,60,61]. After C&D protocols and before the entry of animals, positive bacteria can be sprayed on the building surface and material to colonize the “empty” biotope and help to prevent the settlement of undesirable microorganisms. This concept is illustrated in Fig. 1.

This concept has already turned into practical products and applications. The products currently available on the market are composed of selected bacteria and form a biofilm on the surface of livestock buildings after application of the required C&D protocols. These positive biofilms limit the proliferation of undesirable microorganisms in the buildings through nutritional and spatial competition. Most products are composed of LAB, such as Lactococcus spp, Lactobacillus spp. or Pediococcus spp., often in combination with Bacillus spp. [140]. Large-scale evaluation of those products in field is still sparce due to experimental limitations in livestock buildings, and demonstrations toward pathogens are still mainly performed at lab scale [141].

3.3. *How does it work?*

Several mechanisms of exclusion of undesirable microorganisms by positive biofilms can be invoked. They are detailed in Fig. 2 and in the next sections.

3.3.1. *Competition for substrate*

Within a microbial community, bacteria with competitive advantages to access and consume nutrients essential for their growth will be favored. Nutrients essential for pathogen growth can be consumed by the bacteria within the positive biofilm and thus prevent pathogen proliferation, a phenomenon called the *Jamson effect* [142]. Enzymes can be secreted to metabolize specific substrates in the environment and other secreted proteins can bind to the product before being recognized by the bacteria for transport into the cell [143,144]. Cheating bacteria that can produce cellular receptors homologous to other enzyme-producer bacteria to incorporate specific substrates through proteins will then have an advantage [145]. Substrate availability with coevolution of different species exerts selection pressure to find the most economical way to use substrates in the environment. Interactions like cooperation, competition, or cheating drive the diversity profile of bacteria in the natural environment, which is modified by the availability of the substrate [144].

3.3.2. *Spatial competition*

Motility gives bacteria the capacity to find a favorable place to settle and multiply [146]. The ability to sense food gradients and orientate the cell movement along this gradient is a phenomenon called chemotaxis. It uses two-component systems that detect a molecule by specific chemoreceptors on the membrane allowing the cell to move in the right direction. For planktonic cells, swimming using flagella is the most widespread process of movement. Surface-associated bacteria can migrate through several mechanisms and use flagella to migrate in groups in a highly regulated process named swarming. Secretion of biosurfactants like cyclic lipopeptides can help bacteria to migrate on the surface by reducing the surface tension [147–149], preventing also the adhesion of other species. The most studied molecule is surfactin which can be secreted by *Bacillus* spp. and has been described in a biocontrol effect on plants [150,151]. Another example is *Lysinibacillus fusiformis* S9 which produces a biosurfactant that inhibits biofilm formation and adhesion of other bacteria without any bactericidal activity [152]. Bacteria can also “twitch” on surfaces by anchoring and retraction of dedicated pili [153,154] or colonized surfaces only by cell division [155].

Two-dimensional spatial competition on a surface is transformed into three-dimensional nutritional competition within biofilms; nutrients are consumed faster than they can diffuse through the biofilm matrix, thus generating sharp nutrient gradients [156,157]. These gradients, which influence the competition in biofilms, are influenced by their three-dimensional structures [158]. Flagella and pili participate in the adhesion step inherent in biofilm formation, as well as in biofilm structuration. For example, it has been reported that *Lactococcus lactis* has pili implicated in the structuring of the biofilm [159]. The apparent volume of the cell with a polysaccharide capsule is an advantage in space competition because it allows the bacteria to occupy a larger volume and increase cell fitness and this is implicated in biofilm formation [160]. For the same number of bacteria of the same size, those that will produce a capsule will take up more space and will therefore have access to more nutrients. Bacteria that are able to grow faster will have an advantage in the mixed biofilm because they will colonize surfaces faster, preventing other community members from accessing nutrients [155].

3.3.3. *Interspecies interference*

Interference includes all negative specific interactions other than those of bacterial cells of a given strain with themselves [161,162]. One of the best-known examples of microbial interference is the secretion of antimicrobials by bacteria [163,164]. *Bacillus* strains used as plant biocontrol agents typically contain between 5 and 8% of their total genome dedicated to the biosynthesis of antimicrobials [165]. LAB frequently used in the food industry produce a wide variety of antimicrobials such as bacteriocins [166] or organic acid preventing in particular fungal food spoilage [167]. Similarly, some LAB can inhibit biofilm formation of food-borne pathogens on abiotic surfaces by secretion of bacteriocins or hydrogen peroxide [168–170]. Some of these mechanisms require physical contact between the two cells as shown for a bacteriocin of *Lactococcus piscium* inhibiting *Listeria monocytogenes* [171,172]. *Lactococcus lactis* used in cheesemaking can secrete nisin with strong anti-*Listeria monocytogenes* activity in milk [173].

All bacteria have a growth/no-growth interface in relation to environmental physicochemical parameters (temperatures, pH and a_w (water activity)). LAB can secrete organic acid such as lactic acid that lowers the pH value of their microenvironment thus limiting the growth of pathogens [174,175]. pH fluctuation can be modulated by gradients in biofilms or EPS secretion [176]. The activity of organic acids involves the pH, but also the effects of undissociated acids. For example, for a given pH, the growth of *S. aureus* is more affected if the acidification of the medium is due to the addition of lactic acid rather than HCl [177].

Another example of interspecies interference involves intercellular communication systems. Most virulence factors of *Staphylococcus aureus* are under the control of the *agr quorum sensing* system. *Staphylococcus simulans,* a commensal coagulase-negative *Staphylococcus*, can secrete a peptide that interferes with the *agr* system of *S. aureus* [178]. Similarly *Bacillus licheniformis* DAHB1 shows biofilm-inhibitory activity against the shrimp pathogen *Vibrio parahaemolyticus* by this mechanism [179], as described in several interspecies mechanisms [180,181].

All these competition phenomena can be involved in pathogen exclusion by positive biofilms on the surfaces of livestock buildings.

4. Bottlenecks and trends for positive biofilms in livestock

4.1. Regulatory positions on positive biofilms

As microbial-based products used in animal surroundings can be in contact with the animals, it is appropriate in Europe to refer to the General Food Law (*i.e.* Regulation (EC) No 178/2002, as amended) as regards to the safety of those products. The Classification, Labelling, Packaging (CLP) regulation, the directive on safety of microorganisms as well as other elements such as those of the QSP (Qualified Presumption of Safety) list of the EFSA (European Food Safety Authority) guidance or the GRAS (generally recognized as safe) criteria of the FDA (U.S. Food and Drug Administration (*i.e.* qualified presumption of safety list) on the characterization of microorganisms used as feed additives or as production organisms may also be taken into consideration [182]. Today, C&D in livestock buildings mainly involves chemical products based on detergents and disinfectants. The Biocidal Product Regulation (BPR) (*i.e.* Regulation (EC) no. 528/212) establishes the legal framework for placing on the market new C&D molecules. The chemically oriented requirements of the BPR are, however, not adapted to microbial solutions such as positive biofilms.

4.2. Point of vigilance with the use of positive biofilm

The development of positive biofilm in livestock buildings has some limitations and points of vigilance to consider. The biosafety of the strains used as positive agents must be documented and must comply with national and international regulations (*e.g.* on the list of authorized strains, without AMR genes). Despite biosecurity efforts, bioprotective products can be spread outside the farm environment by water, insects, wind, manure, or humans, making it important to evaluate their safety with standards similar to those for food use. For example, the use of the biocontrol agent *B. thuringiensis,* which has been used for many years in the field, has recently raised questions in the scientific community on their possible involvement in human foodborne infections [183]. In addition, in some conditions, biofilms can protect microbial pathogens from C&D. It has been shown for example that the strain *Bacillus subtilis* NDmed isolated in an endoscope washer disinfector was able to protect *S. aureus* from biocide action in a mixed community [184]. A recent bioinformatics study highlighted the presence of acquired and potentially mobile AMR genes in commercial probiotic strains [185]. The demonstration of intrinsic versus acquired resistance is a complex topic with regulatory agencies around the world having different requirements [185–187]. The identification and monitoring of these genetic elements and the AMR genes are to be considered during the development of the products.

4.3. Rational selection of strains able to form positive biofilms

Environmental conditions on surface livestock buildings are far different from synthetic biofilms grown in laboratories. These synthetic ecosystems are of prime importance to simplify, control and model microbial interactions [188], but on-site field analyses are also required to identify and validate rational screening criteria for strain selection. Data describing natural biofilm structures and composition in livestock buildings are very scarce, due to the lack of adapted tools. Sampling methodology such as scrubbing, rinsing, sonication, scraping and grinding destroys the 3D structure of the biofilm, but is very dependent on the experimenter and leads to only limited community recovery [189]. *In-situ* sampling methodologies on coupons are emerging strategies to capture the whole community with preserved spatial organization [190]. Diversity studies using metabarcoding, metagenomics or other molecular methods such as PCR or qPCR are also interesting to pinpoint community members associated with pathogen prevalence [191–194]. Dynamic models of interaction networks such as the generalized Lotka-Volterra model can be used in an experiment of community dynamics to identify the species involved in a coexistence or species exclusion effect [195]. For example, it has been shown using the Lotka-Volterra model that the resistance of pathogen infection of *Clostridium difficile* was associated with *Clostridium scindens*, a bile acid 7-dehydroxylating intestinal bacterium [196]. One application of these models is to understand the relationship between surface-attached microbial communities and animal disease by studying the microbial communities of healthy livestock buildings and comparing them with buildings where pathogenic bacteria are reported to be present. If there are differences between the two, a selection of positive bacteria from "healthy" livestock buildings identified by the model as barrier effect bacteria against pathogens can be used to guide the microbial ecology in other buildings.

One important criterion for selecting candidate strains for use in positive biofilms is their fitness in the farm environment. Bacterial strain selection is mainly done using laboratory conditions, which is optimal for growth. The physico-chemical and biological parameters of the farm environment, such as temperature, humidity, pH, composition of the culture medium, indigenous microbiota and material, must be taken into consideration to develop a more rational and field-like model [197].

Most current commercial products are mixtures of LAB and *Bacillus* spp., but there are few scientific studies demonstrating synergistic mechanisms between these species. *Bacillus subtilis* biofilm can protect LAB from desiccation and there is a synergy between them against *S. aureus* proliferation in laboratory models [198]. Multispecies positive biofilms can have enhanced properties in comparison to strains cultivated alone [199–201] and new methodologies are emerging to screen strains alone and in combination. High-throughput methods like kChip screening have been developed to screen around 100,000 different communities per day using droplet assay and different optical growth assays. In this case, the communities can be screened quickly for their capacity to inhibit pathogen growth [202].

The application methodology like spraying or atomizing of the agents has also to be carefully adapted to the strains and the environment of application. Nutrients can be added to the formulation to initiate the growth of the positive bacteria. Other molecules like surfactants and sugars can also help to enhance the initial adhesion or surface colonization. Recently, it has been shown that sucrose enhances root colonization by *Bacillus subtilis* by increasing surfactin secretion [203]. One other important parameter is that the selected strains must

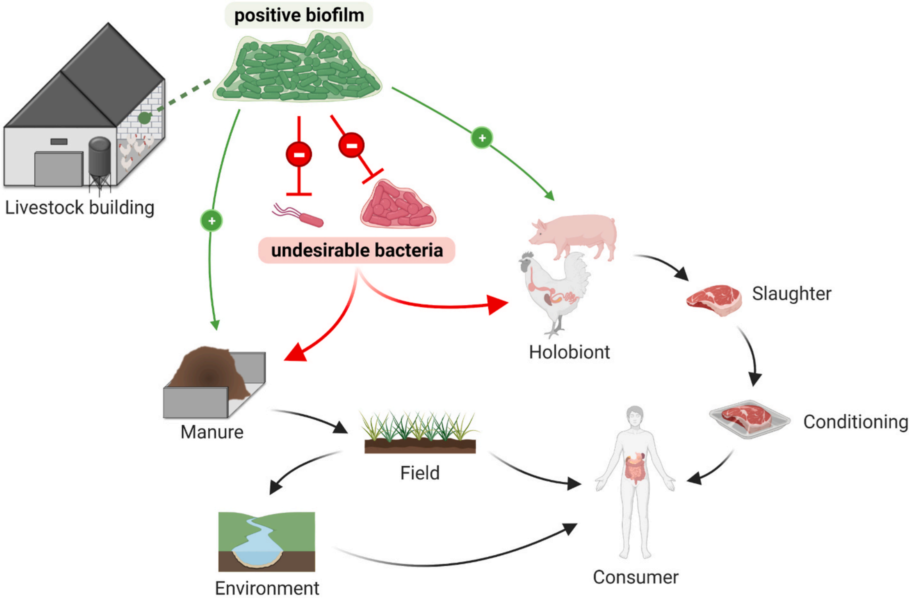

Fig. 3. Potential impact of the addition of positive biofilm in livestock buildings on the spread of undesirable bacteria from farm to fork. Positive bacteria that are applied to livestock building surfaces and that form positive biofilms have an antagonistic effect on undesirable bacteria that can be present as harmful bacteria reservoirs. By limiting the establishment of these undesirable bacteria, animal diseases are directly reduced, as is the abundance of undesirable bacteria in manure used to fertilize the soil (red arrow). Overall, positive biofilm establishment limits proliferation of undesirable bacteria at the beginning of the food chain. This leads to a reduction of spread from animals to consumers via slaughter and the conditioning steps, but also to a reduction of spread via manure to the environment and to fields that finally reach the consumer (black arrow). Finally, the positive biofilm can have a direct positive impact on the fermentation process of manure. Likewise, the positive bacteria that enter in direct contact with animals can also have a positive effect on the holobiont with, for example, a positive effect on the gut like probiotics (green arrow). Figure created with https://biorender.com/. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

be easy to produce on an industrial scale. For example, *Bacillus* can form spores that are less costly energetically to produce than the lyophilization process used for LAB.

4.4. Beyond excluding pathogens on livestock surfaces

Positive biofilms used in livestock buildings prevent the settlement of undesirable microorganisms on the surfaces. Microorganisms from the positive biofilm may also have side effects outside the farm, *e.g.* by reducing the spreading of pathogenic organisms and by seeding beneficial bacteria in the food chain (Fig. 3). In close contact with animals, especially in early life stages, they can modulate their microbiota and be beneficial for their health. In this regard, it has been demonstrated that a probiotic-based cleaning product applied to poultry farm litter can modify the litter's microbial diversity, but also the chicken caeca microbiota compared with an un-exposed control group [204].

Natural surface communities in livestock buildings can harbor bacteria containing genes of antibiotic resistance [205]. The application of a positive biofilm with selected strains dilutes those organisms and limits the spread of antibiotic resistance genes in the food chain.

The organic matter left at the end of a cycle in a livestock building (bedding, manure, slurry) can contain microbial pathogens and is frequently used as fertilizer in crop fields. Bacteria used in the formulation of positive biofilms can enrich the population of strains able to ferment these products (*e.g.* LAB) and prevent the development of pathogens before spreading [206,207]. Bacteria from positive biofilms such as *Bacillus* spp. have also been shown to have beneficial effects on plants by producing growth-promoting factors or by excluding pathogens from the roots [131,165,208].

5. Conclusion

Political and societal demand to reduce the use of chemical products and antibiotics in farms has triggered the development of alternative biosecurity approaches, including the use of microbial positive biofilms to protect surfaces. This biological approach has shifted from labs to farms in recent years with various commercial products now on the market in several countries. However, these promising approaches need a deeper understanding of the associated microbial interactions and their variability in different breeding contexts to be used successfully. Research is also needed to further assess their benefits from the farm to the fork, in particular by limiting the spread of undesirable bacteria in the food chain and the environment. Finally, national and international regulations should adapt to these innovative sustainable solutions to encourage research, development and their use on farms.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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