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A Review On Multi-Species Biofilms In Food Industry

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Abstract
Multispecies biofilms are prevalent in the food industry and pose a significant challenge as they serve as a reservoir for various microorganisms. These biostructures consist of microorganisms embedded in an organic matrix composed primarily of polysaccharides, proteins, and nucleic acids. The matrix facilitates microbial adhesion to surfaces and promotes tolerance to environmental stress. Multispecies biofilms are notoriously difficult to eradicate completely, making them a source of food contamination due to the continuous shedding of cells and spores onto food and other surfaces.
The protective nature of multispecies biofilms arises from the physical barrier they provide, their induction of stress proteins, facilitation of gene exchange, prevention of desiccation, and production of substances that protect against competing microorganisms. Additionally, biofilms exhibit increased resistance to disinfectants when exposed to sub-lethal concentrations, further exacerbating their persistence in food processing environments.In the food industry, where various types of microorganisms coexist, multispecies biofilms readily form on the surfaces of food processing instruments. These multispecies biofilms can lead to the transmission of diseases such as listeriosis, primarily caused by Listeria monocytogens. The formation of mixed-species biofilms enables intermicrobial protection through the development of resistance mechanisms against disinfectants. Quorum sensing, the production of extracellular polymeric substances, and complex microbial interactions, including competitive and cooperative interactions, contribute to the formation of biofilms among different species. Consequently, biofilms can compromise food safety and quality control.
This report provides an overview of the microorganisms responsible for food poisoning and their association with biofilm formation. It further explores the formation of multispecies biofilms and elucidates the roles of quorum sensing, extracellular polymeric substances, and microbial interactions. The harmful effects of multispecies biofilms in the food industry are discussed, emphasizing the need for effective control strategies. Understanding the mechanisms underlying biofilm formation and the protective strategies employed by thesestructures is crucial for developing targeted approaches to mitigate their impact on food safety and quality.
Keywords : *Multi-species biofilms, Biofilms formation and control in food industry.*

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I. Introduction

Bacterial cell to survive in adverse condition make biofilm . Biofilm is the bacterial community in which they survive with lower cellular metabolic rate in poor nutrient conditions . Bacterial cell take nutrient from the microenvironment and release cellular by-product in the biofilm . Most of this biofilm found in food industries are formed by combination of two or more species of microorganisms instead of single species and this type of biofilm made up with more than one species are called multispecies biofilm .

This type of multispecies biofilm made up of species such as *Staphylococcus, Stenotrophomonas, Streptococcus, Pseudomonas* , *Listeria* etc. and this biofilm with pathogenic organism can herm the production unit of food industry, also there may be case of food posing by food infection or food intoxication in food related industries .

Now days this multispecies biofilm are showing resistance to disinfectant in many food industries, like seafood, brewing , dairy processing etc.

Species diversity in the raw materials are responsible for multispecies biofilms in the food contact surface and nonfood contact surface (such as pipes in dairy industry) in meat , fish , dairy and other food posing industries .

Microscopic techniques are there such as microcolonies , layered structure , different layer help in recognizing the bacterial biofilm in food surfaces .

And at last this multispecies biofilm in food industry cause various problems such as blocking of pipes, pathogenic organisms biofilm leads to food born disease, change the flavour and texture of foodetc.

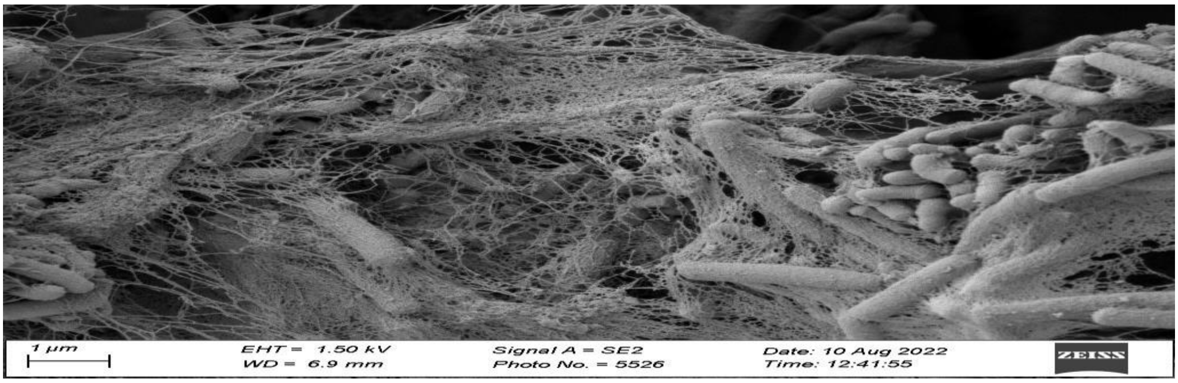


Fig 1 : Multispecies biofilms in food industry [Source – Internet (ilvo.vlaanderen.be)].

Organisms Related To Multispecies Biofilm Formation

In the food industry, biofilm-forming species occur in factory environments and can be pathogenic to humans if they develop biofilm structures. Process environments in the food industry, e.g. Wood glass, stainless steel, polyethylene, rubber, polypropylene, etc. serve as artificial substrates for these pathogens [1][2].
Examples of these relevant biofilm-forming pathogens for the food industry are briefly ingiven below–

Bacillus cereus –

Bacillus cereus is an anaerobic or facultatively anaerobic gram-positive spore-forming bacterium that can grow in a variety of environments over a wide temperature range (4 degree Celsius -50 degree Celsius) . It is resistance to chemicals, heat treatment[3] .

Bacillus cereus mainly found in soil and it also common in food and food industry such as dairy industry meat processing industry . It produce toxin that can cause diarrhoea like sickness in humans.

Bacillus cereus is responsible for the formation of biofilms on food contact surfaces such as stainless steel pipes, conveyor belts and storage tanks. It can also form floating or submerged biofilms that can secrete a variety of bacteriocins, metabolites,surfactants, as well as enzymes such as proteases and lipases into the biofilms that can affect the sensory properties of food[4].

Campylobacter jejuni-

Campylobacter jejuni are rod-shaped or curved, thermophilic and motile gram-negative bipolar flagella containing bacteria. *Camphylobacterjejuni*also known as anaerobic bacteria that can form biofilms under microaerophilic (5% oxygen 10% carbon dioxide) and aerobic (20% oxygen) conditions. Although *Camphylobacterjejuni*is a fickle organism, it can survive outside of the digestive tract of birds before reaching human host[5][6].

A number of environmental elements initiate the formation of biofilms, which are then influenced by a number of internal factors. The European Union’s 2018 one health report on zoonoses classifies this bacteria as as an opportunistic pathogen of bacteria gastroenteritis and was found to be a common eater of animals and edible poultry, particularly turkeys and chickens[7].

When food preparation and processing areas or water are contaminated, e.g. unpasteurized milk. This bacteria reaches the human host, infects and colonizes the gastrointestinal tract and causes disease[8].

Listeria monocytogenes –

It is a gram-positive bacterium. *Listeria monocytogenes* is a ubiquitous foodborne pathogen that can be found is soil, food,and water. Its consumption can cause miscarriages in pregnant women and other serious complications in the elderly and children. The pathogen can be transmitted through various foods such as dairy products, seafood, meat, fruit, ready meals, ice cream, soft cheese, raw milk, frozen vegetables, candied apples and poultry[9][10].
*Listeria monocytogenes*are not resistant to pasteurization[11].

Listeria monocytogenes multiplies at low temperatures and able to form pure culture biofilm for growing in multispecies biofilm community. It can survive in acidic conditions for long periods of time,forming biofilms that grow in the absence of oxygen. Its number will likely increase or decrease in biofilms depending on the presence of competing microbes[12][13].

Staphylococcus aureus –

It is a nonspore-forming, facultative anaerobic bacterium and also it is gram-negative in nature .

*Staphylococcus aureus*can produce enterotoxins at 10-46 degree Celsius. It can multiply on the skin and mucous membranes of people who come into contact with food and can become a serious problem is food processing plants or industries[14].

These enterotoxins are heat stable and can be excreted during the growth of *S. aureus* into foods contaminated by food workers. The bacterium grows well in foods high in salt or sugar with low water activity. Foods commonly associated with foodborne disease include meat and meat products, poultry and egg products, milk and dairy products, baked goods, salads and particularly cream filled foods like cakes and pastries for sandwiches[15].

***Enterohaemorrhagic Escherichia coli* (EHEC) –**

It is a rod-shaped gram-negative bacterium. Most strains of *E.coli* are part of the human gut microbiome and do not pose any health concerns. However, the virulence categories of *E.coli* include enterotoxin(ETEC),enteroinvasive (EIEC),enteropathogenic (EPEC) and Vero cytotoxigenic (VTEC). O157:H7 EHEC is the most common serotype associated with human EHEC infection in the United States[16]. The widespread spread of *E.coli* in the natural environment is largely due to its ability to grow as a biofilm. It should be noted that some strain of *E.coli* is most relevant to food industry. EHEC serotype O157:H7 is the human pathogen that cause outbreaks of bloody diarrhea and hemolytic uremic syndrome(HUS) worldwide. They can be transmitted through raw milk, drinking water or raw meat, fruits and vegetables, examples- melon,tomato, parsley, lettuce etc. [17].

It can use cilia, flagella and membrane proteins to initiate attachments to inanimate surfaces when the flagella disappears after attachment and the bacteria begin to produce an extracellular macromolecule (EPS) help to make bacteriummore resistant to disinfectants [18].

Some studies also indicates EHEC can from biofilms on various surfaces in the food industry and there is no effective way to prevent EHEC biofilm formation .

***Salmonella enterica* –**

*Salmonella enterica*is a common foodborne pathogen related to poultry meat food industry . It cause gastroenteritis [16] and the symptoms related to foodborne disease by this rod shaped gram negative bacteria are nausea, vomiting , fever, abdominal pain etc[19].

*Salmonella enterica*can form biofilm related to stainless steel related to food industry and result in formation of 3D structure with several layers . This pathogenic bacteria can produce different morphological structure depending on the nutrient availability . For example it found in areticulum – shaped when grown in TSB medium [20].

It is also found that the biofilm associated with *Salmonella enterica*can survive on stainless steel more than a year .

This bacterial cell envelope contain lipopolysaccharide , and this lipopolysaccharide can function as endotoxin which is responsible for causing disease like alteration of lymphocyte function , depress myocardial function etc .

In the year of 2016, 94625 cases of food born disease associated with *Salmonella enterica*was reported European Food Safety Authority .

Other organisms related to biofilm formatation –

Gram-positive bacteria like *Anoxybacillusflavithermus*produce spores that are very heat-resistant and *A. flavuthermus* vegetative cells can grow at temperatures up to 65ºCand show significant increase in bacterial adhesion in the presence of skimmed milk, on the surface of stainless steel[21].

Thermophilic bacteria such as *Bacillus stearothermophilus* , can adhere to stainless steel surface on evaporator processing lines and plate heat exchangers, allowing them to grow and produce biofilms, including the ability to release individual cell or aggregate cells into the final dry product[22].Gram-negative bacterium like *Pseudomonas*produce lots off EPS which helpthem to attach and producebiofilms on the surface made of stainless steel. They can live with other pathogens and form multispecies biofilm, this make them more resistant to any agent that can kill them[23].*Pectinatus* non-spore forming anaerobic gram-negative bacterium which form high percentage of biofilm in breweries due to hygiene issues[24].

Figure 2 : Removal bacterial biofilm in milk industry [source - blog.decon7.com] .

Table 1 : Examples of some biofilm producing organisms in food industry .

Name Of Organism	Type Of Food Industry	Biofilm Type	Associated Disease Name	Characteristics	Reference
Listeria monocytogenes	Dairy, meat, and poultry	Multispecies biofilms with other bacteria	Listeriosis	High resistance to sanitizers, ability to grow at refrigeration temperatures	25
Salmonella spp.	Poultry, eggs, meat, and produce	Multispecies biofilms with other bacteria	Salmonellosis	Heat-resistant, ability to survive in diverse environments	26
Escherichia coli O157:H7	Ground beef, produce, and raw milk	Single-species and multispecies biofilms	E. coli O157:H7 infection	Produces toxins that can cause severe illness	27
Campylobacter jejuni	Poultry, unpasteurized milk, and contaminated water	Single-species biofilms	Campylobacteriosis	Heat-sensitive, requires microaerobic conditions	28
Cronobacter spp.	Dairy industry	Single-species and multispecies biofilms	Meningitis	Can survive in low-moisture environments, associated with neonatal infections	29
Vibrio parahaemolyticus	Seafood, especially raw or undercooked shellfish	Single-species and multispecies biofilms	Vibriosis	Halophilic (salt-tolerant), associated with warmer waters	30
Staphylococcus aureus	Dairy, meat, and bakery products	Mainly single-species biofilms	Staphylococcal food poisoning	Produces heat-stable enterotoxins	31
Clostridium botulinum	Canned foods, low-acid foods	Single-species biofilms	Botulism	Produces potent neurotoxins	32

How Multispecies Biofilm Develop In Food Industry

Modern food processing lines are a suitable environment for biofilm formation on food contact surfaces due to the complexity of the production facilities , long production lead times, mass production and large production area[33] .Foodborne diseases cover wide range of diseases and are caused by pathogen ingested with food . As recognized by the World Health Organization (WHO), food contamination can occur at any stage of production of food , from production to consumption and result in environmental contamination, including water, soil or air contamination[34]and also leads to disease related to food consumption .The process of multispecies biofilm formation consists of five sequential steps : (i) reversible attachment, (ii) irreversible attachment, (iii) development of microcolonies, (iv) biofilm maturation and (v) dispersion or separation[35].

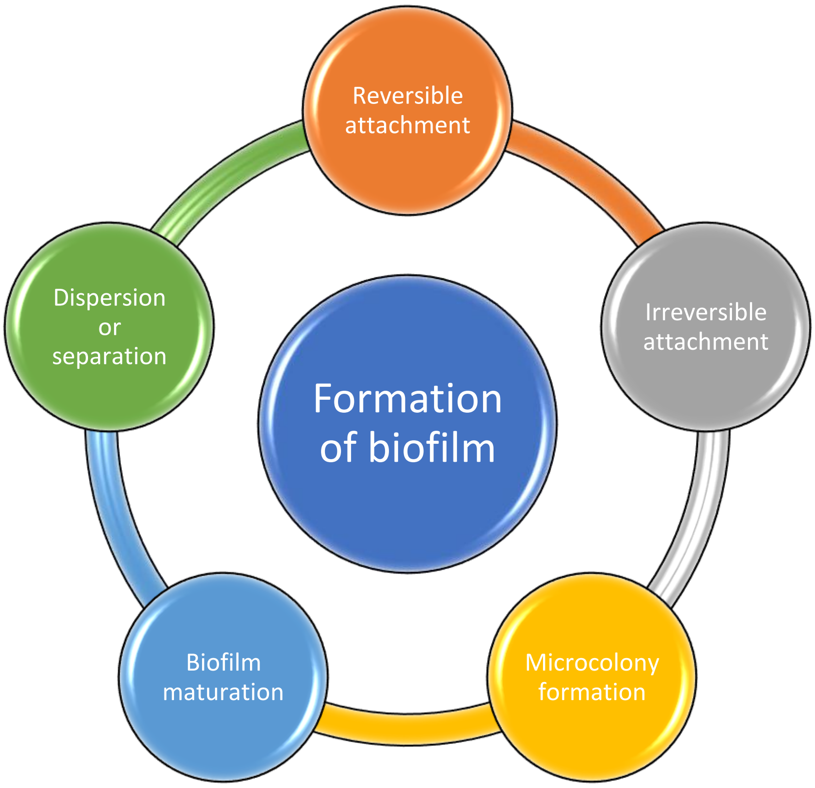
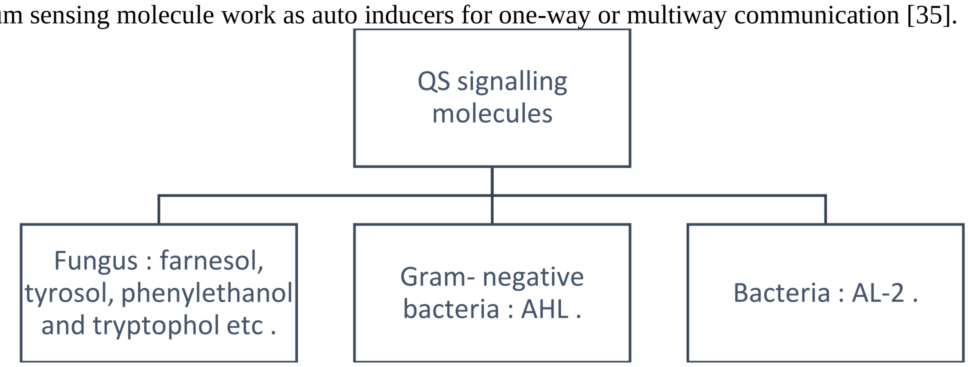


Figure 3 : A imaginary processes for the formation of multispecies biofilms:(1)reversible attachment plankton cells,(2)irreversible attachment, microbial cells attach irreversibly to the surface, cells begin to secrete EPS and QS factors , and the overall population continues to grow in this stage ,(3)Formation of microcolonies, microbial cells interact to form microcolonies, and interspecific andintraspecific cells communicate via QS factors EPS,(4)Biofilm maturation, microcolonies continue to grow and form mature biofilm structure,(5)Scatter or Separationthis is the last step in this step microbial cells actively scatter and revert to planktonic states[35].

Quorum Sensing –

When there is high amount of polysaccharides , lipids , protein and fatty acids the bacteria attach to biological or abiotic surface such as stainless steel of fermenterin food industry this attachment at first is reversible but when the bacteria start to communicate with each other by quorum sensing the attachment become irreversible[36].This quorum sensing is the signalling process by which a bacterial species sense the cell density and by that sensation bacterial species regulate the gene expression [37].

The quorum sensing molecule work as auto inducers for one-way or multiway communication [35].



Among this quorum sensing molecule , forformation multispecies biofilm two molecules acyl-homoserine lactone (AHL) and autoinducer – 2 play a important role. For the maturation of biofilm in case of gram-negative bacteria AHL play a important role it regulate the maturation of biofilm [38] . Now another point comes in play in formation of multispecies biofilm and that is interaction . This interaction may be competitive interaction in which interaction provide disadvantage in coculturing of two or more species or the interaction can cooperative interaction in which bacteria present in multispecies biofilm grow rapidly then the bacteria present in single species biofilm . Now there is role of QS in different type of interaction present in multispecies biofilm .

Example –Competitive interaction occur between *Pseudomonas fluorescens, Shewanellabaltica*[80] , in this case *S. baltica*inhibit the production of AHL in this biofilmor *S. baltica*may consume the AHL secreted by *Pseudomonas fluorescens*[39].

Another molecule is AL-2 . It is a cyclic oligopeptide and produce by both gram-positive and gram negative bacteria . It is also found that the mechanisms of QS *in Staphylococcus aureus* seems to be involved in formation of biofilm of *L. monocytogenes* by the help of AL-2[40].

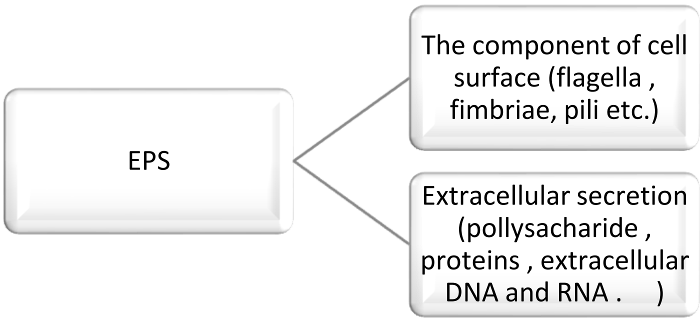
So at last QS is an important mechanisms for communication between multispecies biofilm for formation of biofilm at initial stage and dispersion of biofilm at latter stage.

EPS –

Extracellular polysaccharide is mainly composed of proteins, extracellular DNA and RNA . This EPS help in transporting many molecule such as QS signalling molecule in the biofilm matrix[38].

The extracellular polysaccharide help in the initial step of biofilm formation and that is attachment and it also help in the volume of cell in biofilm[41][42].

If the EPS producing stain is compared with EPS non producing stain then it is seen that EPS producing stains are forming stronger biofilm than the non EPS producing stain [43] . The EPS Producing microbes can be divided into two part :



Some components of EPS such as cellulose , lipopolysaccharide , capsule polysaccharide etcalso help in the increasing the viability of biofilm [44]. One example is given below showing that how EPS from one bacteria help in formation of multispecies biofilm in food industry .

When milk is treated with ultrahigh-temperature , poor biofilm producing bacteria (*Lactobacillus lactis*) improve its attachment (first step of biofilm formation) with the surface (such as stainless steel) by the matrix (EPS) provided by *P . fluorescens*and in return *Lactobacillus lactis*provide nutrient source to*P. fluorescens*[45].

So EPS play a important role in the formation of biofilm and EPS production inhibition can also use as an important key for controlling of biofilm .

Genes associated with multispecies biofilm in food industry -

Due to active or passive dispersion of multispecies biofilm in the production area of in any food industry, may also led to recontaminationof the pasteurized or unpasteurized product, one of the example is *Bacillus cereus* produces endospores, this endospores can not be kill using heat treatment so the shelf life of heat treated product decreased [46]. The common genes responsible for biofilm formation in food industry are-

1. Deg U ,
2. Fla A ,
3. Mot B, etc.

The DegU-P protein regulates the expression of genes involved in various aspects of biofilm formation, including extracellular matrix production, motility, and adhesion. It can activate genes that promote biofilm formation and inhibit genes that may interfere with the process. This regulation helps ensure the coordinated growth and development of the multispecies biofilm. In *L.monocytogenes*for biofilm formation occur rapidly when there are lots of phosphorylation of DegU[47].

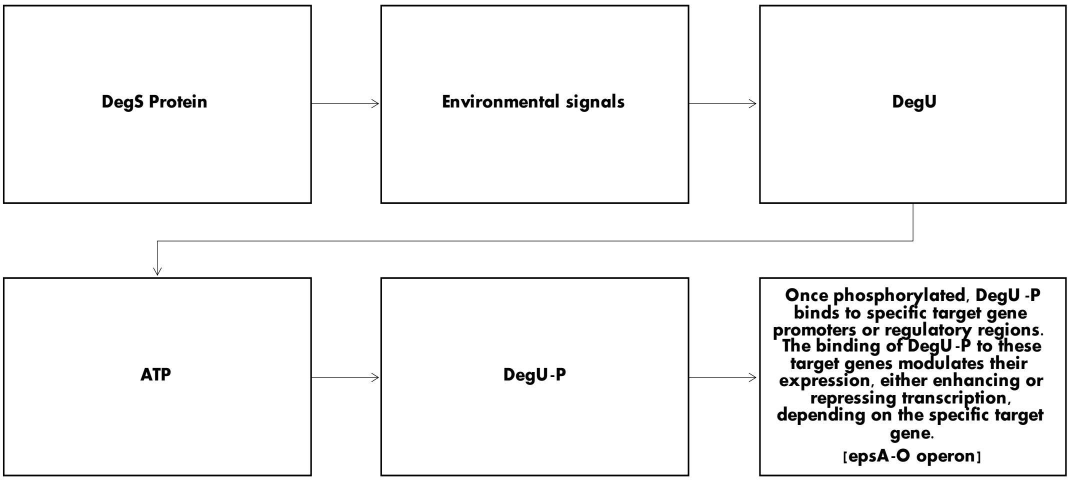


Figure 4 : DegU protein regulation of biofilm production .

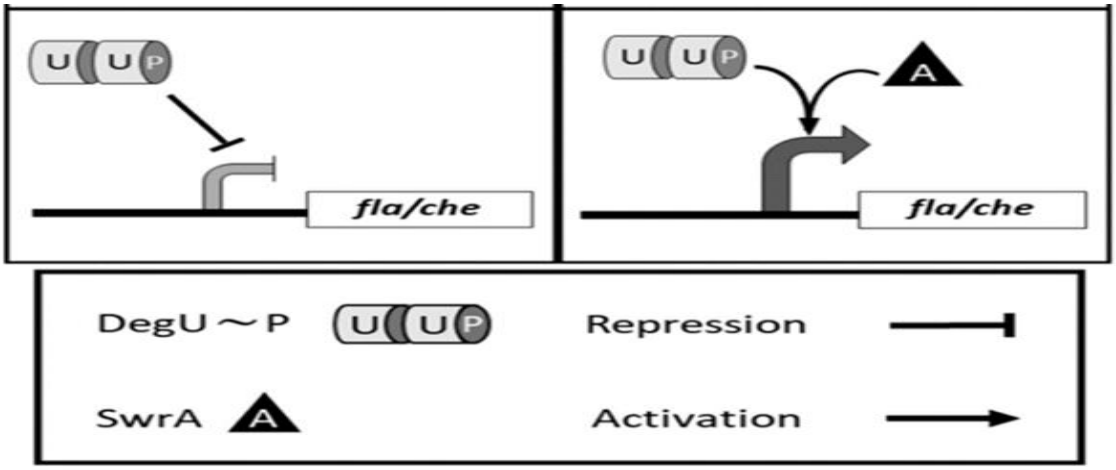


Figure 5 : Fla A gene expression in present of DegU-P and SwrA protein [48].

In the process of multispecies biofilm formation, flagella can contribute to initial attachment and subsequent surface colonization. The working principle of the flaA gene in multispecies biofilm formation involves the regulation of flagellar synthesis and assembly, which impacts the overall behavior and structure of the biofilm. The *fl*aA gene encodes for flagellin, the flagellin is the main component of bacterial flagellum, this help in bacterial attachment[48].

The MotB gene, which is involved in flagellar motility, can play a role in multispecies biofilm formation in the food industry. Multispecies biofilms are complex communities of microorganisms that adhere to surfaces and form protective structures, often found in various food processing and production environments The mot B protein encoded by mot B gene is responsible for transmembrane transport of protons in the aforementioned flagellar motor complex[49].

While the specific role of the MotB gene in multispecies biofilm formation may vary depending on the bacterial species and the specific food industry context, here are some potential ways in which the MotB gene and flagellar motility can impact multispecies biofilms in the food industry:

Initial Attachment: Flagellar motility, driven by proteins like MotB, can facilitate the initial attachment of bacteria to food processing surfaces. Bacterial cells capable of flagellar motility can swim and explore the environment, increasing the chance of encountering and attaching to suitable surfaces for biofilm formation.

Surface Colonization: Once attached, bacteria with functional flagella, regulated by the MotB gene, can contribute to the colonization and growth of multispecies biofilms on food industry surfaces. The ability to move via flagella enables bacterial cells to migrate and position themselves within the biofilm structure.

Interactions and Co-aggregation: Flagellar motility can also influence the interactions between different microbial species within the biofilm. Bacterial cells with functional flagella can exhibit directed movement towards or away from other cells, potentially influencing the composition and structure of the multispecies biofilm.

Biofilm Architecture: The presence of functional flagella can impact the overall architecture and organization of the multispecies biofilm. Bacteria capable of flagellar motility may exhibitdistinct patterns of distribution within the biofilm, contributing to the formation of channels, voids, or other structural features.

Some microorganisms are their which are responsible for release some substances which are responsible for inhibition of biofilm formation this substances are also encoded by some genes present in the substrate releasing organisms. Example *Bacillus cereus* RC6 release two enzymes that degrade casein and show antimicrobial activity against *L.monocytogenes*[50]. So there are some genes that help in the formation of microbial biofilms.

Microbial interaction –

The phenotype of biofilm is affected by interspecies interaction . Various type of interaction are there which influence the growth , abundance and physiological characteristics of organisms in multispecies biofilm, this interaction may be positive interaction (cooperation , mutualism , synergism , syntrophy and altruism) also there is antagonistic interactions [51]among this interaction there are two main type of interaction that also lead to biofilm formation with the help of biofilm formation genes. First competitive interaction , it occur between one or more than one species at a disadvantage in coculturing . one example is interaction between *P. aeruginosa* and *S. aureus* in biofilm formation [52]. Second type is cooperative interaction . In this interaction single species biofilm formation rate is lower thanmultispecies biofilm formation rate . Example – Biofilm formation interaction between *Ralstoniainsidiosa* , *Burkholderiacaryophylli*and *E.coli* [53].There another type of interactioninvolved in multispecies biofilm formation that is neutral state interaction . Example - *Salmonella typhimurium* , *L. monocytogenes*and *Pectobacteriumcarotovorum*did not show any competition with *A. hydrophila,* but is all 3 bacteria show neutral interaction in multispecies biofilm with *A. hydrophila*[54].

Table 2 : Examples of some microbial interaction .

Multispecies biofilms	Surfaces for biofilm formation	Interaction type	Reference
A. hydrophila and *L. monocytogenes*	Crab coupons	Neutral	54
L. monocytogenes and *B. cereus*	Stainless steel coupons	Competitive	55
Listeria innocua , *C. tropicalis* and *Candida Krusei*	Stainless steel coupons	Cooperative	56
E.coli and *L. monocytogenes*	Stainless steel and American	Neutral	57
Salmonella typhimurium and *Hafnia alvei*	96- well round bottom microtiter plates	Competitive	58

Other factor-

Factor such as antibacterial agents , environmental condition , colonization surface, substrate etc.has animportant role in formation of biofilm . Example - When there is whey protein as substrate in dairy industry *Enterococcus faecalis*found as dominant organism in multispecies biofilm but pathogens suchas *Bacillus cereus* , *L. monocytogenes*will not produce biofilm when there is whey protein present as substrate [59]. Some organisms such as *Bacillus* produce bacteriocins ,lipopeptideetcwhich act as antimicrobial agent to fungi .Horizontal gene transfer is another key of multispecies biofilm formation . The movement of genetic material between microorganisms occur between microbial community . Transfer of a resisntce gene tetM from *L. monocytogenes*to *E. faecalis*occur in cheese production industry[60].

The combine affect of QS, EPS , environment factors and microbial interactions are responsible for the formation of multispecies biofilm in food industry . And the biofilm regulated gene are the main playerwhich are responsible for control of QS, motility, metabolism, EPS production and antibacterial substance production .

Effects Of Multispecies Biofilms In Food Industry

In food industries fermentation is carried out with the of microorganisms , this microorganisms coexist as multispecies biofilm. But interaction of different microorganisms can also led to interaction with pathogenic bacteria which can led to destruction of quality and safety of food product . So the problem associated with biofilms in food industry are discussed bellow-

Resistance nature of multispecies biofilm against various disinfectants –

Now days many organisms are resistant to disinfectants , this resistance against disinfection create lot of problems in food industries and the mechanisms for resistance against some bacteria is the new topic of research .The microorganisms are showing resistance against quaternary ammonium compounds (QAC), hydrogen peroxide, sodium hypochlorite, benzalkonium chloride etc(Table 3).

Table 3 : Example of multispecies biofilm that are resistance to disinfectant .

Microorganisms in multispecies biofilm	Disinfectants	Surfaces for biofilm formation	Reference
Pseudomonas aeruginosa and *Listeria innocua*	QAC, tertiary alkylamine and chlorine based	Polycarbonate coupons	61
Salmonella Enteritidis and *P. aeruginosa*	Chlorine	Stainless steel coupons	62
Pseudomonas libanensis and *A. hydrophila*	Hydrogen peroxide , peracetic acid and sodium hypochlorite	Stainless steel coupons	63
Staphylococcus aureus , *Aeromonas spp.* , *Lactococcus lactis* , *C. tropicalis* , and *Lactobacillus spp.*	Peracetic acid	Stainless steel coupons	64
Escherichia coli and *Enterococcus faecalis*	Hydrogen peroxide	8-well glass bottom slides	65
Species isolated from brewery contaminated biofilms (*Pseudomonas* , *Raoultella*)	Sulfathiazole	96-well plates	66

One example, the multispecies biofilm formed by the presence of *L. monocytogenes* and *P. fluorescens*can protect *L. monocytogenes*against various disinfectant [67]. Another multispecies biofilm formed in stainless steel with the combination of *L. monocytogenes*and *Staphylococcus aureus* show high number of *Staphylococcus aureus* in the presence of peracetic acid so presence of one organisms in multispecies biofilm can protect another organisms in food industry[68].

Some time the interaction impact positively in efficiency of disinfectant,

*Acinetobactercalcoaceticus*and*Stenotrophomonasmaltophilia*formed multispecies biofilm that increase the efficiency of sodium hypochlorite when growing in polyvinyl chloride surface(69).

EPS can act as a barrier and can resist the penetration of disinfectant and can lead to resistance of disinfectant and age of biofilm can also lead to disinfectant resistant due to different rate of production of metabolites with the age of biofilm. So multispecies biofilm formed in food industry may increase or decrease the efficiency of disinfectant.*P. fluorescens and S. aures*produce multispecies biofilm in this case the interaction between both the species can increase the production of EPS and this EPS increase the resistance nature of this duel-species biofilm [70].

Age of biofilm is another phenomena for resistance against multispecies biofilm . Mature biofilms can adjust with stress condition because it produce strong three- dimensional structure and this structure block the penetration of disinfectant and act as a physical barrier .Example such as *P. fluorescens*and *L. monocytogenes*multispecies biofilm . In this case *P. fluorescens*pre-existing biofilm on stainless steel can make resistant*of L. monocytogenes*to disinfectant by decreasing the rate of transfer of *L. monocytogenes* on salmon slice[71].

Type of stains also affect the resistance nature of biofilm such as the biofilm formed with the combination of *E.coli* and *Enterobacterfaecalis* . In this case the survival rate of *Enterobacterfaecalis* is increased because in biofilm *Enterobacterfaecalis* cells are covered *with E.coli* cell [72].

Pathogens associated with multispecies biofilm -

Another great problem associated with the multispecies biofilm in food industries is the food-born diseases cause by intoxications or infections caused by food born pathogens .

*Listeriamonocytogenes*associated with multispecies biofilm may cause listeriosis and this organism can grow under high salt conditions or high temperature or refrigeration temperature [73].

Staphylococcus aureus contain icaA gene which lead to biofilm formation by increasing the production of EPS. This organisms can associated in multispecies biofilm with *Aeromonas spp.* , *Lactobacillus lactis and C. tropicalis*[74]can leads to emergence of methicillin-resistance *Staphylococcus aureus* in animal derived food [75].*Salmonella* species can create multispecies biofilms in food production and processing settings, contributing to foodborne outbreaks(76).Certain species of *Vibri*o, such as Vibrio *parahaemolyticus* and *Vibrio vulnificus*, can form biofilms and pose a risk to seafood and other marine-based food products(77).
In 2016, in United States 241994 cases of food poisoning were reported[78].

In below some pathogenic organisms growth were compared and it was found that single species pathogenic bacteria formed more biofilms than mixed species biofilm but they from biofilm in mixed species condition also and leads to drug resistance [79]. By figure 4 and figure 5 it was shown that the growth of *Salmonella agona* biofilm formation rate is lower in the mono-species biofilm formation time . But in case of other pathogens like *Listeria monocytogenes* , *MRSA* , *Enterococcus faecium* the biofilm formation rate is higher in case of mono-speciesbiofilm.

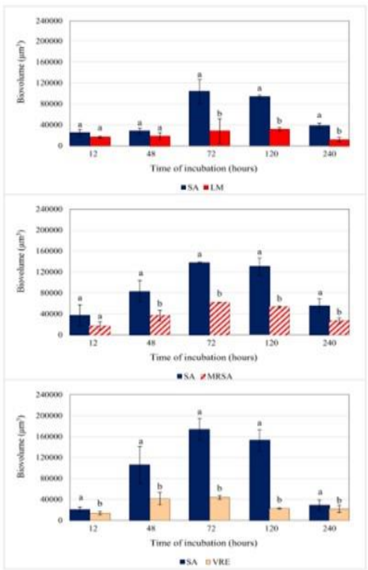


Figure 6 : Biovolume of pathogenic multispecies biofilms on polystyrene after incubation .
SA- *Salmonella enterica* serotype Agona , MRSA - Methicillin-resistant *Staphylococcus aureus* , LM- *Listeria monocytogenes*, VRE- vancomycin-resistant *Enterococcus faecium*[79].

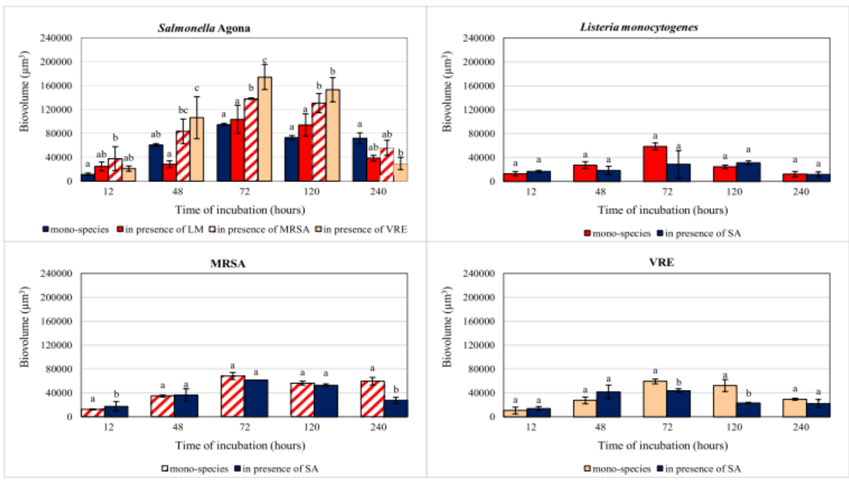


Figure 7: Comparison between mono-species biofilm and multispecies biofilms [79].

So pathogenic organisms associated with food product by multispecies biofilm cause a difficult problem in food sector[79].

Spoilage of food –

Biofilm-associated microorganisms can produce enzymes and metabolites that contribute to food spoilage For. example, enzymes like proteases, lipases, and amylases can degrade proteins, lipids, and carbohydrates present in food, leading to undesirable changes such as texture deterioration, off-flavors, and odor. Metabolites produced by biofilm microorganisms, such as organic acids and volatile compounds, can also contribute to food spoilage.

Teix Biofilm-associated microorganisms can produce enzymes and metabolites that contribute to food spoilage. For example, enzymes like proteases, lipases, and amylases can degrade proteins, lipids, and carbohydrates present in food, leading to undesirable changes such as texture deterioration, off-flavors, and odor. Metabolites produced by biofilm microorganisms, such as organic acids and volatile compounds, can also contribute to food spoilage.Teixeira et al. (2005) isolated bacteria from rubber tubes in milking equipment, even after standard washing procedures. The isolates included potential spoilage organisms and pathogens. The hydrophobic nature of rubber encourages bacterial attachment, and biofilm formation provides protection from cleaning solutions. As rubber ages with repeated cleaning cycles, it can become cracked, providing physical protection for the biofilm [80].

Some examples of multispecies biofilms commonly associated with food spoilage are given below:

Yeast biofilms on the surfaces of brine tanks in cheese manufacturing plants present a persistent source of contamination that is challenging to control. The yeast species found in brines are similar to those in the cheese rind. Biofilms in brine tanks can become extensive, even visible at the air-liquid interface on tank walls[81].

Lactic acid bacteria (LAB) and yeasts: In fermented dairy products like yogurt and kefir, multispecies biofilms consisting of LAB (such as *Lactobacillus spp*. and *Streptococcus spp.)* and yeasts (such as *Saccharomyces spp*.) can lead to flavor changes, gas production, and textural defects[82].

Pseudomonas spp., Acinetobacter spp., and molds: These multispecies biofilms are frequently found in fresh produce, such as fruits and vegetables. *Pseudomonas spp*. and *Acinetobacter spp.* can degrade organic matter, while molds contribute to visible spoilage and mycotoxin production[83].

Enterobacteriaceae and molds: In bakery products like bread and cakes, multispecies biofilms composed of Enterobacteriaceae (e.g., *Enterobacter spp., Citrobacter spp*.) and molds can cause spoilage by degrading sugars, starches, and fats, leading to off-flavors, mold growth, and texture changes[84].

Pseudomonas spp. and*Brochothrixthermosphacta*: This combination is commonly associated with spoilage in meat and poultry products. *Pseudomonas spp*. produce enzymes that degrade proteins and lipids, while Brochothrixthermosphacta contributes to off-odors and slime production[85].

Alicyclobacillus spp. and yeasts: In fruit juices, multispecies biofilms containing *Alicyclobacillus spp*. (acidophilic bacteria) and yeasts can cause spoilage by producing off-flavors, cloudiness, and gas production, impacting the sensory attributes of the product[86].

Pseudomonas fluorescensand Shewanella spp.: Found in seafood products, such as fish and shellfish, this multispecies biofilm combination can contribute to spoilage through protein and lipid degradation, off-odors, and slime production[87].

Lactobacillus spp., Leuconostoc spp., and molds: In sauerkraut and other fermented vegetables, multispecies biofilms consisting of lactic acid bacteria (*Lactobacillus spp.* and *Leuconostoc spp*.) and molds can cause spoilage by altering the pH, texture, and flavor of the product[88].

Bacillus spp., Clostridium spp., and molds: Multispecies biofilms involving spore-forming bacteria *like Bacillus spp*. and *Clostridium spp*., along with molds, can contribute to spoilage in canned food products. These biofilms can produce gas, off-flavors, and visible signs of spoilage due to their metabolic activities[89].

Listeria monocytogenes and various microorganisms: Listeria monocytogenes, a pathogenic bacterium, can form multispecies biofilms with other microorganisms like Pseudomonas spp., Enterococcus spp., and Lactobacillus spp. in ready-to-eat foods, leading to enhanced survival and potential food safety risks.*Listeriamonocytogenes* and *Pseudomonas fluorescens* combination poses a significant risk in ready-to-eat foods, such as deli meats and salads. *Listeria monocytogenes* is a pathogenic bacterium, while *Pseudomonas fluorescens* can enhance its survival and growth by providing a protective environment within the biofilm[90].

Candida spp. and Debaryomyces spp.: This dual-species biofilm is associated with spoilage in fruit and vegetable products, including juices and purees. *Candida spp*. and *Debaryomyces spp.* produce enzymes that degrade sugars and organic acids, leading to fermentation and off-flavors(91).

These examples demonstrate the diverse range of multispecies biofilms that can impact food spoilage in the industry. Understanding their composition and the mechanisms by which they cause spoilage is crucial for implementing effective control measures and maintaining product quality and safety.

Flow Of Pipe -

The presence of multispecies biofilms in pipes used in the food industry can indeed hamper the flow rate of liquids or fluids. Biofilms can accumulate within the pipe, causing a reduction in the effective diameter and increasing the surface roughness, which in turn leads to flow restrictions and pressure drops. Here's an example to illustrate this:

Example: Dairy Processing

In dairy processing, the formation of multispecies biofilms within milk processing pipelines can impact the flow rate. For instance, biofilms composed of bacteria, yeasts, and other microorganisms can attach to the inner surfaces of the pipes over time. As these biofilms grow and mature, they can create a rough, uneven surface, reducing the effective diameter of the pipe and causing frictional resistance to the flow of milk.

The biofilm's presence can lead to increased pressure drop and decreased flow rate, affecting the efficiency of milk processing operations. This can have implications for production capacity, as well as the quality and consistency of the dairy products being processed.

Starter Culture Contamination -

The growth of starter cultures associated with fermented foods can be hampered by multispecies biofilms due to the secretion of metabolites by the biofilm microorganisms. These metabolites can have inhibitory or competitive effects on the growth and activity of the starter cultures. For example, multispecies biofilm of lactic acid bacteria, including *Leuconostoc spp. and Lactobacillus spp*., secretes metabolites such as lactic acid and other organic acids, which create an unfavorable environment for the growth of potential spoilage microorganisms or pathogens. The acidic conditions limit the growth of spoilage bacteria and help preserve the fermented vegetables like this way starter culture growth may be also hamper due to multispecies biofilm[92] .

Biofilms of non-starter lactic acid bacteria (NSLAB), specifically *Lactobacillus spp*., have been found to survive routine cleaning in cheese manufacturing plants. These biofilms can contaminate subsequent manufacturing runs, affecting the quality and consistency of cheese. The presence of NSLAB in cheese is associated with poor hygiene and can influence the flavor of the product [93].

During the fermentation of black olives, a yeast-rich biofilm develops on the epicuticular wax of the olive skin. This biofilm contributes to the production of organic acids. Controlling the biofilm is crucial for maintaining the quality of the product. In this case, the development of a biofilm including yeast is desirable [94].

Other problems –

Contamination of Equipment: Biofilms can contaminate processing equipment, leading to cross-contamination and compromising product safety.Example: Biofilms formed by Listeria monocytogenes and other bacteria in dairy processing equipment[95].

Flavor Alterations: Multispecies biofilms can produce metabolites that impact the flavor profile of food products.Example: Biofilms of lactic acid bacteria and yeasts in fermented dairy products like yogurt and kefir[96]

Biofilm-Induced Corrosion: Biofilms can promote corrosion of metal surfaces, leading to equipment damage and contamination.Example: Biofilms formed by sulfate-reducing bacteria in food processing pipelines[97].

Allergen Cross-Contamination: Biofilms can serve as reservoirs for allergenic proteins, leading to cross-contamination between food products.Example: Biofilms containing allergenic proteins in shared processing equipment in the nut processing industry[98].

Biofilm Controlling Methods In Food Industry Multispecies

Computerised method development is necessary for monitoring the adhesion, growth and removal of biofilm from surfaces in an industrial environment reduces the cost of cleaning operations and minimizes production downtime for maintenance work.

An emerging approaches for biofilms detection, including metagenomics and metatranscriptomics, may shed light on the complex interactions within the biofilm community[99]. For example, the subspecies of *S.aureus* can be classified using MLVA in food industry product samples. This method uses PCR amplification of different *S.aureus* loci showing different tandem repeats (sdr,clfAclfG,ssp,spa) and gel electrophoresis to differentiate detected genotypes. These genotype show different length in the amplified PCR fragments[100][101].

The common detection methods such as agar-agar, qRT-PCR or more specific DNA amplifications are not effective at industrial level due to the previously described presence of VBNC cells in some biofilms (when plated on agar) and the high cost of reagents and equipment (for qRT-PCR and other DNA amplifications)[102]. So the approaches can help in the removal of multispecies biofilm after detection of multispecies biofilm with modern appeoches such as metagenomics are given below -

Surface blocking –

One of the simplest strategies to avoid initial colonization by competing stains is to quickly occupy all available adhesion sites, called "surface coatings". This model can shown by the model of competition experiments between *Pseudomonas aeruginosa* and *Agarobacteriumtumefaciens* . In an experimental mixed-species coculture model , *P.aeruginosa* spread rapidely on the surface due to swarm motility and preventing the attachment of *A.tumefciens*[103]. Like this way in food industries stater culture can be used to block the surface of instruments to prevent the formation of pathogenic organisms biofilms .

Other than this steel coating with nanoparticles including silver, gold, metal oxides(Zno) etc. can be used against biofilm in food industry[104].

Another antimicrobial agent that is silver compounds that can used for surface blocking .One example of surface blocking agent is Ni-P-polytetrafluroethylene which used for coating of stainless steel , this stainless steel is used in many component such as fermenter in used in food industry . And this Ni-P-polytetrafluroethylene is also reduce biofilm formed by

*Bacillus licheniformis and Geobacillusstearothermophilus*and it is also effective against milk deposition in surface [105].

Chemical Treatments –

Different type of chemical sanitizer can be used for biofilm removal in food industry, this chemical sanitizer my be concentration-dependent or time dependent . The main aim of this sanitization process is to reduce number of microorganisms to that levels that are safe for human consumption [106] .

Disinfectants that are chlorine-based are used mostly in food industry but now days many microorganisms are resistance to chlorine treatments such as *S.enterica* are chlorine resistance and it also produce cellulose in stress condition found in food industry[107]. Also *Enterococcus* and *Salmonella* can enter into a VBNC state after chlorine treatment[108].

So alternatively aqueous chlorine dioxide can be used in food industry and chlorine dioxide is more effective against *Bacillus cereus* a major pathogen related to food industry [109]. Chlorine dioxide is also effective against *E.coli* 0157:H7[110].

Hydrogen peroxide react with biofilm structure and knock out the biofilm in concentration of (0.08-5)% without any toxic side effects[111], so it can effectively use in food industry as potent oxidizing disinfectant .

In dairy industry ozone a toxic gas used to prevent the growth of mold on stainless steel , it also knock out different kind of biofilms, viruses, and protozoans by breaking down the organisms cellular envelopes[112].

Other chemicals like salicylate-based polyanhydride ester, interfere and disturbed biofilm formation. This salicylate-based polyanhydride ester can disturb the initial stage of biofilm formation of *S.enterica*[113].Quaternary ammonium compounds also used for removal of biofilms by disruption of bacterial cell membrane causing bacterial cell lysis[114].Some organisms are also resistant to quaternary ammonium compound , example some stains

*L. monocytogenes*isolated from food contain genes such as qacH and bcrABC responsible for resistance against quaternary ammonium compounds .

Enzymatic Disruption

Enzymes can be used against biofilm because it has biodegradable capacity and enzymes has also low toxicity. Biofilm are mainly composed of proteins and polysaccharides, so proteases (example proteinase K , pepsin etc.) and glycosidases (example – amylases , pectinase etc.) are mainly used in food industries [115].

Enzymes such as amylases ,cellulases, lyases, glycosidases and DNAses are also used in food industries against biofilm[116]. Alpha-amylase can take as an example , this alpha-amylase can effectively degrade *Staphylococcus aureus* biofilm[117].

Among all above mentioned enzymes proteases used in most of the food industry because proteases have lower substrate specificity[115]. After partly removable of biofilm by proteases biofilm can easily removed by mechanical treatment process with the action of sanitizer. An example is mix proteolytic enzymes with ultrasonic waves combination can remove upto 96% of *E.coli* biofilms [118].

But enzyme disruption on multispecies biofilm is challenging method for controlling multispecies biofilm is due to the substrate specificity nature of biofilm.

Essential oils –

Essential oil are bacteriostatic in nature , this are non-toxic and have a broad-spectrum antimicrobial activity . This are the mainly derivative of terpenes, oxygenated derivative, terpenoids etc.

Some of these essential oils have biofilm resistance properties. For example, the *S. aureus* biofilm on steel in 24 h was reduced from 10^7CFU/mL to 10^3 CFU/mL by using cinnamon essential oil microemulsions (which are rich in cinnamaldehyde) at 2.5% in medium. TSB for a period of 90 minutes. CFUs were similarly reduced with 5% microemulsion of Salvia officinalis essential oil, rich in thujone, camphor and pinene[119].

The biofilms of three important gram-negative pathogens, *S.enterica, E. coli* and *P. aeruginosa*, were also reduced by up to 80% using a 50 µg/mL extract of the Asian herbal medicines Holarrhenaantidysenterica and Andrographis[120].

Among all of this phenol is most effective followed by aldehydes and ketones. One example is *Murrayakoenigii*, it is effective against biofilms that are associated with *Pseudomonas aeruginosa* [121].

Biosurfactants And Bacteriophages-

This biosurfactant are natural compounds that can change adhesion properties in biofilm formation .

One example is lichenysin . It is non-ribosomal cyclic lipopeptide formed by *Bacillus licheniformis* . Food industries can treated with biosurfactants can reduce binding of MRSA , *C.jejuni*[122].

Fengycin, surfactin are example of another lipopeptides produced by *B.amyloliquefaciens*.

All this compound decrease surface tension and alter the binding capacity and reduce biofilm formation . These biosurfactants ultimately effect the membrane permeability and cause death of cell by swelling .

Bacteriophages and biosurfactants are also used against biofilm [123].*Listeria* phage P100 are used in processed meat products industry for removal of biofilm in working surface [124].

There is limitation with the phage therapy against multispecies biofilm that is ability of phage to target a specific bacteria in a mixed biofilm due to presence of EPS covering of biofilm . But there are some phage which can produce exopolysaccharide depolymerases can over come this problem [125].

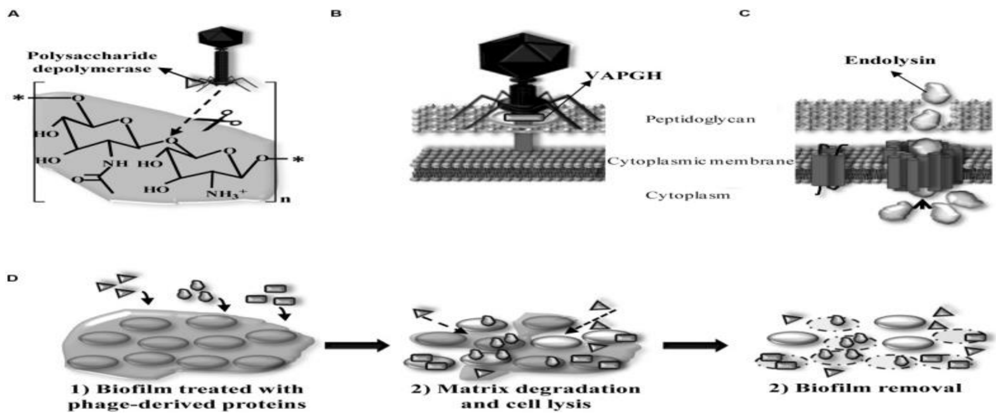
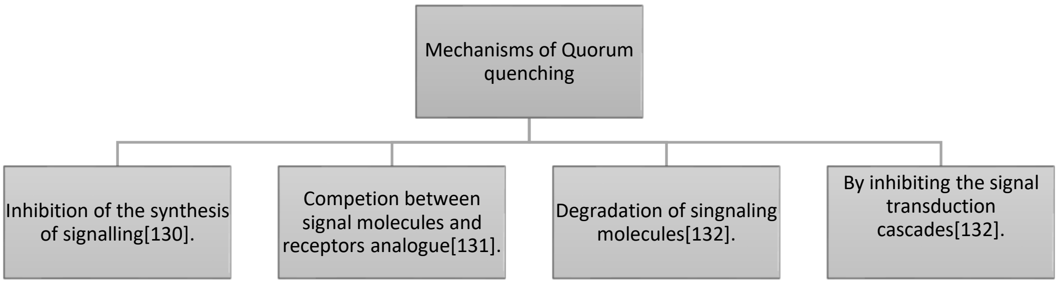


Figure 8: Phage therapy of biofilm . {(A) Location of the exopolysaccharide depolymerase (PIA/PNAG) degrading β-(1,6) bonds of EPS of bioflim formed by staphylococci species and mode of action of phage . (B) Location of virion-associated peptidoglycan hydrolase (VAPGH) at the phage particle and its role in infection. (C) Cell wall structure of Gram-positive bacteria and the role of endolysin in bacterial lysis. (D) Activity of phage-derived proteins when added exogenously and their application as anti-biofilm agents that degrade polysaccharide depolymerase and bacterial lysates (VAPGH and endolysins).} [126].

Quorum quenching as control method of biofilm –

The process of blocking the system of quorum sensing by the inhibition of production of virulence factors is know as quorum quenching. This method of quorum quenching can be used in food industry to kill spoilage or pathogenic bacterial biofilm .

Quorum sensing is how species perceive cell density and adjust gene expression accordingly. There is growing evidence that many microorganisms can sense specific QS signaling molecules, enabling them to recognize and react with other microorganisms in the vicinity[127]. Gram-positive bacteria use oligopeptides as signaling molecules to from biofilms and utilize QS for intraspecific communication [128] . The difficult elimination of biofilms and the increasing resistance of antibiotics require the search for new wats to combat unwanted microorganisms. A promising strategy is aimed at the QS scheme. There are many compounds in the environment that affect communication between microbes[128]. Based on their molecular weight and chemical composition, these compounds belong to one of the groups: macromolecular QQ enzyme andmicromolecular QS inhibitors [129].



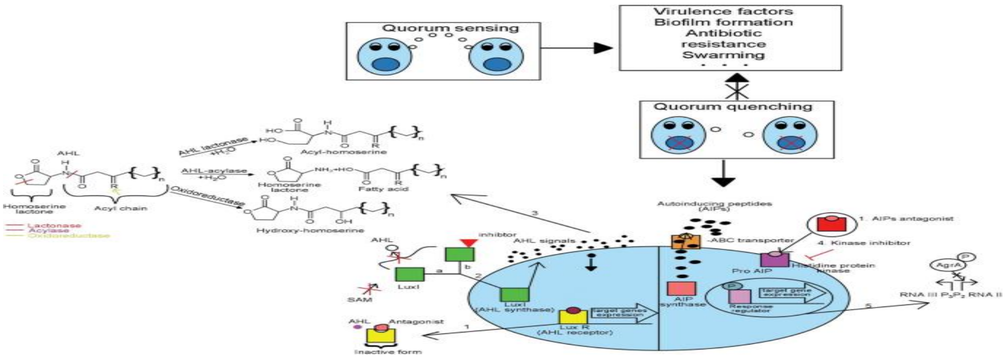
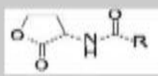
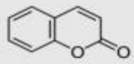
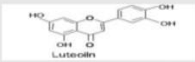
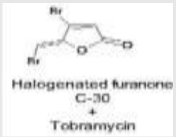


Figure 9 : Mechanisms of quorum quenching[133].

Mechanisms of quorum-sensing blocking in gram-negative and gram-positive bacteria. In many bacteria, cell to cell communication is responsible for the production of various virulence factors. Disruption of quorum recognition inhibits the production of virulence factors. QS systems differ for gram negative and gram positive bacteria. In Gram-negative bacteria, AHL plays the role of autoinducers synthesized by LuxI like enzyme. These molecules penetrate the membrane of the bacterial cell and above a certain concentration threshold the receptor protein LuxR is activated and the target effector gene are transcribed. The signaling molecules of gram-positive bacteria are AIP. They are synthesized in the form of peptides and after modification are exported from the cell by the ABC, ATP binding cassette transport system. Upon reaching a threshold concentration in the environment, self-inducing molecules bind with sensor proteins .The kinase is activated by phosphorylation. The phosphate group is transferred to a transcriptional regulator, resulting in activation of a transcriptional regulator, resulting in activation of target genes mechanisms that interfere in the QS cascades are indicated by numbers in the diagram given above: 1-use of inducing antagonists,2-inhibition of LuxI,3-enzymatic degradation of AHL molecules,4-inhibition of histidine protein kinase activation by a kinase inhibitor,5-by signal transduction cascade blocking.

Table 4 : Example of some quorum quenching molecules.

NAME OF QURUM QUENCHING MOLECULE	SOURCE	SITE OF FUNCTION	PROCESS OF FUNCTION	STRUCTURE
Mon L lactonase	Breastmilk	QS molecule	Degrade of signalling molecules	
Coumarin Norspirmidine	Centellaasiatica Terminalia bellirica extract	Attachment	-Flagella production -EPS production -Swarming(Reduce the attachment) Etc.	
Terminalia bellirica extract	Terminalia bellirica or Bhibhitaki	Microcolony	Inhibit the production of EPS	....
Luteoiln	Vegetable such as celery, broccoli, onion leaves etc.	Mature biofilm	Weaking biofilm, architecture quorum sensing jamming	
Cinnamaldehyde furanone	Cinnamon tree	Mature biofilm	Same as luteolin	
Halogenated furanose є 30+ Tobramycin	Actinomycetes, Streptomyces tenebraius.	Dispersion	Disruption of biofilm architecture	

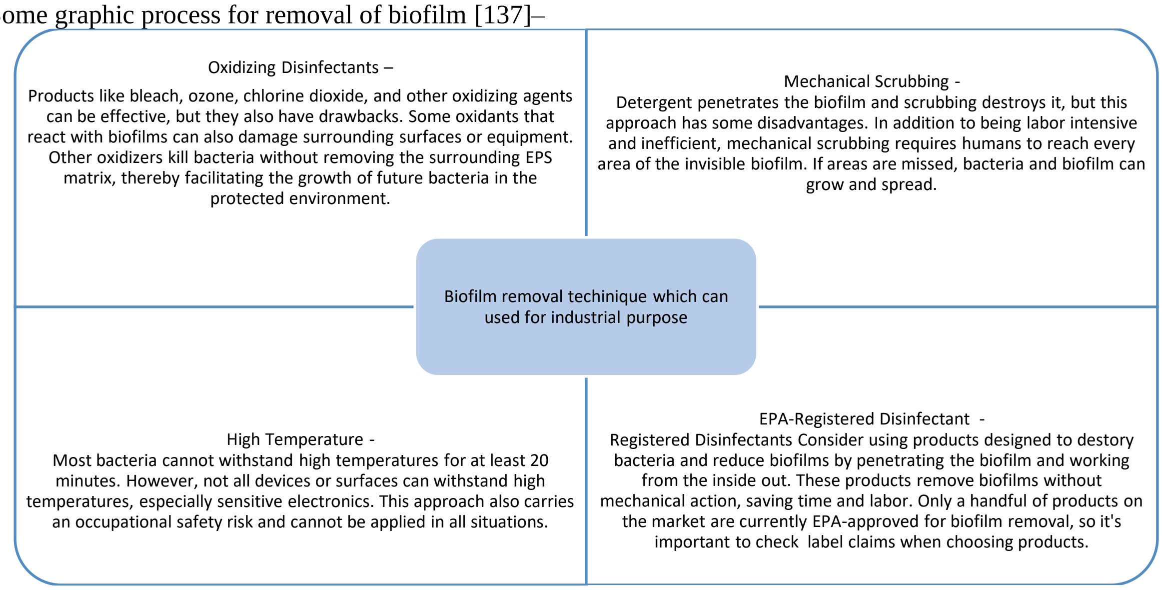
Other mechanisms that are responsible for removal of biofilm -

High hydrostatic pressure is another way of controlling the biofilm . But HHP should be use with the combination with thermal treatments (50°C to 60°C) because HHP technology is not effective against endospore (*B.cereus* spores) [134].

Various types of nanoparticles exhibit photocatalytic properties, where absorption of a specific wavelength is used to induce (accelerate) chemical reactions, including the destruction of cells. usually by generating reactive oxygen species (ROS). In this sense, TiO2 NPs, containing 1%Fe and N, structured as a thin layer on the polystyrene surface, exhibited inactivation of bacterial cells (*E. coli*, *Enterococcus faecalis*, *P. aeruginosa*, *S. aureus*) after sun exposure .When exposed to visible light and especially to ultraviolet light, these NPs also showed antibiotic activity in the case of *E. coli*, *P.aeruginosa* and *S. aureus* bacteria, which had inhibitory value. from 2 to 32 μg/mL[135].

Partially ionized gas with low temperature and interesting antimicrobial properties is known as Non-thermal plasma .It is able to destroy bacterial biofilms of Gram-negative (*S.enterica*, *Pseudomonas spp*) or Gram-positive (*Bacillus spp*.) species in just 10 min . However, its use is still restricted to some laboratory applications, due to its high cost [136] .

Some graphic process for removal of biofilm [137]–



II. Conclusion

Multispecies biofilms in the food industry present a significant challenge to food safety and quality control. Regular monitoring, thorough cleaning and sanitation, surface modifications, and enhanced preservation techniques are crucial in preventing the formation and accumulation of biofilms. Additionally, exploring innovative approaches like quorum quenching can provide new avenues for controlling and mitigating the impact of multispecies biofilms. By understanding the complexities of biofilm formation and implementing appropriate control strategies, the food industry can ensure the production of safe and high-quality food products.Hurdle technology can also be used for control purposes of multispecies biofilm.

To mitigate the multispecies biofilms in the food industry, it is crucial to implement effective control strategies this process can be followed to prevent multispecies biofilm -

- Regular cleaning and sanitation: Establishing thorough cleaning and sanitation protocols targeted specifically at biofilm removal can help prevent the formation and accumulation of multispecies biofilms on food contact surfaces.
- Monitoring and testing: Implementing regular monitoring and testing procedures can aid in early detection of biofilms and spoilage-causing microorganisms. Advanced techniques, such as DNA-based microbial detection or imaging technologies, can assist in identifying and characterizing multispecies biofilms.
- Surface modifications: Using antimicrobial surfaces or coatings that prevent biofilm formation can be effective in reducing spoilage. These modifications can create inhospitable conditions for microbial attachment and growth.
- Enhanced preservation techniques: Employing appropriate preservation methods, such as refrigeration, modified atmosphere packaging, or natural antimicrobial agents, can help control microbial growth and extend the shelf life of food products.

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