



Review Article

Biofilm: An Alarming Niche in Dairy Industry

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Rec. Date:	Feb 07, 2019 06:15
Accept Date:	Mar 04, 2019 06:35
DOI	10.5455/ijlr.20190207061546

Abstract

Biofilm is an aggregation of microbial cells interconnected by extracellular polymeric substances which accelerate growth on different material surfaces adversely affect the dairy industry. This polymicrobial community contains altered phenotype which differs them from planktonic microbes physiologically. It affects the quality and safety of raw materials and their products. Thus, producing serious problems directly affecting human health. Colonisation at the surfaces of open and closed piping systems, floors, waste, walls, ceilings of the production halls play a major role as barrier in the selection of effective sanitation agents for their control. As a result, the sector is forced to face a havoc economical loss. In fact, the condition is aggravating day-by-day. As microbes growing in a biofilm are highly resistant to antimicrobial agents and host's immune system, it is obligatory to employ effective methods to retard the biofilm formation. Therefore, immediate appropriate precautionary measures should be adopted to combat the condition and prevent further complications.

Key words: Biofilm, Biofilm Formation, *Bacillus*, *Pseudomonas*, *Listeria*, *Streptococcus*, *Lactobacillus*, *E. coli*, Prevention and Control of Biofilm

How to cite: Parul, S., Basak, G., Sharma, B., Jain, U., Mishra, R., & Vaishali (2019). Biofilm- An Alarming Niche in Dairy Industry. International Journal of Livestock Research, 9(4), 10-24. doi: 10.5455/ijlr.20190207061546

Introduction

The existence of biofilm has been explored for several years in the food industry. The first documentation was done roughly 75 years back in 1943 (Zobell, 1943). Biofilms are sessile microbial communities where microbes live together in association with each other on biotic or abiotic substrates which are bounded by extracellular polysaccharides, proteins, lipids and DNA (Simões *et al.*, 2010). In other words, simply, biofilms represent an important mode of bacterial life colonizing most of the surfaces in nature. Wet solid



surfaces promote biofilm production as the biotic and/or abiotic materials present in the liquid settle over the solid counterpart and attract active microbes to form biofilms (Mu *et al.*, 2014). Wet exteriors are generally covered with extracellular matrix which further aid the attachment of microbes (Bardiau *et al.*, 2016). This matrix enhances microbial survival and even protects them from antimicrobial agents (Chen *et al.*, 2007). Thus, biofilms act as a crucial defensive biota where microbes possess a secure live, multiply invariably and able to yield thermal-resistant enzymes and spores (Felipe *et al.*, 2017).

Characteristics of Biofilm

Biofilms are complex, dynamic and remarkably heterogeneous structures. Different biofilms exhibit different chemical and electrical properties. Moreover the genetic expression is also different in biofilm bacteria as compared to the planktonic bacteria (Costerton *et al.*, 1999; Fox *et al.*, 2005). The cells are able to coordinate among each other via intercellular communication using biochemical signalling molecules (Flemming *et al.*, 2010). Besides, these are generally impervious to nutritional and oxidative stresses, desiccation, UV light exposure and sanitizing agents (Fatemi and Frank, 1999). Stainless steel, polyvinyl chloride, polyurethane are the prolific surfaces for biofilm attachment when they come in contact with food materials (Mustapha and Liewen, 1989; Midelet and Carpentier, 2002). The biofilm associated microbes are also much less susceptible to antimicrobial agents than those present in planktonic state. As a result, it becomes difficult to get rid of biofilms from the contact surfaces of food (Simões *et al.*, 2006).

Biofilms endorse intraspecies and/or interspecies interaction and some of them may be formed by aggregation of many bacterial genres and are known as mixed biofilms (Simões *et al.*, 2010). A wide variety of foodborne pathogens are also able to attach, colonize and form biofilms, such as the O157 and non-O157 Shiga toxin-producing *E. coli* (STEC), *S. enterica*, *L. monocytogenes*, etc. (Wang, 2019).

Vulnerable Zones of Biofilms Formation

Almost all branches of food industry are affected with it. But it is of utmost concern especially in food processing plants and considerably in dairy industry (Frank *et al.*, 2003; Jessen and Lammert, 2003). In dairy plant, biofilms can develop on floors, walls, drains, interior and exterior of dairy equipment and machines, transporting pipes and even working surfaces are prone to biofilm formation (Krysinski *et al.*, 1992; Hood and Zottola, 1997; Gibson *et al.*, 1999; Sinde and Carballo, 2000; Verran, 2002). These can be seen in almost all product contact surfaces like milk cups at dairy farms to heat exchangers at the processing plants. This poses a great risk to safety, security and quality of dairy products and to the health of consumers who get exposed to such products (Flint *et al.*, 1997; Scheldeman *et al.*, 2005; Burgess *et al.*, 2010., Bayoumi *et al.*, 2012).



Process of Biofilm Formation

The formation process of biofilms by various microbes undergoes a number of phases till its maturation (Stoodley *et al.*, 2002; Breyers and Ratner, 2004; Johnston, 2004). Thus, for accomplishment of the scenario it gradually passes through different steps, viz. 1. Attachment process (reversible and irreversible) 2. Biofilm polymer/microcolony formation 3. Maturation and replication and 4. Cell dispersion or detachment. Under these major steps, the following sequential steps are also involved in biofilm formation. These include- a) substratum pre-conditioning by ambient macromolecule; b) cell deposition; c) cell adsorption; d) desorption; e) cell-to-cell signalling and onset of exopolymer production; f) convective and diffusive transport of O₂ and nutrients; g) replication and growth; h) secretion of polysaccharide matrix, and i) detachment, erosion and sloughing (Simoes *et al.*, 2010).

The process begins with the adhesion of cells to the substratum and this adhesion to the adjoining surfaces is a crucial factor for the entire process (Vieira *et al.*, 1993; Busscher *et al.*, 1995; Donlan., 2002; Chae *et al.*, 2006; Palmer *et al.*, 2007; Patel *et al.*, 2007; Oulahal *et al.*, 2008;). The cell organelles like outer membrane proteins, capsular polysaccharide, lipopolysaccharides, curli, pilli, fimbriae, prosthecae, stalks and flagella create impact on cell charge and hydrophobicity as well as guide the adhesion during biofilm formation (Morris *et al.*, 1997; Harbron and Kent, 1988; Sauer and Camper, 2001; Daniels *et al.*, 2004; De Rezende *et al.*, 2005). Hence, this preliminary conjunction is reversible as the bacterial interaction is weak and the attachment entails numerous morphological changes which are essential for biofilm formation as a result, there lies a possibility of detachment of many cells (Terraf *et al.*, 2012). Thereafter the attachment turns to irreversible one and the bonding is permanent due to exopolysaccharides (EPS) secretion and subsequently the microcolonies form and expand on the surface (Stoodley *et al.*, 2002). These microcolonies form by the cell-aggregation which in turn occurs through growth of microbes, enabling EPS production (Chmielewski and Frank, 2006). Microbial cells remain entrenched within the EPS matrix in multiple layers (cohesion) which provide nutrition for them. A bacterial EPS encompasses polysaccharide, proteins, lipids, nucleic acid, phosphor lipids and humic substances and may also carry the water channels (Jahn and Nielsen *et al.*, 1998). Thus, EPS proves to be a boon for microbes as it provides more resistance within a biofilm (Davies *et al.*, 1998; Sauer and Camper, 2001; Parsek and Greenberg, 2005). Besides, the microcolony aids substrate exchange within species and removes their end-products (Costerton *et al.*, 1994). Progressively maturation and ordered construction occurs in a time period and ultimately detachment of cells occurs and the colonization at new areas crop up (Sauer *et al.*, 2002; Stoodley *et al.*, 2002).





Fig.1: Flow diagram showing the major steps of biofilm formation

Factors Affecting Biofilm Formation

Although the process of biofilm formation does not go so smooth every time. Sometimes it may influence by multiple factors like bacterial strains, pH, nutrient concentration or level, surface texture, surface hydrophobicity, temperature, speed of liquid stream, osmotic pressure etc which affect the biofilm creation (Donlan and Costerton, 2002; Donlan, 2002; Nilsson *et al.*, 2011). The degree of biofilm formation also varies highly with the inter-strain variation, increase in temperature, flow velocity or nutrient concentration. Though these factors may impart negative effect on the formation process if their critical levels are being exceeded (Stoodley *et al.*, 1999; Pan *et al.*, 2009). A pictorial presentation of factors which affect to biofilm formation is given below:

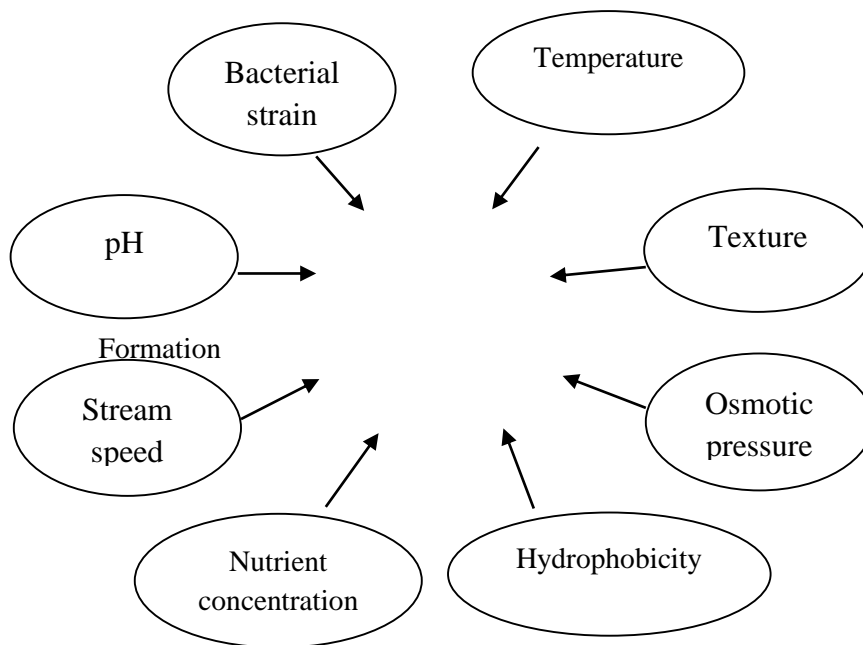


Fig. 2: Factors affecting biofilm formation

Biofilm Forming Microbes of Dairy Industry

Microorganisms occurring in the food industry could be a source of secondary contamination in food products. Their attachment to food contact surfaces in dairy plants and subsequent biofilm formation pose

a risk of contamination in milk and milk products (Flint *et al.*, 1997; Sharma & Anand, 2002). Formation of multispecies bacterial biofilms is took place by many bacterial genera like *Staphylococcus* and *Pseudomonas* which allow the adhesion of other significant pathogens like *Listeria monocytogenes* (Sasahara & Zottola, 1993). In such biofilms, these microorganisms express an increased resistance to environmental stresses and can persist on food processing equipment (Zottola & Sasahara, 1994; Campanac *et al.*, 2002). The other important biofilm forming genera of dairy industry are *Bacillus*, *Pseudomonas*, *Listeria*, *Lactobacillus*, *Staphylococcus*, *Streptococcus*, *Salmonella enterica* serovar Typhimurium and *Coronobacter sakazakii* etc. (Durango *et al.*, 2004; Seifu *et al.*, 2004; Kandhai *et al.*, 2010).

Bacillus

Among the different bacterial genera the *Bacillus* is the predominant bacteria of the dairy plants as present in raw and even pasteurized milk because of its capability to produce heat-resistant spores (Wilkinson and Davies, 1973; Meer *et al.*, 1991; Sharma and Anand, 2002; Ranieri *et al.*, 2009; Shaheen *et al.*, 2010). Within the *Bacillus* species, *B. subtilis* is the classical one which is able to form vigorous biofilms in dairy industry (Chu *et al.*, 2006; Vlamakis *et al.*, 2013). It requires mainly carbon and energy to make the biofilm and use a number of sugars, organic acids and different organic compounds for this task (Stanley *et al.*, 2003; Chu *et al.*, 2008; Fujita, 2009).

Pseudomonas

The genus *Pseudomonas* is another varied bacterial genera in which *P. fluorescens* is the most common one which is responsible for biofilm formation in the dairy processing units. It is well-known for this cause because of its high heat resistance and short generation time and these characteristics make it a successful biofilm former (Wiedmann *et al.*, 2000; Pereira and Vieira, 2001; Dogan and Boor, 2003; Olofsson *et al.*, 2007).

Listeria

Listeria monocytogenes is the chief player of the biofilm within this genera and its biofilm formation capability varies significantly among serotype/lineage and origin (di Bonaventura *et al.*, 2008; Mu *et al.*, 2014). Though the relationship between lineage and biofilm formation is controversial, as some of researcher states that strains of lineage I have more biofilm producing capacity than those of lineage II while som states just opposite to the above said (Djordjevic *et al.*, 2002; Harvey *et al.*, 2007; Combrouse *et al.*, 2013). The biofilm creation by *L. monocytogenes* is mainly affected by temperature, strain origin and nutrient level (Nilsson *et al.*, 2011). The *L. monocytogenes* also has the property of attachment to surfaces passively and its biofilms are primarily comprised of teichoic acids which can grow on polypropylene, steel, rubber and/or glass surfaces (Lemon *et al.*, 2007; Silva *et al.*, 2008; Tresse *et al.*, 2009).

Staphylococcus

Staphylococcus is well recognised bacteria that may form biofilms on food contact surfaces in milk processing plants and especially *Staphylococcus epidermidis* has been recently described as a main biofilm forming species (Sharma & Anand, 2002; Vuong & Otto, 2002; Piette & Verschraegen, 2009). In the process of staphylococcal biofilm formation, the accumulation and development of a mature stage depend mainly on the polysaccharide intercellular adhesions (PIA) that promote bacterial accumulation, especially polysaccharide poly-N-succinylb- 1-6 glucosamine (PNAG) (Felipe *et al.*, 2017).

Streptococcus

Mostly the cheese section of the dairy industry suffers the most due to the biofilms produced by *Streptococcus*, more specifically *Streptococcus. thermophilus*. In the heating chamber of the section where temperature remains within 30 to 73⁰C lies, the maximum degree of biofilm formation occurs by the *Streptococcus* and thus pasteurized milk get contaminated (Couvigny *et al.*, 2015). As a result the defects in milk and cheese quality like acidic flavour and undesirable texture are spotted (Hup *et al.*, 1980; de Jong *et al.*, 2002; Hood and Zottola, 1995; Palmer *et al.*, 2007). Moreover, different strains of *S. thermophilus* from different dairy products show variable intensity of ability to produce biofilm ((Lortal *et al.*, 2009; Couvigny *et al.*, 2015; Scatassa *et al.*, 2015).

Lactobacillus

Few strains of *Lactobacillus* like *L. rhamnosus* can form biofilms in vitro on the abiotic surfaces (glass or polystyrene) (Lebeer *et al.*, 2007; Leccese *et al.*, 2016). Unlike other bacteria, biofilm formation by *Lactobacillus* spp. is relatively beneficial because of its property of colonization and longer mucosal permanence of the host as these help in avoiding pathogenic bacterial colonization (Terraf *et al.*, 2012).

E. coli

E. coli possesses the capacity to form biofilm structures both in vivo and in vitro. In fact among the facultative anaerobic bacteria of the GI tract, *E. coli* can bloom in a multispecies biofilm environment having their structural characteristics (Costerton *et al.*, 1995; Probert and Gibson, 2002). The autoinducer-2 (AI-2) of *E. coli* O157:H7 act as supplementary force for biofilm production as AI-2 signals regulate chemotaxis, flagellar synthesis and motility of genes (Pillai and Jesudhasan, 2006). The *E. coli* O157:H7 yields exopolysaccharides (EPS) which helps in cell attachment and formation of 3D structures of biofilms (Ozer and Demirci, 2006).

Miscellaneous

Besides the above one there are various other microbes which form the biofilms in dairy like obligate and facultative thermophiles. The Obligate thermophiles include *Anoxybacillus*, *Flavithermus*, *Geobacillus* spp.

which prefer higher temperatures (40-68°C) to grow and the facultative thermophiles *B. lichenformis*, *B. coagulans*, *B. pumilus*, *B. sporothermodurans* also involve in biofilm formation (Ronimus *et al.*, 2003; Schelderman *et al.*, 2005; Scott *et al.*, 2007).

Public Health Significance of Biofilms

The biofilms contribute in the deterioration of food quality due to the action of various microbial enzymes like lipases, proteases. In the dairy sector proteases enzyme play the major role in this regard and it is produced enormously by the different genera of bacteria (Celestino *et al.*, 1997; Santos *et al.*, 2003). Because of the catalytic reactions the biofilms persuade corrosion of metallic food surfaces (Vieira *et al.*, 1993). Also, the microbial quality of the products becomes inferior due to remarkable decrease in the heat transfer efficiency of the surfaces (Mittelman, 1998). The bacterial biofilms can persist in dairy plants and can potentially reduce the self-life of the pasteurized milk, cream, cheese and the other milk products (Gopal *et al.*, 2015; López *et al.*, 2015; Tschiedel *et al.*, 2015). Thus, majority of the food borne bacterial contamination leads to gastroenteritis creating a serious threat to public health and disbalance to the livelihood of mankind.

Prevention and Control Strategy of Biofilms

The easiest, cheapest and the most common measure of arresting the biofilm in the dairy industry is cleaning and disinfecting of the all sites, equipment and instruments especially in the alarming zones (Simões *et al.*, 2006). It prevent the development and spread of biofilms on the surfaces and in the food items (Gibson *et al.*, 1999; Verran, 2002). The efficiency and intensity of cleaning directly affect the final quality of the prepared products (Bremer *et al.*, 2006). The poor cleaning procedures are the causes of retention of biofilms as disinfectants are unable to enter the matrix of biofilms (Simões *et al.*, 2006).

In recent times, many natural compounds like various plant extracts, honey, essential oil (EO) etc. are found effective against microbial biofilm formation. These different natural products are used efficiently against different microbial biofilms in various successful experiments. For example, honey is a natural product having antimicrobial properties against about 60 species of bacteria and fungi (Massocks *et al.*, 2012; Santangelo, 2013; Molan, 2013). It was found that honey helped in inhibiting *Enterococcus* spp. biofilm production and reducing biofilm formation of EHEC O157:H7 (Lee, 2011; Ng *et al.*, 2014). Even a low concentration of honey can arrest the curling QS expression and virulence genes in bacteria (Lee, 2011). Similarly, essential oils (EOs) are also used against a wide range of pathogens since time immemorial as no antimicrobial resistance occurs (Hammer *et al.*, 1999; Ohno *et al.*, 2003; Ali *et al.*, 2005). But now these are recognised too as an antibiofilm agent (Isman, 2000). As cumin oil and cinnamon oil, both are used in food industry for their aroma, are now also a potent antibiofilmers (Chang *et al.*, 2001; Iacobellis *et al.*, 2005). A few of such natural products are listed in the below table.

Table 1: Different natural elemental extracts effective against various microbial biofilms

Element(s)	Extract	Against	References
<i>Epimedium brevicornum</i>	Plant extract	<i>Propionibacterium acne</i>	Coenye <i>et al.</i> , 2012
<i>Malus pumila</i>			
<i>Polygonum cuspidatum</i>			
<i>Rhodiola crenulata</i>			
<i>Dolichos lablab</i>			
<i>Melia dubia</i>	Bark extract	<i>E. coli</i>	Ravichandiran <i>et al.</i> , 2012
<i>Capparis spinosa</i>	Plant extract	<i>E. coli</i>	Issac <i>et al.</i> , 2011
		<i>Serratia marcescens, Pseudomonas aeruginosa</i>	
		<i>P. mirabilis</i>	
<i>Lagerstroemia speciosa</i>	Fruit extract	<i>Pseudomonas aeruginosa</i> PAO1	Singh <i>et al.</i> , 2012
Apiaceae family	Cumin seed EO	<i>Klebsiella pneumoniae</i>	Safoura <i>et al.</i> , 2010
Cinnamon	Cinnamon oil	<i>S. mutans & Lactobacillus plantarum</i>	Filoche <i>et al.</i> , 2005
Cinnamon	Cinnamon oil	<i>Staphylococcus epidermidis</i>	Nuryastuti <i>et al.</i> , 2009
<i>Cinnamomum cassia</i>	EO	Enteropathogenic <i>E. coli</i> (EPEC) &	Oliveira <i>et al.</i> , 2012
		<i>L. monocytogenes</i>	
Oregano	Oregano EO	<i>Staphylococcus & E. coli</i>	Nebahat <i>et al.</i> , 2010
Brazil nut	Brazil nut oil	Dental biofilm	Filogônio <i>et al.</i> , 2011

Besides the plant extract, bio-cleaners (enzyme-based detergents) or green chemicals help extensively in this field and practice of mixture of enzymes promote quicker degradation of biofilm (Simões *et al.*, 2010). Likewise, phages having polysaccharide degrading enzymes can destroy biofilms rapidly and up to 80% of *Pseudomonas fluorescens* biofilms can be destroyed by phages and the bacteriophage T4 found to be effective against *E. coli* biofilms (Doolittle *et al.*, 1995; Sillankorva *et al.*, 2004). Biosurfactants also use as preventive measures as biosurfactants from *Lactococcus lactis* 53 inhibit biofilm formation on silicon rubber of the equipments (Rodrigues *et al.*, 2004). The Surfactin produced by *Bacillus subtilis* prevents *Salmonella enterica*, *E. coli* and *Proteus mirabilis* biofilms (Mireles *et al.*, 2001). In dairy processing sectors biopreservatives like nisin, lauricidin, reuterin, pediocin protect from biofilm formation by various microbes including *L. monocytogenes* (Dufour *et al.*, 2004; Zhao *et al.*, 2004). Moreover, quorum sensing property of the microbes can be inhibited in order to prevent biofilm production at its very early stage (Dong *et al.*, 2002). The use of ultrasound is one of the most recent advances to control and prevent microbial biofilm generation (Kallioinen and Manttari, 2011).

Conclusion

Biofilm formation possesses profound implications and throws a major challenge to the dairy sector where they act as the principal reservoir of microbial contamination. These lead to financial crisis by impairment of raw material and its products. Therefore, choosing of a profound, prominent and efficient measure is in an urge in order to safeguard the whole sector from further deficiency and mitigating the present problem

judiciously. The decision adopted should be in harmony with science, capital and time so that no turbulence can slow down its way.

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