Journal of Food Engineering and Technology 2023;12(1):21-28
https://doi.org/10.32732/jfet.2023.12.1.21

Microorganism Carrier-Surface Method as an Efficient Model for Microscopic Characterization of Biofilm Structure and Dispersion in Dairy Associated Spore-Forming Bacteria

Fadila Malek

Department of Biology, SNV-STU Faculty, University of Tlemcen, Tlemcen, 13000, Algeria
E-mail: malekfad@yahoo.fr

Received: 9 February 2023; Accepted: 24 March 2023; Available online: 25 May 2023

Abstract: Dispersion, defined as the release of planktonic cells is the final stage of biofilm development and of major significance in clinical and industrial settings. Currently, biofilm dispersion is considered as a promising avenue for biofilm control and an important topic research. However, a problem facing such research projects is how to induce planktonic life in a biofilm. Numerous systems are used for the investigation of biofilm dispersion, including dynamic continuous or static batch systems. This mini-review describes the usefulness of the microorganism carrier-surface method as a simple biofilm growth model which successfully allowed microscopic characterization of biofilm structure and dispersion in dairy-associated spore-forming bacteria and should be an efficient model for studying dispersion process.
Keywords: Biofilm growth model; Biofilm structure; Dispersion in *Bacillus cereus*; Dispersion in thermophilic bacilli; Microscopic characterization; Dairy industry.

1. Introduction

Biofilms are a multicellular complex formed of micro-organisms that are attached to a surface and embedded in a matrix, consisting of exopolymeric substances (EPS), protecting them from harsh influences from the environment [1, 2]. As shown in figure 1, the formation of biofilms is a developmental process that is initiated by planktonic (free-living) organisms transitioning to a surface-associated lifestyle, and is completed when cells escape from the biofilm structure in a process referred to as dispersion to return to planktonic mode of growth [4]. The biofilm dispersion process constitutes the final stage of biofilm development, and a necessary step for bacteria to leave the biofilm macrostructure and colonize new environments [5]. This process is highly regulated and can occur spontaneously or be induced by intrinsic or environmental factors.

Biofilm dispersion has a crucial meaning in the field of food quality and safety as well as in medicine, with regard to cross contamination and disease transmission issues [6, 7]. In the dairy industry, the biofilms formed on equipment surfaces are recognized to be a major source of contamination of processed milk and dairy products with both spoilage and pathogenic bacteria [8, 9]. Similarly, in medicine, the release of dispersed bacteria, often with enhanced virulence into the host, is responsible of systemic infections, by promoting the dissemination of contaminations through the organism [10, 11]. That is why, in recent years, dispersion is considered as an interesting target for biofilm prevention and control strategies, in industrial and clinic settings, as the planktonic state is considered to be more vulnerable to antimicrobial agents and immune responses [12-14]. In this regard, Inducing biofilm-dispersal has been suggested as a means to fight against biofilm and it has been recommended in some instances to clean surfaces by applying a signal or an effector of biofilm dispersal in close proximity to the biofilm [5, 15]. Hence, efficient methods for the investigation of biofilm dispersal and recovery of dispersed cells are necessary to achieve such purposes. This mini-review describes a simple biofilm model that successfully allowed microscopic visualization of authentic biofilm structures and dispersion, in dairy-associated spore-forming bacteria.

2. Methodological approaches for investigation of biofilm dispersion

Taking into account that biofilm dispersion is an important topic research, the need for technics that induce planktonic life in a biofilm is required. Numerous systems used for studying biofilm dispersion, including static (microtiter plates, glass tubes.) and dynamic models (flow cells, microfermenters.), are reported (table 1). Some of these *in vitro* assays are easy to set up and allow high-through put assessments; however they are species-

dependent and differ from laboratory to laboratory. As an illustration, a relationship between dispersion and medium flow was found in dynamic systems, the biofilm diameter increased with increasing flow rate, for *Pseudomonas aeruginosa* [4]. While, for other bacteria, biofilm dispersal can be simply induced by reducing the shaking speed after an initial step of biofilm formation [5].

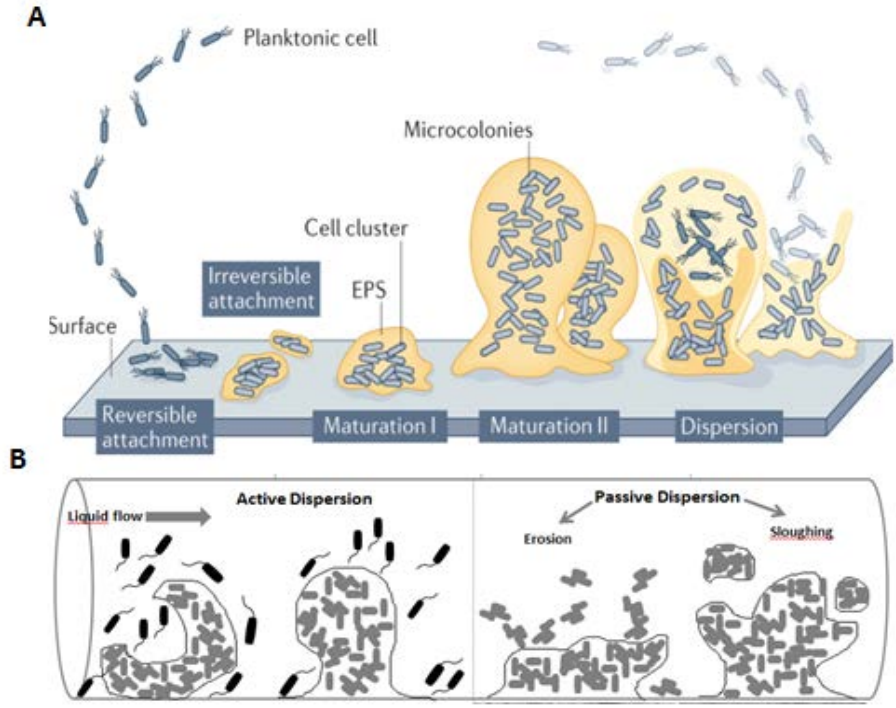

Fig. 1. Biofilm formation and dispersion styles in a milk pipe. (A): Five stages of biofilm development according to Sauer et al. [3]. (B): Biofilm dispersion styles that may occur in milking systems of dairy processing plants.

Static batch models were also suitable for studying biofilm dispersal. The microorganism carrier-surface method previously described to test sanitizers' effectiveness [21], allows rapid biofilm dispersal in mesophilic and thermophilic dairy-associated spore forming bacteria [20]. The formation of non-submerged biofilms on open surfaces is a practical method that was also used in several works [22, 23]. The microorganism carrier-surface method combined to environmental scanning electron microscopy imaging (ESEM) or simply crystal violet staining revealed an efficient model for the investigation of biofilm structure and dispersion in dairy-associated spore forming bacteria [20]. Large biofilms that exceeded a minimum diameter of 40 μm, required for dispersion to occur in the way called seeding dispersal, previously described in flowing systems [24], are developed with this static batch model, by mesophilic and thermophilic spore forming bacteria (Fig. 2). Another aspect that emerges from the literature is the problem of the recovery of dispersed cells in dispersion assays. In this regard, this biofilm model can provide additional data and measurements, since samples examined in ESEM can be used with a range of downstream methods directly after viewing [25]. Accordingly, in-depth investigations of biofilm dispersal notably the recovery of dispersed cells and identification of dispersal compounds are possible with this biofilm model after ESEM imaging. In addition, this microscopic approach enables the visualization of biofilms in their native state and thus, should contribute to depict how biofilms really develop especially *in vivo* in clinical and industrial settings, as expressed in the literature.

Table 1. Examples of methods for investigation of biofilm dispersion

Methods	References
Application of microturbulences in polystyrene Petri dishes	[16]
Confocal laser scanning microscopy (CLSM)	[17]
Assessment of biofilm biomass in a microtiter-based batch system	[18]
Cristal violet staining of ring biofilm in a glass tube	[19]
Microorganism carrier-surface method combined to Environmental scanning electron microscopy (ESEM) or crystal violet staining	[20]

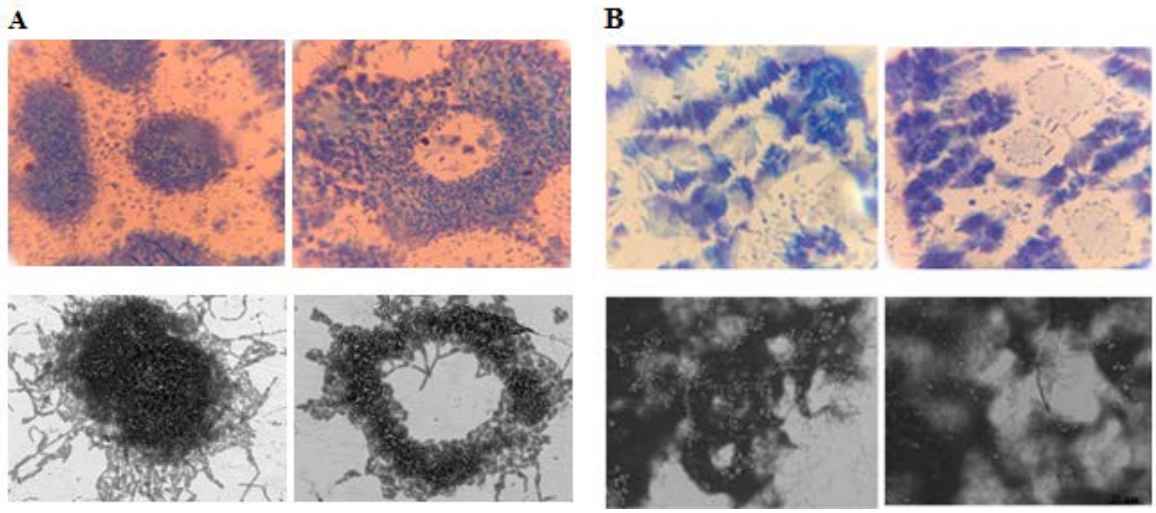

Fig. 2. Active dispersion in 20 h old biofilms formed according to microorganisms carrier-surface method after crystal violet staining (images above) or ESEM imaging (images below). The formation of large transparent cavities, or central hollow structures, characteristic of seeding dispersal are obvious in both *B. cereus* biofilms (A) and more extensive amorphous biofilm matrix of thermophilic bacilli (B).

3. Adaptation and persistence of spore-forming bacteria in the dairy environment

Bacteria in raw milk arriving at dairy processing facilities are highly diverse, but the pressure exerted by food processing conditions should select some taxa [26]. Accordingly, restrictive processes such as heat treatment result in reducing the diversity of the processing plant microflora. This is consistent with the concept of the in-house microflora [27], which is partly a reflection of the raw material used and partly a reflection of processing conditions namely heat treatment and cleaning procedures in dairy product manufactures. As shown in table 2, the microbiota of dairy processing equipment comprise various Gram+ and Gram- bacteria, however the bacteria belonging to the Genus *Bacillus* are often predominant [28-31]. Due to their spore forming properties, *Bacillus* and related genera (*Brevibacillus, Paenibacillus, Geobacillus, Anoxybacillus*) survive industrial pasteurization and form on stainless steel equipment biofilms which are difficult to eradicate [32,33]. These microorganisms can contaminate either in the form of vegetative cells, spores, or detached biofilm clumps that adhere to the stainless steel components. Thus, the ability of bacteria to form biofilms is responsible for their persistence onto technological equipment surfaces, and constitutes a major microbiological challenge for the dairy industry. The protection of strains within biofilms formed inside pipe milking systems has been described as a survival strategy for *B. cereus* recurrent genotypes that have been shown to persist on equipment surfaces in a dairy plant, for several years [34]. In order to better understand their persistence strategies, the spore surface and biofilm characteristics of this recurring *B. cereus* were characterized [35]. Interesting findings were that cleaning procedures (cleaning-in-palce, CIP system) may affect the spore surface hydrophobicity and hydrophilic spores were best able to withstand chemical cleaning, and form specific biofilm features on stainless steel surfaces (Fig. 3).

Table 2. Constitutive microflora of biofilms in dairy processing equipment [30]

	Frequency of isolation (%)					Biofilm formation (%)
Analyzed bacteria	Tanks-coolers	Bactofuge units	Pasteurizers	Cheese baths	Packaging machines	
Bacillus	100	51	65	77	37	100
Lactobacillus	100	34	45	69	12	57
Enterococcus	79	37	43	25	5	79
Staphylococcus	71	24	12	17	2	87
Streptococcus	53	9	2	8	5	29
Pseudomonas	69	11	3	5	0	74
E. coli	77	23	38	39	9	82

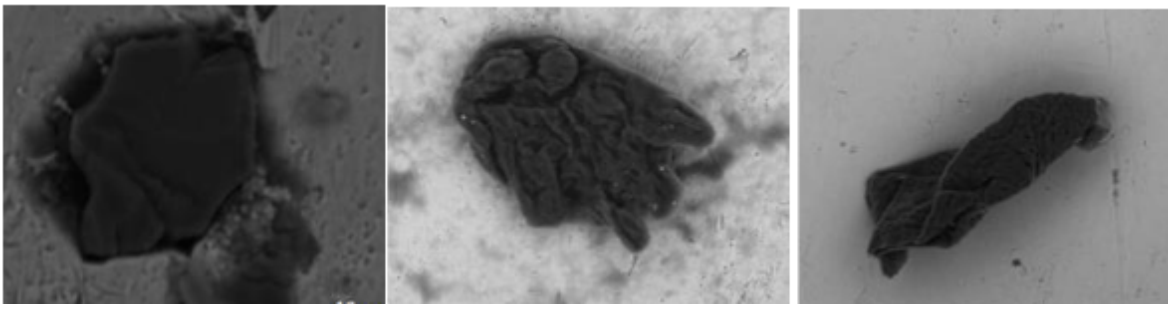

Fig. 3. Specific biofilm features characterized by high resistance to chemical cleaning and disinfection. ESEM images showed smooth and wrinkled matrix surface topographies of 7 days old biofilms formed by hydrophilic *B. cereus* spores on soiled (left and center images) or non-soiled (right image) stainless steel surfaces, according to microorganism carrier-surface method [35].

On another hand, thermophilic bacilli such as *Geobacillus stearothermophilus*, *Anoxybacillus flavithermus* and *Bacillus licheniformis* are also important contaminants in the dairy industry and one of the most common groups of biofilm-forming organisms in milk powder processing plants [36, 37]. Overall thermophilic bacilli are generally not pathogenic, their presence in dairy products is an indicator of poor hygiene and high numbers are unacceptable to customers. In addition, their growth may result in milk product defects caused by the production of acids or enzymes, potentially leading to off-flavours [38]. These bacteria are able to grow in sections of dairy manufacturing plants where temperatures reach 40–65°C. According to Gopal et al. mesophilic spore-forming bacteria are a primary cause of concern for manufacturers of powdered dairy ingredients with thermophilic spore-forming being more prevalent in the end product. Furthermore, adaptation of milk-associated mesophilic and tehrmophilic bacilli species to the dairy environment results in the formation of robust biofilm in recombined milk processing lines [39]. Disruption of such biofilms is the cause of the dissemination of resistant spores in the dairy environment and subsequent contamination of finished products. Hence, dispersion is largely involved in cross contamination problems and has a crucial meaning in recombined milk processing lines, since skimmed milk was reported to be the substrate in which bacteria such as *B. cereus* can easily disperse and disseminate in the dairy environment [40].

4. Biofilm characteristics of dairy-associated spore-forming bacteria

Both mesophilic and thermophilic spore forming bacteria are known as efficient biofilm formers on dairy processing stainless steel equipment. The most recalcitrant biofilm associated with dairy processing plants, are those which form at critical locations such as heat exchanger [41,42] or dead ends, corners, cracks, crevices gaskets, valves and the joints of stainless steel milking pipes [33]. The formation of biofilms is initiated by the attachment of both vegetative cells and spores. Several factors affect the attachment of microorganisms to dairy processing line surfaces and the subsequent biofilm development will occur most readily on surfaces that are rougher, more hydrophobic, and coated by surface conditioning films [43]. Regarding the last item, dairy biofilms are predominated by bacterial extracellular polymeric substances (EPS) and milk residues, referred to as biofouling [44, 45]. Moreover, they are characterized by rapid development (< 12 h) [45]. According to Burgess et al. thermophilic bacilli such as *Geobacillus* or *Anoxybacillus flavithermus* are characterized by a fast growth rate (generation time of approximately 15–20 min) and have a high propensity to readily form biofilms on stainless steel equipment. While the making of a mature biofilm takes several hours to several weeks, depending on the system under development [46], in dairy processing environments only a few hours are required for thermophilic bacilli to form young mature biofilms, approximately 6 h both *in-vitro* [38]and *in- situ* inside milk pipelines (Fig. 4). As previously outlined [43], the time available for biofilm formation will depend on the frequency of cleaning and disinfection regimes.

Furthermore, as previously reported [20], biofilm which develop on dairy processing equipment are characterized by a high structural diversity in both mesophilic and thermophilic bacilli, reflecting adaptations to specific niches (Fig. 5), as well as rapid dispersal which occurs in lesser than 20 h (Fig. 6). Both characteristics are significant resistance factors to cleaning procedures. Indeed, the variability in the biofilm structure of the mesophilic *B. cereus* [47, 48], or the thermophilic *Geobacillus stearothermophilus* [36], is assumed to influence the control of these bacteria in the dairy industry, whereas, dispersion is involved in cross-contamination problems.

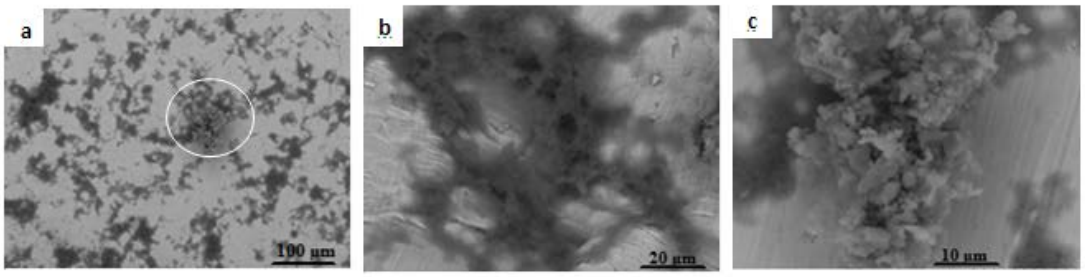

Fig. 4. Young and old biofilms formed *in situ* in recombined milk processing lines. (a) an overview of 7 days old biofilm, developed on stainless steel coupons introduced inside milking pipes at post-pasteurization locations. (b and c) points of (a) at high magnification, (c) 6 h old biofilm formed between two production runs on the bottom of (a) (white circle).

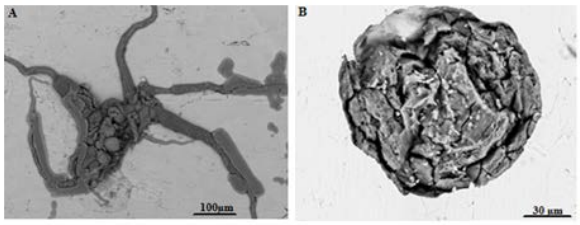

Fig. 5. Structural diversity of *in-vitro* dairy-associated spore- forming bacteria biofilms developed on stainless steel. (A) An overview of a heterogeneous extensive *Geobacillus* biofilm, at low magnification. (B) Compact tree-dimensional mushroom-like structure of *B. cereus* biofilm.

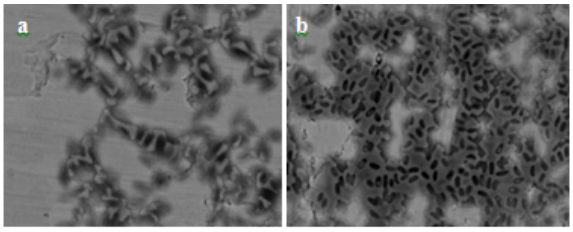

Fig. 6. ESEM micrographs of 20 h old biofilms on stainless steel at advanced dispersal stage. The end of biofilm dispersal imaged as cell-free EPS-matrix debris in *B. cereus* (a) and EPS-matrix fragments crossed by deep cellular imprints devoid of dispersed cells in thermophilic bacilli (b).

5. Dispersion vs cross-contamination and control of biofilms

Dispersion is a generalized term used to describe the release of cells, either individually (active dispersal) or in groups (passive dispersal) from a biofilm or substratum (Fig. 1b). In active dispersal, planktonic cells are released from the biofilm, in response to an antibiofilm stimulus like nutrient starvation or dispersal signal release, while passive dispersal is induced by an external force, like shear forces which cause the complete or partial destruction of the biofilm (2,4]. Detachment or dispersion as the final stage of biofilm development is an essential step of the biofilm cycle life, which acts as a potent mean of disseminating bacteria with enhanced colonization properties in the surrounding environment [5]. Thus, the transfer of bacteria from biofilm through dispersion has a crucial meaning regarding cross contamination in the food industries. Cross-contamination occurs when adhered bacteria detach (dispersed cells) and contaminate the product as it passes the surface, making biofilms the main source of bacterial contamination of the final products in dairy processing plants [49, 50]. Dispersion process is also important as a potential control point for the manipulation of biofilm development and novel biofilm dispersal strategies that can more effectively release biofilm-associated microbes from the protection of the EPS could improve anti-biofilm therapeutics or industrial biocides [50, 51].

6. Dispersion styles in dairy-associated spore-forming bacteria

Microorganism carrier-surface method allowed the observation of dispersion process in young biofilms. Indeed, non-submerged biofilms developed on stainless steel surfaces by *B. cereus* and thermophillic bacilli strains underwent rapid dispersion, as they were at advanced dispersion stages after 20 h cultivation (Fig. 6). This indicates that this biofilm model supported fast growth of cell-rich biofilms resulting in rapid dispersal, following nutrient starvation, inasmuch as chemical gradient within biofilms is assumed to be the driving force of dispersion [4]. In addition, dispersion occurred in the way called seeding dispersal or central hollowing, previously described for *P. aeruginosa* biofilms cultured in flowing systems [52, 53]. Indeed, non-submerged biofilms developed under static conditions were substantial structures with high colony diameters of > 40 µm, previously reported as a threshold required for hollow cavity formation to occur in *P. aeruginosa* biofilms [24]. It is worthy to note that structural and dispersal similarities recorded for biofilms formed in batch system and biofilms in dynamic systems increase the score of the non-submerged assay as an efficient biofilm cultivation system. In addition, various dispersion styles were obtained by this simple biofilm model, from more conventional seeding dispersal to an original

dispersion style not previously described and observed in thermophillic bacilli strains (Fig. 7). Cells were released from large compact biofilms through small holes performed in the matrix using sharped tools. This strategy to leave the biofilms was also observed in *B. cereus* compact mushroom-like biofilm (unpublished data). Unlike recognized dispersing mechanisms, this unusual strategy to escape from the biofilm appears not to rely upon the well-documented biochemical matrix degrading [12-15], but on a physical process which consisted of piercing the matrix surface using well-defined geometrical sharped structures most likely of crystalline nature. Crystal structures in biofilms have mainly been ascribed to mineral formations in specific biofilms characterized by high rates of minerals [54]. Such high content in minerals has neither been reported for *B. cereus* matrix biofilms, mainly composed of polysaccharides, proteins and eDNA [55], nor for thermophilic bacilli biofilms. Thereby, the observed piercing tools should rather be organic formations mainly polysaccharides or/and proteins. More in-depth investigations are required to elucidate such dispersion style.

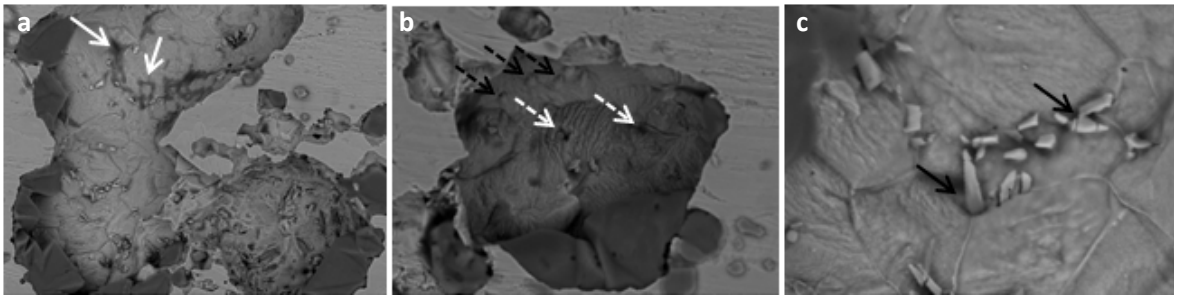

Fig. 7. An original dispersion style in thermophilic bacilli biofilms. (a) large compact biofilms formed in stainless steel crevices. (b and c) areas of (a) at higher magnification. White arrows showed vegetative cells released from the biofilm through small holes in the EPS-matrix. Other small holes are obvious at different points of the upper surface of these biofilms (white dashed arrows). Black arrows show various well-defined sharped structures emerging from the matrix or still piercing it (black dashed arrows). Adapted from [20].

7. Conclusion

The use of microorganism carrier-method as a simple static batch biofilm model combined with ESEM imaging or simply crystal violet staining enabled the investigation of biofilm structure and dispersion in dairy-associated spore forming bacteria. Biofilm structures developed according to this methodological approach were successfully resolved either in optic microscopy or high resolution ESEM which allowed the observation of authentic biofilms, in their native state. Dispersion process was also well resolved in both microscopic techniques. The recovery of dispersed cell, which is a limitation in dispersal assays, is possible following ESEM imaging. Knowing that techniques to properly harvest dispersed cells are needed, this simple and easy-to-control methodological approach should meet this requirement.

7. References

[1] Wijman JGE, de Leeuw PPLA, Moezelaar R, Zwietering MH, Abee T. Air liquid interface biofilms of Bacillus cereus: Formation, Sporulation and Dispersion. Appl. Environ. Microbiol. 2007;73:1481-1488.
[2] Flemming HC, Wigender J, Szewzyk U, Steinberg P, Rice SA, et al. Biofilms: an emergent form of bacterial life. Nat Rev Microbio. 2016;14: 563-575.
[3] Sauer K, Stoodley P, Goeres DM, Hall-Stoodley L, Burmølle M, Stewart PS, Bjarnsholt T. The biofilm life cycle: expanding the conceptual model of biofilm formation. Nat Rev Microbiol. 2022;20:608–620.
[4] Rumbaugh KP, Sauer K. Biofilm dispersion. Nat Rev Microbiol. 2020;18: 571–586. doi:10.1038/s41579-020-0385-0
[5] Guilhen, C., Forestier, C., & Balestrino, D. Biofilm dispersal: multiple elaborate strategies for dissemination of bacteria with unique properties. Molecular microbiology. 2017;105(2) 188-210.
[6] McDouglad D, Rice SA, Barraud N, Steinberg PD, Kjelleberg S. Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. Nat Rev Microbiol. 2011;10: 39-50.
[7] Friedlander, A., Nir, S., Reches, M., & Shemesh, M. Preventing biofilm formation by dairy-associated bacteria using peptide-coated surfaces. Frontiers in Microbiology. 2019;10, 1405.
[8] Austin JW, Bergeron G. Development of bacterial biofilms in dairy processing lines. J. dairy res. 1995;62:509-519.

[9] Marchand S, De Block J, De Jonghe V, Coorevits A, Heyndrickx M, Herman L. Biofilm formation in milk production and processing environments; influence on milk quality and safety. Comprehensive Reviews in Food Science and Food Safety. 2012; 11(2):133-147.
[10] Marks LR, Davidson BA, Knight PR, Hakansson AP. Interkingdom signaling induces *Streptococcus pneumonia* biofilm dispersion and transition from asymp-tomatic colonization to disease. mBio. 2013;4: e00438–13
[11] Stacy A., Everett, J., Jorth, P., Trivedi, U., Rumbaugh, K.P.,and Whiteley,M. Bacterial fight-and-flight responses enhance virulence in a polymicrobial infection. Proc Natl Acad Sci. 2014;111: 7819–7824.
[12] Lister JL, Horswill AR. Staphylococcus aureus biofilms: recent developments in biofilm dispersal. Front Cell Infect Microbiol. 2014;4: 178.
[13] Fleming D, Rumbaugh KP. Approaches to dispersing medical biofilms. Microorganisms. 2017; 5(2): 15.
[14] Ramakrishnan R, Singh AK, Singh S, Chakravortty D, Das D. Enzymatic dispersion of biofilms: An emerging biocatalytic avenue to combat biofilm-mediated microbial infections. Journal of Biological Chemistry. 2022;102352.
[15] Kaplan, J. B. Therapeutic potential of biofilm-dispersing enzymes. The International journal of artificial organs, 2009;32(9): 545-554.
[16] Kaplan, J.B., Fine, D.H. Biofilm dispersal of *Neisseria subflava* and other phylogenetically diverse oral bacteria. Appl Environ Microbio. 2002;l68: 4943–4950.
[17] Wang R, Khan BA, Cheung GYC, Bach et al. Staphylococcus epidermidis surfactant peptides promote biofilm maturation and dissemination of biofilm-associated infection in mice. J Clin Invest. 2011;121: 238–24
[18] Barraud, N., Moscoso, J.A., Ghigo, J.-M., and Filloux, A. Methods for studying biofilm dispersal in *Pseudomonas aeruginosa*. Methods Mol Biol Clifton NJ. 2014 ;1149: 643–651.
[19] Stacy A, Abraham N, Jorth P, Whiteley M. Microbial community composition impacts pathogen iron availability during polymicrobial infection. PLoS Pathog. 2016;12: e1006084
[20] Malek F. Evaluation of a non-submerged cultivation assay combined to ESEM imaging for analysis of biofilm formed by dairy-associated spore-forming bacteria. African journal of microbiology research. 2016a;10:1263-1273
[21] Maris P. Biofilm and disinfection of microorganism carrier-surface method. Sciences des aliments. 1992;12:721-728
[22] Sommer P, Martin-Rouas C, Mettler E. Influence of the adherent population level on biofilm population, structure and resistance to chlorination. Food Microbiol. 1999;16:503-515.
[23] Leriche V, Carpentier B. Limitation of adhesion and growth of Listeria monocytogenes on stainless steel surfaces by Staphylococcus sciuri biofilms. J. Appl. Microbiol. 2000; 88:594-605
[24] Purevdorj-Gage B, Costerton WJ, Stoodley P. Phenotypic differentiation and seeding dispersal in non-mucoid Pseudomonas aeruginosa biofilms. Microbiology. 2005;151:1569-1576.
[25] Bergmans L, Moisiadis P, van Meerbeek B, Quirynen M, Lambrechts P. Microscopic observation of bacteria: review highlighting the use of environmental SEM. Int endod J. 2005;38:775-788
[26] Carlin F, Brillard J, Broussolle V, Clavel T, Duport C, Jobin M, Nguyen-Thé C. Adaptation of Bacillus cereus, an ubiquitous worldwide-distributed foodborne pathogen, to a changing environment. Food Res Int. 2010;43:1885–1894.
[27] Bagge-Ravn D, Hjelm M, Christansen JN, Johansen C, Gram L. The microbial ecology of processing equipment in different fish industries - analysis of the microflora during processing and following cleaning and disinfection. Int. J. Food Microbiol. 2003; 2718: 1-12.
[28] Sharma M, Anand SK. Characterization of constitutive microflora of biofilms in dairy processing lines. Food microbial. 2002; 19:627-636.
[29] Malek F, Boudjemaa M B, Khaouani- Yousfi F, Kihel M. Microflora of biofilm on Algerian dairy processing lines: An approach to improve microbial quality of pasteurized milk. African Journal of Microbiology Research. 2012;6: 3836-3844.
[30] Kukhtyn M, Berhilevych O, Kravcheniuk K, Shynkaruk O, Horiuk Y, Semaniuk N. Formation of biofilms on dairy equipment and the influence of disinfectants on them. Eastern European Journal of Advanced Technologies. 2017;5: 26-33.
[31] Weber M, Liedtke J, Plattes S, Lipski A. Bacterial community composition of biofilms in milking machines of two dairy farms assessed by a combination of culture-dependent and–independent methods. PLoS One. 2019;14(9), e0222238.
[32] Bremer PJ, Fillery S, McQuillan AJ. Laboratory scale Clean-In Place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. Int. J. Food Microbiol. 2006;106:254-262.

[33] Gopal N, Hill C, Ross PR, Beresford TP, Fenelon MA, Cotter PD. The prevalence and control of Bacillus and related sporeforming bacteria in the dairy industry. Front Microbiol. 2015;6:1418
[34] Malek F, Boudjemaa MB, Métri AA, Kihal M. Identification and genetic diversity of *Bacillus cereus* strains isolated from a pasteurized milk processing line in Algeria. Dairy Science and Technology. 2013;93:73-82
[35] Malek F. Hydrophobicity and specific biofilm features of *Bacillus cereus* spores subjected to pH stresses. African Journal of Microbiology Research. 2016b;10: 744-751.
[36] Burgess SA, Flint SH, Lindsay D. Characterization of thermophilic bacilli from a milk powder processing plant. J Appl Microbiol. 2013;116:350–35
[37] Zhao Y, Caspers MPM, Metselaar KI, de Boer P, Roeselers G, Moezelaar R, Groot MN, et al. Abiotic and microbiotic factors controlling biofilm formation by thermophilic sporeformers. Appl. Environ. Microbiol. 2013;79:5652-5660.
[38] Burgess SA, Lindsay D, Flint SH. Thermophilic bacilli and their importance in dairy processing. International J. food microbial. 2010;144:215-225.
[39] Malek F. Spore-forming bacteria and biofilm: A recurring problem in reconstituted or recombined pasteurized milk processing lines. Canadian journal of microbiology. 2019;65: 405–420 (in French).
[40] Alonso VPP, Kabuki DY. Formation and dispersal of biofilms in dairy substrates. Int J Dairy Technol. 2019;70:1–7
[41] Te Giffel M, Beumer RR, Langeveld LPM, Rombouts FM. The role of heat exchangers in the contamination of milk with B. cereus in dairy processing plants. Int. J. Dairy Technol., 1997;50(2): 43-47.
[42] Svensson B, Ekelund K, Ogura H, Christiansson A. Characterization of *Bacillus cereus* isolated from milk silo tanks at eight different dairy plants. Int. Dairy J. 2004;14:17-27.
[43] Simoes M, Simoes LC, Vieira MJ. A review of current and emergent biofilm control strategies. Food Sci. Technol. 2010 ;43: 573-583.
[44] Flemming HC, Ridgway H. Biofilm control: conventional and alternative approaches. Marine and industrial biofouling. 2009;103-117.
[45] Flint S, Bremer P, Brooks JD, Palmer J, Sadiq FA, Seale B, Zain SNM. Bacterial fouling in dairy processing. Int Dairy J. 2020;101:104593
[46] Mittelman MW. Structure and functional characteristics of bacterial biofilms in fluid processing operations. J Dairy Sci. 1998;81:2760–2764
[47] Majed R, Faille C, Kallassy M, Gohar M. Bacillus cereus biofilms— same, only different. Front Microbiol. 2016;7:1054
[48] Lim ES, Baek SY, Oh T, Koo M, Lee JY, Kim HJ, Kim JS. Strain variation in *Bacillus cereus* biofilms and their susceptibility to extracellular matrix-degrading enzymes. PLoS One. 2021; 6(6): e0245708.
[49] Munsch-Alatossava P, Alatossava T. Potential of N2 gas flushing to hinder dairy-associated biofilm formation and extension. Frontiers in Microbiology. 2020;11, 1675.
[50] Zhu T, Yang C, Bao X, Chen F, Guo X. Strategies for controlling biofilm formation in food industry. Grain & Oil Science and Technology. 2022.
[51] Kaplan JB. Biofilm matrix-degrading enzymes. Microbial Biofilms: Methods and Protocols. 2014;203-213.
[52] Boles BR, Thoendel M, Singh. Rhamnolipids mediate detachment of Pseudomonas aeruginosa from biofilms. Mol. Microbiol. 2005;57:1210-1223.
[53] Kirov SM, Webb JS, O'May CY, Reid DW, Woo JKK, Rice SA, Kjelleberg S. Biofilm differentiation and dispersal in mucoid Pseudomonas aeruginosa isolates from patients with cystic fibrosis. Microbiology. 2007; 153:3264-3274
[54] Holling N, Dedi C, Jones CE, Howthorne JA, Hanlon GW, Salvage JP, et al. Evaluation of environmental scanning electron microscopy for analysis of Proteus mirabilis biofilms in situ on urinary catheters. FEMS Microbiol. Lett. 2014;355(1):20-27.
[55] Vilain S, Pretorius JM, Theron J, Brözel VS. DNA as an Adhesin: *Bacillus cereus* Requires Extracellular DNA To Form Biofilms. Appl. Environ. Microbiol. 2009;75:2861-2868