Biofilm and Food Processing

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Advocating science-based food safety law and trade

Outline

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2- Biofilm formation

3-Biofilm control/removal

4-Cleanliness of sanitized surfaces

5-Suggested standards for dairy equipment surfaces

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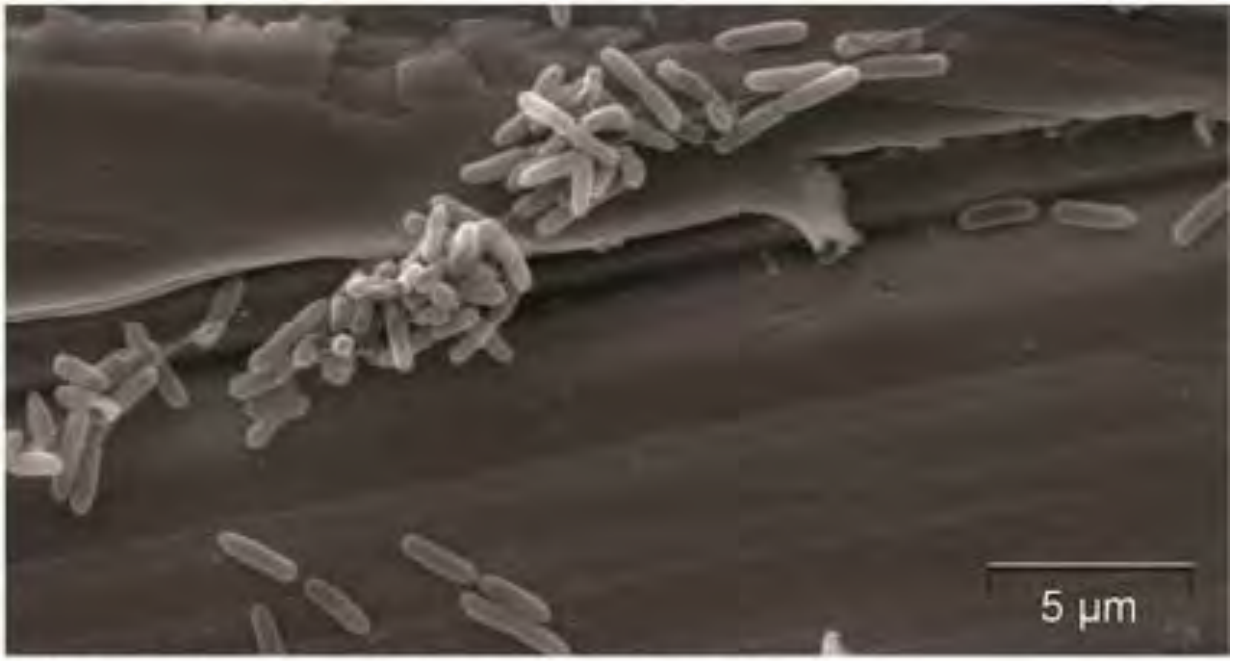


Organic residue/bacteria

Awad et al., 2018 (Used with permission)

Definition of biofilm

Biofilm formation in food processing environments is of special importance because it may have a huge impact on the

-**hygiene**,

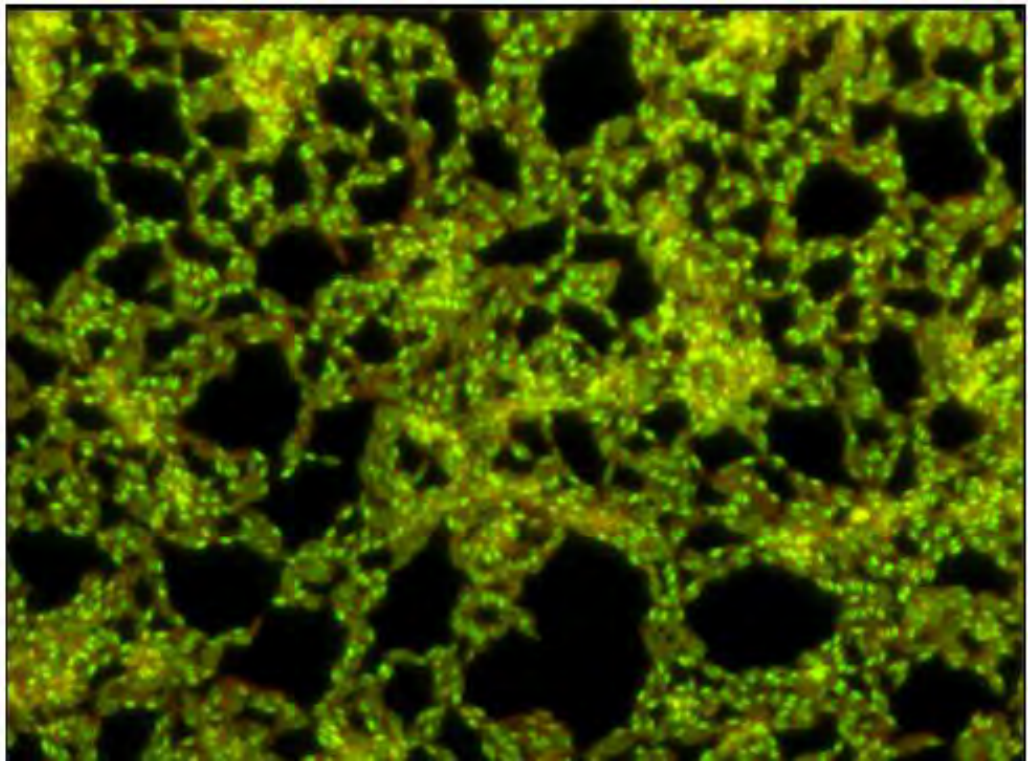
-**food safety** and

-**quality** of food products.

Definition of biofilm

A biofilm may be defined as "*a complex community of microorganisms, attached to a surface interacting with each other, producing an polymeric substances (EPS) matrix slime*".

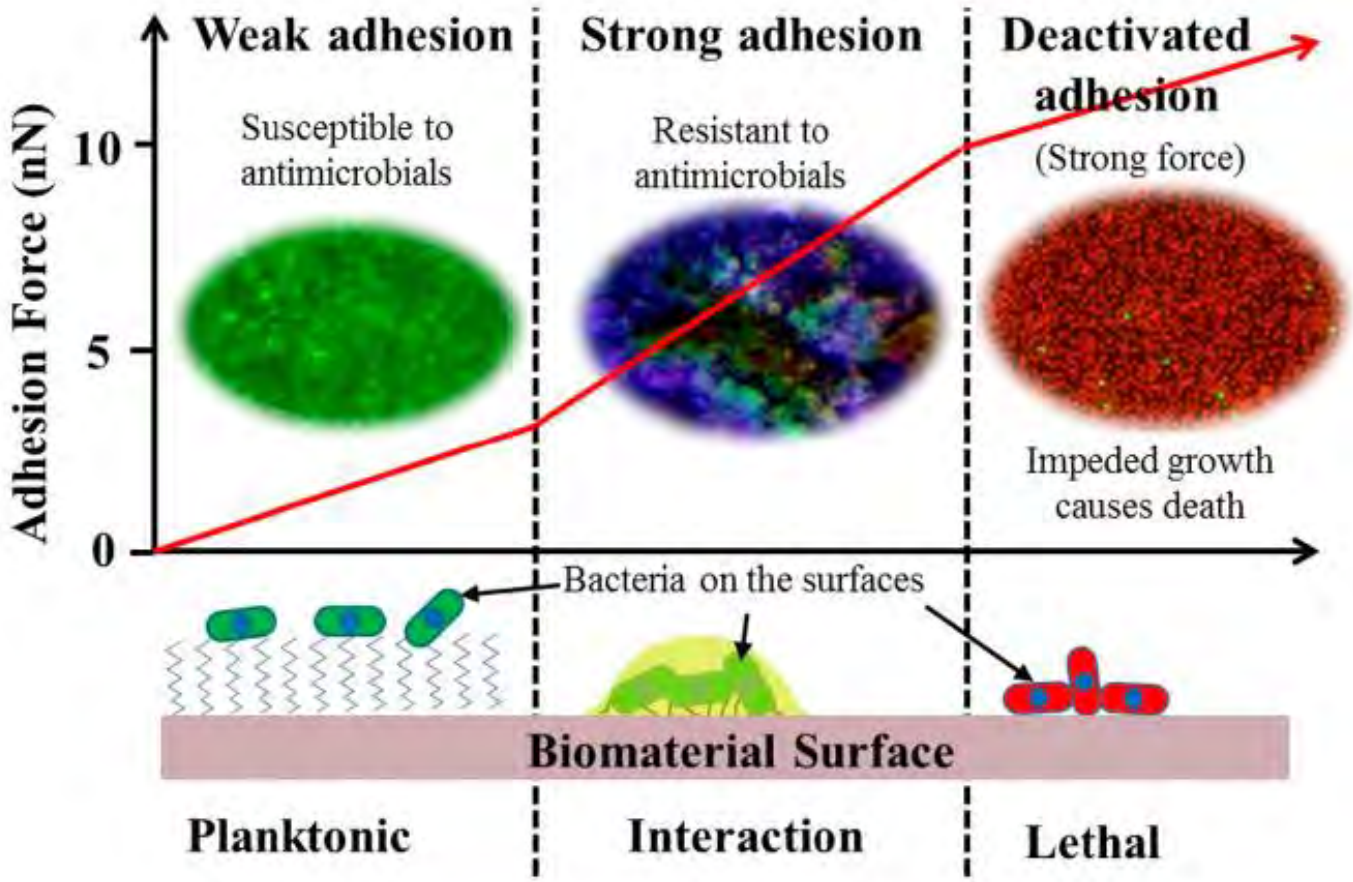
Microscopic images of a *B.cereus* biofilm grown for 48h in TSB1/10. Observation by epifluorescence after staining with the Live/Dead stain (magnification × 400). Endospores produced within the biofilm are stained in green, cells are stained inorange-green.



Majed et al., 2016 (Used with permission)

Biofilm formation

Food-borne pathogens and spoilage organisms can **attach** to and **produce** EPS on food contact surfaces and other food environments.



Alam et al., 2019 (Used with permission)

Biofilm formation

Schematic representation of listerial extracellular biofilm matrix.

The major components:

extracellular polymeric substances

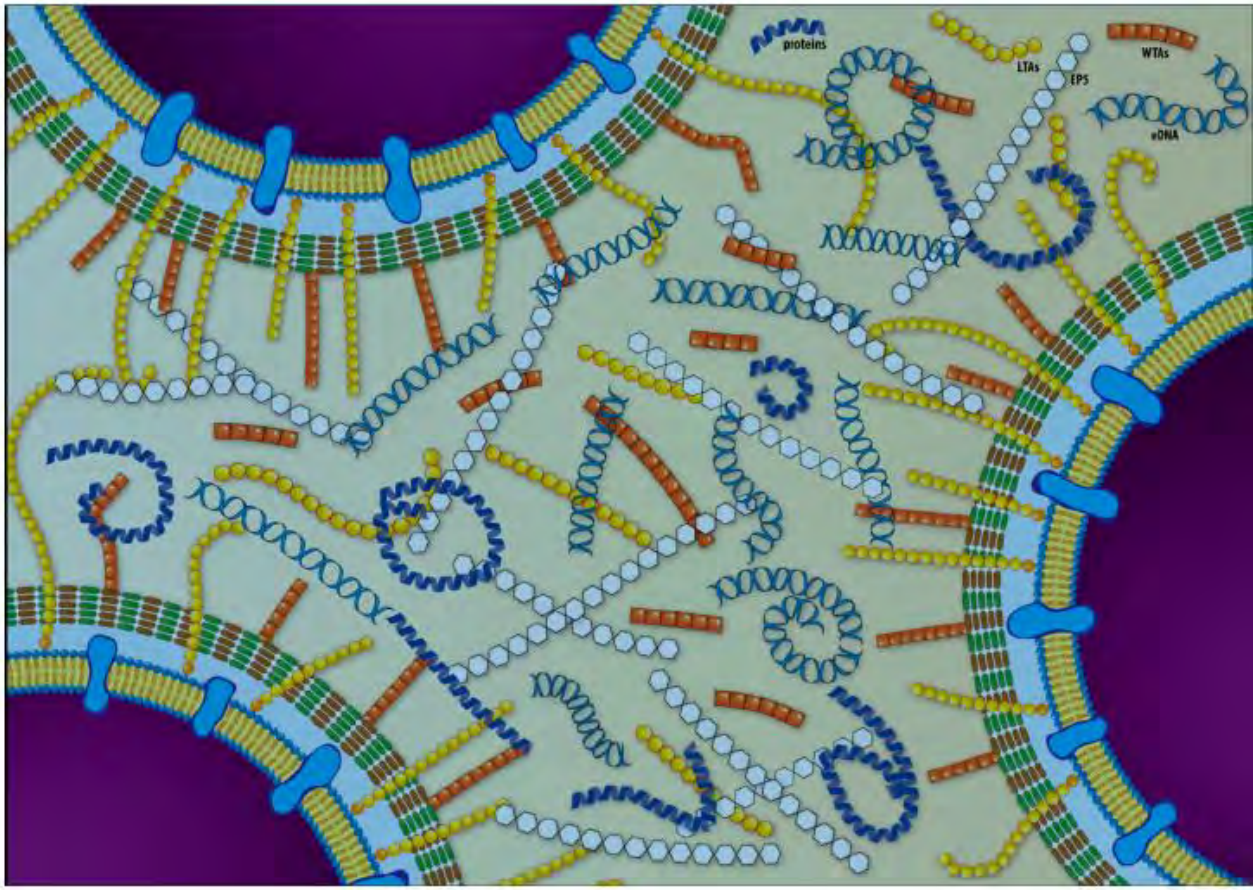
wall teichoic acids

lipoteichoic acids

proteins, and

eDNA

are distributed heterogeneously within the matrix.



Colagiorgi et al., 2016 (Used with permission)

Definition of biofilm

This matrix also provides:

- **protection** to the innermost cells being the most susceptible
- channels throughout the whole biofilm to **get in nutrients** or **get rid of waste**
- adhesion to the associated surface.

In the food industry, **surfaces and equipment** (both food-contact and non-food-contact) are frequently colonized by microorganisms forming biofilms.

Definition of biofilm

In most cases, this represents a challenge and a **concern**, as biofilms formed by

- spoilage or
- pathogenic microorganisms

can serve as a

- source of cross-contamination in foods,
- reducing the effectiveness of food processing strategies and
- compromising food quality and safety.

Definition of biofilm

There is debate as to whether microbial persistence in food processing environments is due to

- **the presence of harborage sites**, which are **difficult to clean and disinfect**, or
- to the colonization of these environments by microorganisms showing particular abilities **to survive in the harsh conditions** prevailing during food processing.

A significantly higher biofilm-forming ability on contact surfaces is linked to ***a lower susceptibility to common sanitizers***.

Biofilm formation

Biofilm development is a dynamic process. These are the most important steps:

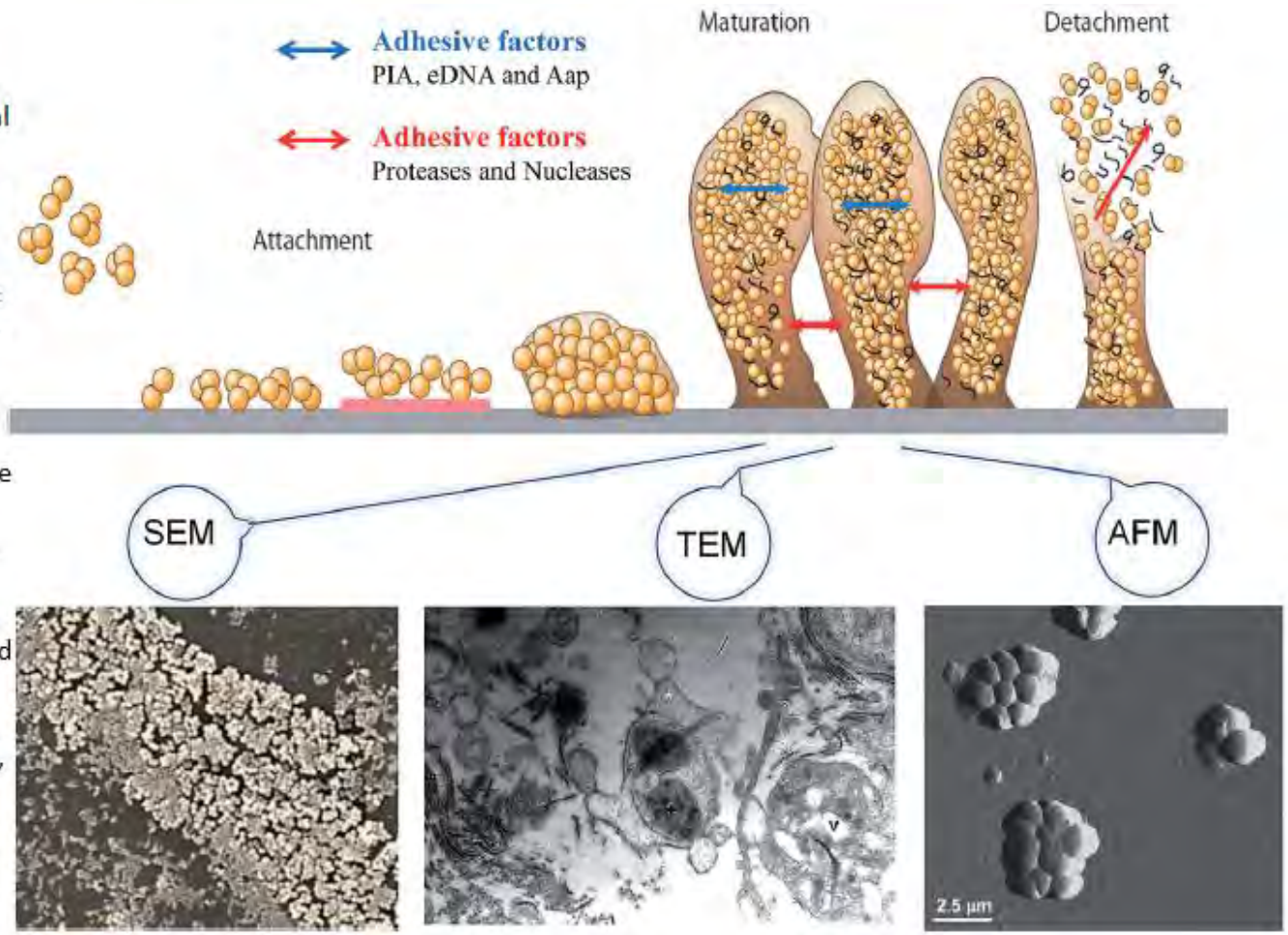
1. Planktonic (**free-living**) microbial cells attach to a surface
2. Using their **signaling system** (Quorum sensing) they will try to find out if they are alone or with others
3. If the **concentration** of cells has **reached a certain level**, they will start to produce extracellular polymeric substances (EPS)
4. This leads to an **irreversible attachment of the cells** anchoring them to the surface.

Micro-colony development results from

- simultaneous aggregation and
- growth of microorganisms,
- accompanied by EPS production.

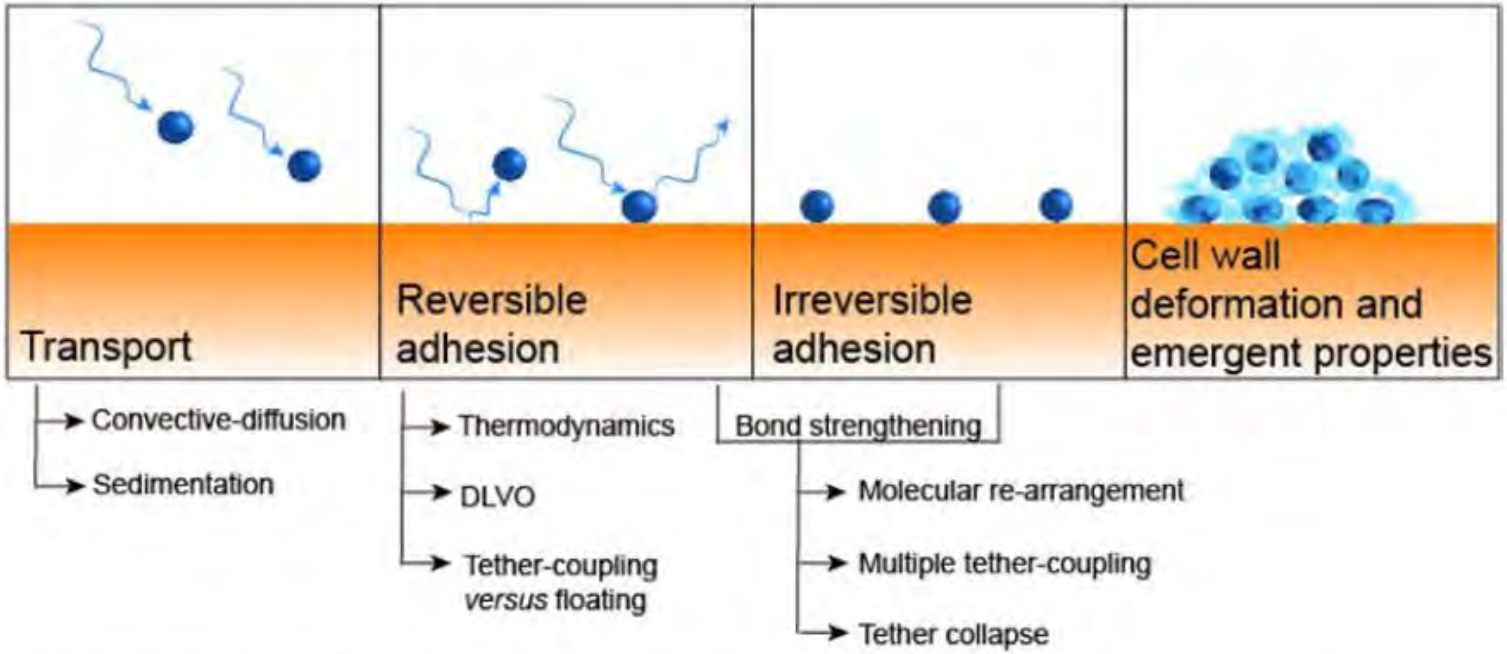
Biofilm formation

Evolution model of biofilm over 48 h, with changes in microcolony formation, maturation, and dispersion. The bacterial cells attach to a surface (the cells are depicted as yellow circular types). After attachment on the red surface, the bacteria come together to form a microcolony. The extracellular polymeric matrix is depicted as the orange outline around the microcolony. Then, biofilms maturate with 3D biofilm structures. Finally, the adhesive factors provide higher stability for the biofilms, while the biofilm is disrupted by proteases and other enzymes, changing into free cells. Planktonic cultures of *S. epidermidis* were grown for up to 48 h incubation at 37 °C, and biofilm growth was examined at various stages of development by scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy micrographs (AFM). The biofilm was shown to form within 6 h, and became established after 24–48 h.



Zhao et al., 2018 (Used with permission)

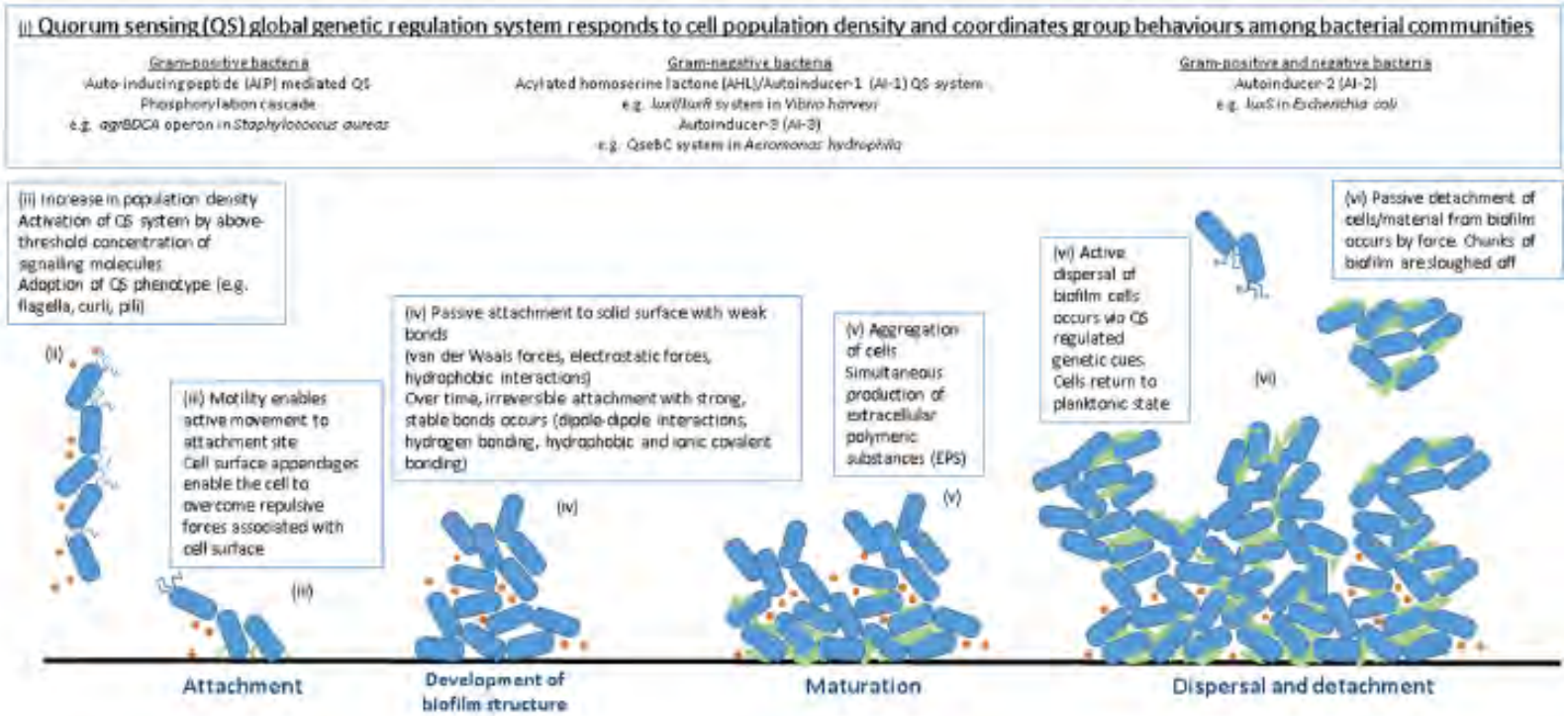
Biofilm formation



Four distinct, physicochemically controlled steps in biofilm formation.
(1) Transport of bacteria towards a substratum surface, occurring through convective-diffusion or sedimentation.
(2) Reversible bacterial adhesion to a substratum surface, that can be modeled by surface thermodynamics, Lifshitz-Van der Waals and electrostatic double-layer interactions as in the DLVO-theory and tether-coupling or "floating" adhesion models.
(3) Transition from reversible to irreversible bacterial adhesion through physicochemical bond strengthening mechanisms.
(4) After bond-strengthening, cell wall deformation occurs yielding emergent properties, characteristic of a mature biofilm.

Carniello et al., 2018 (Used with permission)

Biofilm formation



Stages of biofilm formation. I. QS signaling molecules, II. High population density, high QS signal, III. Attachment to solid surface, IV. Increase in cell numbers, irreversible attachment, development of biofilm structure, V. Biofilm maturation and EPS production, VI. Dispersal.

Coughlan et al., 2016 (Used with permission)

Biofilm formation

A **mature biofilm** consists of microorganisms in EPS-enclosed micro-colonies interspersed with less dense regions of the polymer matrix that include **water channels transporting nutrients and metabolites**.

Individual cells of the biofilm may also be actively released into the surrounding environment to attach and colonize other surfaces.

It is important to note that cells within biofilms are **physiologically distinct from their planktonic counterparts**.

Biofilm formation

Modern food processing plants support and select for biofilm-forming bacteria on food contact surfaces due to

- highly automated systems,
- lengthy production cycles and
- vast closed surface areas in processing lines.

Areas in which biofilms most often develop are those which are the most **difficult** to

- rinse,
- clean and
- sanitize.

Biofilm formation

- Dead ends,
- gaskets,
- joints,
- pumps,
- grooves,
- surface roughness due to **surface defects**,
  - by-pass valves,
  - abraded equipment parts,
  - sampling cocks,
  - overflow siphons in filters and
  - corrosion patches, etc.

are hard-to-reach areas.

https://www.imi-critical.com/product-type/bypass-valves/

Biofilm formation

The presence of

- ✓ **nutrients** or
- ✓ even microscopic food residues, and
- ✓ frequent stress conditions from
- **cleaning**,
- sanitizing or
- **processing treatments** may

individually or collectively influence

- ➢ biofilm development and
- ➢ biofilm structure.

Biofilm formation

It is apparent that various simple carbohydrates can modulate biofilm formation in bacteria; for example, **milk lactose**, shown to enhance biofilm formation in both *S. aureus*, predominantly by inducing production of polysaccharide intercellular adhesin protein,

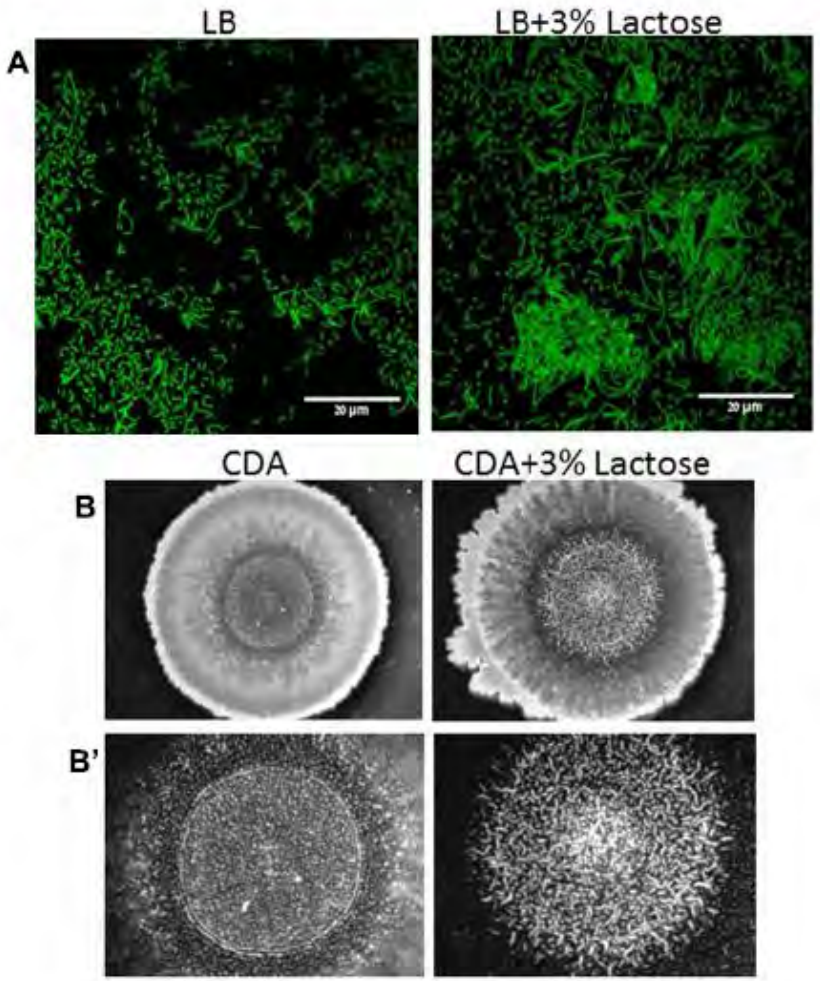
- **L-leucine** in *L. monocytogenes*,
- **butyric acid**, released during milk lipolysis, in *Bacillus* spp.,
- **Iron** in *Bacillus cereus* on **the stainless steel** compared with polystyrene,
- **Ca2+ and Mg2**+ in *Geobacillus* spp. and
- **milk proteins** in *Streptococcus thermophilus* biofilm formation on stainless steel.

Biofilm formation

Lactose may induce biofilm formation by *B. subtilis*:

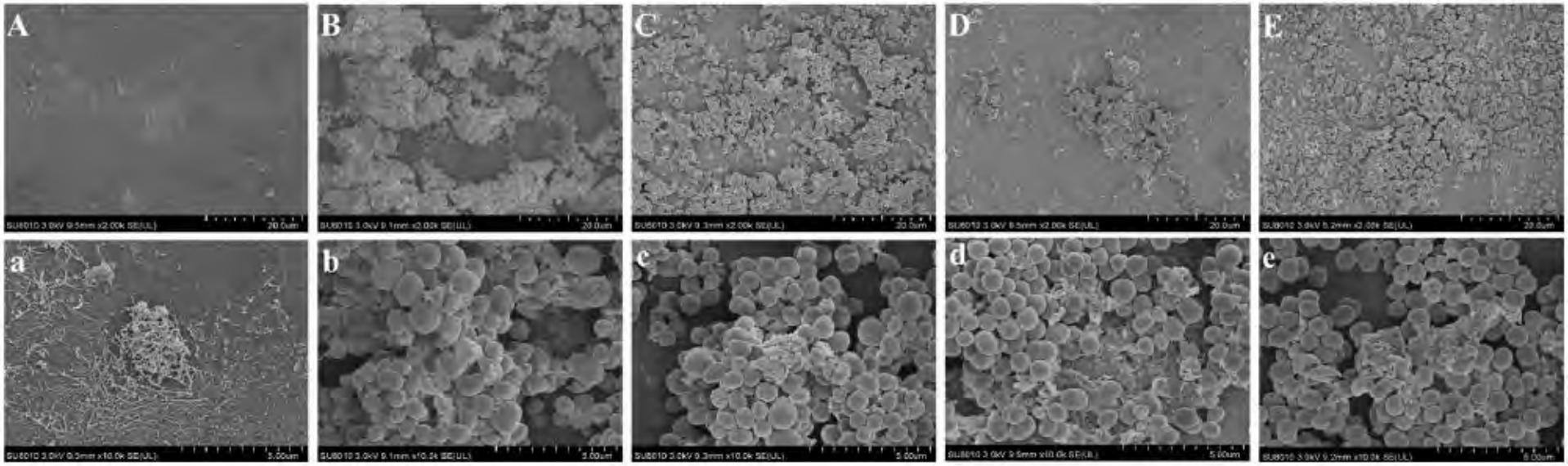
(A) CLSM images of bundles formation. Overnight cultures of *B. subtilis* were diluted into LB or LB supplemented with 3% lactose. Cultures were then incubated for 5h at 37◦C and 50rpm. A sample from each culture was then analyzed using a confocal microscope. Images are representative of three biological repeats.

(B) Colony biofilm was generated on chemical defined agar (CDA) and CDA supplemented with 3% lactose. (B') Zoomed images of the center of generated biofilm.



Duanis-Assaf et al., 2016 (Used with permission)

Biofilm formation

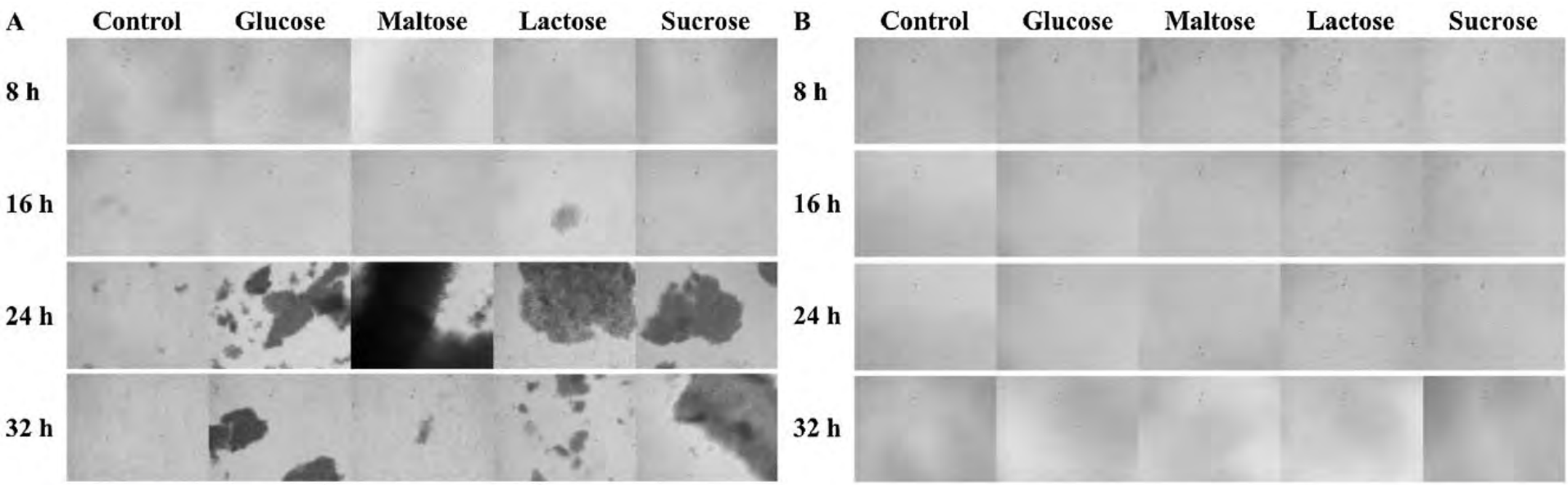


SEM images of biofilm formed by *S. epidermidis* in nutrient broth (NB) supplemented with different carbon sources (2.5 mg/mL) at 37°C on coverslips for 24 h.

A-a: NB. **B-b:** Glucose. **C-c:** Maltose. **D-d:** Lactose. **E-e:** Sucrose.

Zou & Liu, 2020 (Used with permission)

Biofilm formation



Light microscopic photographs of biofilm formed by *S. epidermidis* grown in nutrient broth supplemented with different carbon sources (2.5 mg/mL) at 37◦C (A) or 55◦C (B).

Zou & Liu, 2020 (Used with permission)

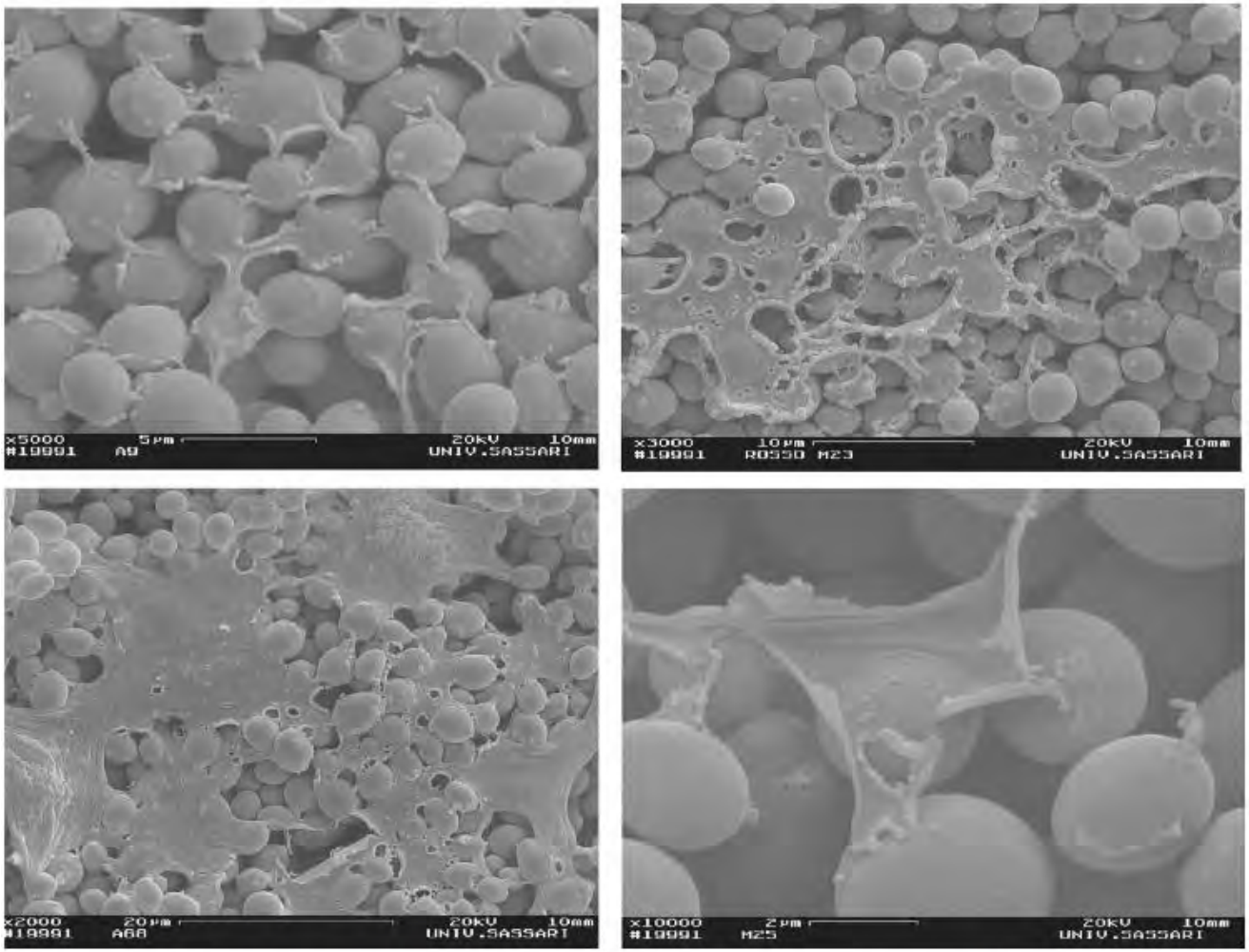
Biofilm formation

- ✓ **Seventy nine** percent of isolates were **Gram-negative rods**,
- ✓ 8.6% Gram-positive cocci,
- ✓ 6.5% Gram-positive rods and
- ✓ 1.2% yeast strains.

The most common organisms were

- *Pseudomonas*,
- *Staphylococcus* and
- *Enterobacter* spp.

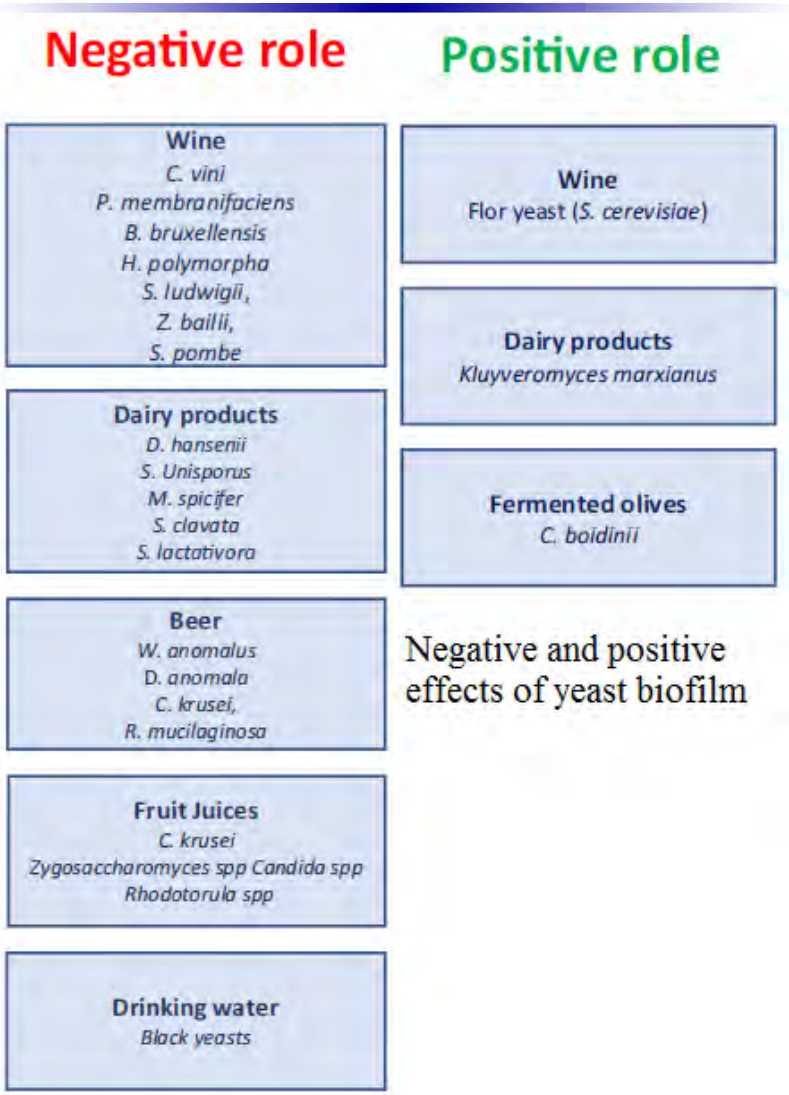
Biofilm formation



Extracellular matrix from different *S. cerevisiae* biofilm-forming

Zara et al., 2020 (Used with permission)

Biofilm formation



Zara et al., 2020 (Used with permission)

Biofilm formation

Summary of the frequency of genera among isolates identified in 16 factory sites (n=78)

Genus	*Percentage*	*Genus*	*Percentage*
Pseudomonas	**23**	***Staphylococcus***	**8.6**
Enterobacter	**8.6**	*Flavobacterium*	7.7
Acinetobacter	7.7	***Bacillus***	**6.5**
Serratia	5.1	*Klebsiella*	5.1
Aeromonas	3.8	*Vibrio*	2.4
Citrobacter	2.4	*Kluyvera*	2.4
Agrobacterium	2.4	*Hafnia*	2.4
Providencia	1.2	*Escherichia*	1.2
Pasteurella	1.2	*Proteus*	1.2
Yersinia	1.2	*Trichosporan*	1.2

Biofilm formation

Speers et al. (1984) found

✓ ***Pseudomonas*** spp.

and

✓ ***Micrococcus*** spp.

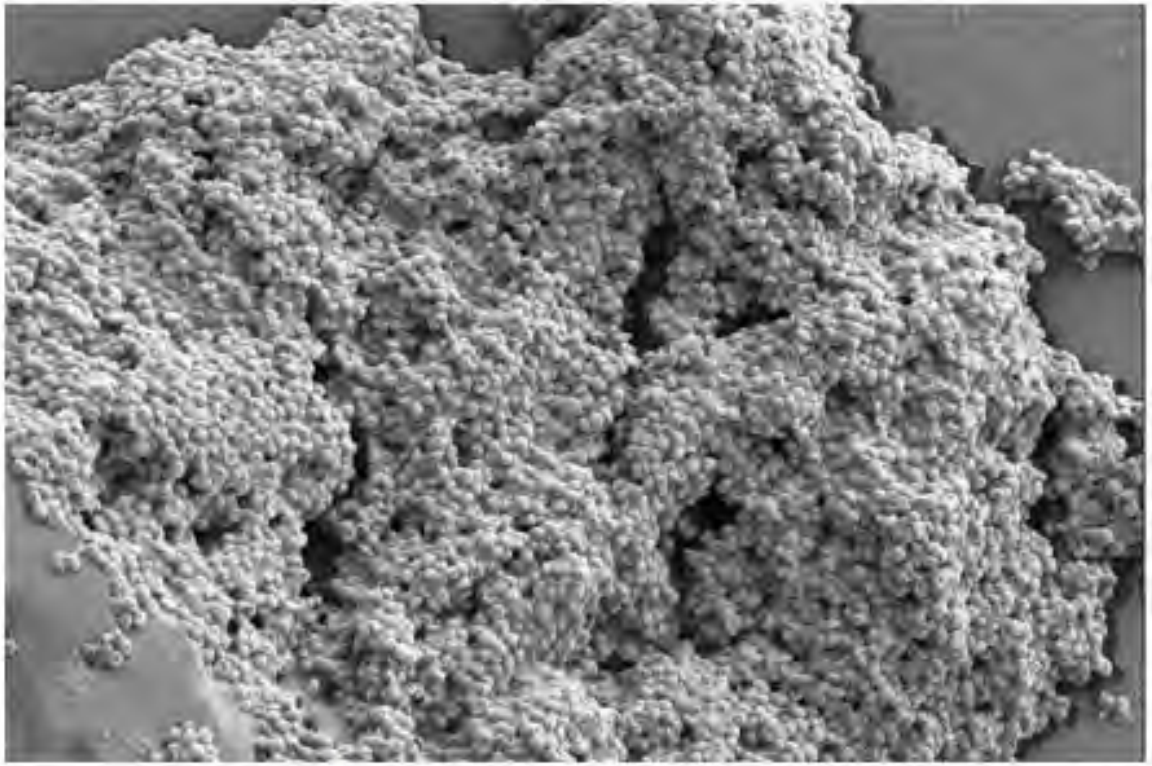
and

Zoltai et al. (1981) detected

- ***Staphylococcus aureus***

and

- ***Streptococcus cremoris.***



SEM image of *S. aureus* biofilm on the surface of un-washed stainless steel pipeline (5000x)

Miao et al., 2019 (Used with permission)

Biofilm formation

Hood and Zottola (1997) isolated a variety of **micro-organisms** associated with test surfaces in **four meat processing plants** with

- ***Pseudomonas*** and
- ***Klebsiella*** species being **the most common** and
- *Aeromonas* spp.,
- *Citrobacter freundii* and
- *Hafnia alvei* also detected.

These authors noted that the most common organisms were **mucoid**, indicating prolific EPS production.

Miao et al., 2019 (Used with permission)

Biofilm formation

Mettler and Carpentier (1998) studied the microflora associated with the surfaces in

- milk,
- meat

and

- pastry sites

and concluded that it was specific to the processing environment.

Biofilm formation

Pseudomonas spp. predominated in

- **the low temperature meat site**

and

- yeasts and *Leuconostoc* spp. in the pastry site.

Pseudomonas spp. were found at all three sites and have been found in almost all food factory environments where biofilms have been studied.

Biofilm formation

Pseudomonas are environmental psychrotrophic organisms that readily **attach to surfaces** and are **common spoilage organisms in chilled foods.**

Other common Gram negative bacteria that have been associated with surfaces are **coliforms** which are **widely distributed in the environment** and may be indicators of inadequate processing or post-process contamination.

Biofilm formation

Staphylococcus spp. were also found at all three sites. In addition, other studies have found ***Staphylococcus*** sp. associated with surfaces.

Staphylococci are associated with **human skin** and therefore their presence on surfaces may be as a result of transfer from **food handlers**.

https://www.worldfoodinnovations.com/innovation/surfacehygiene-monitoring-using-atp-amp-bioluminescence

Biofilm formation

These studies primarily rely on swabbing and traditional microbiology and therefore **only represent a proportion of the culturable organisms** that can be recovered from accessible sample areas.

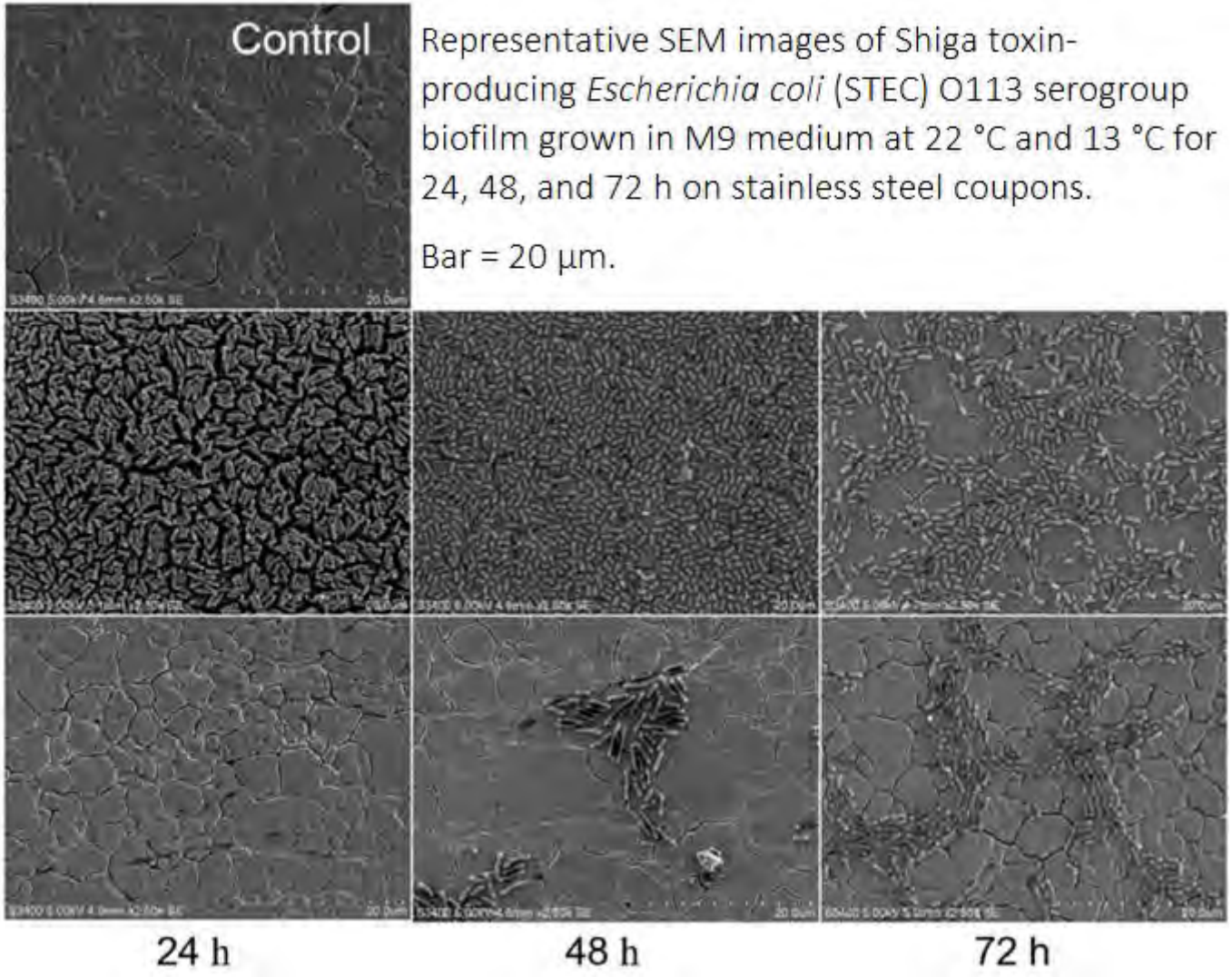
https://www.worldfoodinnovations.com/innovation/surfacehygiene-monitoring-using-atp-amp-bioluminescence

Biofilm formation

Andrade et al. (1998) found that the thermoduric psychrotrophic lactic acid bacteria involved in **milk spoilage** readily attached to **surfaces**.

Farrell et al. (1998) demonstrated the transfer of ***Escherichia coli* O157:H7** from spiked **meat** samples to stainless steel surfaces in a meat grinder, thus demonstrating that **the food product can be a source of pathogenic organisms that attach to surfaces** and remain at low levels after cleaning treatments (50% of surfaces).

Biofilm formation

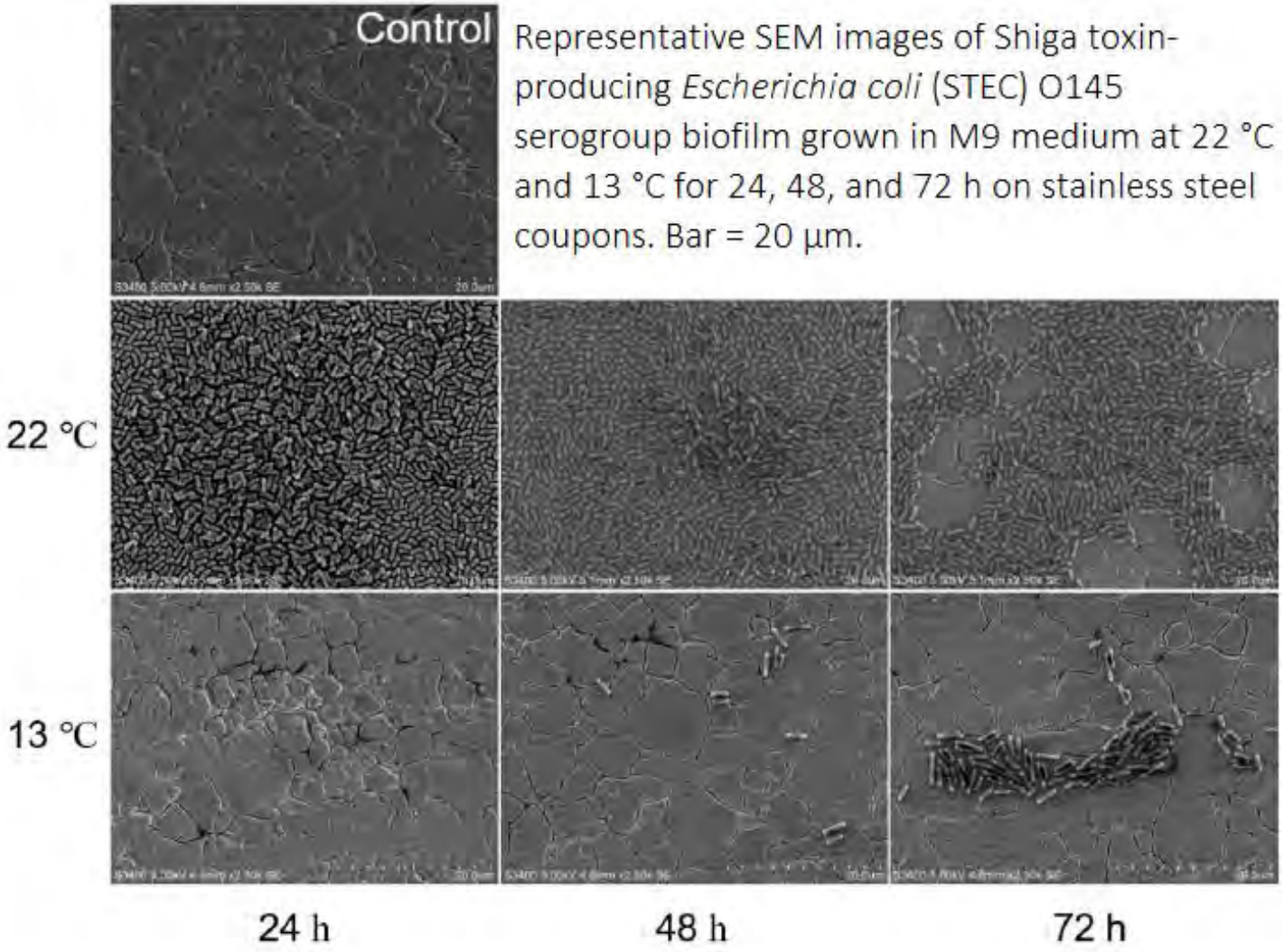


Representative SEM images of Shiga toxin-producing *Escherichia coli* (STEC) O113 serogroup biofilm grown in M9 medium at 22 °C and 13 °C for 24, 48, and 72 h on stainless steel coupons.

Bar = 20 µm.

Ma et al.,, 2019 (Used with permission)

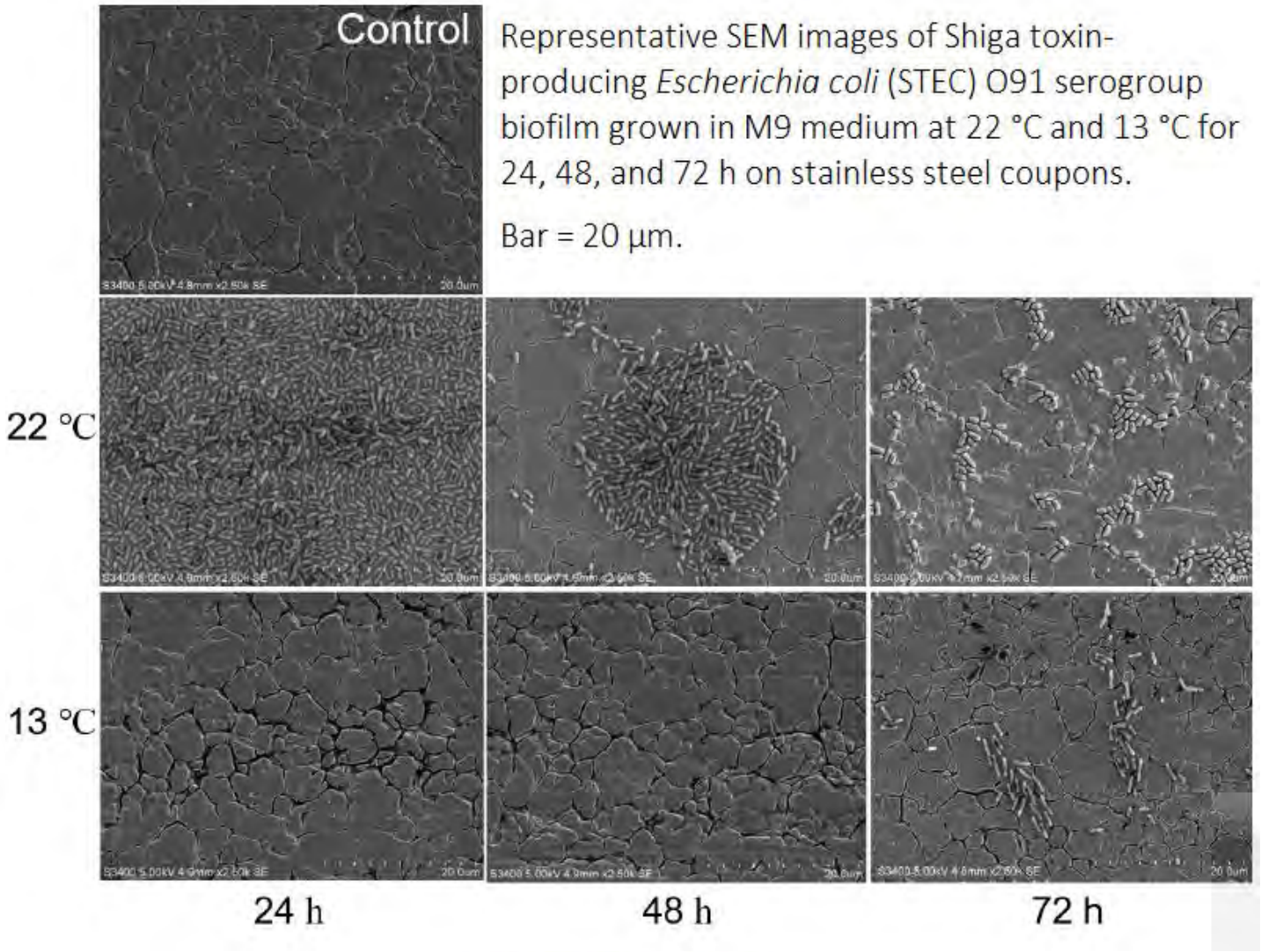
Biofilm formation



Representative SEM images of Shiga toxin-producing *Escherichia coli* (STEC) O145 serogroup biofilm grown in M9 medium at 22 °C and 13 °C for 24, 48, and 72 h on stainless steel coupons. Bar = 20 µm.

Ma et al.,, 2019 (Used with permission)

Biofilm formation



Representative SEM images of Shiga toxin-producing *Escherichia coli* (STEC) O91 serogroup biofilm grown in M9 medium at 22 °C and 13 °C for 24, 48, and 72 h on stainless steel coupons.

Bar = 20 µm.

Ma et al.,, 2019 (Used with permission)

Biofilm formation

Listeria monocytogenes is a well-adapted pathogen with the ability to proliferate in **cold wet conditions** that are ideally suited for biofilm formation in various environments.

Listeria spp. have been isolated from wooden shelves in

- cheese-ripening rooms,
- processing

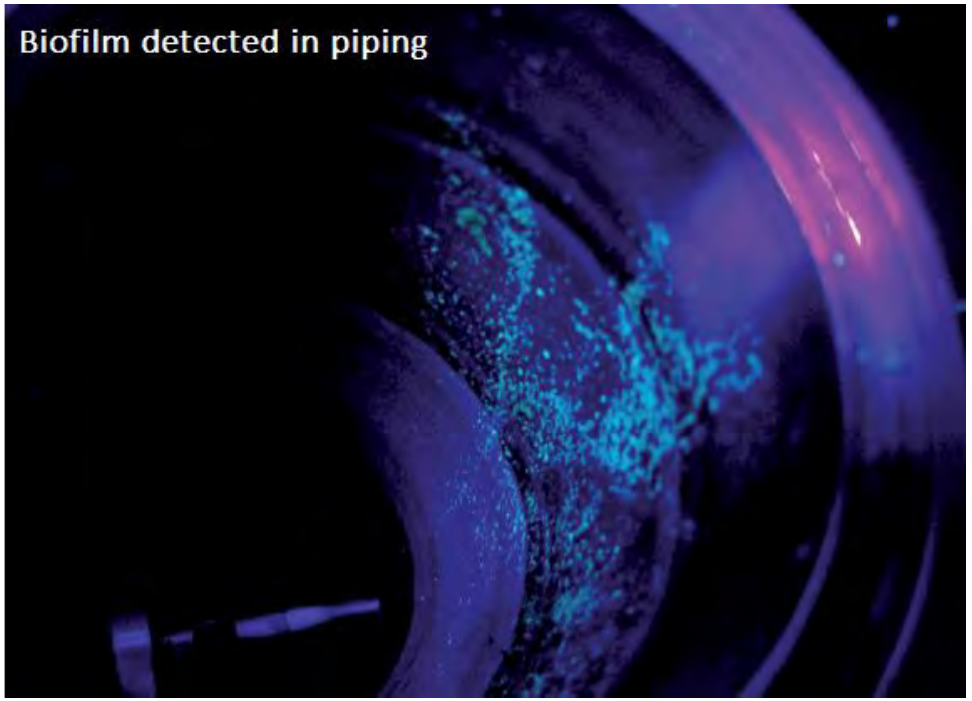
and

- packaging equipment,

and especially

- **wet**,
- **difficult-to-clean** environments

such as conveyor belts, floor drains, condensate, storage tanks, etc.



https://www.eit-international.com/wp-content/uploads/2020/06/Bactiscan-bacteria-datasheet-EIT.pdf

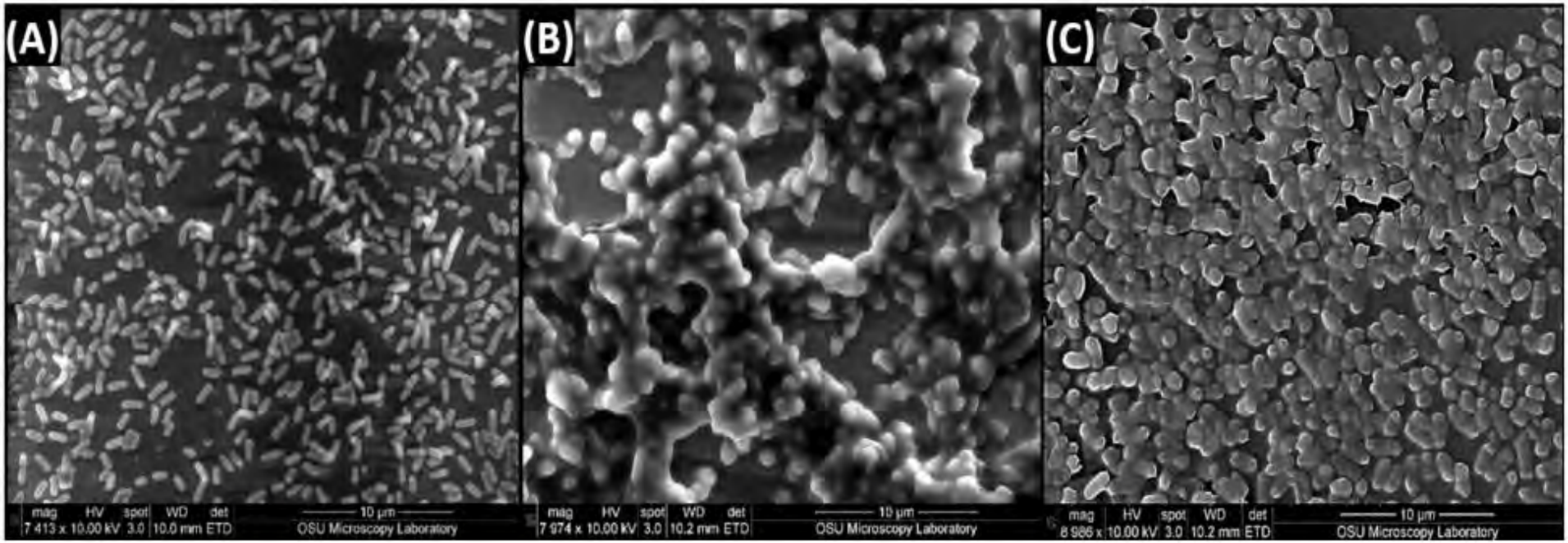
Biofilm formation

The growth of ***L. monocytogenes*** in food plant biofilms increases the general contamination level in the plant and may be an indication of unsatisfactory cleaning/sanitization procedures.

https://www.silikalamerica.com/clean-room-flooring.html

Biofilm formation

Outbreaks of **listeriosis** and **salmonellosis** have been implicated to **post-pasteurization /processing contamination of milk, cheese and ice-cream** as a contributing factor.



Scanning electron microscopy (SEM) of enhanced 7-day biofilms prepared on slide chambers from (A) *Listeria monocytogenes* 99-38, (B) *E. coli* O157:H7 F4546, and (C) S. Montevideo FSIS 051. Approximately 7000–9000-fold magnification.

Aryal & Muriana, 2019 (Used with permission)

Biofilm formation

Heat-resistant spore-forming organisms are commonly found in food/dairy processing plants and even in extreme environments such as in hot (80°C) alkaline solutions in reuse CIP systems.

Bacillus and other thermoduric bacteria may form a biofilm if hot fluid continuously flows over a surface for 16 h or longer.

Biofilm formation

Pathogenic bacteria can also **coexist** within a biofilm with other organisms; for example, ***Listeria***, ***Salmonella*** and **other pathogens** have been found in established ***Pseudomonas*** biofilms.

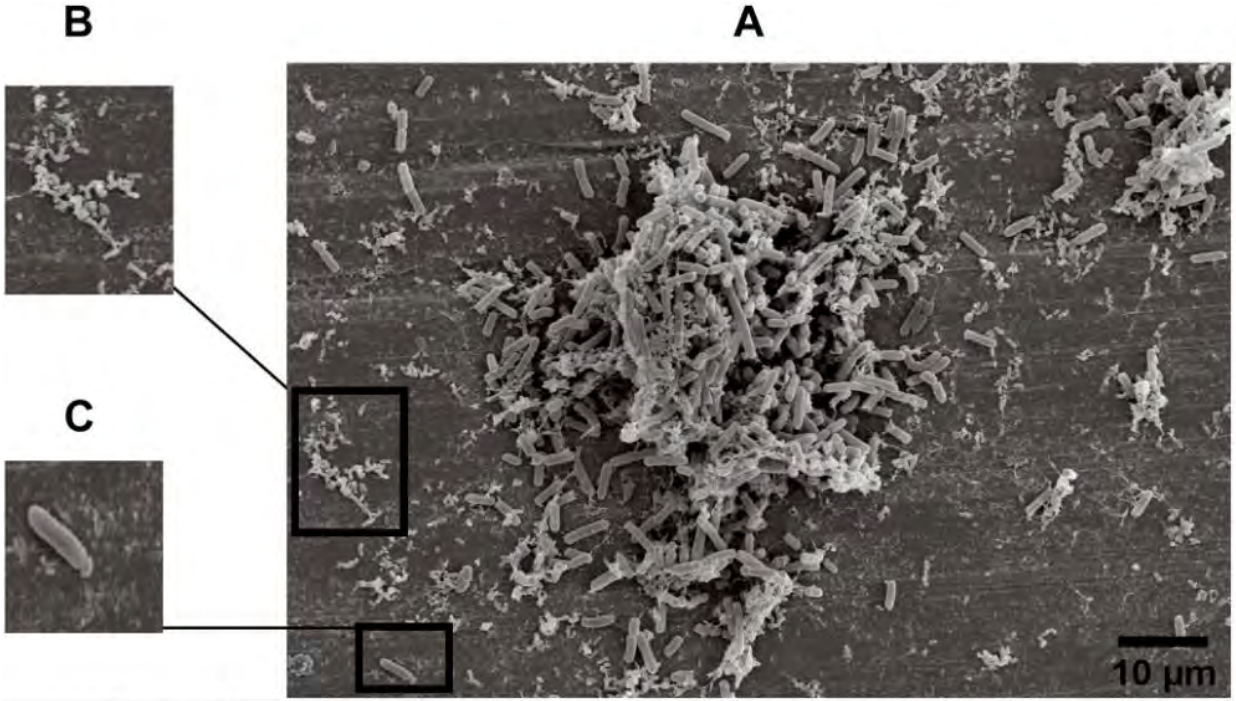


Image obtained by SEM (JEOL) of microbial cooperative interaction of *L. monocytogenes* and *B. cereus* on stainless steel (A); *L. monocytogenes* (B); and *B. cereus* (C).

Alonso et al., 2020 (Used with permission)

Biofilm formation

Cooperative and competitive interactions were found between *L. monocytogenes* strains isolated from dairy products and *B. cereus*.

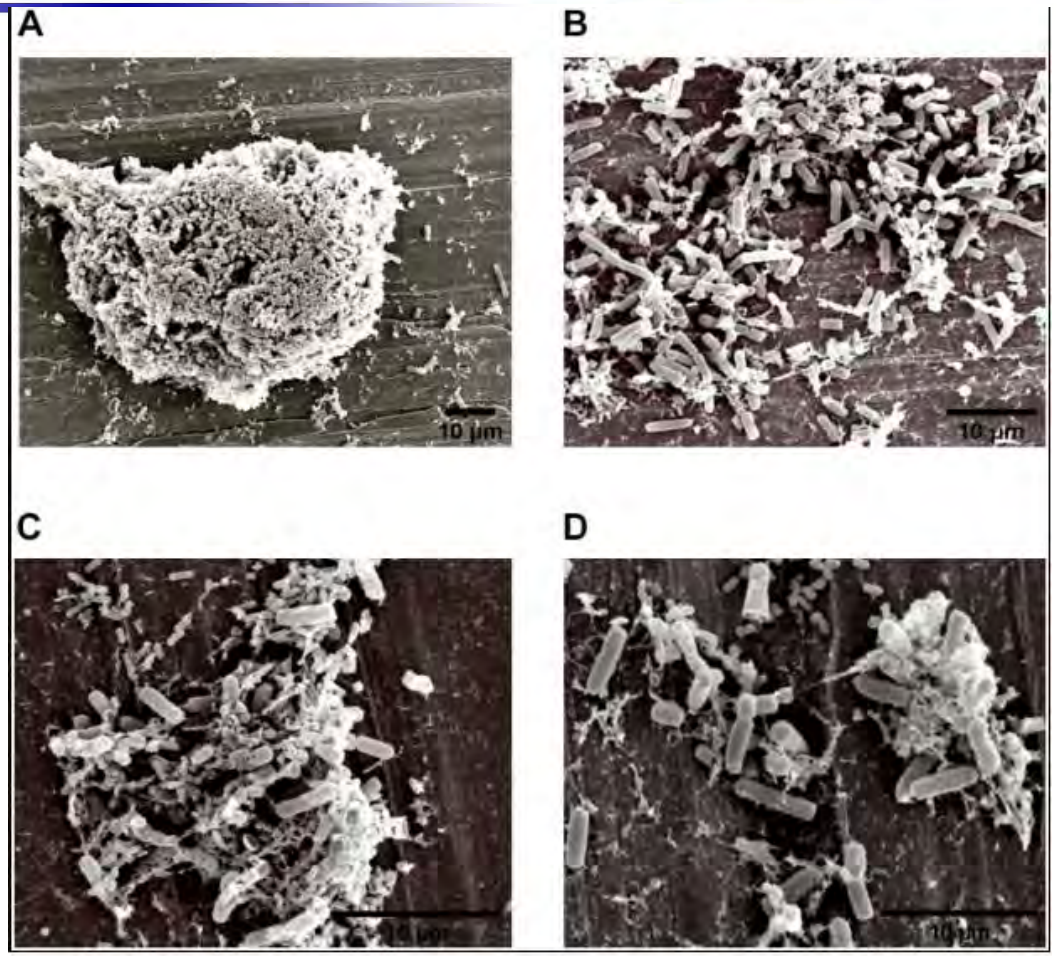


Image obtained by SEM (JEOL) of the interaction between *L. monocytogenes* and *B. cereus* in the biofilm on stainless steel. Agglomerates of dual-species biofilms (A); Biofilm cells (B, C); and low EPS production (D).

Alonso et al., 2020 (Used with permission)

Biofilm formation

Although the presence of ***Salmonella*** spp. is not well documented, various studies suggested that ***Salmonella*** can establish themselves in biofilms on food surfaces.

The significance of the **growth** and **activity** of bacteria at **solid–liquid interfaces on food product contact surfaces** has been emphasized previously.

Biofilm formation

It was suggested that **proteolytic enzymes may be produced and released from established *Flavobacterium* biofilms**.

It has also been found that the production of **catalase** by attached populations of ***Pseudomonas aeruginosa*** biofilms may be **partly responsible for increased resistance to sanitizers containing hydrogen peroxide**.

Biofilm formation

The organisms present on food processing surfaces can, therefore, be inoculated

- from the environment,
- from people and
- from the product.

It is not clear under what circumstances the survival and development of microorganisms from each source are favored, but the results to date suggest that

- ***pseudomonads*** and
- ***Staphylococci***

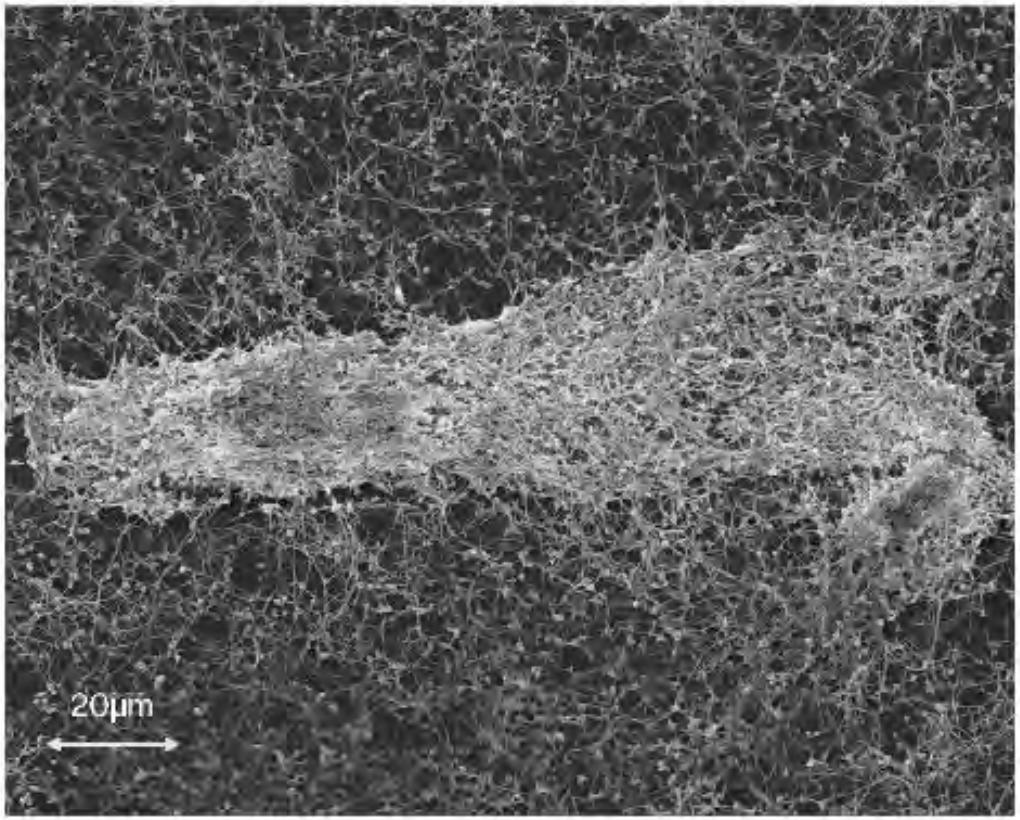
most frequently found and thus the **environment** is the most common source **rather than** the raw ingredients.

Biofilm formation

Campylobacter jejuni has been shown to form biofilms under a variety of conditions and plays a large role in survival under harsh conditions.

A scanning electron micrograph of biofilm formed by C. jejuni strain 11168-O under 800× magnification.

These biofilms exhibit the archetypal biofilm architecture with cells encased in an exuded extracellular matrix.

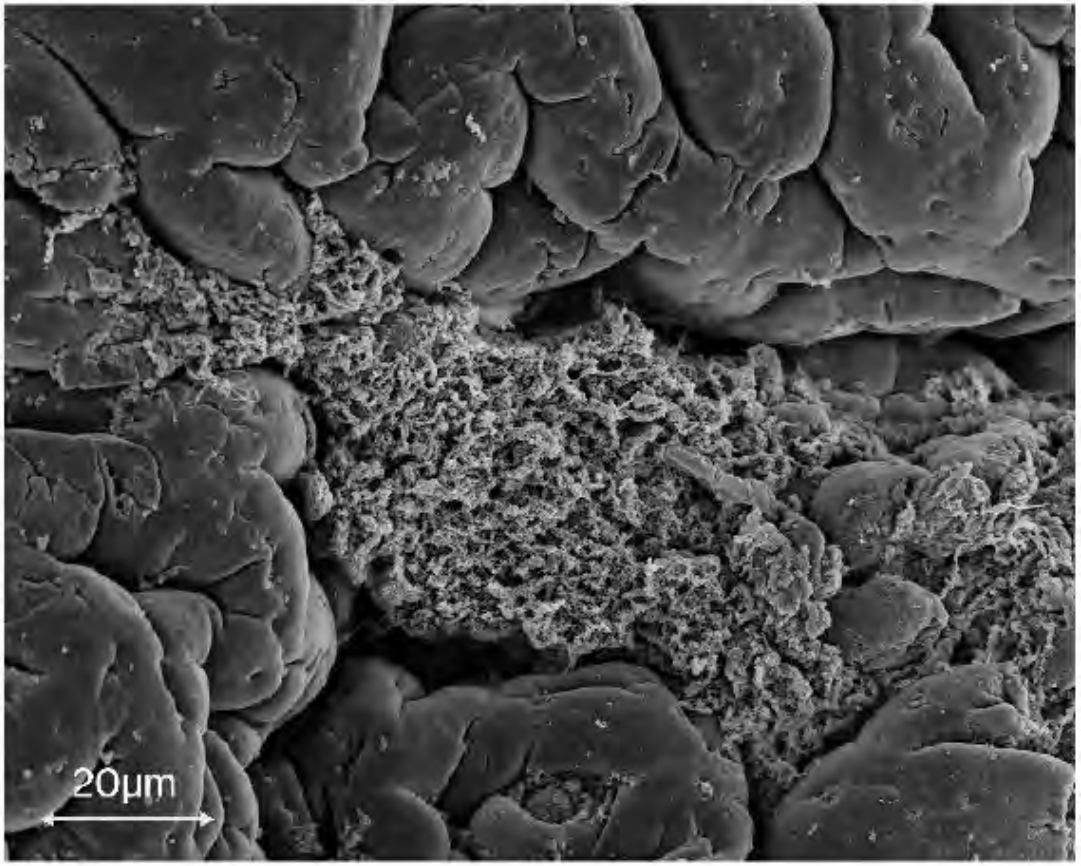


Tram et al., 2020 (Used with permission)

Biofilm formation

A scanning electron micrograph of *C. jejuni* biofilm formed by strain 11168-O in chicken caecum at 200× magnification.

These biofilms were formed throughout the caecum and suggest that biofilms formed by *C. jejuni* affect survival in the avian intestinal tract.

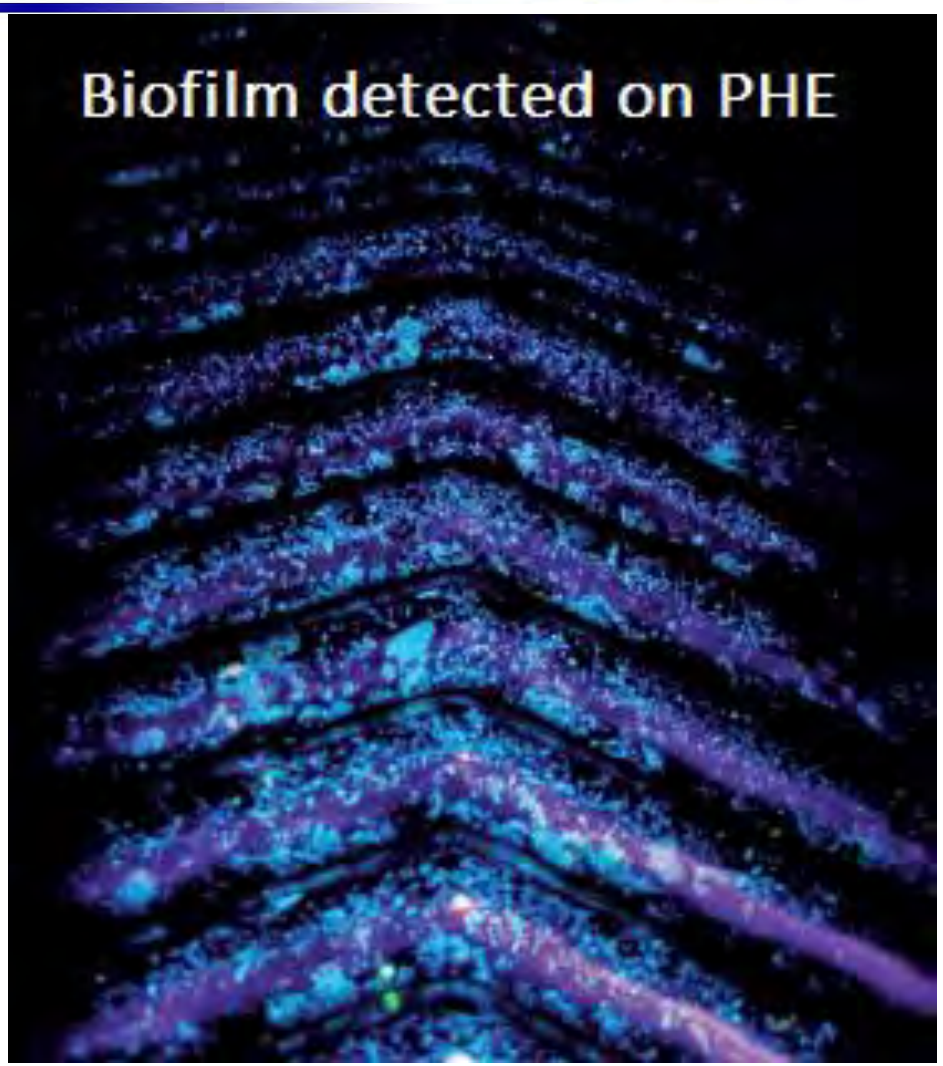


Tram et al., 2020 (Used with permission)

Biofilm formation

Biofilms may develop in environments that have

- **a high microbial diversity** (e.g. floor drains) or
- in environments dominated by one or a few microbial species, such as on **plate heat exchangers**.



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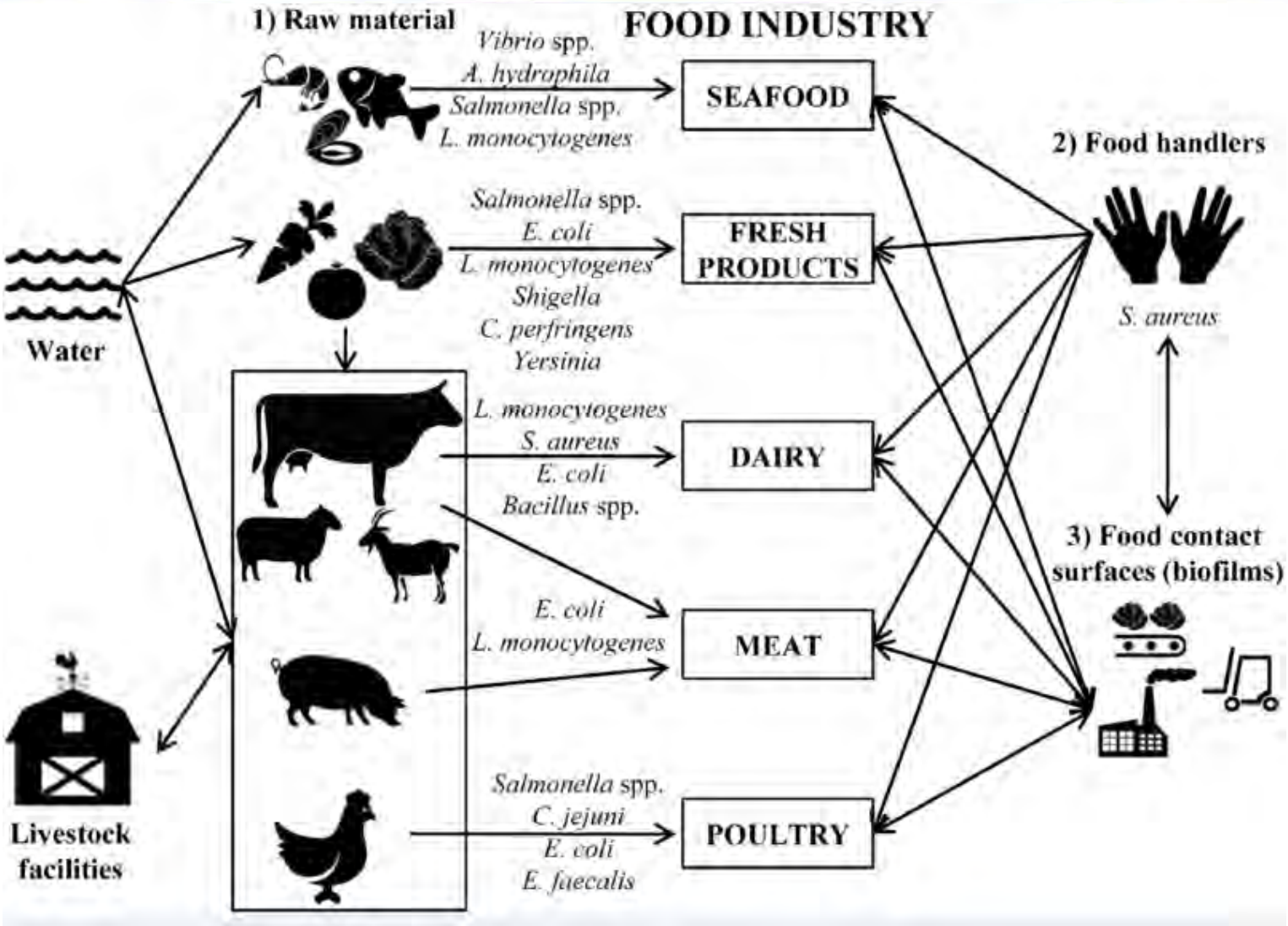
Biofilm formation



Gutiérrez et al., 2016 (Used with permission)

Biofilm formation

Microorganisms in established biofilms are highly resistant to treatment with antimicrobial agents (e.g. antibiotics, disinfectants, etc.).

It has been suggested that adhered cells in a biofilm can tolerate antimicrobial compounds at concentrations of **10–1000 times** that needed to kill genetically equivalent planktonic bacteria.

Biofilm formation

Biofilm cells have the ability to survive harsh environmental conditions such as

- fluctuating pH,
- extreme heat or cold,
- low nutrient concentrations,

and they are highly resistant to exposure to

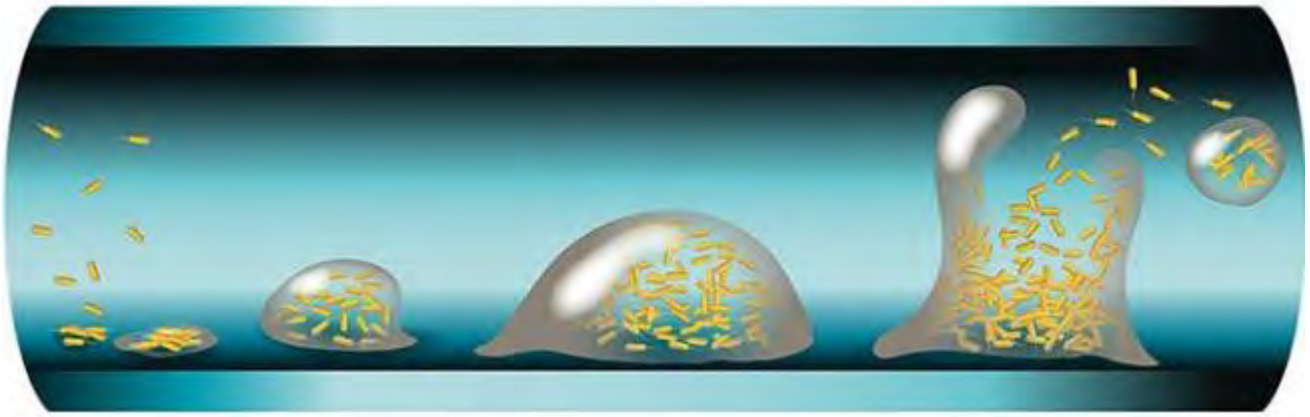
- UV light,
- chemical shock,
- starvation and
- dehydration.

Biofilm formation

They may cause:

- Post-pasteurization contamination,
- decreased shelf-life, or
- potential spoilage of products.

Attached cells become irreversibly adsorbed to the surface, which enables the organisms to resist mechanical cleaning procedures.

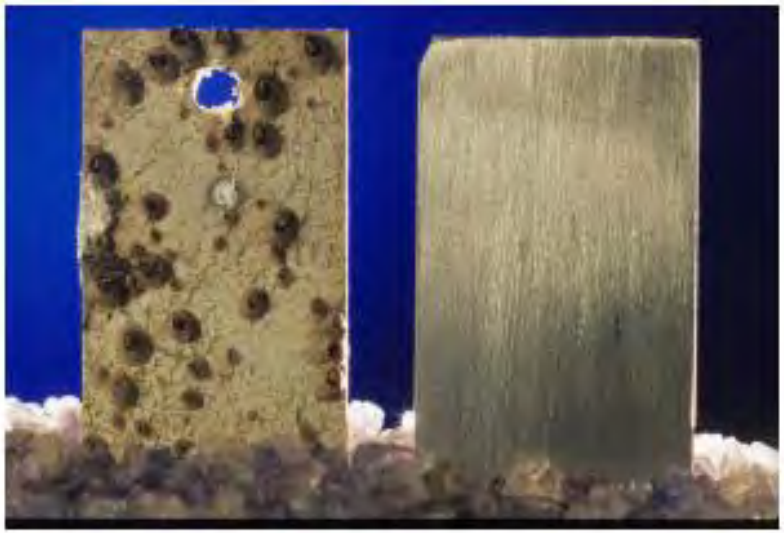


https://www.wattagnet.com/articles/39357-ways-to-manage-biofilm-in-poultry-drinking-water?v=preview

Biofilm formation

Reduction in the efficiency of heat transfer occurs if biofilm accumulation becomes sufficiently thick at locations such as **plate heat exchangers**.

Biofilm microorganisms may also be responsible for the corrosion of metal milk pipelines and tanks due to chemical and biological reactions.



http://www.alvimcleantech.com/cms/en/about-biofilm/biofilm-related-issues/mic-prevention

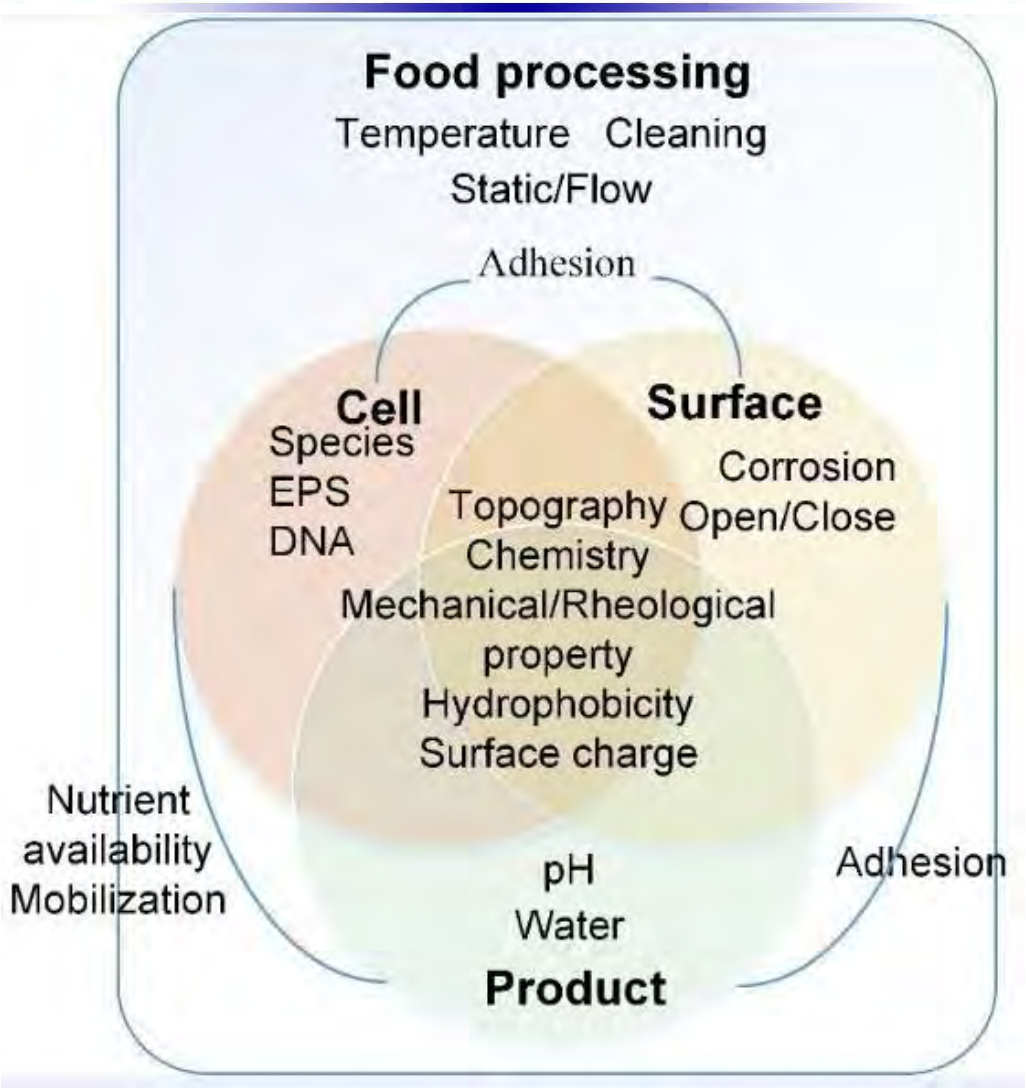
Biofilm formation

Biofilm accumulation in the food environment, and especially the development on ***food contact surfaces***, is important.

Biofilms in food processing environments have, for example, the following potential implications:

- Microorganisms in biofilms are **highly resistant to treatments**
- Biofilm cells have the ability to survive harsh environmental conditions
- **Post-pasteurization contamination**
- Attached cells become irreversibly adsorbed to the surface
- **Food-borne pathogens** and **spoilage organisms**
- Heat-resistant spore-forming organisms
- The presence of ***Salmonella*** spp., ***Flavobacterium*** and ***Pseudomonas aeruginosa***.
- Reduction in the efficiency of heat transfer

Important factors in biofilm formation and their relationship



Huang et al., 2020 (Used with permission)

Biofilm control/removal

The most important factors that contribute to biofilm formation are

- **inadequate removal of residual soil from surfaces** (cleaning)

and

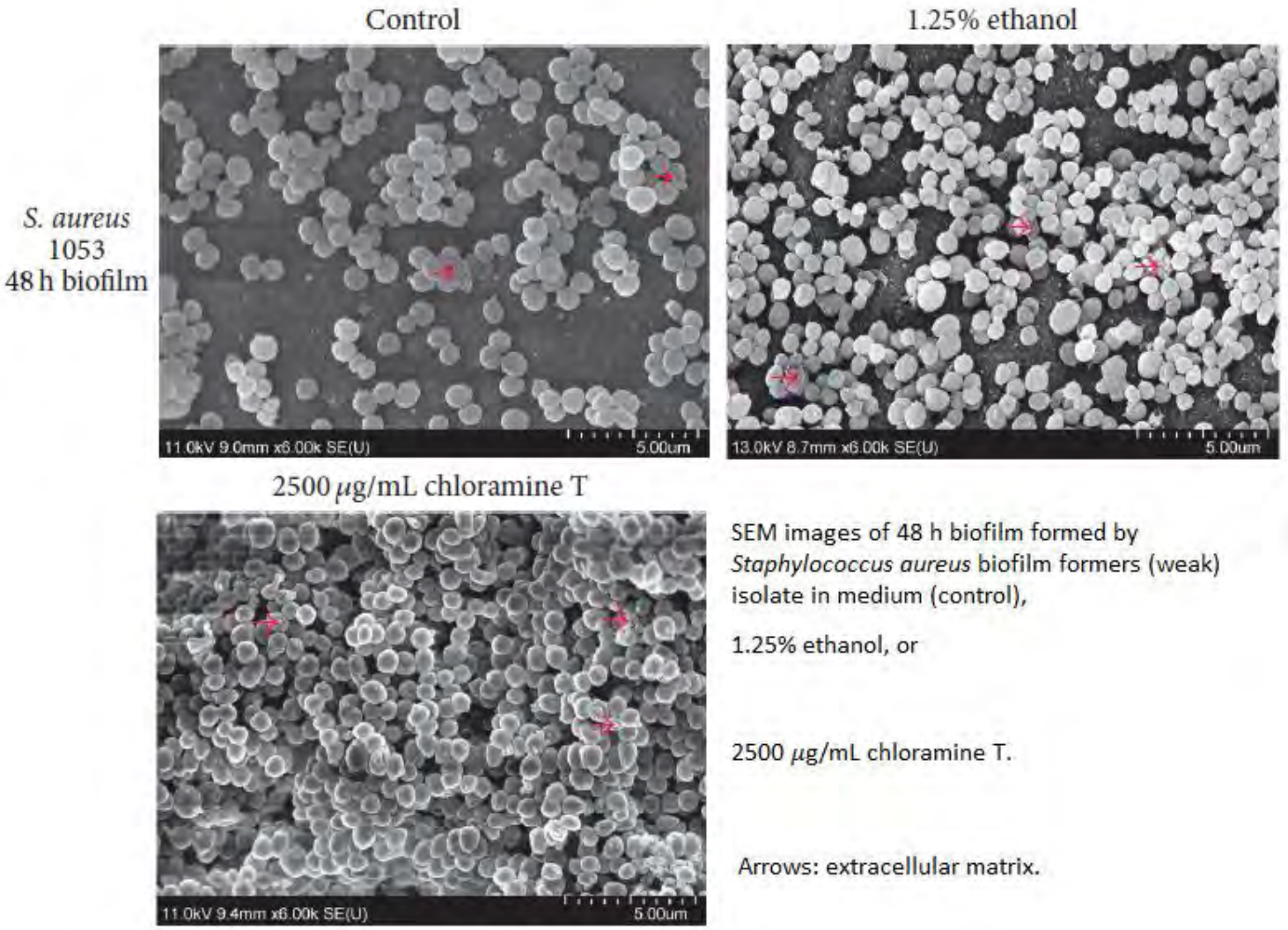
- **incorrect sanitation and sterilization of food contact surfaces**.

Microorganisms remaining on equipment surfaces may **survive for prolonged periods** depending on the **amount** and **nature** of the

- ✓ **residual soil**,
- ✓ **temperature** and
- ✓ **relative humidity**.

For example, milk is a highly nutritious medium, so any residue not removed can promote **bacterial growth**, **bacterial adhesion to the surface** and, consequently, **biofilm development**.

Biofilm control/removal



SEM images of 48 h biofilm formed by *Staphylococcus aureus* biofilm formers (weak) isolate in medium (control),

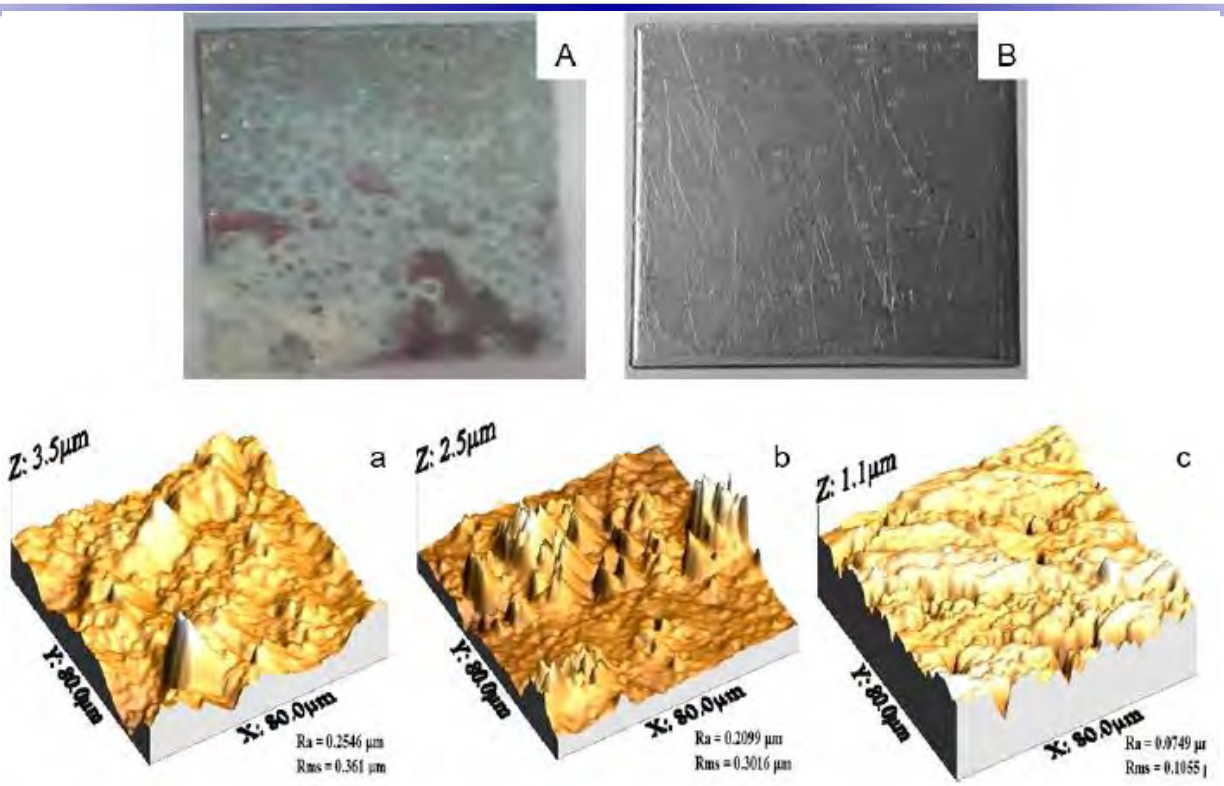
1.25% ethanol, or

2500 μg/mL chloramine T.

Arrows: extracellular matrix.

Cincarova et al., 2016 (Used with permission)

Biofilm control/removal



Appearance of SSP with deposits type (**A**) (panel A), and type (**B**) (panel B). AFM micrographs of SSP plates: (**a**) type A deposits; (**b**) type B deposits; (**c**) SSP without deposits.
SSP: Stainless steel plates 304 (2.5 × 2.5 cm) surface finish 2B

Without deposits: All plates were submerged in 100 mL of raw milk, and heated at 70 ˚C for 15 min in a constant water bath to promote protein denaturation and surface adhesion.

Deposits type (A): For type A deposits the temperature increased to 90 ˚ C, and kept for 30 min

Deposits type (B): For type B deposits the temperature increased to 121 ˚ C, and kept for 5 min

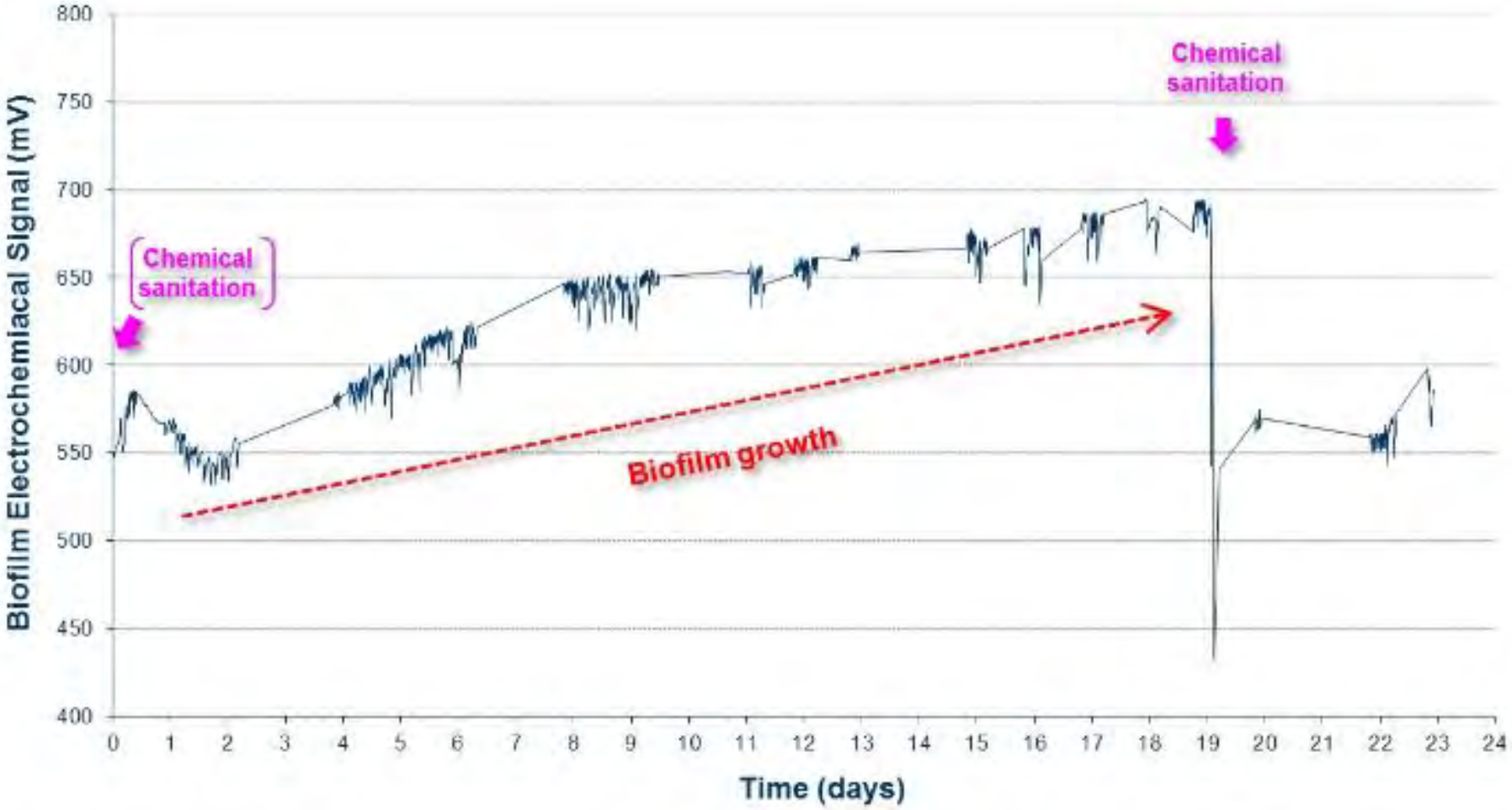
Jiménez-Pichardo et al., 2021 (Used with permission)

Biofilm control/removal

It is not practical to clean and sanitize frequently to prevent attachment of microbes to surfaces, since **cell attachment may occur within a few minutes to hours**.

However, it is important to clean and disinfectant (if needed) **after a short time** to avoid the forming of resistant biofilms that are **more difficult to remove than those recently formed**.

Biofilm formation



http://www.alvimcleantech.com/cms/en/biofilmsensors/application-cases/food-production

Biofilm control/removal

It has been suggested that removal of biofilms during cleaning is significantly enhanced by applying mechanical force to a surface, such as

- high-pressure sprayers

and

- scrubbers.

Non-aerosol-generating detergents, such as **foam**, as well as the use of **sanitizers**, will result in a **higher bacterial kill when used in conjunction with mechanical methods**.

Biofilm control/removal

The **formation of aerosols** or **small droplets** is often found during washing and spraying of surfaces, floor and drains. Care should be taken **not to contaminate clean areas or sanitized processing equipment**.

High-pressure, low-volume water is normally used to rinse surfaces; however, it has been found that flow above a pressure of 17.2 bar does not enhance biofilm removal.

https://blog.istc.illinois.edu/2019/11/11/safer-sanitation-in-food-and-beverage-manufacturing-and-processing/

Biofilm control/removal

Ideally, plant layout and equipment **should be designed** to prevent the accumulation of soil and water, and **to allow** for easy cleaning and sanitation operations.

Problems often occur at locations such as **dead ends**, **pumps** and **joints** where **gaskets must be used**, and areas where surfaces may not receive sufficient exposure to cleaning and sanitizing chemicals.

In addition, **the modification of equipment surfaces by anti-microbial coatings** and **new ideas to improve surface hygiene** may ultimately aid in inhibition of biofilm formation.

Biofilm control/removal

Micrographs of SSP with biofilms on type A deposits (a, b) and type B deposits (c, d); ELP with biofilms on type A deposits (e, f). Green fluorescence indicates viable cells, while red fluorescence indicates damaged cells. Panels (a, c, e) show 40 × magnification; Panels (b, d, f) show fluorescence intensity.

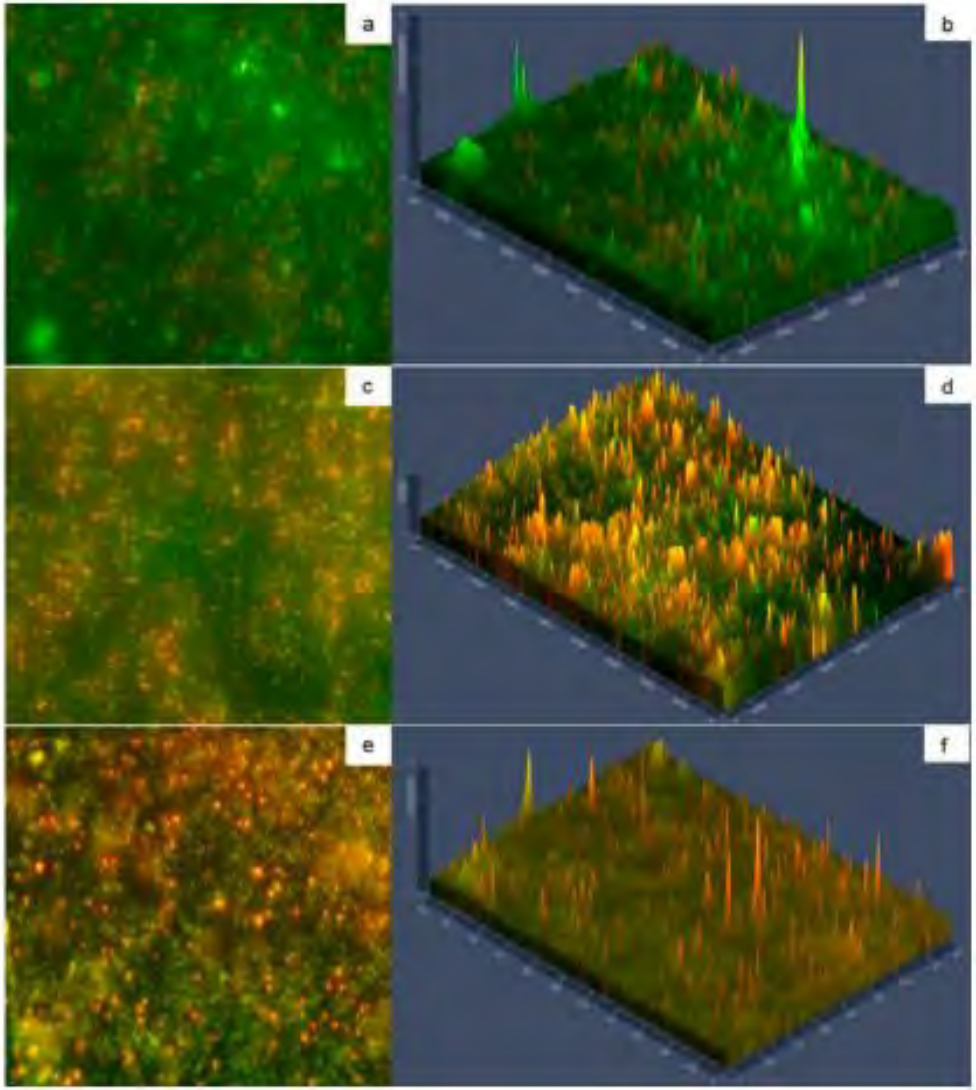
SSP: Stainless steel plates 304 (2.5 × 2.5 cm) surface finish 2B

ELP: Electropolished stainless steel plates 304 (2.5 × 2.5 cm) surface

Without deposits: All plates were submerged in 100 mL of raw milk, and heated at 70 °C for 15 min in a constant water bath to promote protein denaturation and surface adhesion.

Deposits type (A): For type A deposits the temperature increased to 90 ° C, and kept for 30 min

Deposits type (B): For type B deposits the temperature increased to 121 ° C, and kept for 5 min

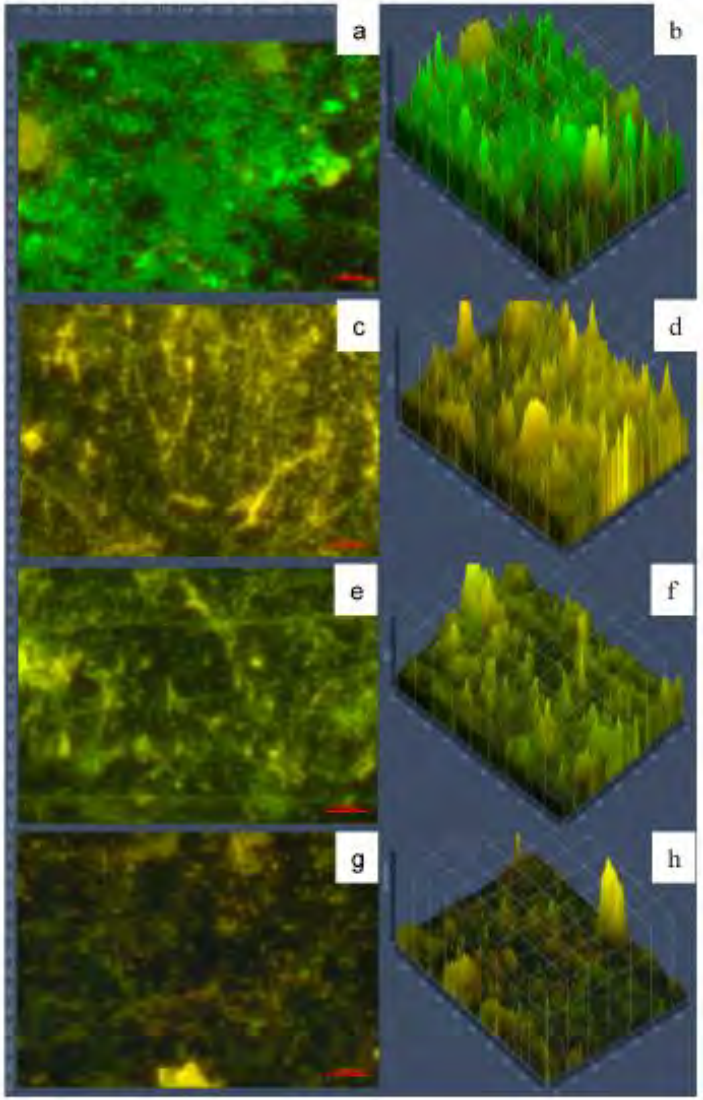


Jiménez-Pichardo et al., 2021 (Used with permission)

Biofilm control/removal

Micrographs of SSP with biofilms on type A deposits. Panels (a, b) show 5-day biofilms. Panels (c, d) show the cleaning stage of 50 mg NaOH/L of AEW at 30 °C for 10 min, only. Panels (e, f) show disinfection using 50 mg/L total available chlorine of NEW at 20 °C for 5 min, only. Panel (g, h) show cleaning followed by disinfection stages. Green fluorescence indicates viable cells, while red fluorescence indicates damaged cells. Panels (a, c, e, g) show 40 × magnification; panels (b, d, f, h) show fluorescence intensity.

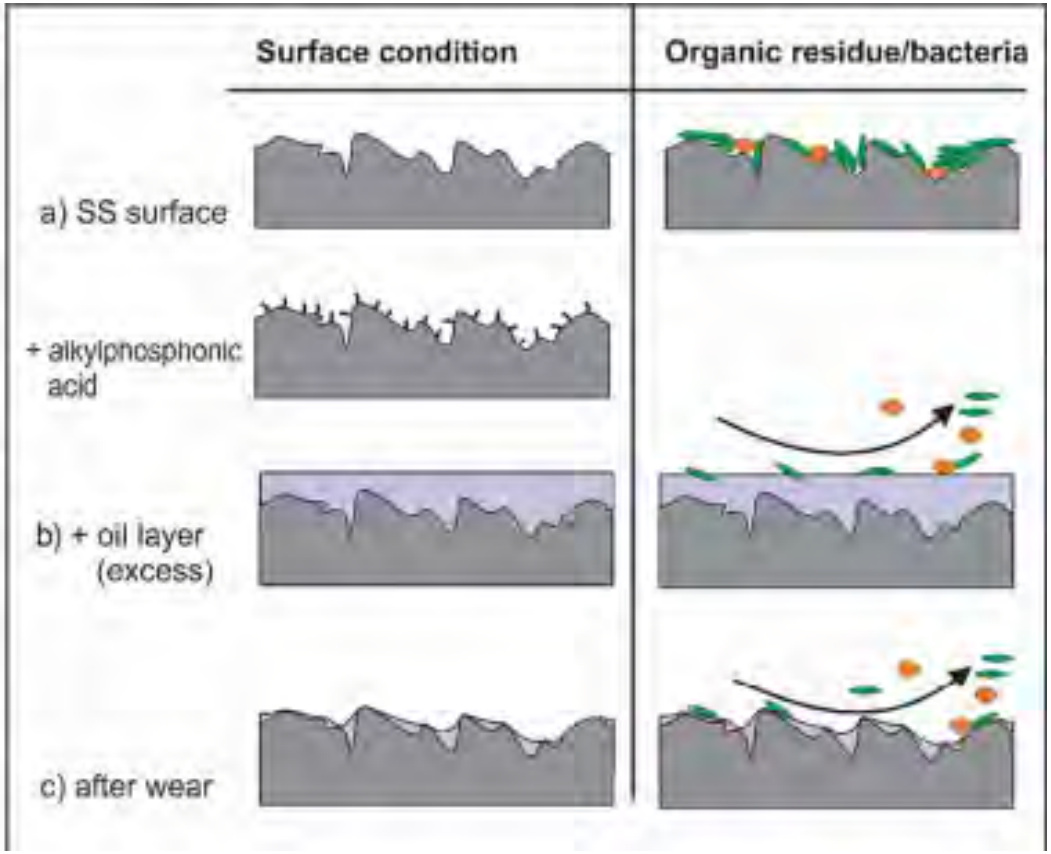
SSP: Stainless steel plates 304 (2.5 × 2.5 cm) surface finish 2B
AEW: Alkaline electrolyzed water
NEW: Neutral electrolyzed water
Without deposits: All plates were submerged in 100 mL of raw milk, and heated at 70 ˚C for 15 min in a constant water bath to promote protein denaturation and surface adhesion.
Deposits type (A): For type A deposits the temperature increased to 90 ˚ C, and kept for 30 min
Deposits type (B): For type B deposits the temperature increased to 121 ˚ C, and kept for 5 min



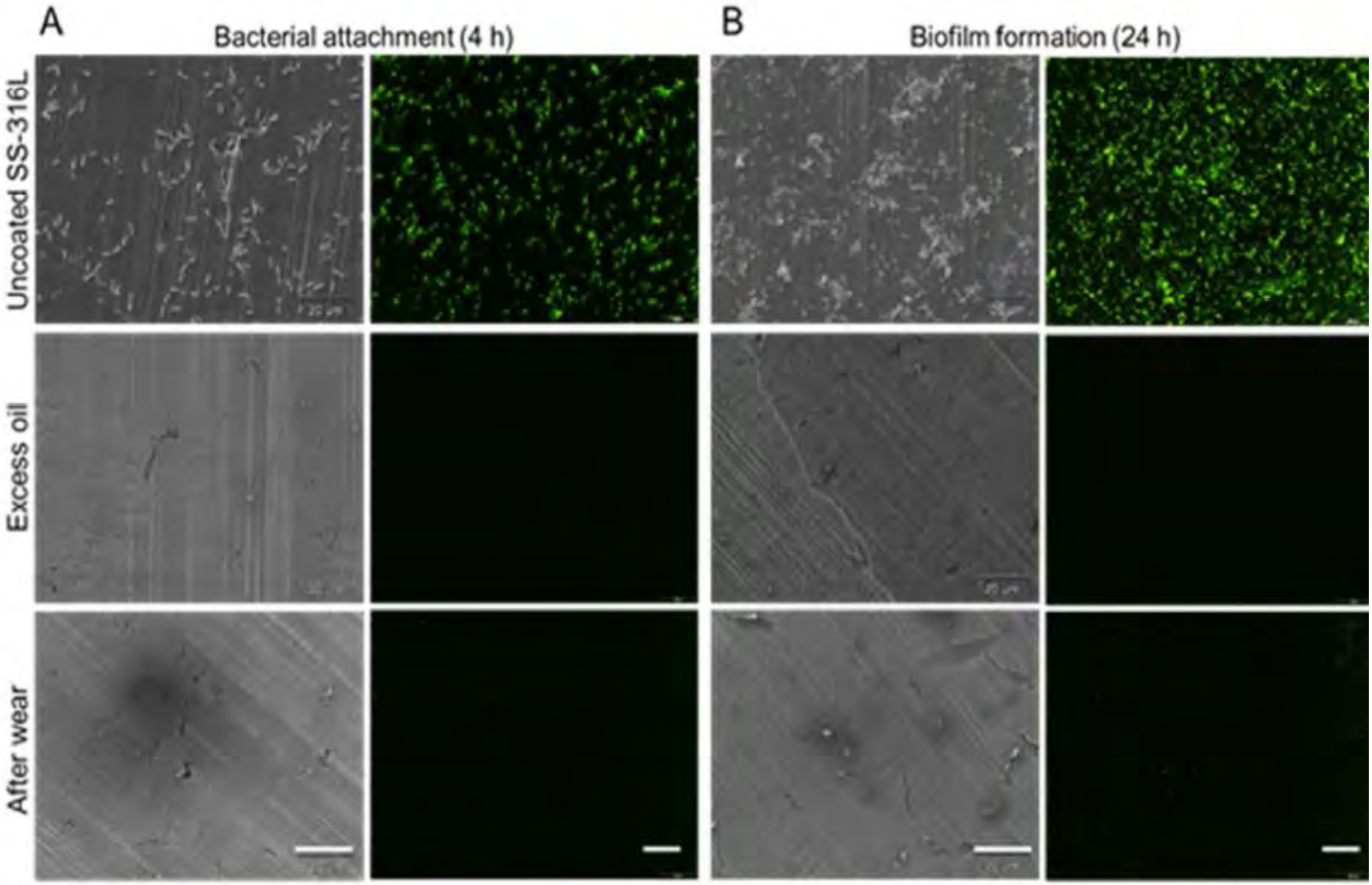
Jiménez-Pichardo et al., 2021 (Used with permission)

Modified equipment surface

Suppression of organic residue and bacterial and biofilm formation on untreated and oil-coated SS surfaces. Bare SS (a) was functionalized with C8- and C18-phosphonic acid, then coated with food-safe cooking oil (b). Surface defects enhance the adhesion of organic matters and bacterial cells (top right) while oil coated surface prevent the adhesion cells (middle right). After exposure to physical wear conditions (c) remaining oil fills the concave surface defects, blocking those sites from organic accumulation and bacterial adhesion.



Awad et al., 2018 (Used with permission)

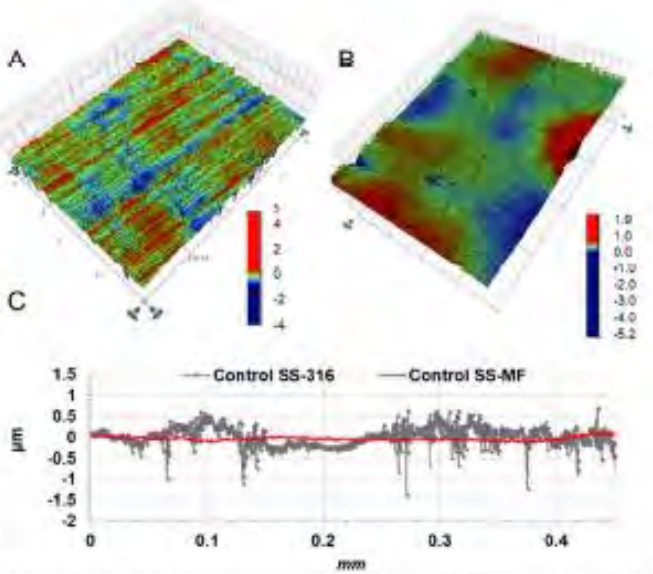


Reduction of *P. aeruginosa* attachment (A) and biofilm formation (B) on mineral coated SS-316 after growth in LB broth for 4h and 24h, respectively. SEM (panels 1 and 3) and fluorescence (panel 2 and 4) micrographs of cells on uncoated, oil coated (excess) and after wear SS-316 surfaces. (Bar: 50 µm)

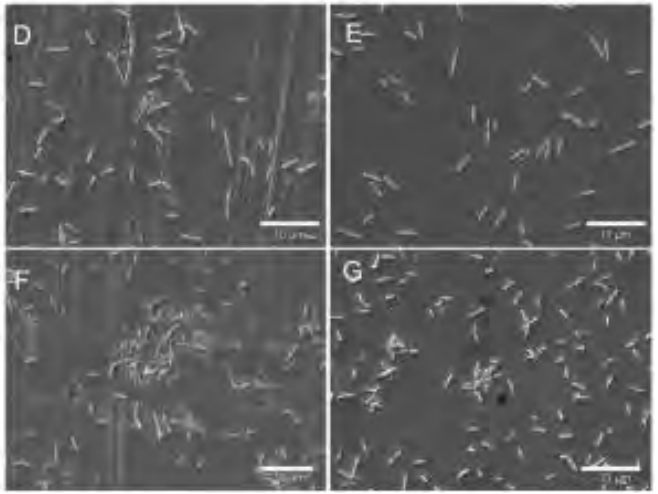
Awad et al., 2018 (Used with permission)

Modified equipment surface

Effect of SS surface roughness on bacterial cell adherence and biofilm formation.3D Optical profilometer images of SS-316 (A); SS-MF (B) and example surface profiles (C).



SEM micrographs showing attached cell attachment after 4 h (D, E) and biofilm formation after 24 h (F, G) on untreated SS- 316 (left panel) and SS-MF (right panel) after immersion in *P. aeruginosa* PAO1 culture at room temperature. The projected area



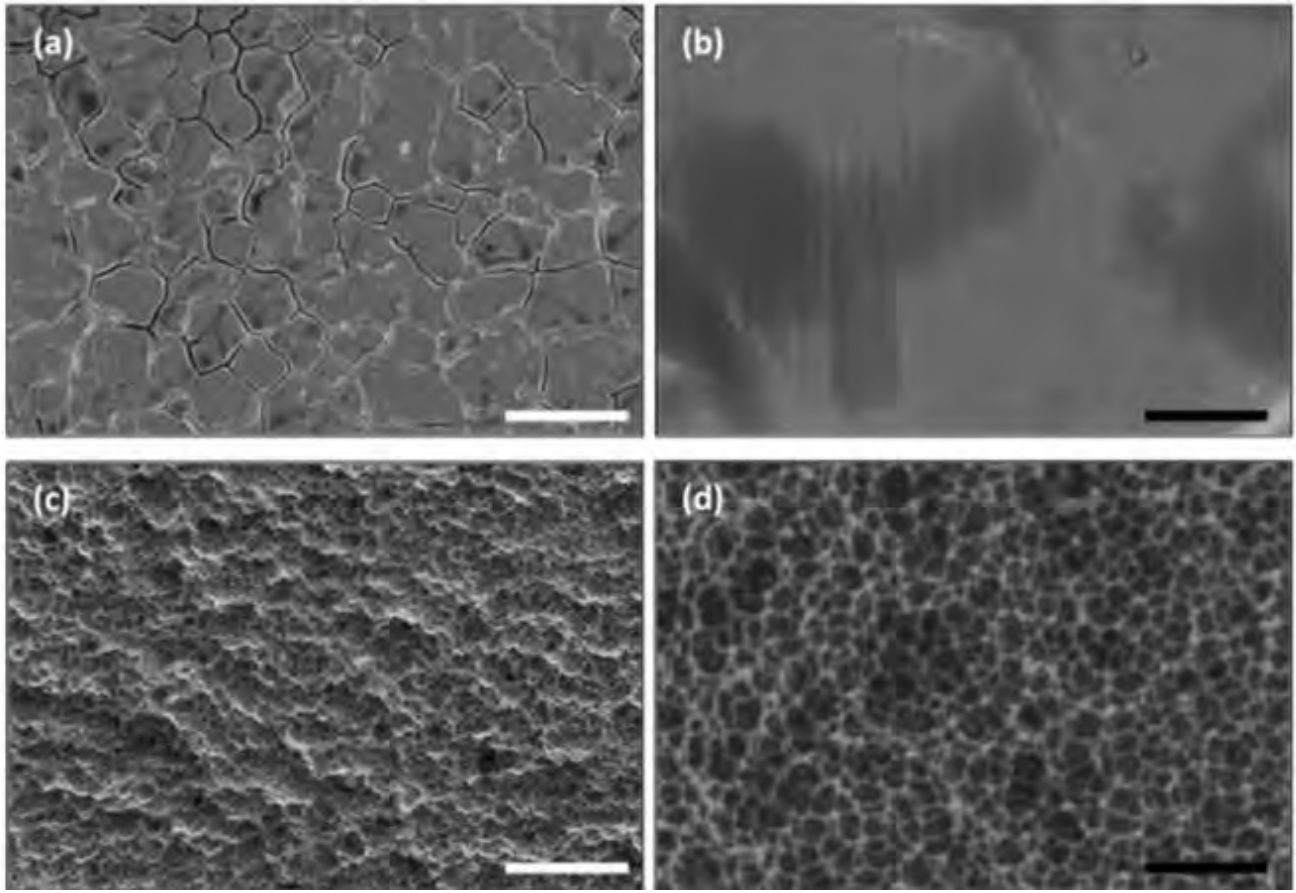
Awad et al., 2018 (Used with permission)

Modified equipment surface

FE-SEM micrographs of bare stainless steel (a, b) and

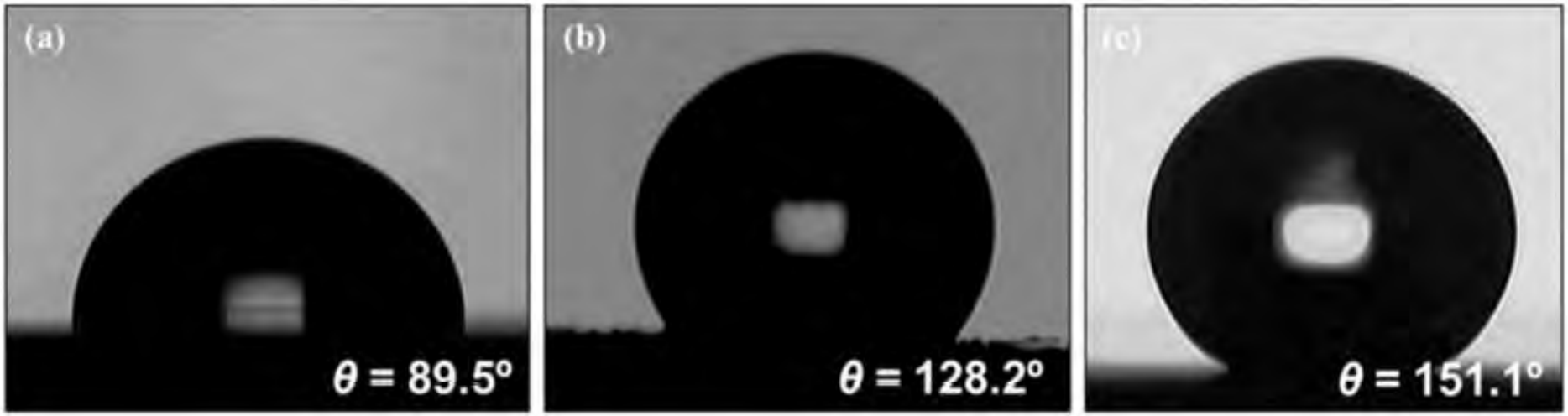
stainless steel electrochemically etched at 10 V for 10 min (c, d).

White and black scale bars in (a–d) indicate 25 µm and 500 nm, respectively.



Ban et al., 2020 (Used with permission)

Modified equipment surface



Surface wettability of (a) bare stainless steel, (b) stainless steel electrochemically etched at 10 V for 10 min, and (c) stainless steel electrochemically etched at 10 V for 10 min with Teflon coating.

Ban et al., 2020 (Used with permission)

Modified equipment surface

Comparison of bacterial attachment and biofilm formation of *E. coli* O157:H7 on bare stainless steel (a, c, e)

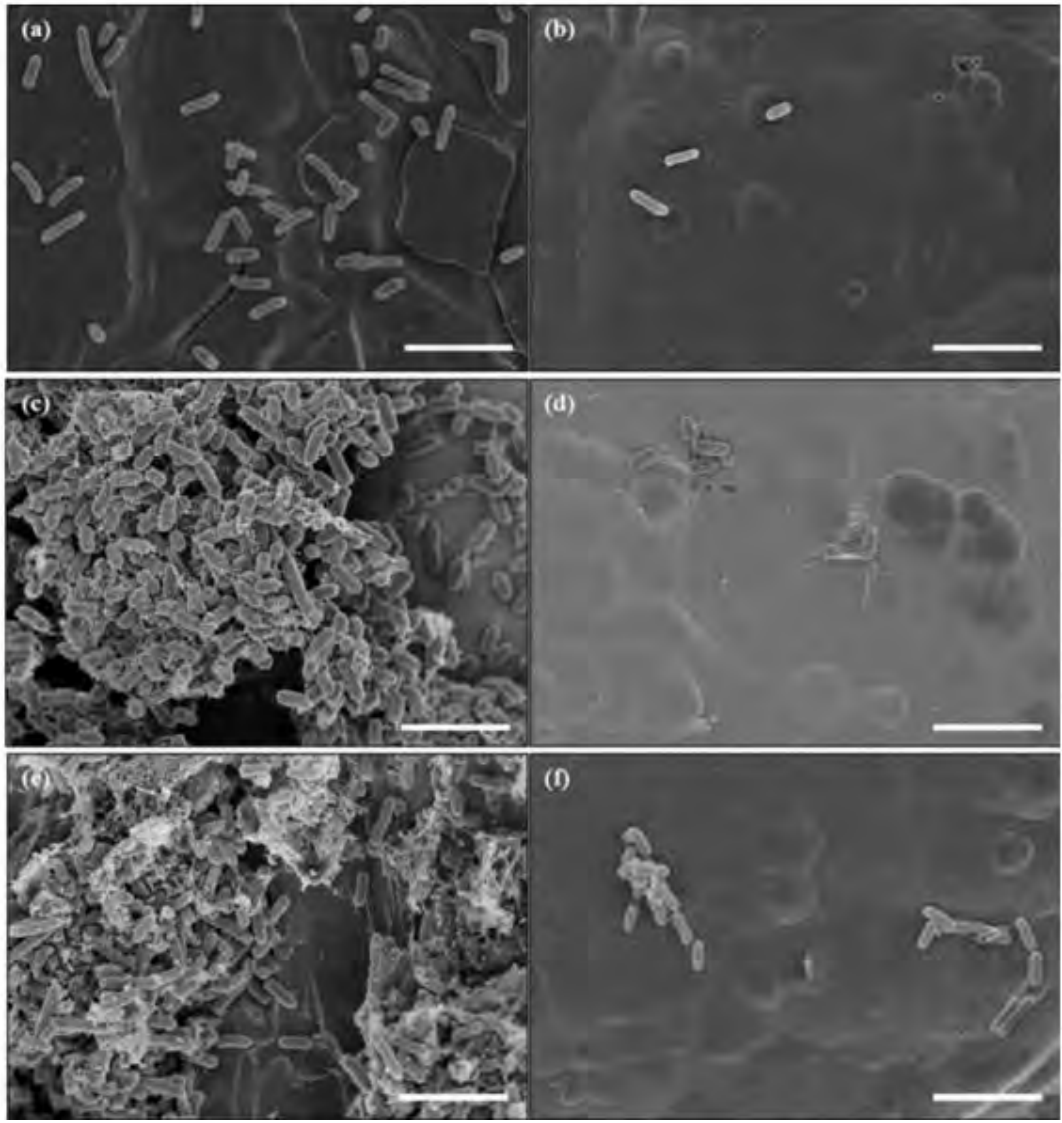
and

nanoengineered stainless steel (stainless steel electrochemically etched at 10 V for 10 min with Teflon coating) (b, d, f) observed by FE-SEM.

Bacteria were attached on the surfaces after being submerged in bacterial cell suspension for 4 h (a, b), under static (c, d)

and flow conditions (e, f).

White scale bars in (a–f) indicate 5 µm.



Ban et al., 2020 (Used with permission)

Biofilm control/removal

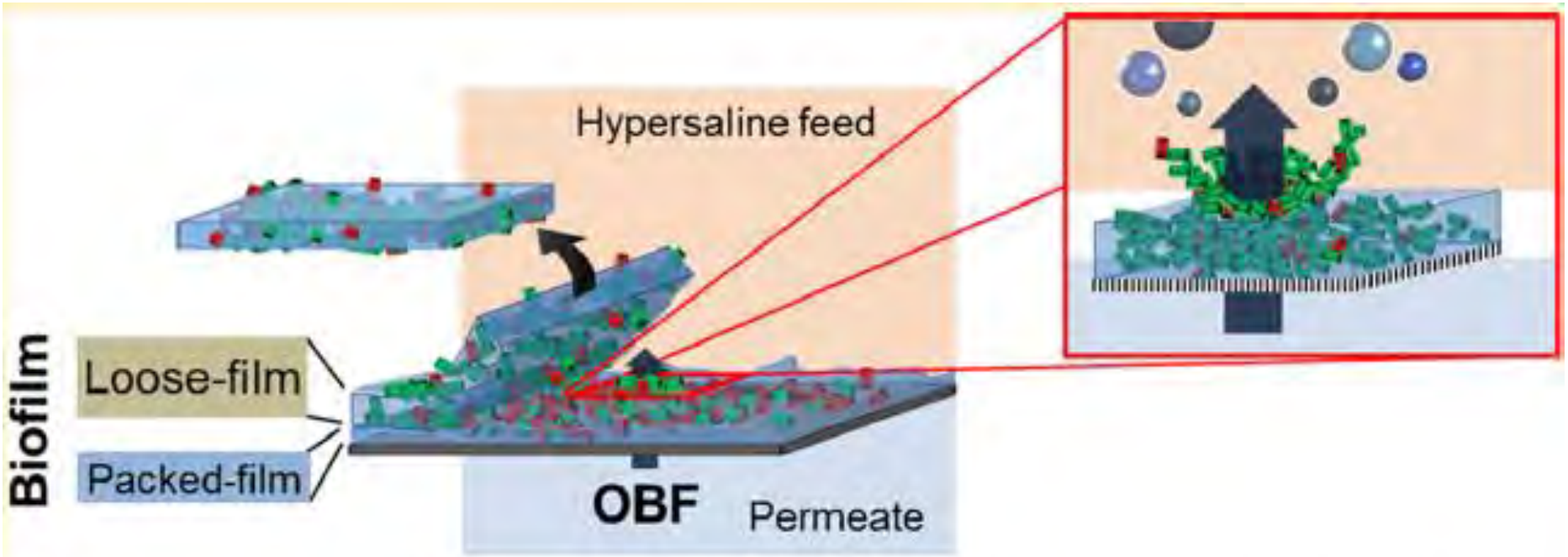
Cleaning procedures **should effectively remove food residues and other soils** that may contain microorganisms or promote microbial growth.

Most cleaning regimes include **removal of loose soil with cold or warm water** followed by the **application of chemical agents**, **rinsing** and **sanitation**.

Cleaning can be accomplished by using **chemicals** or **combination of chemical and physical force** (water turbulence or scrubbing).

High temperatures can reduce the need for physical force.

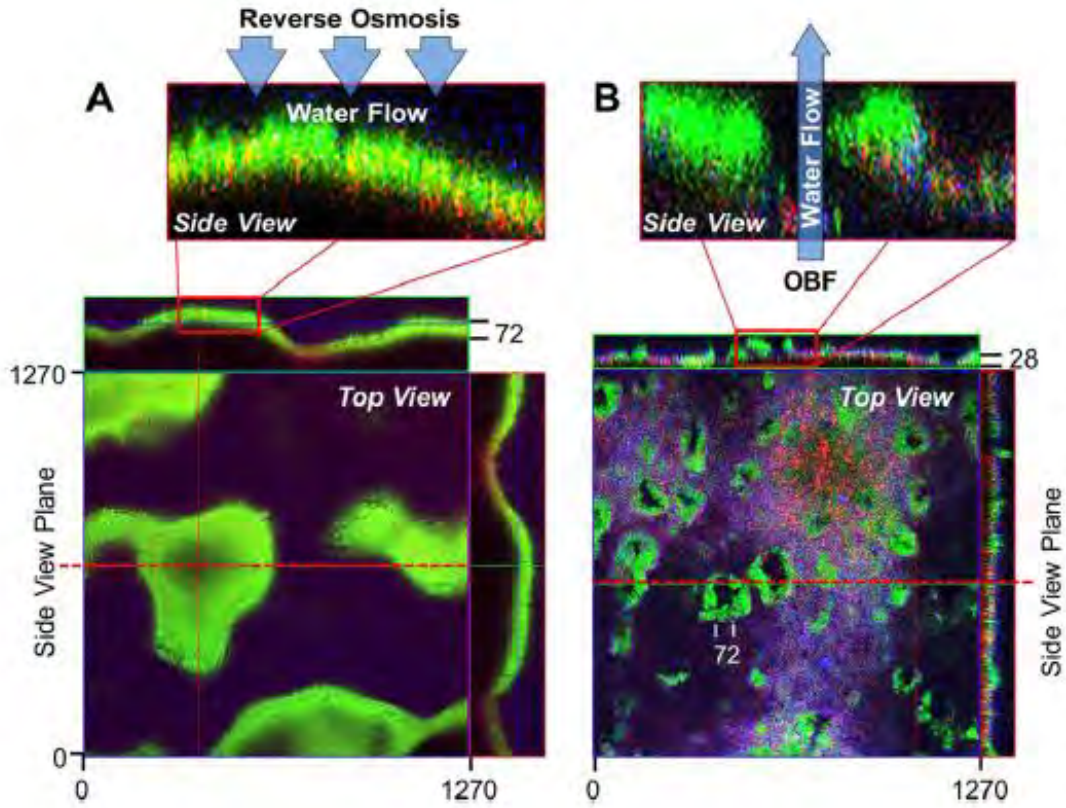
Biofilm control/removal



The application of osmotic back-flushing (OBF) for the removal of biofilms from reverse osmosis (RO) membranes resulted in significant biofilm detachment, leaving a thin, perforated bacterial film (24 μm thickness) with vertical cavities ranging from 15 to 50 μm in diameter. Application of OBF led to significant reduction in the biovolume (70–79%) and substantial removal of total organic carbon and proteins (78 and 66%, respectively), resulting in 63% permeate water flux recovery.

Bar-Zeev et al., 2014 (Used with permission)

Biofilm control/removal



(A) CLSM orthogonal views of *P. aeruginosa* biofilm structures developed on the RO membrane after it had been biofouled for 24 h. (B) Biofilm architecture after an osmotic back-flushing procedure.

Top insets are matching enlargements of the biofilm layer before and after OBF (A and B, respectively) with a schematic illustration of the flow of water through the membrane and/or biofilm.

Blue: EPS (polysaccharides), Green: live cells, Red: dead cells. All axis units are micrometers.

Bar-Zeev et al., 2014 (Used with permission)

Biofilm control/removal

Chemical cleaners

- suspend

and

- dissolve food residues

by

✓decreasing surface tension,

✓emulsifying fats

and

✓peptizing proteins.

Biofilm control/removal

Problems such as

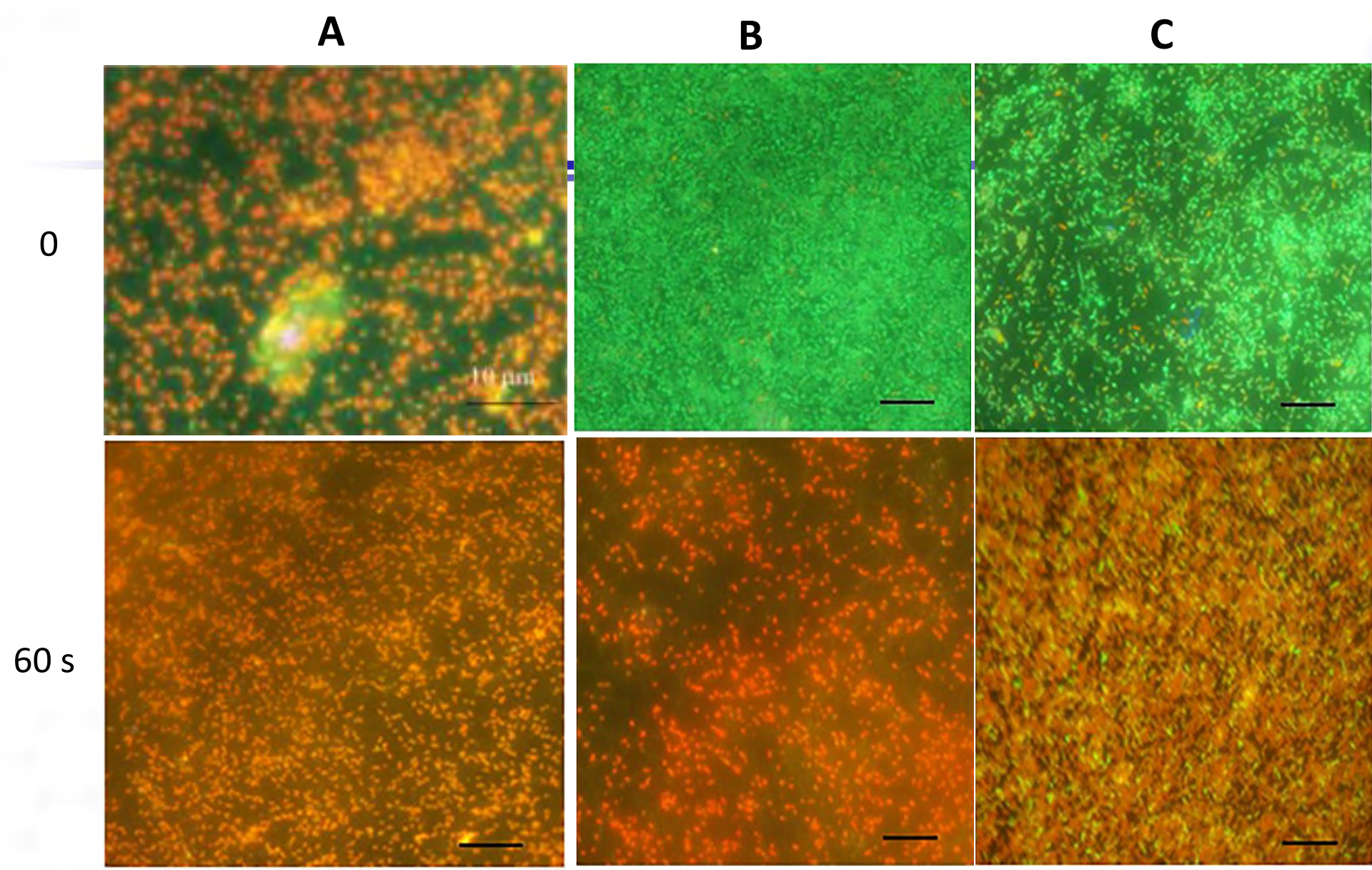
- **corrosion**

and

- **biofouling**

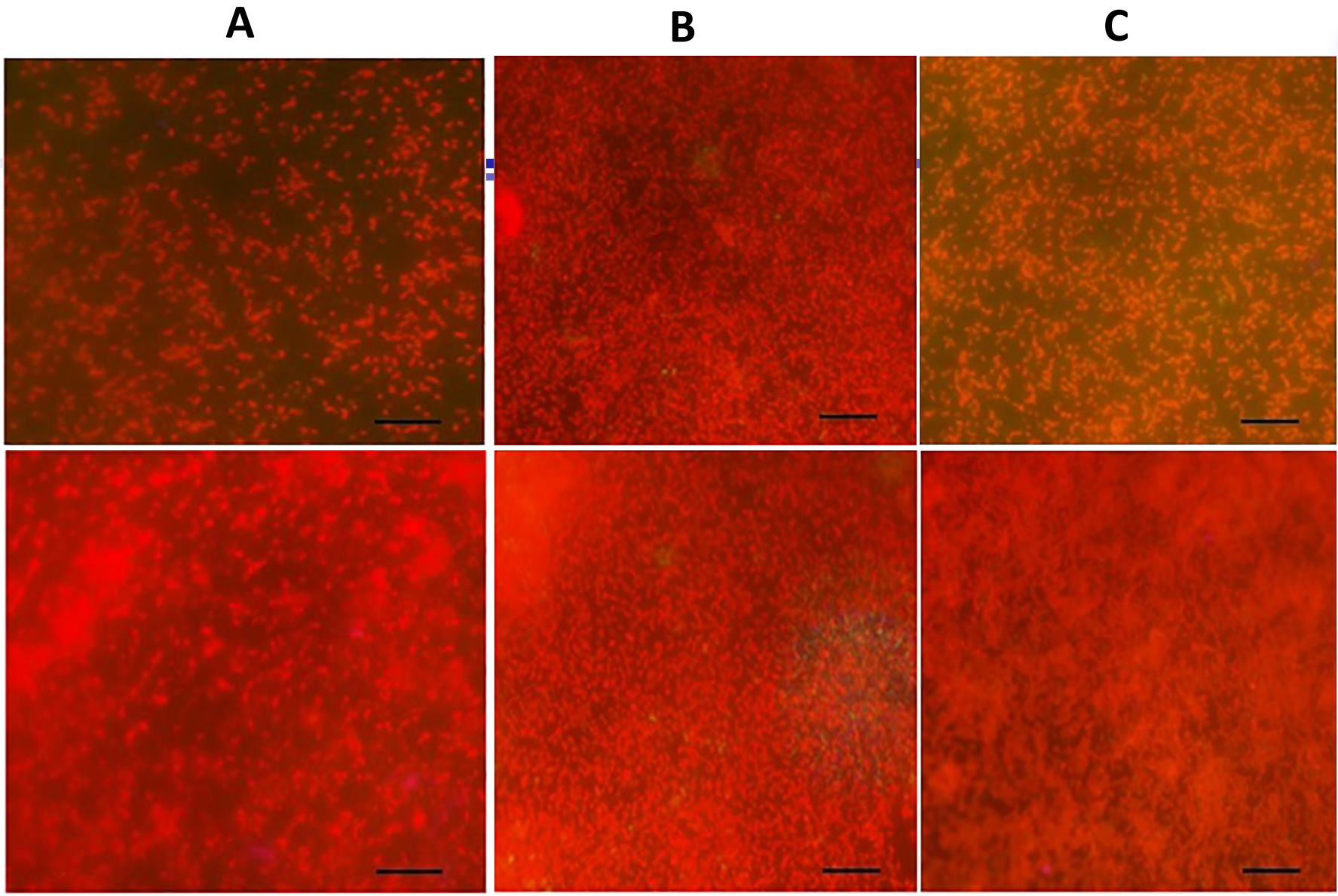
in **cooling systems** by microbial biofilms are **normally prevented/controlled by chemical treatment**.

Research concerning the complex molecular mechanisms that regulate **the synthesis of EPS**, the attachment of microorganisms, as well as the development and detachment of biofilms will ultimately lead to improved strategies for the control of biofilms.



Photomicrographs of biofilms formed by biofilm-producing *L. monocytogenes* isolates (isolates A, B and C) on stainless steel surfaces for 48 hours at 35 ºC, after treatment with peracetic acid (0.5%, v/v) at 60, 120 and 180 s. **Viable cells** are fluorescent **green** and **non-viable cells** are fluorescent **red**. Magnification: 1,000x. Bar = 10 µm.

Lee et al., 2017 (Used with permission)



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Lee et al., 2017 (Used with permission)

Biofilm control/removal

Arias-Moliz et al. (2015) observed that the effect of **Peracetic acid (PAA)** on *Enterococcus faecalis* biofilms was lower than that of **sodium hypochlorite (NaClO).**

They postulated that, although **PAA** was able to diffuse inside the biofilm clusters, its lower antimicrobial effect compared with that of **NaClO** could be explained by the resistance of *Enterococcus faecalis* to PAA oxidative stress.

In contrast, the bacterial strains tested in the present study were damaged by PAA after 15 s, with almost 100% of cells damaged after **30 s (*L. monocytogenes*)** or **60 s (*S. aureus*)**.

Biofilm control/removal

Lytic life cycle of phages inside a biofilm.

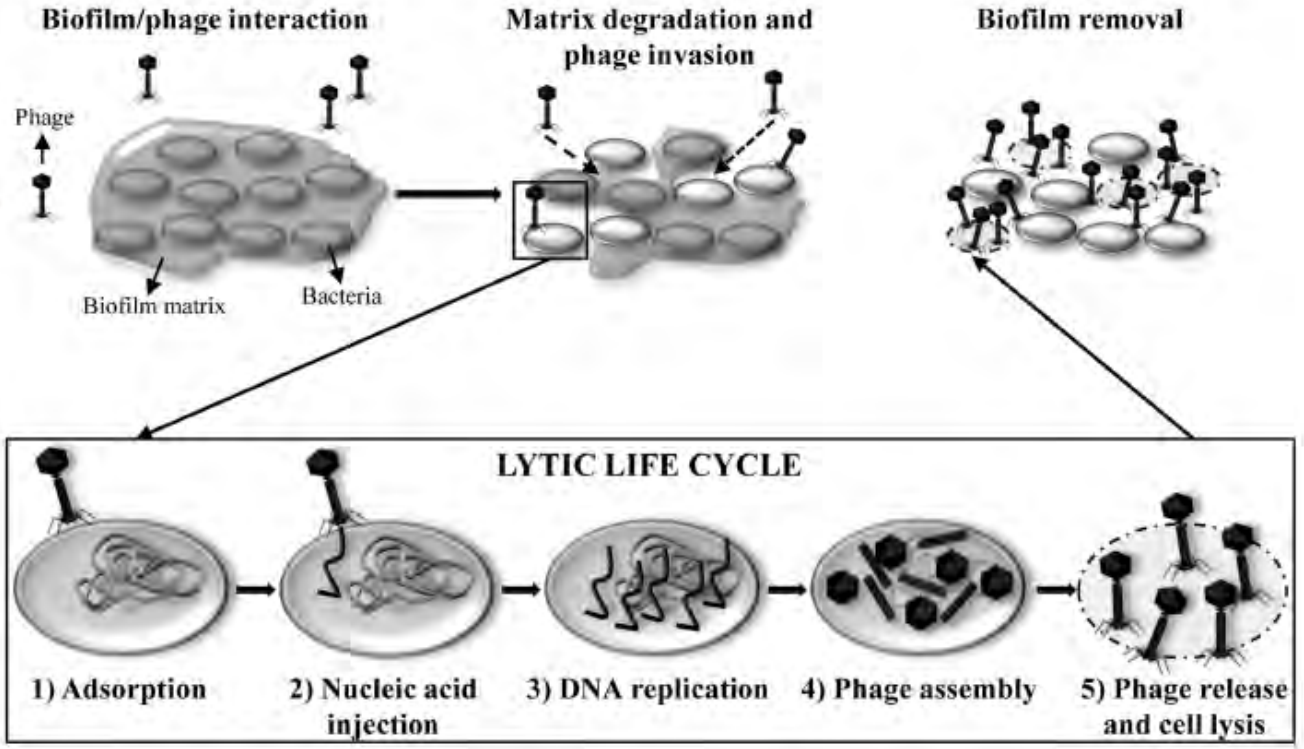
(1) Adsorption of the phage particle onto the host bacterial cell surface. Tail fibers bind to specific receptors on the cell surface.

(2) Injection of the nucleic acid into the cytoplasm of the host bacterium.

(3) Replication of the phage genome in multiple copies. Phage early genes are expressed to regulate the host metabolic machinery to be subjected to phage propagation.

(4) Formation of new phage particles by expression of the phage late genes and assembly of the phage heads and tails, packaging of the nucleic acid inside the heads and maturation of the virions.

(5) Lysis of the host bacterium and release of the new phage progeny ready to infect other cells in the biofilm and start a new cycle.



Gutiérrez et al., 2016 (Used with permission)

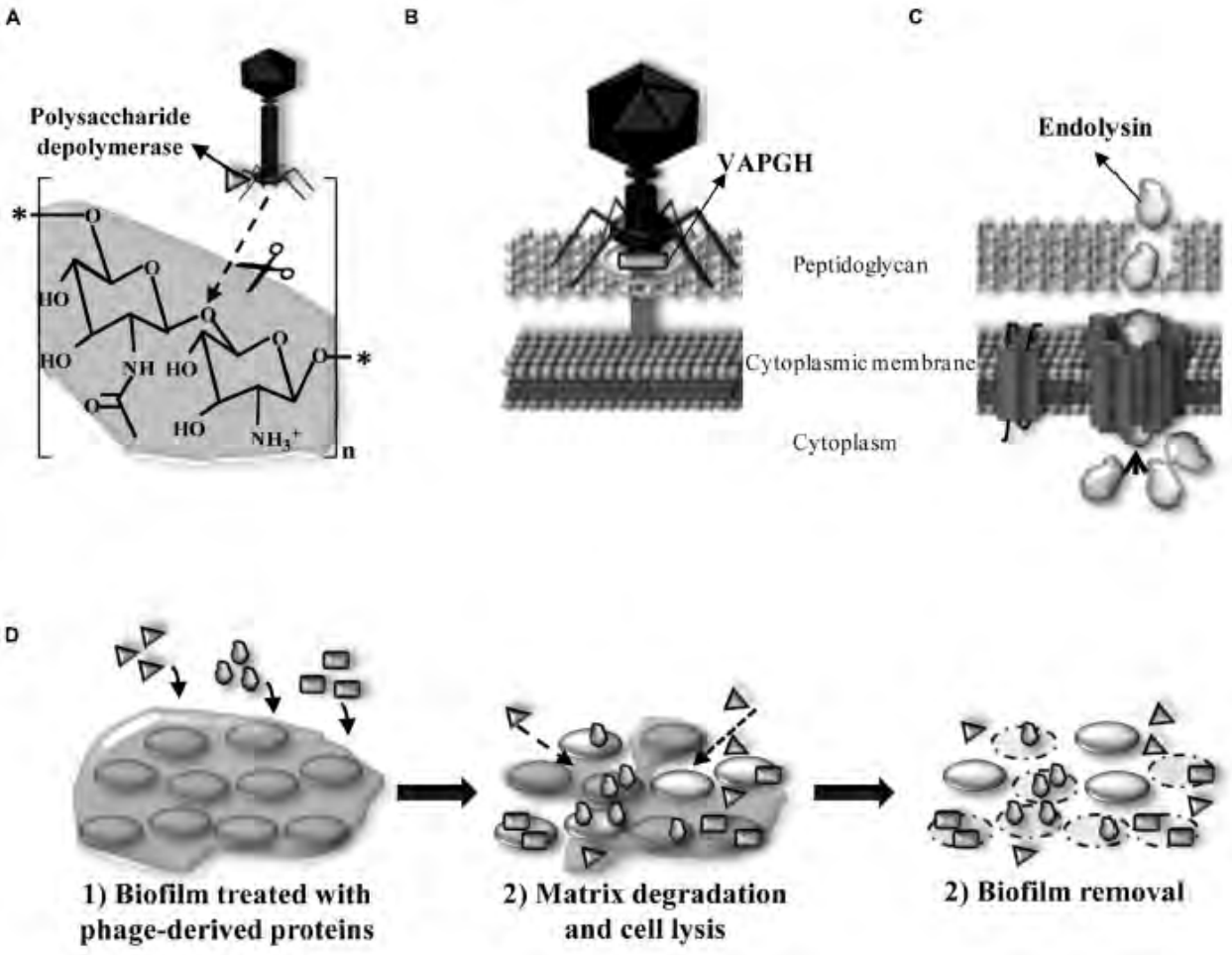
Biofilm control/removal

(A) Location of exopolysaccharide depolymerase degrading β-(1,6) bonds of the biofilm extracellular matrix (PIA/PNAG) of staphylococcal species in the phage particle and mode of action.

(B) Location of virion-associated peptidoglycan hydrolase (VAPGH) at the phage particle and its role in the infection process.

(C) Structure of Gram-positive bacteria cell wall and role of the endolysin during the bacterial lysis.

(D) Activity of phage derived proteins when added exogenously and their application as anti-biofilm agents degrading polysaccharidic matrices (polysaccharide depolymerases) and lysing bacteria (VAPGHs and endolysins).

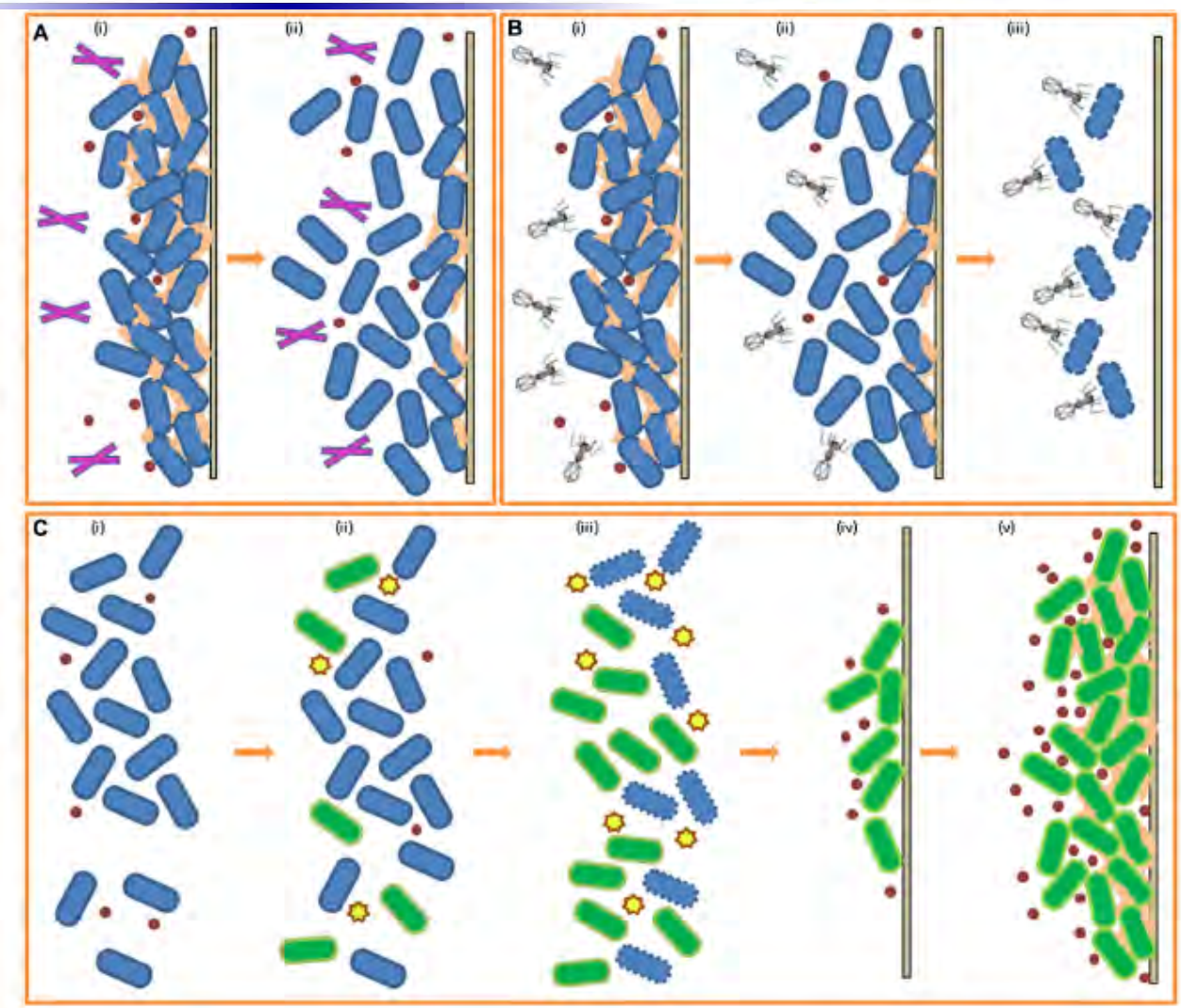


Gutiérrez et al., 2016 (Used with permission)

Biofilm control/removal

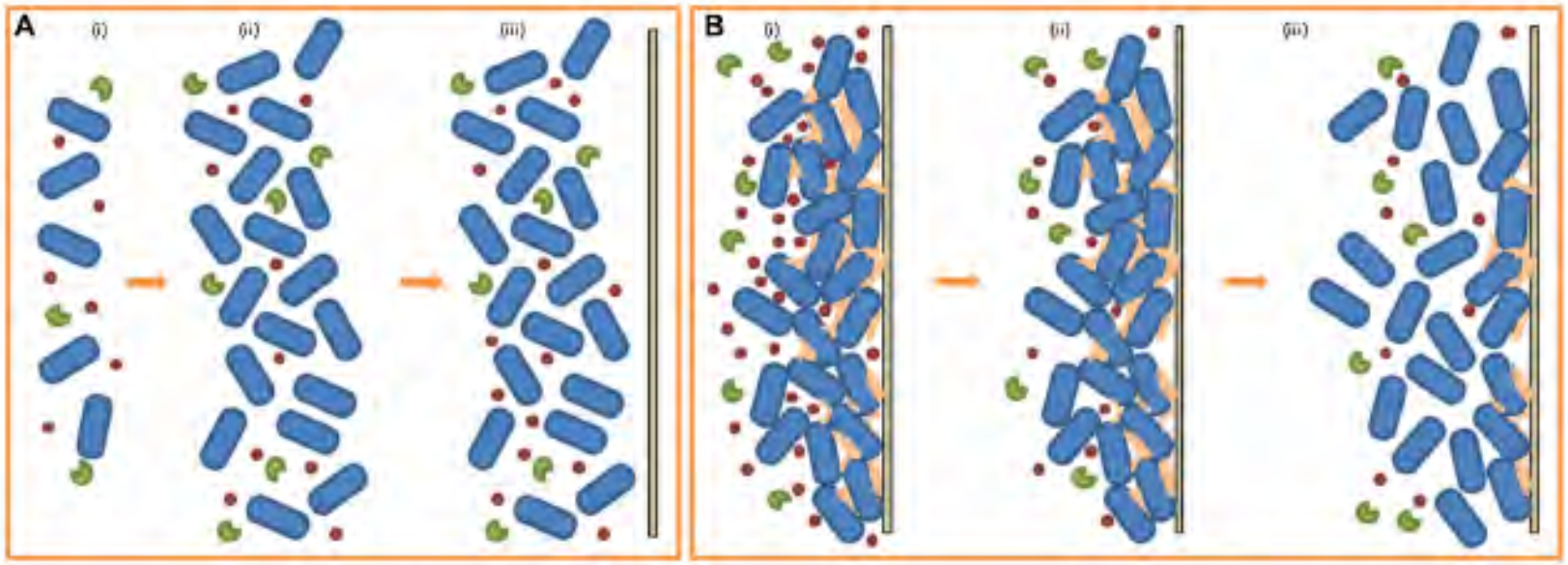
Biofilm control through enzymes, phage, and bacteriocins.

(A) Effect of enzymes on pre-existing biofilm (i) biofilm formed, EPS production, addition of enzymes (ii) break down of EPS and biofilm reduction by enzymatic action. **(B)** Effect of bacteriophage on pre-existing biofilm (i) biofilm formed, EPS production, addition of phage (ii) degradation of EPS by phage, reduction of biofilm (iii) bacterial cells in biofilm targeted by targeted for infection by phage. **(C)** Effect of bacteriocins and competitive exclusion on biofilm-forming cells (i) planktonic cells of species A (blue)(ii) addition of bacteriocin-producing species B (green) (iii) targeting of species A by bacteriocins, increase in number of species B cells (iv) increase in QS molecule concentration for species B, attachment to solid surface (v) biofilm formation of species B in place of species A.



Coughlan et al., 2016 (Used with permission)

Biofilm control/removal



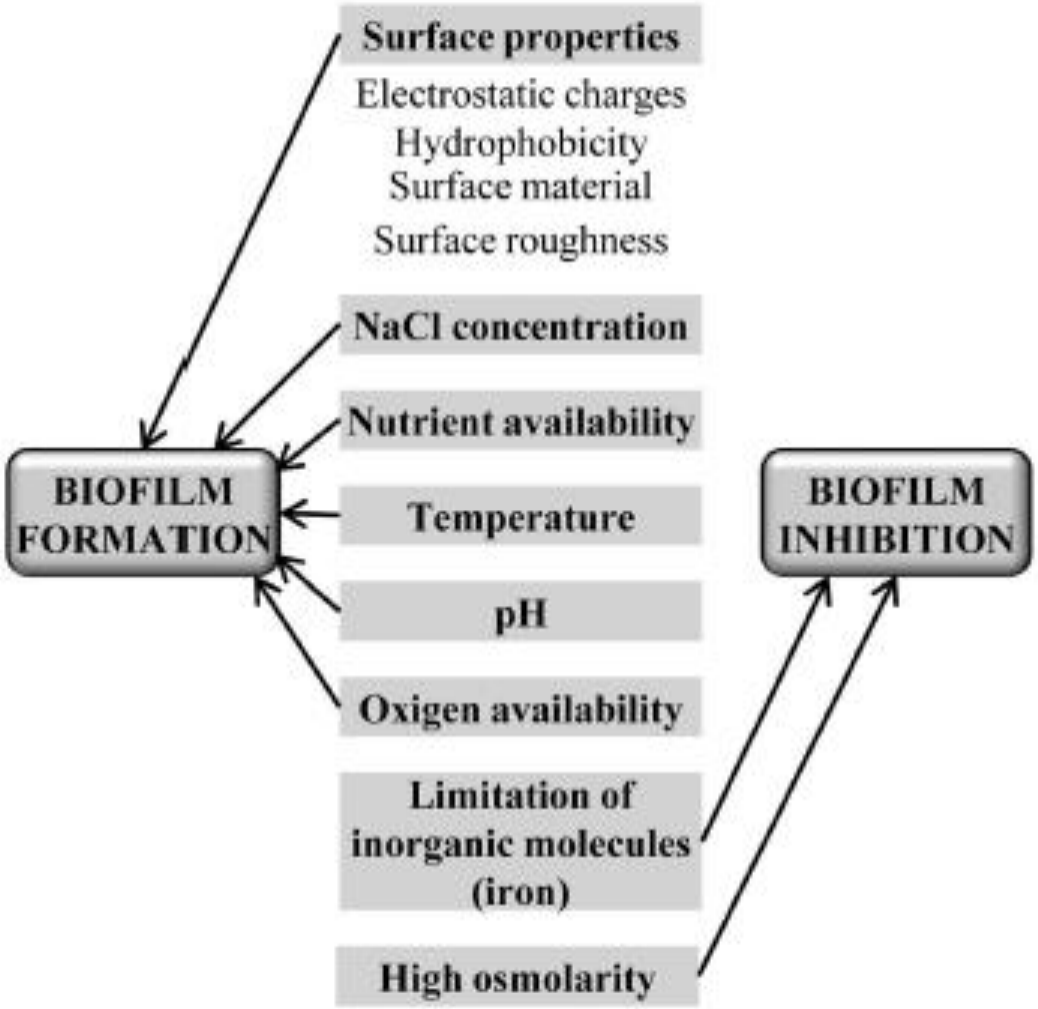
Quorum quenching (QQ) and biofilm formation.

(A) Effect of QQ molecules on early stage biofilm formation (i) low population density, low QS signal, addition of QQ molecules (ii) high population density, low QS signal, QS molecules degraded by QQs (iii) absence of attachment to solid surface, biofilm formation does not occur. **(B)** Effect of QQ molecules on early pre-existing biofilm (i) biofilm formed, high QS signal, addition of QQ molecules (ii) QS molecules degraded by QQs, reduction of QS signal (iii) decrease in EPS production, release of cells, return of released cells to planktonic state (i.e., reduced biofilm).

Coughlan et al., 2016 (Used with permission)

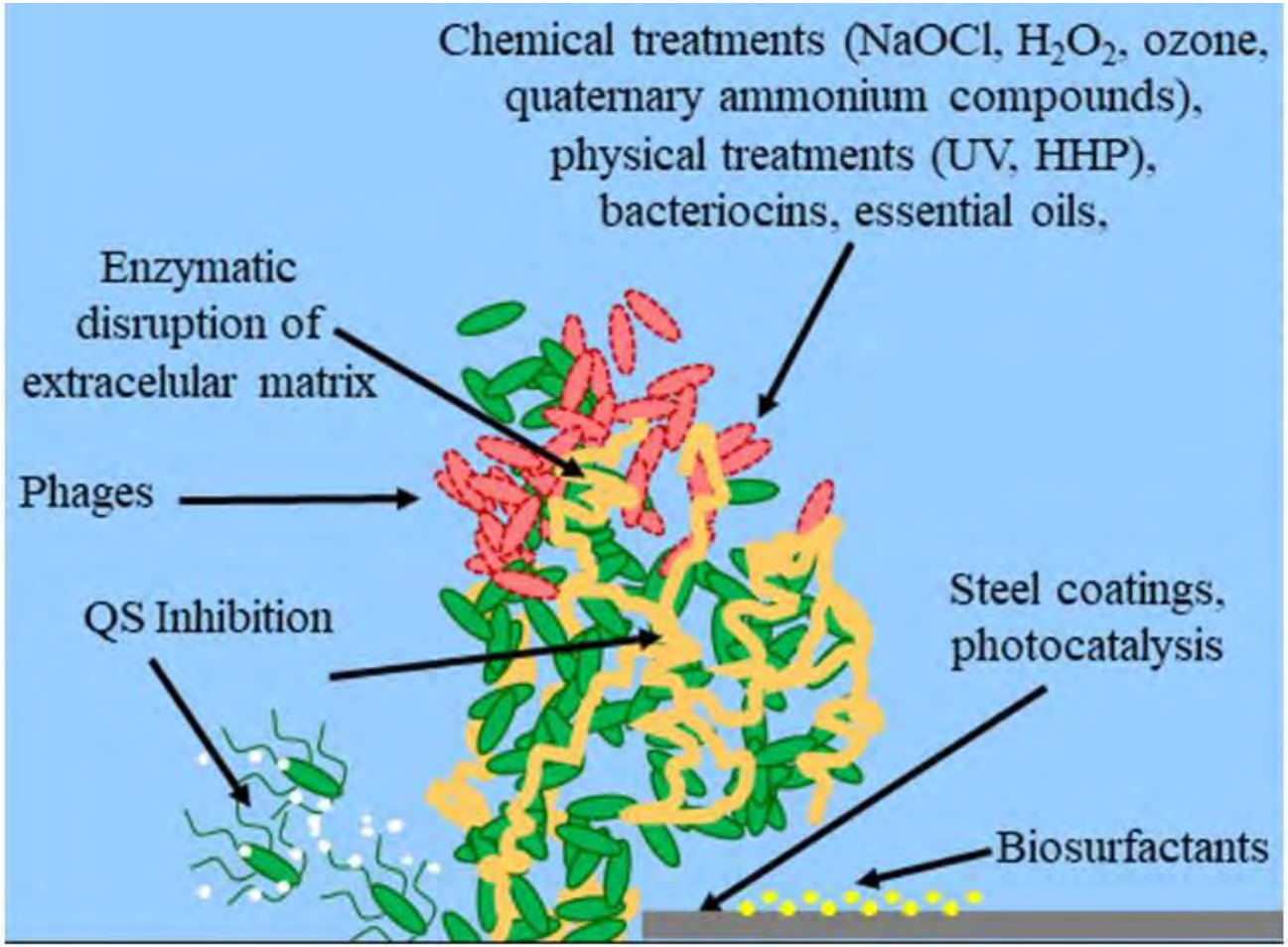
Biofilm control/removal

Main food industry's parameters that can influence biofilm development:



Gutiérrez et al., 2016 (Used with permission)

Biofilm control/removal



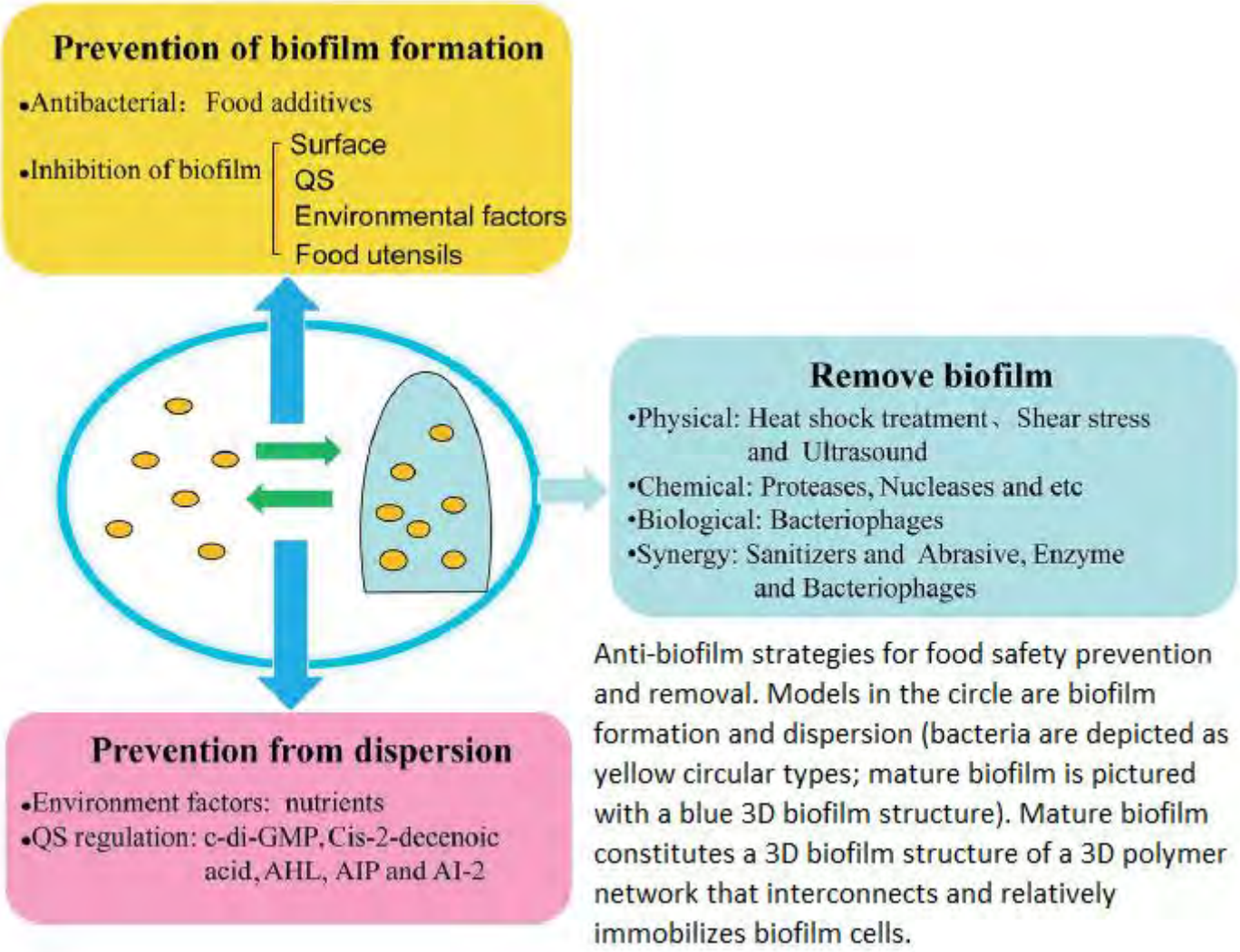
Control methods for biofilm establishment, development and eradication.

Red bacterial cells indicate dead cells. White dots indicate Quorum Sensing (QS) signals. Yellow dots indicate the treatment of the surface with biosurfactants. The extracellular matrix is indicated in orange.

Arrows indicate the site of action for methods targeting bacterial cell integrity (chemical treatments, physical treatments, bacteriocins, essential oils), extracellular matrix (enzymatic disruption), cell-to-cell communication (QS inhibition), or physical properties

Galié et al., 2018 (Used with permission)

Biofilm control/removal



Anti-biofilm strategies for food safety prevention and removal. Models in the circle are biofilm formation and dispersion (bacteria are depicted as yellow circular types; mature biofilm is pictured with a blue 3D biofilm structure). Mature biofilm constitutes a 3D biofilm structure of a 3D polymer network that interconnects and relatively immobilizes biofilm cells.

Galié et al., 2018 (Used with permission)

Cleanliness of sanitized surfaces

Enumeration of total bacterial counts, coliforms, yeasts and molds are **the most common microbiological examinations** carried out to assess the hygiene of food/dairy equipment surfaces.

The types of **microorganisms** present reflect to some extent the standard of plant hygiene.

Selective and differential culture media may also be used to test specifically for given groups of organisms.

Cleanliness of sanitized surfaces

The conventional methods include:

1- **Swab/swab–rinse plating methods**

2- **Agar contact plate methods**

- RODAC (Replicate Organism

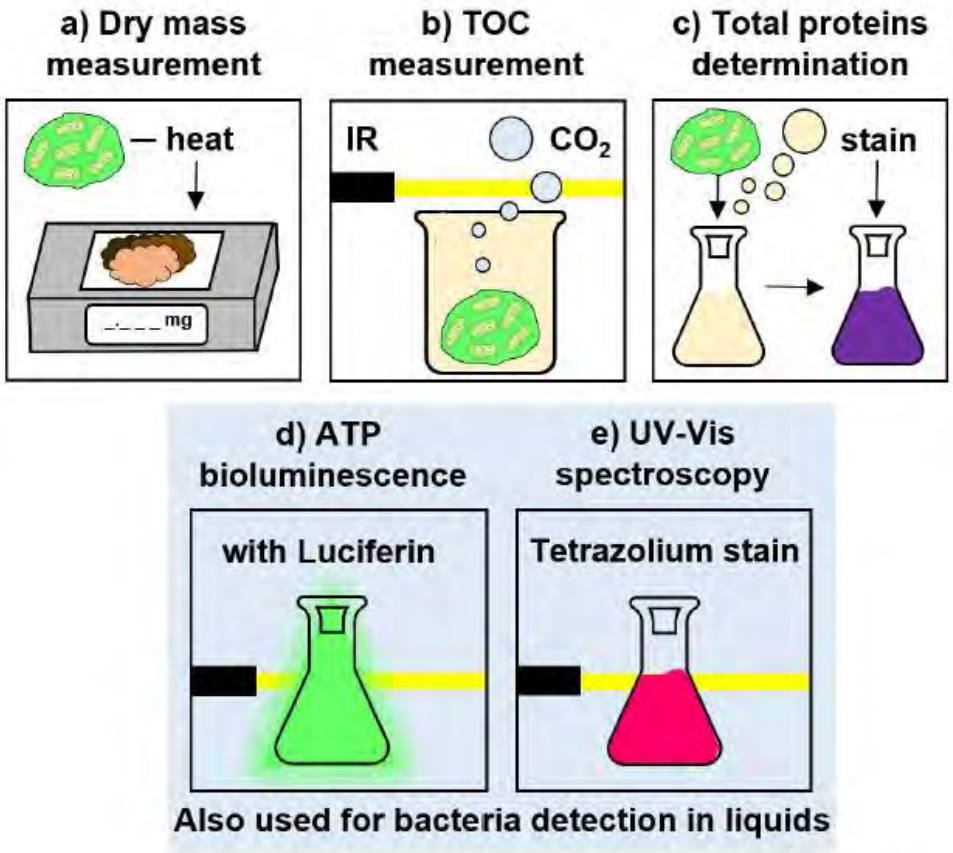
Detection and Counting)

- Agar slice methods
- Dry rehydratable film method

3- **ATP-bioluminescence test**

4- Visual inspection

5- Other methods



Indirect methods for quantitative offline analysis of biofilm

http://www.alvimcleantech.com/cms/en/about-biofilm/white-papers/bacteria-detection

Biofilm detection methods

Direct and indirect biofilm detection and enumeration methods for food processing settings

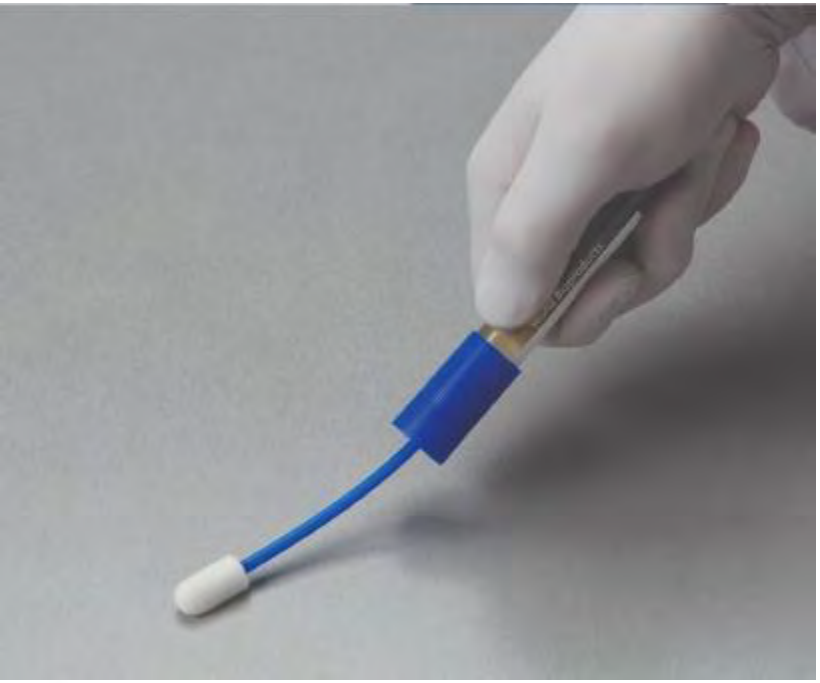
Test	Type	Method
Direct		
BioFinder	Qualitative	Direct observation of color change due to dying of biofilm components.
Contact plates	Quantitative	Sterile agar plate is placed on surface of interest and biofilm is detected via conventional culture methods.
Direct epifluorescence microscopy	Quantitative	Automatic cell quantification using computer software on digital images.
REALCO Biofilm Detection Kit	Qualitative	Direct observation of color change due to dying of biofilm components.
TBF® 300/ TBF® 300S	Qualitative	Direct observation of color change due to dying of biofilm components.
Indirect		
BacTrac 4300	Quantitative	Total viable counts calculated via impedance.
Plate count	Quantitative	Culture plating to determine the number of colony forming units (CFU).
TEMPO®	Quantitative	Cell counts from biofilms are calculated using most probable number (MPN) system based on fluorescence.
Abcam XTT tetrazolium salt and resazurin assay kit	Quantitative	Metabolic assays combined with spectrophotometry can be used to quantify biofilm.

Cadena, et al., 2019 (Used with permission)

Swab/swab–rinsing plate methods

This method may **also be supplemented by the bioluminescence test for total ATP**.

These methods are applicable to any surface, especially **hard-to-reach areas** such as surfaces with:



- cracks,
- corners or
- crevices

that can be reached by hand.

https://www.charm.com/criticality-environmental-sampling/

Swab/swab–rinsing plate methods

A moistened swab or sponge is rubbed over a designated area to remove the microorganisms from the surface.

The sample liquid, or decimal dilutions, if necessary, is then examined by the plate-count method.

The reproducibility of the swab techniques is variable due to the unreliable efficiency of swabbing and the proportion of bacteria removed from the surface is unknown.

Furthermore, it is time-consuming (results available within days) and highly operator dependent.

Despite their limitations, **the swab methods are very useful and almost universally applied in the dairy industry**.

Agar contact plate methods

The **agar contact plate methods** are simpler than swabbing, but it is not possible to sample irregular or rough surfaces that are indeed niches that **harbor biofilms**.

In addition, microorganisms do not quantitatively adhere to the agar surface upon application, again resulting in selection for a specific micro-population or underestimating microbial numbers on the sampled surface.

https://www.pharmamicroresources.com/2016/04/what-is-impact-of-disinfectants-on.html

Agar contact plate methods

Flat or **slightly bent surfaces** which are smooth and non-porous can be sampled by pressing a solidified piece of appropriate nutritive agar against a surface.

A number of commercial products are available in this regard:

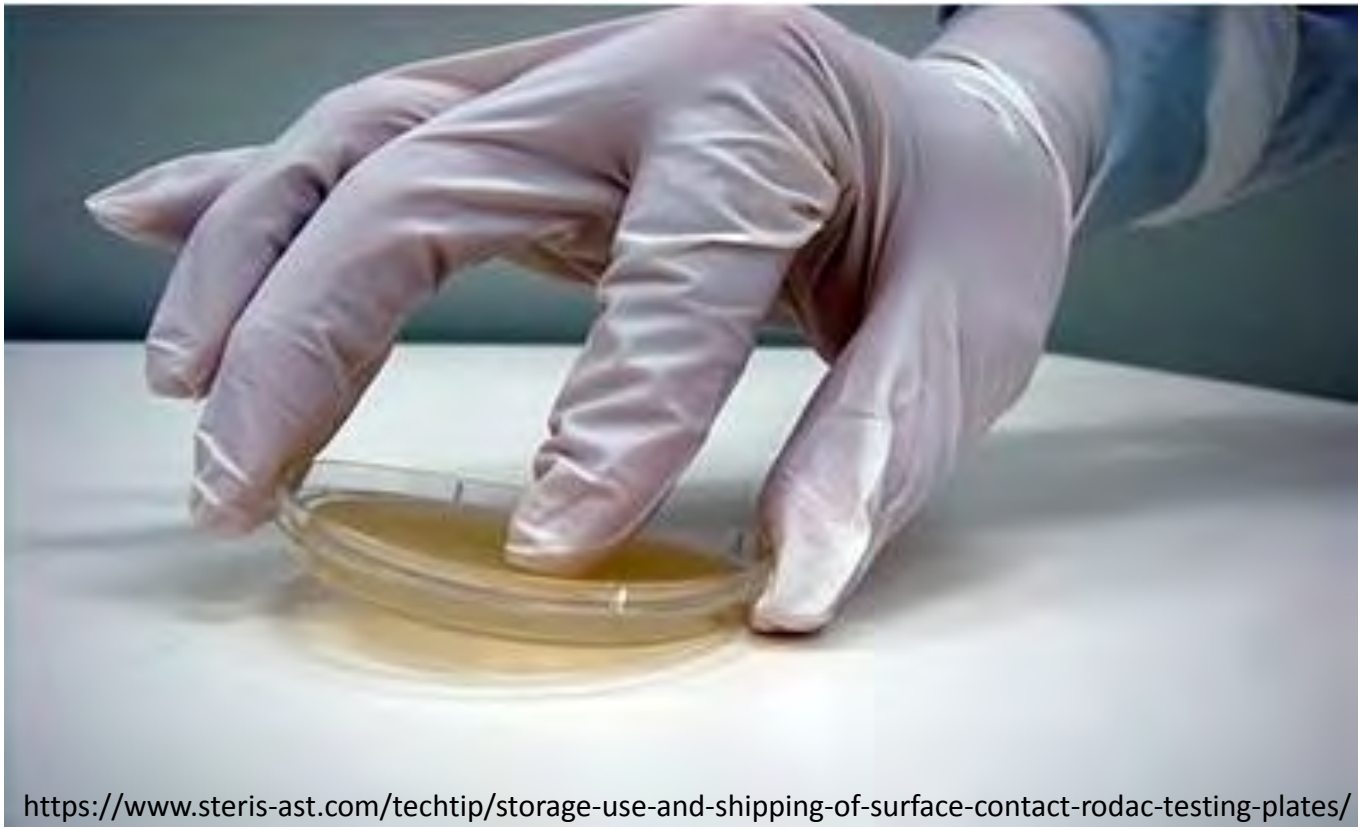
- **RODAC plate count**
- **Agar slice methods**
- **Dry rehydratable film method**

https://www.pharmamicroresources.com/2016/04/what-is-impact-of-disinfectants-on.html

Agar contact plate methods

RODAC plate count:

The replicate organism direct agar contact (RODAC) method employs special commercially available **plastic plates** in which the agar medium protrudes slightly above the rim.
The agar surface is pressed onto the test area, **removed** and **incubated**.

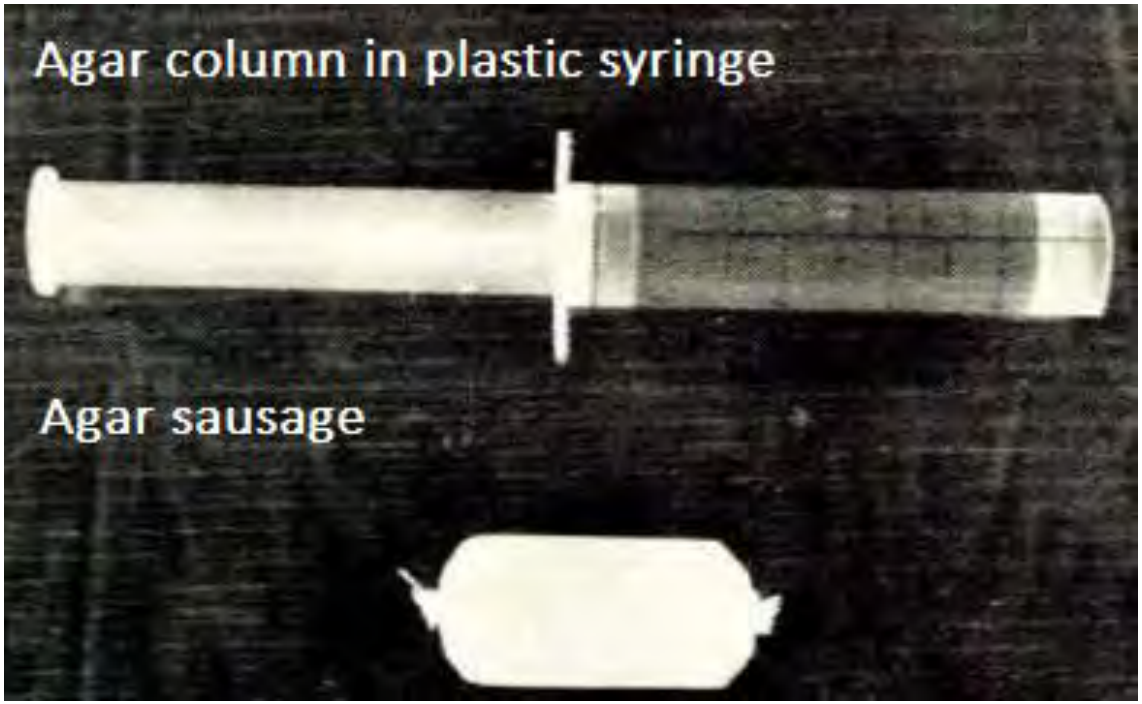


https://www.steris-ast.com/techtip/storage-use-and-shipping-of-surface-contact-rodac-testing-plates/

Agar contact plate methods

Modified large syringes or **plastic sausage casings** can be filled with **agar medium** and a portion pushed out and pressed onto the test surface, cut off and incubated.

Unless caution is taken to apply agar to the sample surface with constant pressure and time, reproducibility of sampling can be questionable.



Horwitz, 1974 (Used with permission)

Agar contact plate methods

Dry rehydratable film method:

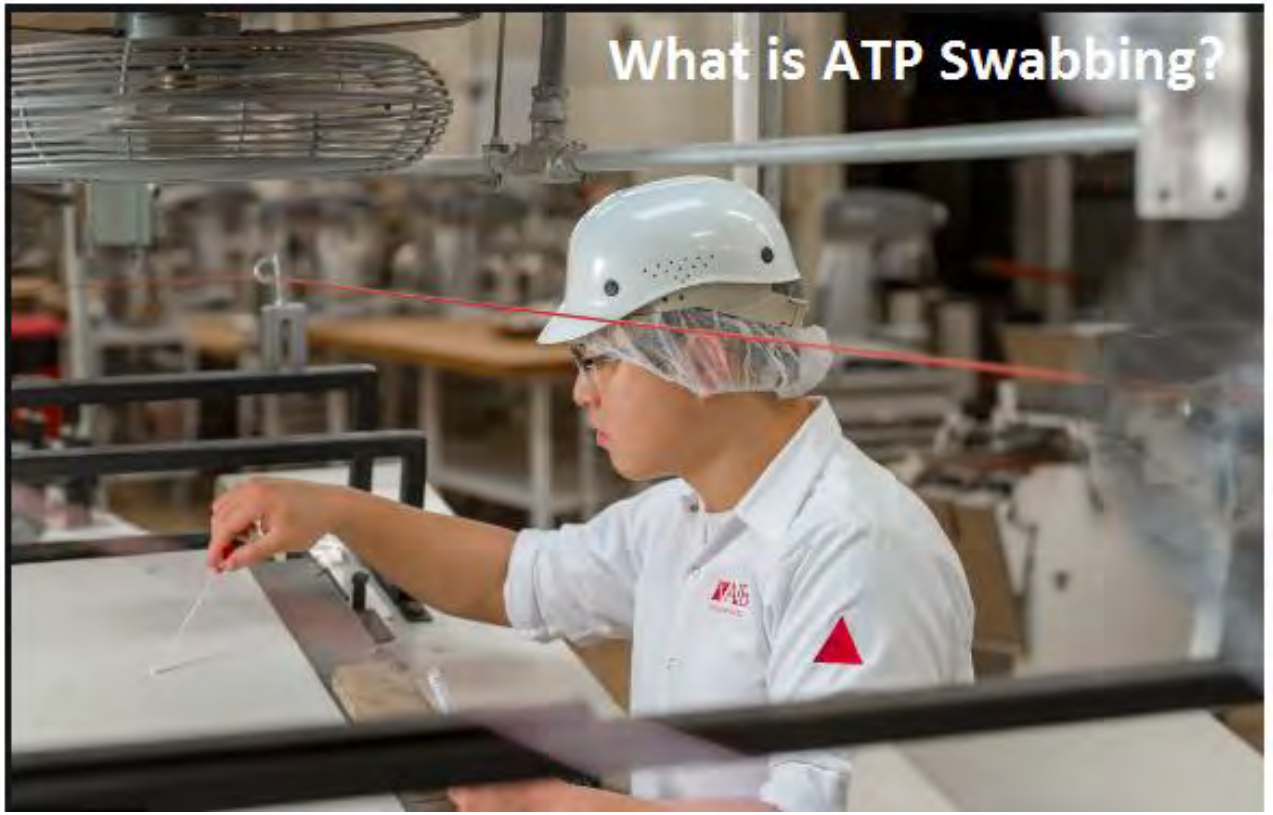
This (Petrifilm aerobic count) method also provides a simple direct-count technique on both flat and curved surfaces.

This procedure **is less applicable for surfaces with cracks or crevices**.

https://multimedia.3m.com/mws/media/241111O/environmental-monitoring-procedures-article.pdf

ATP-bioluminescence test

The most rapid biochemical method to detect biofilms, or the effective removal thereof, can be monitored by the **ATP-bioluminescence test**. This test is a biochemical method for estimating total ATP collected by swabbing a surface.



https://www.aibinternational.com/en/Food-First-Blog/PostId/1204/tip-of-the-week-what-is-atp-swabbing

ATP-bioluminescence test

Total ATP is related to the amount of product residues left behind on surfaces and also to microbial contamination, **collected by the swab**.

Results can be obtained **within 5–10 min** and is also a rapid method to determine cleaning effectiveness and the state of hygiene of plant surfaces.

Iwawaki et al., 2019 (Used with permission)

ATP-bioluminescence test

The method must be used carefully and with a sufficient number of tests to obtain meaningful results.

The readings **are not intended to correlate with the microbial count**, but there **is an excellent correlation between clean surfaces and low levels of ATP**.

Step 1

Use special swab to sample surface

Step 2

Place swab in reaction tube

Step 3

Place tube in luminometer
Results: Relative Light Units

Boyce, 2016 (Used with permission)

Visual inspection

Inefficient cleaning usually results in a visual build-up of a residual film(s) on surfaces.

Some of these films have a characteristic appearance which can help to determine the cause of the cleaning failure.

The Bactiscan Highlights Problem Areas in Processing Facilities

https://www.rapidmicrobiology.com/news/instant-reliable-detection-of-biofilm-using-the-bactiscan

Visual inspection

Films containing **fat** are **soft** when wet and dry, while **protein** films are **hard** when wet or dry and have a **light brown color**.

Inorganic/mineral films are hard when wet or dry, usually have a **rough porous texture** and are **invisible** when wet and **white** when dry.

Other methods:

- The adhesive (sticky) tape method
- Rapid methods for monitoring the hygiene of dairy equipment surfaces.

The Bactiscan Highlights Problem Areas in Processing Facilities

https://www.rapidmicrobiology.com/news/instant-reliable-detection-of-biofilm-using-the-bactiscan

Suggested standards for dairy equipment surfaces prior to pasteurization/heat treatment

Total colony (or coliform) count 100 cm^{-2}	Conclusion
500 (coliforms <10)	Satisfactory
500–2500	Dubious
>2500 (coliforms >100)	Unsatisfactory

Nowadays, with improved cleaning and sanitation programs, a total colony count of

- **200 cfu 100 cm−2**

would be expected, and

- **below 50 cfu 100 cm−2**

for equipment containing **pasteurized products**.

Global Harmonization Initiative
Advocating science-based food safety law and trade

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