Chapter 2. *Listeria* Biofilm formation on abiotic surfaces related to food processing environments

[2.1].   Introduction

In the recent years behavior of bacteria in complex communities or consortia, which are naturally more diffuse in natural environments than freely planktonic single cells, has been increasingly studied. Main reason for this interest is due to the "methodological holistic approach", which changed object of study from single individuals to interaction between bacterial species and within multispecies complex communities (Kuramitsu, et al. 2007).

The technical advances in microscopic techniques and the molecular methods of investigation has revealed that more than 90% bacteria in natural environments exist as biofilms adhered to different substrates, instead of freely floating cells, even if this phenomenon was already noted in first half of twentieth century (Hall-Stoodley, et al. 2004). Earlier biofilms were defined as passive cellular aggregates simply adhered on surfaces.

Currently there is a strong conviction that biofilms represent dynamic biological systems, that are highly structurated. This kind of association is also possible to be observed in human body, where it is correlate to medical devices (lenses, catheters or prostheses) leading, as final result, to chronic infections. Similar structures can be found also in food processing environments, where, if composed of pollutant or pathogen species, biofilms can cause food spoilage and foodborne diseases (Hall-Stoodley, et al. 2004; Ilyina, et al. 2004; McLandsborough, et al. 2006; Palmer and Stoodley, 2007).

[2.2].   Defining biofilms and understanding their architectural complexity

The most complete definition of biofilms is "spatially and metabolically structured microbial communities embedded in an extracellular polymer matrix and located at a phase interface". Currently there are four different known types of interphase confines, where biofilms can develop (Nikolaev and Plakunov, 2007):

- Liquid (or any aqueous medium)-solid surface
- Liquid-air
- Between two immiscible liquid systems
- Solid surface-air

Many experiments focused on exploiting bacterial biofilms and their characteristics reported in literature have focused on slid surface immersed in liquid systems, but within this category several forms are included:

1) *Monolayer*: single layer of cells of one or more microbial species, within which it is not possible to distinguish any morphological differentiation apart form an internal and an external part;

2) *Mats*: they are multispecies communities, in which different bacteria establish association between each other leading to stratificated structures. The thickness of mats can reach even ten centimeters and is regulated on gradient of key factors involved (e.g. light, nutrients or oxidation potential);

3) *Plaques*: dental biofilms are bacterial communities extensively studied and described, where hundreds of bacteria can coexist simultaneously;

4) *Film*s in turbulent flow with band-like appearance due to flow tensile strength;

5) *Fungi*: in this three dimensional structure bacterial create an articulate and complex architecture rich in channels, voids and pores. Many researchers have also studied role of quorum sensing process in similar phenomenon;

6) Benthic and river sediments (Nikolaev and Plakunov, 2007).

From above mentioned categories it is possible to conclude that biofilm spatial architecture and organization of biofilm can be strongly related to environmental conditions to which cells are exposed and thus biofilms can be thought as best survival strategies adopted by microorganisms: if cells grow in turbulent aqueous flows, it is more probable that adopted form could be filamentous streamers, while, in case of backwater-like conditions, tower-like appendices are likely to be formed. Environment does not select just for more appropriate forms and/or architectures, because its influence is more wide-ranged:

- Environment includes also chemical gradients of molecules which can affect gene expression patterns resulting in a modified cellular phenotype more suitable to survive in special conditions;

- Chemical and physical attributes of natural locations can also be thought as selective agents for the detection of more suitable and competitive microbes in given conditions;

- Natural factors can also modify tolerance against bactericidal agents and improve environmental adaption of microbes (Stewart and Franklin, 2008).

[2.3].   Stages of biofilm formation

Biofilm development results from several steps of a dynamic process (Fig. 2.1): solid surfaces, once submerged in liquids, change their superficial attributes because they are covered by different materials (conditioning films). In this phase superficial properties of materials are radically modified in order to allow the attachment of living microorganisms. An example comes from dental plaque, where proteins of saliva play an important role in the attachment of different bacteria: Rosan and Lamont (2000) considered the layer of salivary pellicle onto teeth to be a "biologically active substrate to which the pioneer organism may attach". This conclusion was confirmed by Rudney (2000): this author reported that, because they possess negatively charged domains, salivary proteins formed a thin pellicle (or enamel), which act as a conditining layer for bacterial attachment on oral cavity surfaces. The importance of the enamel in the bacterial attachment onto oral cavity surfaces was confirmed also by Marsh (2004) and by Jenkinson and Lamont (2005).
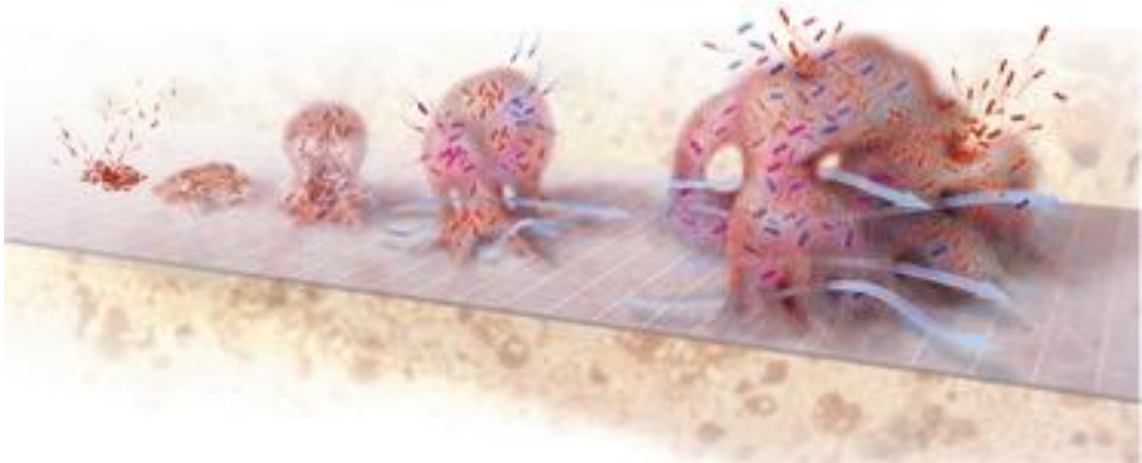
Fig. 2.1: Biofilm formation in turbulent flow with relevant stages of the process. [from http://wvlc.uwaterloo.ca/biology447/Biofilms/biofilmsoverview.htm, last acces 12/10/2010]

Next step is reversible adhesion, in which nonspecific interaction forces and cell superficial elements are involved and bacteria are not strongly linked to the surface material. After this, cells lose their intrinsic motility and begin the excretion of extracellular polymeric substances (EPS), which form organic amorphous matrix in which cells are embedded. At this point microcolonies are formed on surfaces (Irreversible adhesion). The secondary colonizers starts attaching on surfaces of the primary colonizers leading to thickning of the biofilm and to the formation of specific architectural elements (like channel, cavities and tower-like appendices) in the biofilm.

These structural elements play an important role in nutrient movements and waste removal strengthening the idea of spatially organized community in which form is perfectly linked with the assumed function (deBeer, et al. 1994). Mature biofilm can increase its thickness and complexity for long period of time, after which structural degradation and cell dispersal take place. This last step has been seen in biofilm formation in turbulent condition, where, once reached its maximum of growth, cells and aggregates of organic matter detach from the biofilm and colonize new environments.

[2.4]. Adherence ability and biofilm formation of *Listeria*

The bacterial adherence onto surfaces, as well as growth and main growth-related parameters of different strains (Lianou, et al. 2006), is differently expressed within *Listeria*, even from strains isolated in the same environmental niche: Kuswahara and Muriana (2009) examined *Listeria* spp. isolates from three different meat processing plants observing different prevalences. Within isolates from the same plant authors observe large variation in terms of the capacity of *Listeria* to adhere on available surfaces, even if two of the three examined plants involve only weak and moderate adhesive strains. The third

plant, which was also the oldest one, included also very firmly attached *L.monocytogenes* probably related to its age of construction and level of sanitation practices adopted .

The origin of *Listeria* strains was investigated for its influence on bacterial adhesion to surfaces and consequent biofilm formation. Tresse et al. (2007) found weak but significative variations among 101 examined strains in function of colonized surface and origin of the strain. Clinical isolates showed better adhesive ability on polystyrene than stainless steel, while the industrial strains were more adherent than clinical isolates. Unfortunately no possible relation with belonging to a specific genetic lineage was observed, even if 4b strains are reported as less adhered than 1/2a and 1/2b. This observation was confirmed also by Moltz and Martin (2005), Lianou et al. (2006) and Bonaventura et al. (2008).

Despite the large amount of studies regarding *Listeria* spp. biofilms, there are still many questions and unclear aspects. The present study aims at studying the development mechanisms of *Listeria* spp. biofilms on different materials. Moreover, adhesion under static conditions will be compared with adhesion in turbulent conditions; factors influencing surface attachment (e.g.: temperature, nutrients) will also be investigated.

[2.5].   Materials and methods

I.   Test materials

Stainless steel coupons were used to test *Listeria* adhesion in static conditions of incubation. 0,1 mm thickness wire of stainless steel (SS), polyethylene terephtalate (PET), and Copper (C), which were acquired from local supermarkets, were used as surface test materials in dynamic regime tests, covering a large range of possible materials present in food transforming locations.

II.   Bacterial strains

Listeria spp. strains were reported in Tab. 2.1. Each strain was grown on ALOA (Agar Listeria Ottaviani & Agosti, Biolife Italiana Srl) plate and in Blood Agar (LABM, United Kingdom) at 37°C for 48 and 24 hours, respectively. These two media were used for species identification and β-hemolysis

assay, respectively. Single colonies from the plates were transferred into Brain Heart Infusion (BHI; LABM, United Kingdom) and incubated for 16-18 h at 37°C and 200 rpm in a shaking bath. Stock cultures were kept at -70°C.

III.   Static biofilm on stainless steel

Stainless steel coupons (Fig. 2.2) were used as test surface: each coupon has ten squares (1 cm x 1.cm x 0.1 cm), organized in two rows, for an overall superficial area of 1 cm^2. Selected strains reported in Tab. 2.1 were subcultured in BHI broth for 18 h at 200 rpm at 37°C. Each strain was harvested at 4000 *xg* for 5 min and washed twice with sterile peptone water. Cell numbers were determined by serial dilutions and plating on TSA (Tryptone Soy Agar, LABM, United Kingdom) plates, after incubation for at 37°C 24 h. In parallel to plate counting, optical density at 600 nm (OD$_{600}$) was measured on the same dilutions, in order to have a rapid evaluation of bacterial density. Aliquots were taken from aqueous cell suspensions and inoculated into 25 ml BHI Broth. The volume of aliquots to be added into 25 ml BHI broth, was calculated in order to provide a density of 10^5-10^6 CFU/ml. Inoculated medium was added into a Petri plate containing a previously washed and autoclaved steel coupon. After 24 h incubation at 37°C, the coupon was transferred into a new Petri plate, into which 25 ml of 10% diluted BHI (dBHI). The so treated coupon was incubated for 7 days at 37°C. At defined periods (0, 3 and 7 days) coupons were scraped with wet cotton swabs. Swabs were serially diluted into sterile peptone water and plated onto TSA plates, which were incubated as above. Three replicates were performed for each strain.
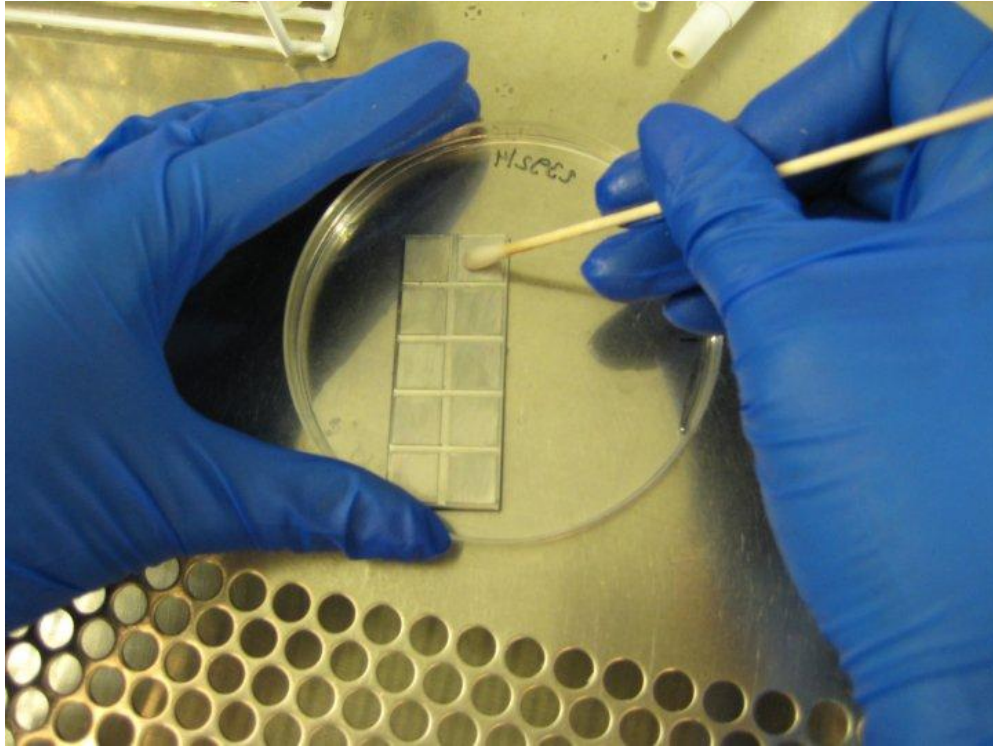
Fig. 2.2: Stainless steel surface used for monitoring bacterial adhesion.

Strains	Serotype	B-hemolysis	Description
UC 8410	-	-	*L. innocua* cheese brine isolate
UC 8409	-	-	*L. innocua* cheese brine isolate
EELA Oulu ba 2392/4	1/2a	-	*L.monocytogenes* Fetal bovine brain
EELA Hki L211	1/2a	-	*L.monocytogenes* Cold smoked rainbow trout foodborne out break Food isolate
EELA Hki L627	3a	-	*L.monocytogenes* Butter foodborne outbreak Food isolate
KTL IHD 42526	1/2a	-	*L.monocytogenes* Cold smoked rainbow trout foodborne out break Clinical isolate
KTL IHD 42573	3a	-	*L.monocytogenes* Butter foodborne out break Clinical isolate

Tab. 2.1: *Listeria* **spp. strains with relevant properties of interest and their origin of isolation.**

IV.   Biofilm formation test under dynamic conditions

The apparatus (Fig. 2.3) was composed by 5 glass tubes (10 cm long, internal diameter 0. 4 mm), into which the wire test material was introduced, linked with teflon junctions to a solution dispenser (30 cm long, i.d. 20 mm). The apparatus was chemically disinfected with an succession of disinfection

solutions (NaOH 0.5 mol and 50% EtOH) and washed with sterile physiological solution. An over-night culture of *L. innocua* strains in skimmed milk was introduced into the system at 1 ml/min flow rate and, when the pipeline was full, the flow was stopped and the whole system was incubated at 37°C for 1h. A washing step with physiological solution was performed to remove only slightly adhered cells. After this step, the nutrient solution was flown inside the appatatus at a flow rate for 1 ml/min. At defined time points (0, 3, 6, 18, and 24 h), the flow was stopped and aliquots of both tested materials and eluate were taken and subjected to serial dilutions and plating on BHI agar, to count both sessile and plancktonic cells, respectively. All the experiments were performed in triplicate.
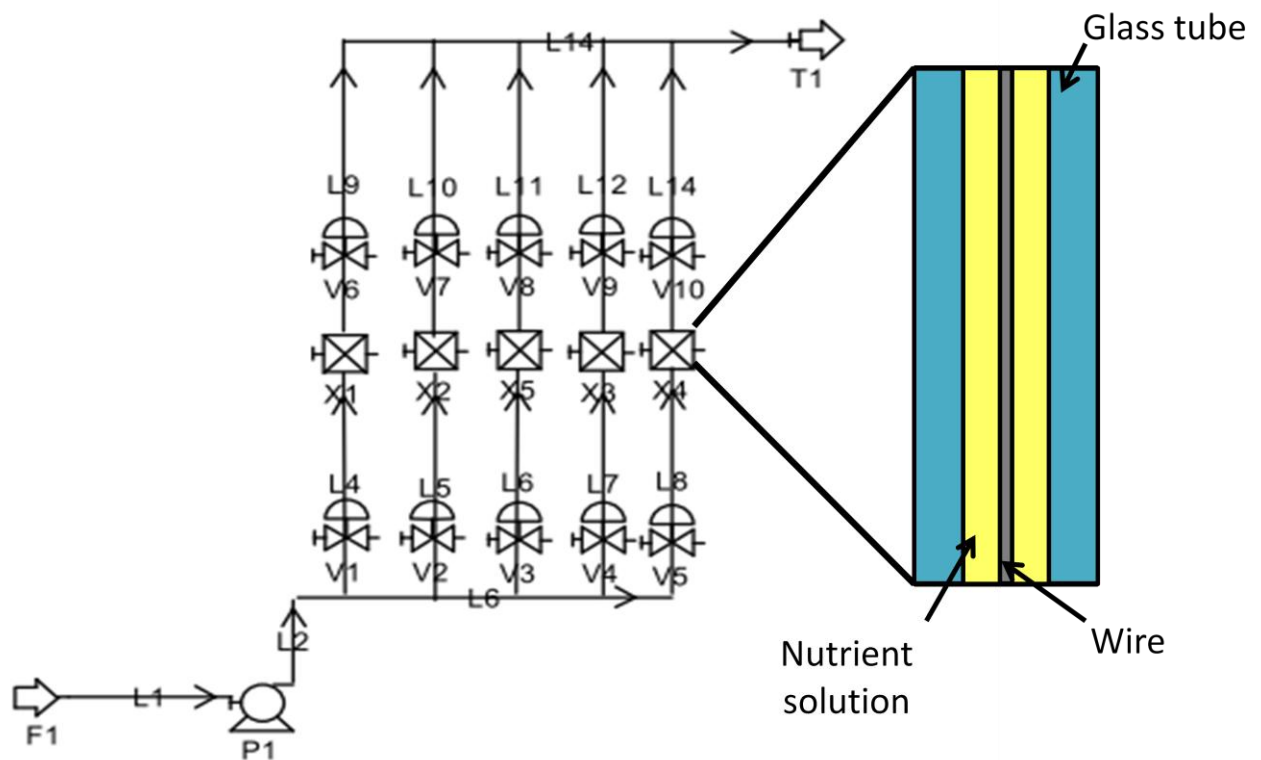

Fig. 2.3: Schematic representation of employed system with enlargement of glass tube (X_n) in which the wire of testing material was inserteed. F1: nutrient solution (skimmed milk) input, T1: exhausted nutrient solution, V_n is referred to valve to open and close the flow of milk, while L_n represent the tubes to allow flow moving.

V.   Sample preparation for SEM observation

Each sample was subjected to 8 h dehydration steps with increasing concentration of ethanol (70, 85, 95 and 100 %). The samples were then put into a Critical Point Dryer (Bal Tec CPD 030, Pubish), where several washings with liquid CO_2 (99,5%, Sapio) were performed until the CO_2 critical point (40°C and 7 atm was reached. The so treated sample is completely dehydrated and stored under vacuum.

Further steps were metallization by doration through Top Autocoater SC20 (Pubish) and storage in dessicator. Each sample was examined by Scanning Electron Microscope (SEM) FEI ESEM XL30.

VI.   Microbial adhesion to solvents (MATS)

This assay was performed as described by Brianet et al. (1999): old liquid culture was harvested at 2500 *xg* for 5 min and washed twice with 0.85 % NaCl in water. Cells suspension was divided into 2.4 aliquots, to which 0.4 ml of hexadecane (Sigma-Aldrich) was added. The mixture was vortexed for 1 min and then incubate at room temperature for 15 min, to allow complete separation of the two phases composing the mixture. 1 ml was taken from the mixture and optical density at 400 nm was measured. Results were collected from three replicates obtaining by using two indipendent subcultures and were expressed as percentage of affinity with solvent by using the equation:

$$\% \text{ affinity with the solvent} = 100 \times [1 - (A/A_0)]$$

VII.   Statistical analysis and treatment of data

Data were statistically analysed by SPSS 14.0, to One-way ANOVA, *t* test and post-hoc analysis in order to detect significant parameters and quantitative definition of influence of selected parameter (i.e. strain, temperature, nutrients, surface material) .

[2.6].   Results

I.   Adhesion under static conditions

Different strains of *Listeria* from 7 up to 8 Log were examined for their capacity to adhere on stainless steel and their ability to survive on this surface with limited amount of nutrients. Compared to TSB, BHI supports better microbial growth and thus supposely helps to distinguish more clearly any eventual difference between tested strains. dBHI was chosen to assess survival and biofilm formation of examined strains under limited presence of nutrients. Dilution factor of growth medium was, instead of 0.5 or 0.2x, 0.1x, to simulate nutrient depletion. Obtained results are reported in Fig. 2.4. *L. innocua* UC

8410 (isolated from cheese brine) was compared with two *L. monocytogenes* isolates from European cases of foodborne listeriosis. *L. monocytogenes* EELA HKi L211 was isolated from a sample of the cold smoked rainbow trout involved in the listeriosis outbreak occurred in 1994-1995 in Sweden, while *L. monocytogenes* KTL IHD 42526 was isolated from a hospitalized patient of same episode. *L. monocytogenes* EELA Hki L627 and *L. monocytogenes* KTL IHD 42573 were food and clinical isolates, respectively, from butter-related listeriosis outbreak. *L. monocytogenes* Oulu ba 2392 is referred to clinical animal case.

After 24 h ours of incubation *Listeria* spp. were present at high levels (more than 10^7 CFU/cm^2), even if the extent of adhesion was slightly different among tested isolates. EELA Hki L211 was revealed the least adherent strain, while Oulu ba 2392/4 and EELA Hki L627 exhibited the highest adhesion on stainless steel, reaching levels greater than 10^8 CFU/cm^2. Strong similarity in surface adhesion was observed in the both clinical isolates (KTL IHD 42573 and 42526), while corresponding food isolates presented different extent of adhesive properties. Despite the quantitatively different extent of adhesion, investigated strains did not significantly differ from each other, demonstrating that both the species and origin of isolation cannot completely account for diversified bacterial attachment on surface.

When the strains were grown under nutrient depletion, a geneally common trend of behavior was observed: incubation in these conditions led to a 3-fold stronger overall reduction at 168 h (1.5 Log reduction factor) than 72 h (0,4 LOG). Nevertheless, bacteria were not completely killed by the extended incubation, and the counts of the most susceptible strain, UC 8410, never decreased at levels lower than 10^3 CFU/cm^2 after 168 h). Neither foodborne outbreak-related clinical isolates were strongly affected by prolonged limited source of nourishment (0.5 LOG reduction factor for both the isolates) unlike the food isolates. The animal clinical isolate Oulu ba 1283/2 had shown to be notably susceptible to nutrient depletion. Even if it showed higher values, EELA Hki L627 was 1-fold more susceptible than EELA Hki L211 within 168 h (0,25 LOG reduction compared with 1.5 LOG, repsectively). Statistical analysis did not detect any significant differences between the strains of different origins.
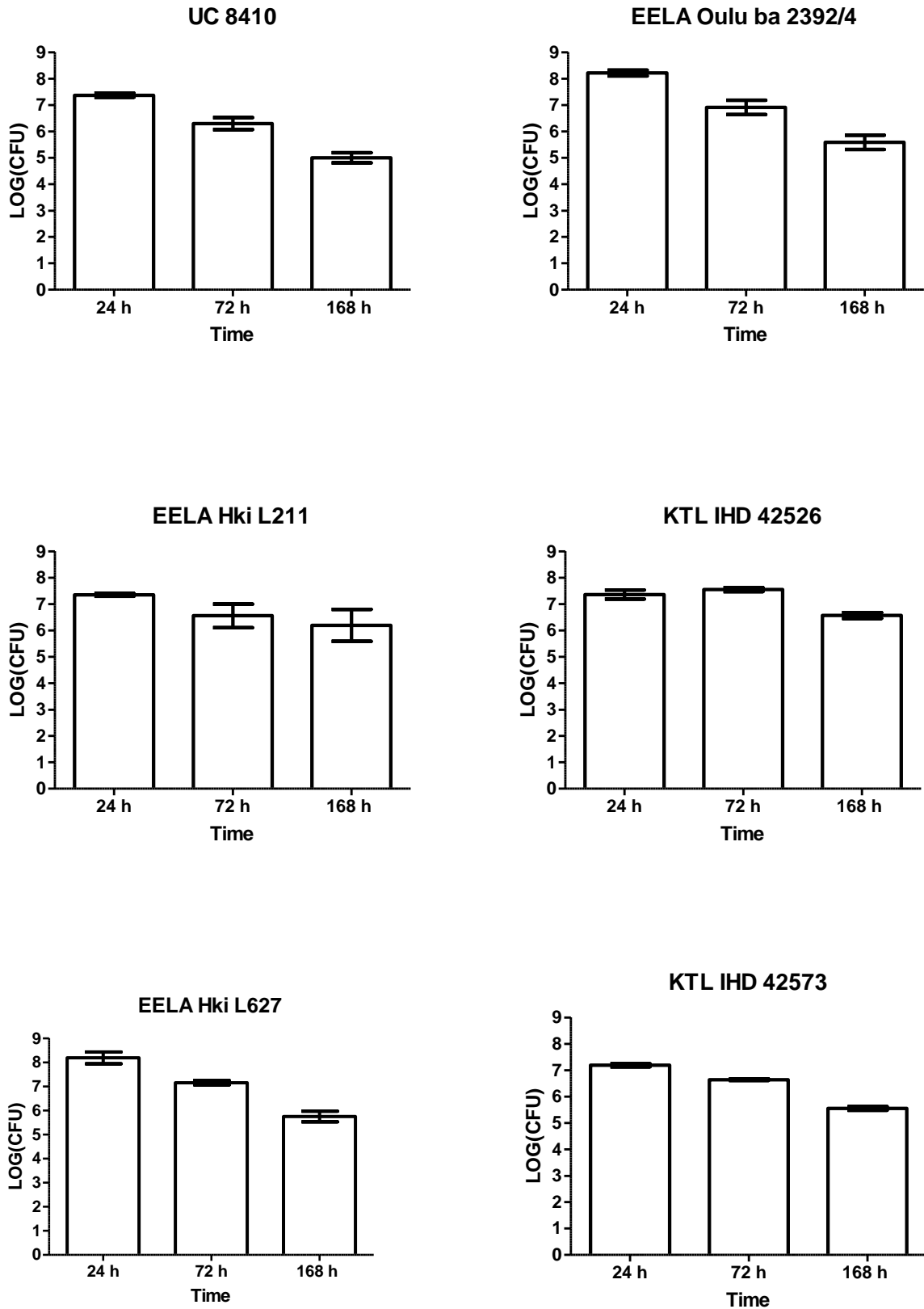
Fig. 2.4: Adhesion assay of *Listeria* spp. strains for 7 days at 37°C. Bars represent LOG(CFU) referred to an overall superficial area of 1 cm². Data are expressed as mean of three replicates ± standard error mean.

II.   Bacterial adhesion in turbulent flows

In the scientific literature static apparatus has been commnonly usedto test the biofilm forming ability of strains. There are instead few reports about biofilm formation under moving flows. For this purpose a suitable apparatus was designed (Fig. 2.3). Among the studied strains *L. innocua* UC 8410 was chosen for biofilm formation assay under dynamic regimen together with *L. innocua* strain UC 8409 (isolated from same source), in order to verify if strains isolated from the same environmental niche could possess the same adhesion ability. These strains were also selected as a model of the pathogenic species *L.monocytogenes*.

The first experiment was conducted with stainless steel wire (20 cm long, 1 mm i.d): stainless steel was chosen because of its common use in food manufacturing plants. Milk was employed as nutrient solution as a model of food, which supports the surface adhesion and functions as a nourishment source for bacteria. Experiment was performed with skimmed milk to avoid any effect attributable to fats and lipids. The apparatus used allowed the analysis of biofilm under nutrient flow on different materials. A wire of tested materials (in this case SS) was introduced into a glass pipe and then exposed, after initial inoculum (i.e. static incubation of the wire for 1 h in presence of an over-night bacterial culture) within the incubation cell itself, to skimmed milk flow for 24 h. At defined periods of time, the flow was temporarily stopped, in order to allow sampling of aliquots of tested surfaces and nutrient solution, which were used to monitor sessile and planktonic cells, respectively (Fig. 2.5).

After 1 h incubation, UC 8410 showed a gradually increasing attachment to the SS wire, of which the maximum reached level was 10^8 CFU/cm^2 after 6 h of monitoring in presence of a milk flow. Despite the high density of cells freely suspended in growth medium, UC 8409 was demonstrated not to be able to adhere on tested surfaces in the same experimental conditions. From these results, it was observed that UC 8410 possessed a higher adhesive capacity than UC 8409 and thus was able to form biofilms under dynamic regimen.
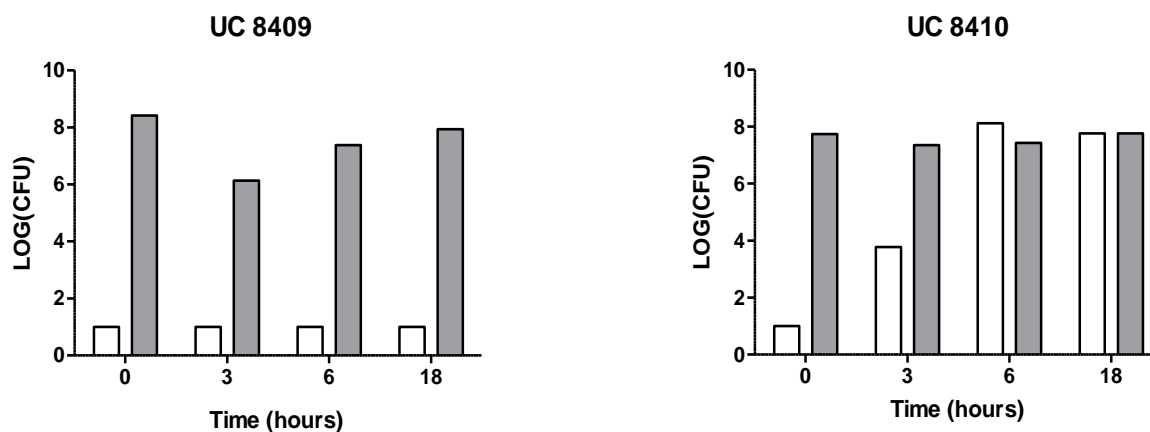
UC 8409

UC 8410

Fig. 2.5: Surface attachment of *L. innocua* strains on stainless steel wire at 37°C. Sessile cells (white bars)are expressed as LOG(CFU/cm^2) superficial area, while planktonic cells (grey bars) are expressed as LOG(CFU/ml). 0 h is referred to 1 h incubation of over-night colture in skimmed milk, while value obtained until 18 h demonstrate adhesion capacity of the two employed strains during 18 h testing period under dynamic conditions. 1 LOG(CFU) is to be intended as less than 10 CFU.

III.   Cell superficial hydrophobicity

Based on the data obtained using SS in the presence of a milk flow, cell hydrophobicity of both *L. innocua* UC 8409 and UC 8410 was measured, in order to obtain an explanation to the differing adhesive capacity of the analysed strains. The test was conducted using protocol described by Briandet et al. (1999). Microbial adhesion to solvents assay (MATS) was performed in triplicate for the two strains and the results are shown in Fig. 2.6.
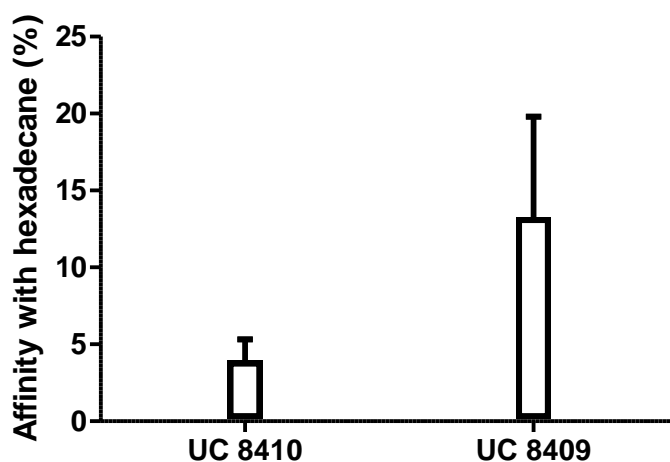

Fig. 2.6: Percentage of affinity of *Listeria innocua* strains with hexadecane. Bars represent the average of three replicates ± standard error of mean.

UC 8410 exhibited low affinity with hexadecane, demonstrating strong hydrophilic properties of overall cell surface, while UC 8409 showed a better comnpatibilty with the employed solvent. As reported by Bellon-Fontaine et al. (1996), Briandet et al. (1999) and Takahashi et al. (2010), *L. innocua* UC 8410 was demonstrated not to possess relevant electron-donating and Lewis acid properties, which were instead much more relevantly shown in UC 8409. The great quantitative difference among the two isolates suggested that the adhesive capacity of a strain could partially be related to intrinsic superficial properties of the cell itself, although it cannot be excluded the contribution of other factors involved in this phenomenon.

IV.   Surface materials

The second aim was to study if the test surface could affect *L. innocua* UC 8410 adhesion and thus biofilm formation in the presence of shear forces due to flowing solution. Three different materials were tested (SS, PET, C). The analysis of bacterial counts conducted on all tested material (shown in Fig. 2.7) indicated that UC 8410 exhibited relevant adhesive capacity, whose reached levels depended on employed materials.

L. innocua UC 8410, in presence of SS, showed gradually increasing denisties on wire until to levels almost comparable with the corresponding ones observed for the planktonic cells. When PET was employed as testing surface, constant cell recoveries, which were lower than those obtained with SS, were observed with regard to sessile cells, altough planktonic cell density was almost 10^9 CFU/ml in both these samples. C was suggested not able to support the adhesion of milk protein or *Listeria* spp., because absence of colonies have been observed even at the lowest dilution since 3 h sampling (Fig. 2.7).

 Statistical analyses on the data indicate that the tested materials affected significantly the surface bacterial attachment ($p < 0.01$). Post hoc tests further revealed that both SS and PET showed strong similarities ($p > 0.01$), but moved away significantly from C ($p < 0.01$).
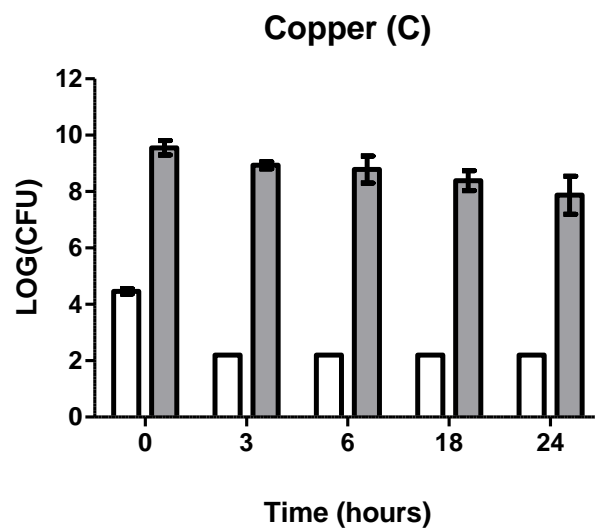
Stainless steel (SS)

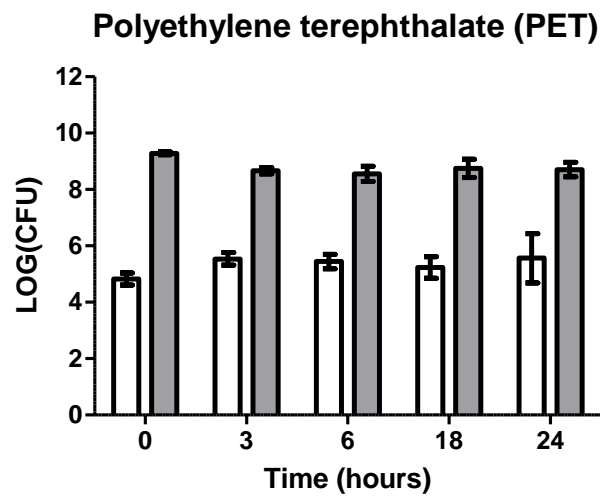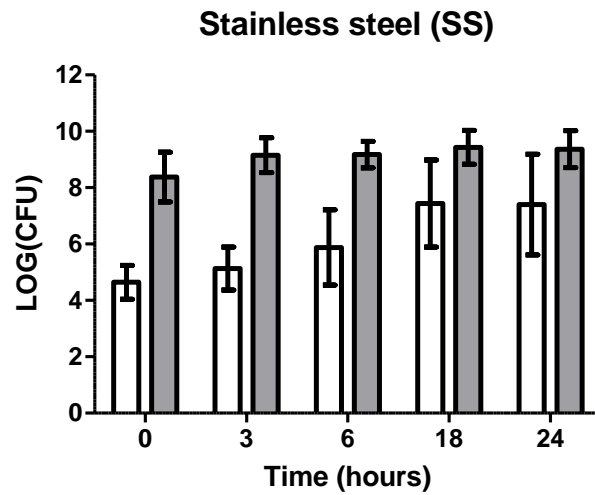## Polyethylene terephthalate (PET)

Copper (C)

**Fig. 2.7: Adhesion on different materials of *L. innocua* under dynamic conditions. Data are expressed as mean of three replicates ± standard error mean (SEM). White bars: sessile cells, gray bars: planktonic cells. Sessile cells are expressed as LOG(CFU/cm^2), while planktonic cells are expressed as LOG (CFU/ml). 1 LOG(CFU) is to be intended as less than 10 CFU.**

V. SEM examination of inoculated wires

All tested materials were examined with SEM, in order to observe growth of biofilm on tested wires during the 24 h testing period (Fig. 2.8 to Fig. 2.12). SS showed a slightly rough surface, whose irregularities could be used as contact point for cells to establish sessile consortia (Fig. 2.8). Deposition of organic matter was observed in samples after 1 h of incubation with *Listeria* in milk suspension, which lead to a complete coverage of the whole available surface (Fig. 2.9). 3 h samples showed the presence of mushroom- like structures connected to each other (Fig. 2.10). After 6 h it was possible to observe a multi-layered architecture of relevant thickness along the whole sample surface. The complex matrix of biofilm covered the whole surface and seemedto hide bacterial cells, which were thus not observed with SEM.
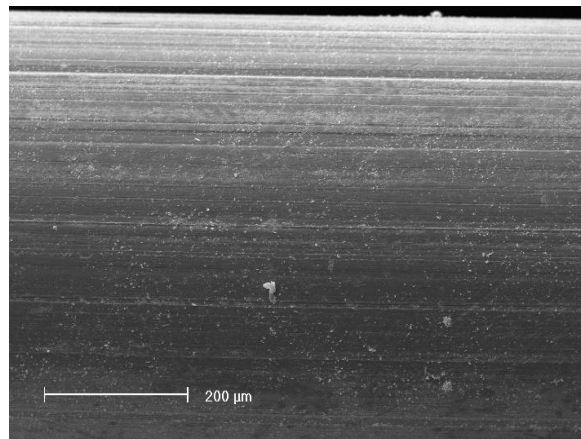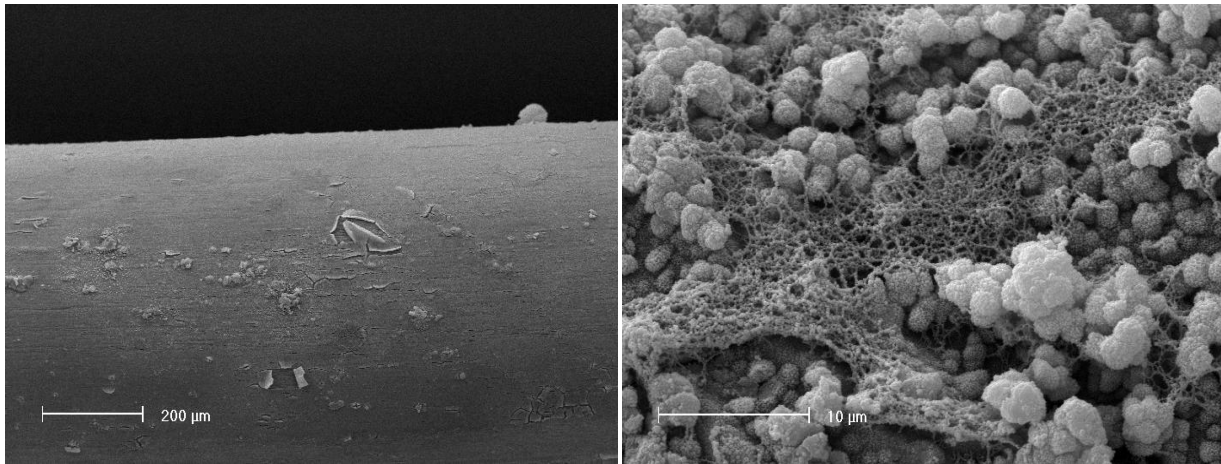

Fig. 2.8: Images of stainles steel coupon after chemical disinfection steps obtained at magnitudo of 150 (leftt image).
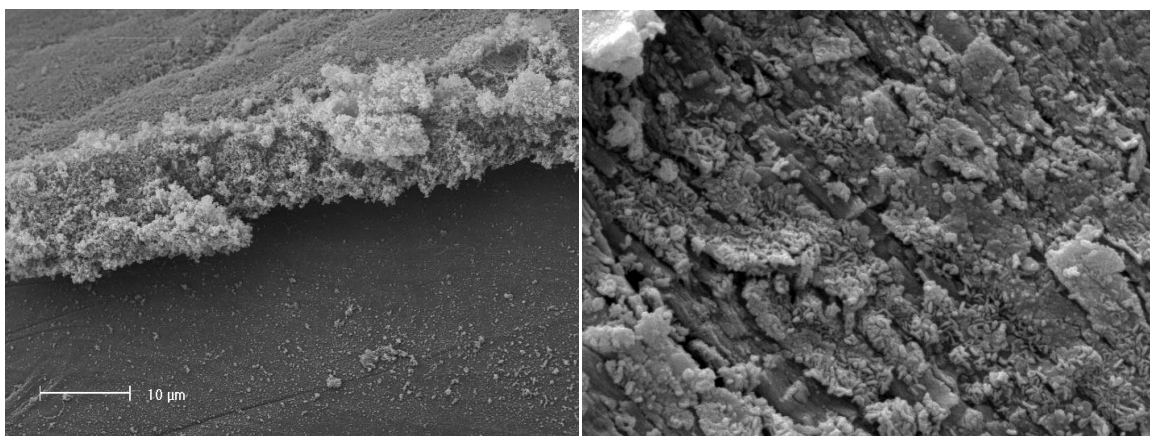

Fig. 2.9: SS sampled 0 h after initial inoculum. Magnitudo was 1900.000 and 4500.0000 for left and right image, respectively.

Fig. 2.10 SS specimen after 3 h from test beginning [magnitudo of 100 (left image) and 3052.311 (right image)].

PET specimens (Fig. 2.11) examined with SEM instead showed that organic matter was not only able to cover the whole available surface, but a spontaneous detachment of deposited material took place after reaching a certain thickness, although the detachment of these layers could be due to specimen preparation procedure. Observing on C tested in the same experimental conditions at SEM, skimmed milk protein covered homogenously all available area, altough, despite the bactericidal action attributed to copper (corresponding plate countigs were less than 100 CFU/ml), cells were present in a sort of cavities throughout the all examined material surface, demonstrating that copper can successfully limit bacterial attachment but it is not sufficient to solve this problem (Fig. 2.12).
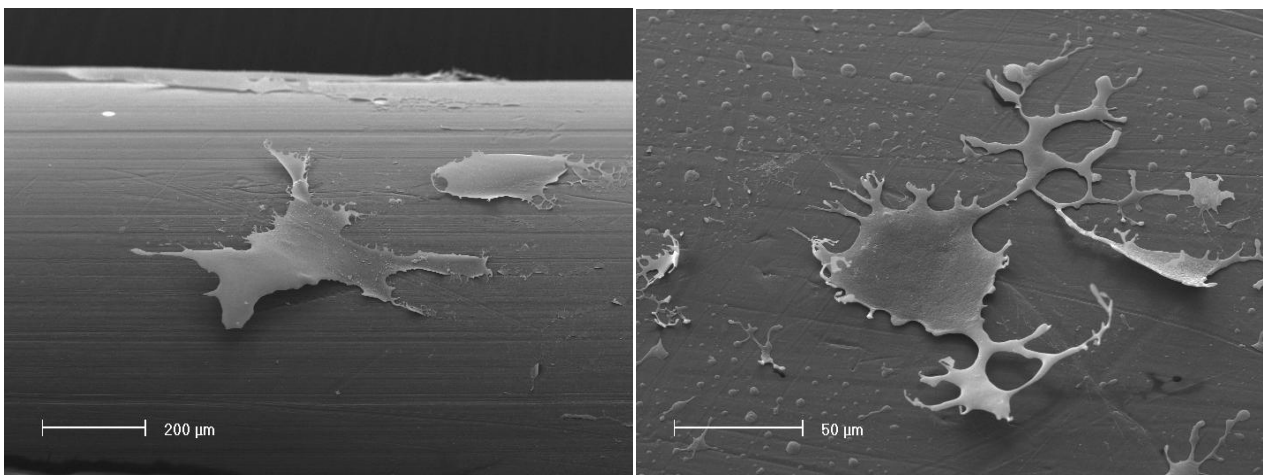
Fig. 2.11: PET specimen at 3h (left image, magnitudo = 100) and 6 h (right image, magnitudo = 500).
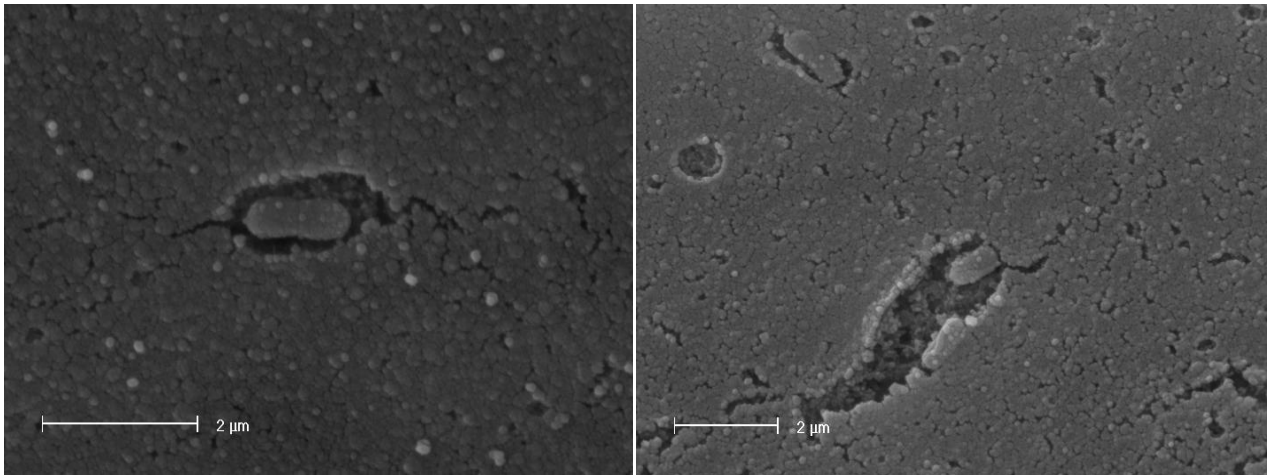
**Fig. 2.12: C specimen at 3 (left image, magnitudo = 15000) and 6 h (right image, magnitudo = 10000).**

VI.   Temperature

In order to assess the role of temperature on *Listeria* biofilm formation, further experiments were performed. SS was choosen as a test surface, because, based on already obtained results, it gave quantitative results concerning bacterial adhesion under the test conditons. The studied temperatures (20, 30 and 37°) allowed the comparison with results from former studies, in which static apparatus were employed.

Both adhered and planktonic cells were evaluated with the same experimental procedure discussed in par. III, sect. 2.5. Results are shown in Fig. 2.7. At 37°C the cell present in the flow of nutrient ranged between 10^7-10^9 CFU/ml. Shifting the temperature from 37°C to 30°C did not result in a significant amount of planktonic cells, which were kept almost constant (10^9 CFU/ml), while slight decrease was observed in the recoveries of planktonic cells at 20°C both after 3 and 24 h.

Regarding the adhered cells, increasing recoveries were observed at 37°C up to 24 h, when the number of recovered cells reached the maximum level (ranging between 10^6-10^8 CFU/cm^2). When the dynamic apparatus was incubated at 30°C, the adhesion of the strain was notably modified: cells adhered to SS wire were 10^6-10^8 CFU/cm^2 after initial inoculum and then raised up to 10^9 CFU/cm^2 at 3 h. A sharp reduction until 10^4 CFU/cm^2 was observed at 6 h. Elapsing the time, cells present on taken specimen of wire showed higher levels until to restore the density observed at 3 h (10^8-10^9 CFU/ cm^2 at 24 h), which slightly diverged from the value corresponding to planktonic cells at the same time period.

A similar trend was observed when temperature was reduced when temperature was lowered at 20°C, even if cell recoveries were significantly lower (2-fold lower than the corresponding one at 30°C or 37°C). These reduced values could be attributed to a not efficient cellular metabolism when bacteria were grown at non- optimal temperatures.

37°C

30°C

20°C
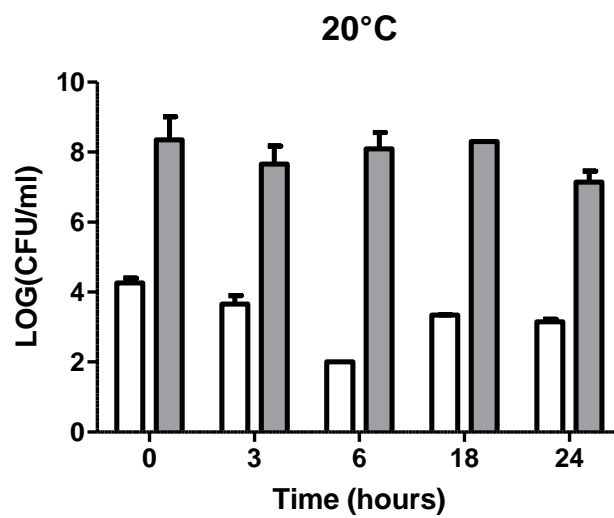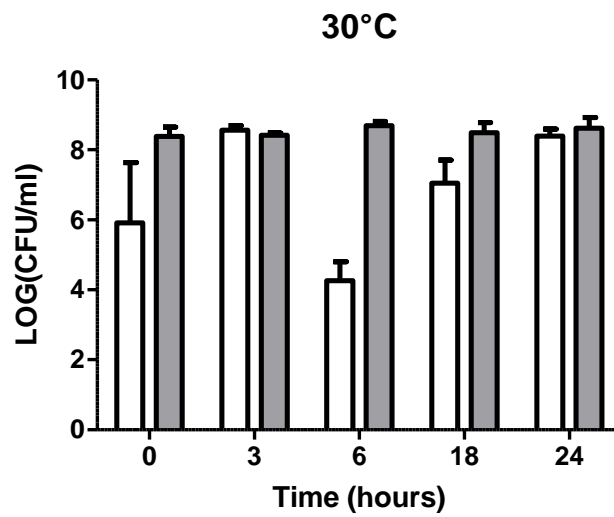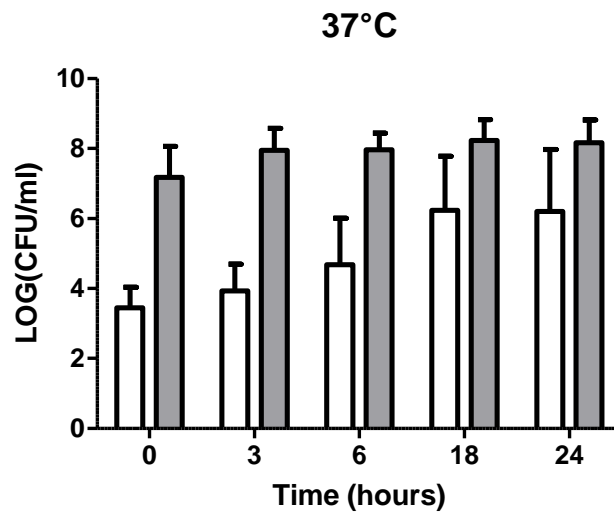
Fig. 2.13: Effct of temperature on stainless steel. Data are expressed as average of three replicates ± standard error mean. White bars represent adhered cells, while gray bars sho density of planktonic cells.

VII.   Influence of the medium on biofilm formation

In order to obtain a more detailed prospective of establishment of sessile communities in the presence of turbulent flows, adhesion monitoring of diverse substrates was performed. Most of the earlier studies have used growth medium such as BHI or TSB broth (diluted or not) to simulate adhesion to food processing surfaces (Cheok and Schraft 2000,Kalmokoff et al. 2001Moltz and Martin 2005, Tresse et al. 2007, Harvey et al. 2007) BHI was also included in the present study in order to compare the collected results with previous findings. In parallel the same experimental conditions were applied using skimmed milk, which reproduces realistical bacterial contamination of plants processing or handling liquid foodstuffs. Beside comparison of these two mentioned media, role of microbial activity in the formation of above mentioned multilayered architectures was investigated: in this case assessment was conducted with skimmed milk supplemented with 10 μg/ml.

SS was employed as a test material due to its previously demonstrated ability to support bacterial adhesion in the presence of shear forces, while inoculum was obtained through static incubation of the wire in presence of an over-night culture of *L. innocua* UC 8410 (this treatment was not performed with chloramphenicol supplemented milk). All these media were used into apparatus used in par. III for 24 h at flow rate of 1 ml/min and, at defined time point, flow was interrupted to allow sampling of aliquots of tested wire These specimen were examined through observations at SEM (Fig. 2.15).

In the presence of BHI (image A), no deposition of organic matter took place and no colonies were detected even at lowest dilution, suggesting the incapacity of BHI to support bacterial attachment on surfaces under dynamic conditions. When skimmed milk (image B) was used as a nutrient solution, it was possible to observe tower-like appendices, which were then substituted by very thick multi-layered structures, within which bacterial cells were probably embedded (although they were not found in any sample examined at SEM). Even if there was a relevant deposition of organic materials on wire surfaces, 10 μg/ml chloramphenicol-supplemented milk did not achieve the same matrix architecture (also in terms of number of observed structures typical of bacterial biofilms and strucutral complexity) observed when *L. innocua* UC 8410 was grown with a flow of sterile milk. These architectural elements could therefore be attributed to bacterial metabolic activity, instead of being considered as mere artefacts due to milk protein deposition on SS wire.
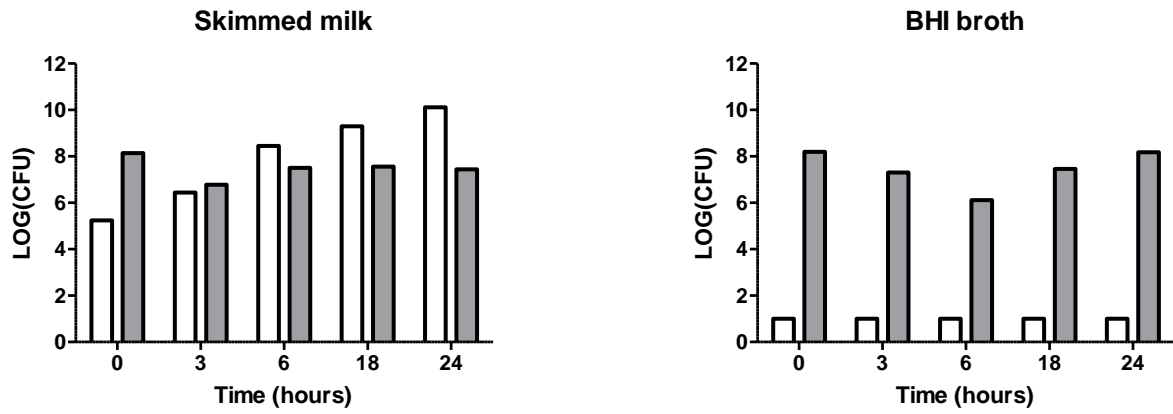
Fig. 2.14: Adhesion of *L. innocua* **UC 8410 on SS wire in presence of different substrates. White bars: sessile cells, gray bars: planktonic cells. Sessile cells are expressed as LOG(CFU/cm²), while planktonic cells are expressed as LOG (CFU/ml). 1 LOG(CFU) is to be intended as less than 10 CFU. Error bars are omitted for clarity.**
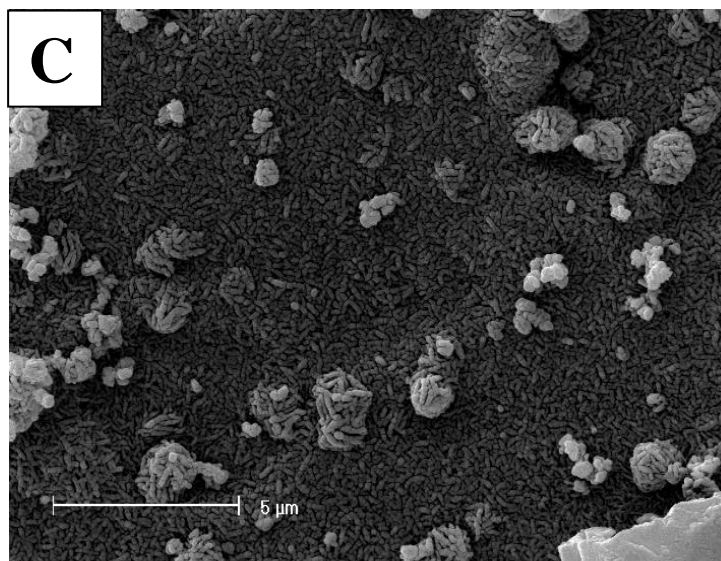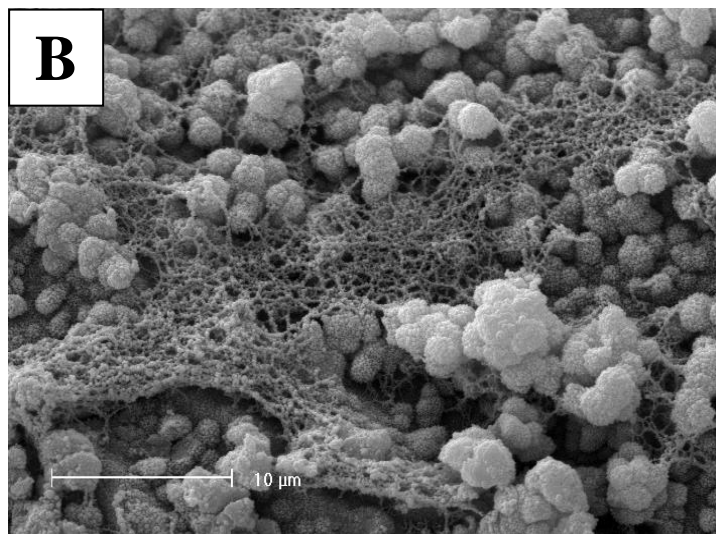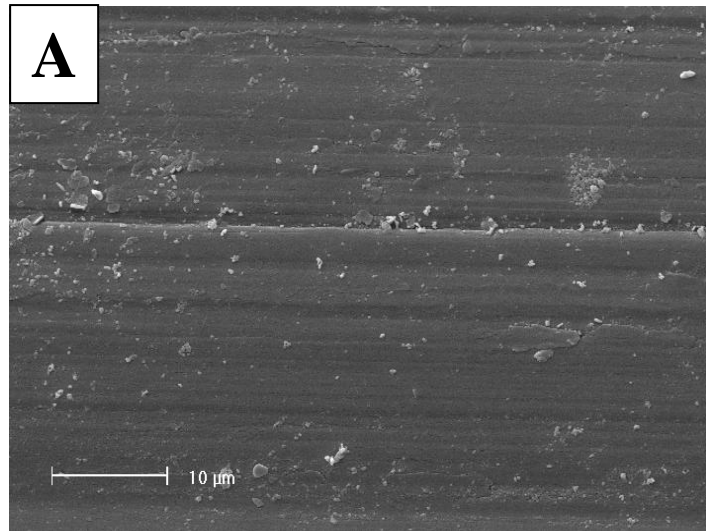
Fig. 2.15: SEM images of SS under flow of skimmed milk after 3h. A: SS wire in BHI broth (magnitudo = 2000, B: SS sample inoculated with *L. innocua* UC8410 (magnitudo = 3052.311), C: SS wire exposed to 1 h contact with milk supplemented with 10 μg/ml chloramphenicol (magnitudo = 6250).

[2.7].   Discussion

Seven *Listeria* spp. strains (six *L. monocytogenes* and one isolate of *L. innocua*) were studied for their capacity to attach and survive on stainless steel. To mimic the starvation conditions, the medium was diluted up to 0.1% of the usual concentration, to assess its influence of strictly stringent depletion of nutrients on survival capacity and surface adhesivity was one of investigated parameters. Limited presence of nutrients resulted more notably significantly at 7 days than 3 days, altough differences among employed strains were observed. Clinical isolates were much more tolerant to limited amounts of nutrients than the food isolates, and *L. monocytogenes* proved to be much more robust than *L. innocua*, although both the species were grown in non-optimal conditions. Possible association between the source of isolation, species identity and bacterial adhesion were investigated, but no clear conclusions could be drawn. Apparently neither the species or the origin can account for the differences detected. This finding is in agreement with the results of Lianou et al. (2007) and Tresse et al. (2007).

Bacterial adhesion onto surface is a multifactorial phenomenon, in which several aspects were investigated in the present research. The strain of the same species do not possess similar adhesivity. This property is differently expressed among sporadic or persistent strains (Harvey et al. 2007, Kushwaha and Muriana 2009). This data is in accord with previous results of Borucki et al. (2003), which confirms the presence of interstrain variability within *Listeria monocytogenes* in biofilm formation, which is not attributable to a certain serotype or phylogenetic division. Similar conclusion were drawn by Moltz and Martin (2005), Bonaventura et al. (2008) and Lianou et al. (2006).

Most of the previous studies on biofilms have employed static apparatus (like microtiter plates or stainless steel coupon), which can give useful information about strain adhesion, but, as underlined by Perni et al. (2007), cells deposition is more likely to take place, instead of biofilm formation, in similar conditions. Moreover flow cells repeat better the conditions of food processes. For this purpose a suitable laboratory-sized system was constructed in order to monitor bacterial attachment onto the surfaces in presence of a flowing solution, simulating bacterial contamination within a plant processing liquid foodstuffs like milk.

Examination of hydrophobicity revealed major differences between the two *L. innocua* strains used in the first trials of biofilm development under dynamic conditions. This fact could at least partially be explained by the differences of adhesivity observed on stainless steel wire. This result is in agreement with conclusions drawn by Briandet et al. (1999): the authors suggested that both electrical and Lewis acid-base interactions play a role in the adhesion of *L. monocytogenes* Scott A on stainless steel coupon. Analyzing their results, Takahashi et al. (2010) hypothesized that hydrophobicity and thus properties of

cellular surface are mainly involved in surface adhesion, while Lewis acid-base characteristics seem not to be so markedly important , but not enough to be completely excluded.

In the present investigation attention was given on few selected parameters, in order to test the compliance of the used apparatus with the devices used in literature by other authors: obtained results seem to be in agreement with most of former reports not only in terms of final quantitative data obtained, but also about significance of the monitored factors (Borucki, et al. 2003, Lianou et al. 2006, Mai and Conner 2007, Kushwaha and Muriana 2009). Tested materials were chosen to reflect the variety of possible equipment and surfaces present in food processing environments, ranging from stainless steel to plastics (such as PET) and showing different capacities in supporting adhesion: it was interesting that PET is closer to SS, although this latter one was considered markedly different from C. Furthermore, copper has been known to be bactericidal, but in our experiments it did not demonstrate to be able to eradicate completely microbial contamination: SEM images show, when copper is used, very few cells were enveloped within a very thin layer of organic matter superficially attached, even if counts are less than 160 CFU/cm^2.

Varying typology of nutrient, a different trend in surface colonization is shown: while plate counts using BHI as growth substrate showed ineffective attachment to tested materials, skimmed milk supported better the cells in the establishment of sessile communities. This was probably due to the presence of amphoter proteins, which represent both a source of nourishment and physical support for colonization. Parallel experiments in the same analytical conditions with BHI as a nutrient solution did not produce the multi-layered structure. Trials with skimmed milk supplemented with 10 μg/ml chloramphenicol did not bring about the same superficial tower-like architecture, in terms of both amount and structural complexity, confirming the role of microbial activity in the formation of similar complexes.

In literature it has been reported that milk and its components compete with *Listeria* cells for the attachment on surfaces. Wong (1998) showed that milk and milk proteins significantly reduced cell recoveries from stainless steel surfaces, even if milk could support the growth of bacteria on not adequately cleaned surfaces. Similar conclusion were reported by Barnes et al. (1999): the authors hypothesized that milk protein can inhibit bacterial attachment by surface characteristic modification and by the establishment of a conditioning layer on tested material. Our results suggest instead an opposite effect: milk did not only support the attachment and growth of *Listeria* in turbulent regime, but also acted as a building material for multilayered structures, which could reduce significantly cellular recoveries if a suitable disruptive method for such matrices is not applied. A similar phenomenon has been observed with dental plaque (Rosan and Lamont, 2000; Marsh, 2004; Jenkinson and Lamont, 2005; Filoche et al., 2010,)

Among the investigated parameters, temperature significantly affected the bacterial attachment, in agreement with the findings of Mai and Conner (2007). The range of temperature used was chosen in order not to create too strongly limiting condition (lowest temperature used was 20°C). When 37°C was used as incubation temperature, an increasing adhesion to SS within 24 h was observed, while a stronger variability was observed with lower temperatures. Both 30 and 20°C showed similar trends: after initial attachment adhesion was reduced at 6 h and then increased after 12 h, even if obtained results are quantitatively different (maximal difference observed was 4 LOG). Statistiscal analysis revealed that 37°C and 20°C did not significantly differ between each other and could be assumed as top and bottom temperature concerning bacterial attachment under dynamic regimen. 30°C significantly move away from other two investigated values despite sharing similar kinetic of attachment on SS.

Even if an effect attributable to the shift from optimal conditions for growth cannot be excluded, temperature modified sensibly the entity of adhered cells maybe because of thermo-dependent regulation of superficial attributes such as flagella. It has been suggested that flagellum-mediated motility is required for initial cell attachment of *Listeria* during biofilm formation to overcome any repulsive interfacial force. Flagella have also reported to act as adhesive support facilitating early attachment of *Listeria* on stainless steel, even if Lemon et al. (2007) reported that for *L.monocytogenes* the real critical factor for adhesion to and biofilm formation on abiotic surfaces is flagellum-mediated motility. In agreement with findings by Djordjevic et al. (2002), Bonaventura et al. (2008) found negative correlation between biofilm formation on stainless steel and swimming motility, suggesting that motility and related characteristics are not required for the biofilm formation of *L.monocytogenes* and that other changes in superficial structures apart of flagella contribute to attachment of the bacterium to solid surfaces.

Microbial cell surface charge and hydrophobicity play an important role in the initial steps of microbial adhesion (van Loosdrecht et al., 1990). Bonaventura et al. (Bonaventura et al., 2008) suggested that growth temperature can positively modulate the hydrophobicity of *L.monocytogenes*. Despite th quantitative differences experimentally observed among employed strains in this investigation, no significant difference was observed. Similar conclusions could be found in Takahashi et al. (2010), where a positive correlation was found (even if less than 0.90). All these observations could suggest that bacterial surface properties could be involveld in the attachment of *Listeria* on surfaces along with other potential parameters, which should be further investigated.

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