Chapter 5. Conclusions

The current research activity was mainly focused on biofilm formation in dynamic conditions. In literature several authors have studied the microbial adhesion on several surfaces of different microbes, although these researches were based on static apparatus, where cells deposition is more likely to take place instead of the complex tower-like structures of biofilms. Other scientists performed their research by using a flow cell, by which it was possible to monitor, through a suitable microscopic technique, the bacterial surface adhesion: the only disadvantage of this technique was the not accurate quantification of this phenomenon. Through this apparatus it was possible to have a quantitative evaluation of the bacterial adhesion in presence of a turbulent flow of nutrient solution, investigating different materials, which could be found in food processing environments (such as stainless steel or plastic materials as PET).

Beside a quantitative assessment of bacterial adhesion, some parameters of interest were investigated for their role in the process of biofilm formation on abiotic surfaces in presence of flowing solutions. The collected results have allowed to demonstrate that there is a certain variability among strains belonging to the same environmental niche: two strains of *L. innocua* isolated from cheese brine have demonstrated markedly different capacity to adhere on stainless steel in presence of a moving flow of skimmed milk, demonstrating an interspecific variability of behavior, concerning the bacterial adhesion on abiotic surfaces, among strains belonging to the same environmental niche and even to the same species.

The surrounding environment, in which bacteria grow, play an important role with regard to the bioflm formation on inert surfaces. Brain Heart Infusion, which has been routinely used for culturing bacteria such as *Listeria* , has been used as growth medium for testing bacterial adhesion on static approaches, but, when used within the above mentioned apparatus as nutrient solution, it cannot support the attachment of tested bacteria on used surface materials. In experiments conducted with skimmed milk, *L. innocua* UC 8410 was able to adhere in great numbers on the wire of stainless steel until to be quantitatively comparable with the numbers of cell present in planktonic state in the flowing growth medium.

These results demonstrated that, in presence of shear forces due to the applied flow of milk, milk proteins acted as supporting material for bacterial adhesion on the wire as well as source of nourishment, although they were considered by other authors as competitors of bacteria for adhesion to stainless steel and other kind of materials. This conclusion was further confirmed by SEM observations of taken inoculated stainless steel wire, on which thick multilayered structures composed of organic matter covered homogenously the whole available surface. Further tests conducted with the same apparatus have allowed to confirm that observed structures are the result of metabolic activities of bacteria and not mere deposit of milk proteins.

Beside stainless steel, adhesion on PET and copper was investigated, in order to demonstrate any influence attributable to the tested material. The strain *L. innocua* UC 8410 has demonstrated to be able to adhere on PET specimen at high level, although the cell recoveries were lesser than the values observed with stainlees steel. When copper was used as tested surface, milk proteins could form just a very thin layer as well as a limited extent of bacterial adhesion was observed. Intrinsic properties of the material (like roughness or presence of superficial irregularities) could favor the deposit of organic matter and the attachment of pioneer bacteria. While smooth surface like PET or copper demonstrated to be a less favorable site for biofilm formation, by SEM it was possible observing that the wire of stainless steel possessed a rough surface, where the deposition of milk protein and the attachment of bacteria were more probable to take place.

Another important factor influencing the bacterial surface attachment could be found in the tested material and its interaction with organic matter. In presence of hydrophilic surface like stainless steel, the milk proteins could modify the superficial properties of the material and led to the formation of a superficial layer of organic matter, to which cells adhere. The completely different behavior of hydrophobic materials such as PET could be attributable to the incapacity of the same milk protein fraction to adhere firmly on these surfaces. Milk proteins could therefore act as a conditioning layer (having the role of salivary proteins in plaque formation) and modify sensibly the superficial properties of the material, making it possible the formation of a bacterial biofilm. The most abundant protein fraction is consisted of casein, which are present as colloidal submicelles freely suspended in milk and could be involved in bacterial attachment onto surfaces, due to the simultaneous presence of both polar and apolar portions within their structure. Further investigations are required to give a complete elucidation of the role of proteins, with special mention of casein, concerning bacterial attachment to surfaces.

Temperature was hypothesized to be another factor influencing the phenomenon of bacterial surface adhesion: by reducing temperature, there was a proportional reduction of bacterial adhesion capacity, probably because main metabolic pathways could be slowed down at temperatures far from their optimum, where bacterial adhesion capacity is better shown. The effect of temperature could be thus translated into a reduction of the overall bacterial metabolic efficiency, which is hindered by low temperatures. An interesting topic of research could be evaluating the extent and the regulation of genes, during the growth in biofilm state, in function of the incubation temperature, focusing on main metabolic features such as the carbohydrate metabolism.

Biofilm grown under dynamic conditions with the above mentioned device were tested also for resistance to biocides, because bacterial biofilms are known to confer protection against biocidal agents used for the disinfection of food processing surfaces . In this study we have focused our attention on two different substances, like benzalkonium chloride (BC) and peracetic acid (PAA). The treatment with BC has sanitation efficiency between 1 and 30% and our results allowed to highlight the relevance of biofilm age on stainless steel against this bactericidal substance: survivors numbers to BC increased in function of elapsed time,

reaching their maximum 24 hours after the inoculums. A possible explanation could be the incapacity of BC to penetrate the whole thickness of organic matrix on the wire. Towards PAA, bacteria have a totally different response: the bactericidal effect is stronger than the one performed by BC, and the higher bactericidal effectiveness of PAA than BC was probably attributable to its strong oxidative properties, which can corrode the organic matrix and be effective against the cells embedded within it. As above mentioned, PET and C did not offer a favorable superficial support for the formation of bacterial biofilm, as confirmed by the higher efficiency of both BC and PAA on these materials.

An antimicrobial compound is routinely tested through European Standard such as the standard EN 1040:2006, which expresses the capacity of a molecule or a formulation to reduce a bacterial suspension until a defined value (e.g. for vegetative cells this value is five logarithms). Another investigated parameter is the sensitivity of a strain or a bacterial species to a substance, and this evaluation is obtained through the determination of the minimum bactericidal concentration (MBC). MBC corresponds to the antimicrobial concentration above which bacterial growth is completely inhibited. However, the effect of lower dosages on bacteria as well as the kinetic of adaption during the 24 h of incubation has been investigated.

For this purpose the apparatus BioscreenC was used: this consists of a turbidity reader with the possibility to monitor simultaneously and periodically the growth of bacteria under different conditions. In parallel to the two above mentioned substances, pinosylvin and resveratrol, two stilbenes abundantly present in grapes and in knotwood, respectively, were tested for their bactericidal effectiveness against the tested isolates. Through MBC determination, both pinosylvin and resveratrol have demonstrated not to be effective antimicrobials in comparison with BC and PAA. Both the polyphenols were tested on different *Listeria* strains through BioscreenC apparatus to verify their antimicrobial properties on the planktonic cells.

Not all the tested concentrations of polyphenols were able to inhibit the bacterial growth, because certain dosages of these compounds (until 32 μg/ml and 42 μg/ml of pinosylvin and resveratrol, respectively) could have a protective effect against EtOH, which was used to dissolve these apolar compounds. The so obtained data confirmed that both pinosylvin and resveratrol did not possess any relevant antimicrobial properties and cannot be considered therefore antimicrobials. Then different concentration of pinosylvin were investigated for their effect on bacterial biofilms of different isolates of *Listeria*. While concentrations higher than 200 μM were effective against planktonic cells, pinosylvin was able to interfere in the development of bacterial biofilms on stainless steel only at 400-500 μM. Further research should be required to clarify the mechanism by which polyphenols could interfere with growth of bacteria as adhered communities.

Role of quorum sensing in biofilm development process was also investigated. In *L. innocua*, like in *L. monocytogenes*, there is a gene which encodes for LuxS, the enzyme necessary for synthesis of autoinducers 2 (AI-2) signaling molecules, which act as cell density sensors and activators of trascription mechanisms in

response to modified environmental conditions. By using an integrative plasmid, the *luxS* gene of an adhesive *L. innocua* was inactivated by using a Campbell-like integration.

The so-constructed *luxS*-null mutant of *L. innocua* UC 8410 was tested, in comparison with its wild type, for adhesion to the above mentioned surfaces in dynamic conditions: in presence of shear forces, due to the turbulent flow of milk, the *luxS*-null mutant have demonstrated a strongly reduced capacity to attach even on stainless steel, the most propitious material, among the tested surfaces, for the bacterial adhesion in such conditions.

In parallel to the bacterial adhesion, bactericidal effectiveness was evaluated on the adhered cells of *L. innocua* UC 7117 on a stainless steel wire immersed in a flow of milk. Also planktonic cells of *L. innocua* UC 7117 were investigated in the same experimental conditions for their sensitivity to BC, which revealed an increased bactericidal efficiency. This suggested that AI-2 played an effectively important role concerning the adhesion to abiotic surfaces because of the demonstrated strong reduction of adhered cells as well as a markedly reduced tolerance to biocidal compounds. Further investigation should be performed in order to describe which specific metabolic pathways could be affected by *luxS*.

L. innocua UC 7117 was compared, through BioscreenC, with its parental strain for the growth kinetic within 24 h and the sensitivity to the polyphenols pinosylvin and resveratrol. Because of the reduced obtained values as well as the extended time required for growing, *luxS* disruption was demonstrated to change sensibly the overall metabolic efficiency of the bacterial cell. Furthermore, the genetic modification increased the sensitivity of the *luxS*-null mutant to EtOH as well as to concentrations of the two tested stilbenes higher than 200 μM, while concentrations ranging from 50 up to 150 μM were confirmed to have a protective effect on bacterial cells against 3% EtOH. *L. innocua* UC 8410 was also compared, with its *luxS*-null mutant, for adhesion capacity on stainless steel coupons in static conditions. In parallel also sensitivity of recovered cells on stainless steel to pinosylvin was investigated. While *L. innocua* UC 8410 biofilm was able to tolerate up to 100 μM of pinosylvin, *luxS*-null mutant, when it grew on stainless steel, was strongly decreased at such concentration after only 72 h. The inhibitory effect of the stilbene increased its effectiveness with correspondent increase of used concentration, confirming that inhibitory effect of pinosylvin on biofilm formations was dose-dependent. The interference effect operated by polyphenols on bacterial biofilms as well as transcription gene profile should be further investigated.