Biofilms formation by *Listeria monocytogenes* isolates under different growth conditions at refrigeration temperature

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Listeria monocytogenes continues to be one of the major microorganisms responsible for severe food contamination worldwide leading to serious, and potentially fatal, food born diseases in human and animal. Like many others, these bacteria have the ability to form biofilms and live as communities that confer protection face to stressful ambient conditions as well as resistance to disinfectants and antimicrobial agents. Therefore, biofilms are considered one of the most relevant virulence factors of *Listeria* spp. and it is of utmost importance to study all the mechanisms and processes involved on their formation under as many different conditions as possible. Since the survival and growth of *L. monocytogenes* at low temperatures make difficult the control of this foodborne pathogen, and attending to the fact that refrigeration is widely used to improve shelf life of foods, the aim of this work was to evaluate the growth and activity of *L. monocytogenes* biofilms formed at refrigeration temperature as under batch and fed-batch growth systems.

The *L. monocytogenes* strains assayed were four food isolates (747, 925, 930, 994) and one clinical isolate (1562). Biofilm formation was performed during 5 days in 96-weel microtitre plates, at 4ºC with shaking at 120 rpm and using TSB with 0.25% of glucose. All experimental conditions were the same when performing batch or fed-batch assays, except that in fed-batch the medium of all wells was removed twice a day and replaced by an equal volume of fresh medium. The biofilm biomass was assessed by the Crystal Violet method and the cellular activity evaluated by the XTT colorimetric method. These evaluations were performed after the first 12 hours and at each 24 hours since the beginning of incubation.

The monitoring of biofilm development along the 5 days revealed an increase of biofilm biomass from the 4th day and only in batch growth conditions, since that in fed-batch system the biomass values remained low and almost with no variation during all the 120 hours. These considerable differences can be related with the cells wash-out that takes place every time the medium is substituted. This fact, together with the slower replication of bacteria at low temperatures, may diminish the biofilm development at refrigerated environments. Additionally, the lack of nutrients in batch conditions must lead to a stressful environment for the bacterial cells and therefore trigger the biofilm formation as a protection mechanism. On the other hand, XTT results showed that the cellular activity tends to be higher in the fed-batch growth system, which is in agreement with the fact that these conditions provide a more favorable environment by supplying the bacterial cells with a larger amount of nutrients. All the results were similar for the five strains of *L. monocytogenes*.

In general, this work not only shows that *L. monocyogenes* is able to form biofilm at refrigeration temperature under batch conditions but also puts into evidence the importance of the choice of the biofilm growth system, since different conditions lead to different biofilm formation abilities. This is a very important aspect to considerate since most authors seem to test bacteria's ability to form biofilm based on only one type of growth conditions.

Keywords: *Listeria monocytogenes*; biofilm formation; refrigeration temperature; growth conditions