# Process Validation: Microorganisms are stressed too!

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## Why study the efficacy of antimicrobial systems used in meats?

Meat is rich in many nutrients and is part of the daily diet of many individuals including microorganisms! Indeed, meat is highly perishable because it allows the growth of a multitude of these microorganisms. Meat spoilage costs the Canadian industry an estimate $200 millions per year (11) while food-borne illnesses account for a total economic cost (ex. health care, lost of productivity, value of deaths) of $2.1 billion (20). In fact, 11 millions Canadians are affected every year by food-borne and water-borne diseases (4, 20). The etiologic agents responsible for the diseases are totally unknown in 50% of the cases. When the etiologic agent is known, the presence of pathogenic microorganisms and their toxins represent the vast majority of the reported cases (7, 22). According to the *Centre québécois d'inspection des aliments et de santé animale* (CQIASA; 7), meat and poultry is the type of food most often incriminated (35.2%) in episodes of food-borne diseases[1]. Hence, research related to the detection and the control of microorganisms is important for public health and for its socio-economical impact on the meat industry. Furthermore, meat and meat products are the 4$^{th}$ most important manufacturing industry in Canada (3).

## Microbes are living organisms, not inert substances

In order to survive, microorganisms react to the antimicrobial systems that we use to control them. They can modify their genetic pool by spontaneous mutation or by acquiring foreign pieces of DNA. Furthermore, we now know that survival to an inhibitory treatment, such as heat or acid, can be improved by prior exposure to sub-lethal conditions (21). At the molecular level, stress proteins, induced by a sub-lethal heat treatment, have been identified in several eukaryotic and prokaryotic organisms. The response associated with heat shock can be induced also by other factors (ethanol, UV, DNA-gyrase inhibitors) and many proteins induced by various stresses have already been identified (21). Cross protections between different stresses have also been observed (2, 13). Interestingly, heat shock proteins protect *Escherichia coli* cells against freezing but not chilling conditions (6). Bacteria also sense and communicate their exposition to extracellular chemical stresses such as pH. For example, Extracellular Sensing Components (ECS) are activated by stress into Extracellular Induction Components (EIC) that act as "alarmones" to warn unstressed cells to prepare for the upcoming danger. Theses EIC are small size molecules that diffuse readily in the environment and the same EIC can be activated by more than one stress. Whether there is an ESC-EIC couple for every stress is still a matter of debate (17). These evidences highlight the complexity of the bacterial stress response and the need to better understand, the role, the mode of action, and the impact of stress proteins on the efficacy of antimicrobial systems used in foods (18).

The efficacy of antimicrobial systems is traditionally evaluated by cell enumeration on solid growth medium and food composition is known to influence microorganism resistance (ex. with heat, higher humidity is detrimental to survival, whereas higher fat content is beneficial (1, 12)). Furthermore, viable organisms too stressed or injured don't directly grow

[1] Currently in Canada, the yearly statistics on food-borne diseases lie within the provinces although Health Canada has published national reports on specific concerns from time to time.

and form distinct colonies on solid media. These viable but not culturable organisms may recover during the storage period in foods and resume growth. Hence, determination of the efficacy of an antimicrobial treatment can be overestimated if solely based on cell counts. As indicated by Yousef and Courtney (23), method to evaluate adaptation and stress response of microorganism in food matrices is urgently needed. Our current knowledge on the bacterial stress response raises a public health concern with respect to minimally processed foods developed to meet consumers' demand for more natural foods, without preservative and longer shelf life during which microorganisms may have the chance to recover and grow.

The use of several less severe microbiological barriers has been successful in controlling microorganisms in foods and is referred to as the "hurdle technology" concept (14). However, cells submitted to sub-lethal treatments survive better to subsequent lethal conditions (21). These evidences have prompted us to evaluate if the survival level is different when the antimicrobial systems are applied in different orders. Our work on *Lactobacillus alimentarius*, a meat isolate (13), indicates that cells survive better when they are exposed to a sub-lethal osmotic shock (NaCl) prior to an acid stress (citric or glutamic acid) compared to the reverse. Lowest survival is obtained when the treatments are applied simultaneously. Hence**,** the sequence of events during food processing is important and will influence the overall efficacy of the treatments and the level of microbiological control obtained.

Fundamental research on bacterial physiology is essential to develop new tools (ex. biomarkers) for process validation and the control of microorganisms in foods. DnaK, a heat shock protein studied in *E. coli*, has been successfully used in our laboratory for monitoring cooking (19). Using a competitive ELISA test, the intracellular concentration of DnaK was evaluated in *E. coli* ATCC 25922. Our results indicate that for a given process lethality ( $F_{70}^{10}$ of 1, 3 and 5 min), the intracellular concentration of DnaK in *E. coli* varied with the heating temperature (50 or 55°C). At a temperature of 60°C and higher, DnaK and cell counts were below detection level suggesting that the treatments were severe enough to avoid *E. coli* cell adaptation. Furthermore, a higher intracellular concentration of DnaK allows the cells to eventually recover from the treatment and to resist better to a subsequent more severe stress (19). This research demonstrates that process lethality values are truly equivalent only once the treatment is severe enough to prevent cell adaptation. Although DnaK is induced to a higher level by a heat shock, this protein is also present in cells growing under optimal conditions and acts as a chaperone protein. It is implicated in the folding of nascent polypeptides, repair of denatured proteins, and degradation of non-functional ones (8). Because other stresses, such as ethanol, UV and DNA gyrase inhibitor also cause an increase in intracellular DnaK (21), we are currently investigating if this chaperone protein may have a more general role in the bacterial stress response.

Our research on bacterial stress response should lead to the determination of conditions where cells are able to adapt and to resist, and to the identification of biomarkers for the efficacy of antimicrobial systems. Concurrently, it also determines the conditions under which cells can no longer adapt and start to die; in other words, when a process begins to be effective. To achieve this, we must work on real food/meat matrices and with various organisms including pathogenic and spoilage organism.

The meat-borne outbreaks related to *Escherichia coli* O157:H7 in fermented dry sausages (9) have prompted modifications of the Meat Hygiene Manuel (5) imposing on processors to demonstrate that their process control *E. coli* O157:H7. Of course, no processors will purposely introduce *E. coli* O157:H7 in their facilities. Working with pathogenic organisms requires special installation that shall meet the "Laboratory Biosafety Guidelines" of the Public Health Agency of Canada (15). Hence, proper pilot laboratories had to be set up. The first one to be established is located at the Faculty of Veterinary Medicine of the Université de Montréal. A room in their biosafety level 2 facilities was adapted to accommodate processing equipments, and experiments are conducted in collaboration with

scientists from the Food Research and Development Center of Agriculture and Agri-Food Canada in St-Hyacinthe (Fig. 1). A second laboratory is located at the Canadian Research Institute for Food Safety (CRIFS, University of Guelph) and two other facilities are under construction (University of Alberta, Université Laval). To my knowledge these are the four pilot facilities available in Canada to process food with pathogenic organisms.

Although thorough process validation could become cumbersome, especially when pathogenic organisms are targeted, changes in procedures, and their impact on the microflora, must be tested and analysed (10). Indicator organisms (ex. coliforms, *E. coli*) are still used for that purpose and compliance with the Meat Hygiene Manuel of Procedures (5) should be demonstrated even with changes that are not commonly perceived to have an effect on carcass hygiene such as animal behaviour and welfare (16). So, better be safe than sorry and take the time and energy to analyse your process. Microorganisms are amazing creatures that will always find a way to surprise us!

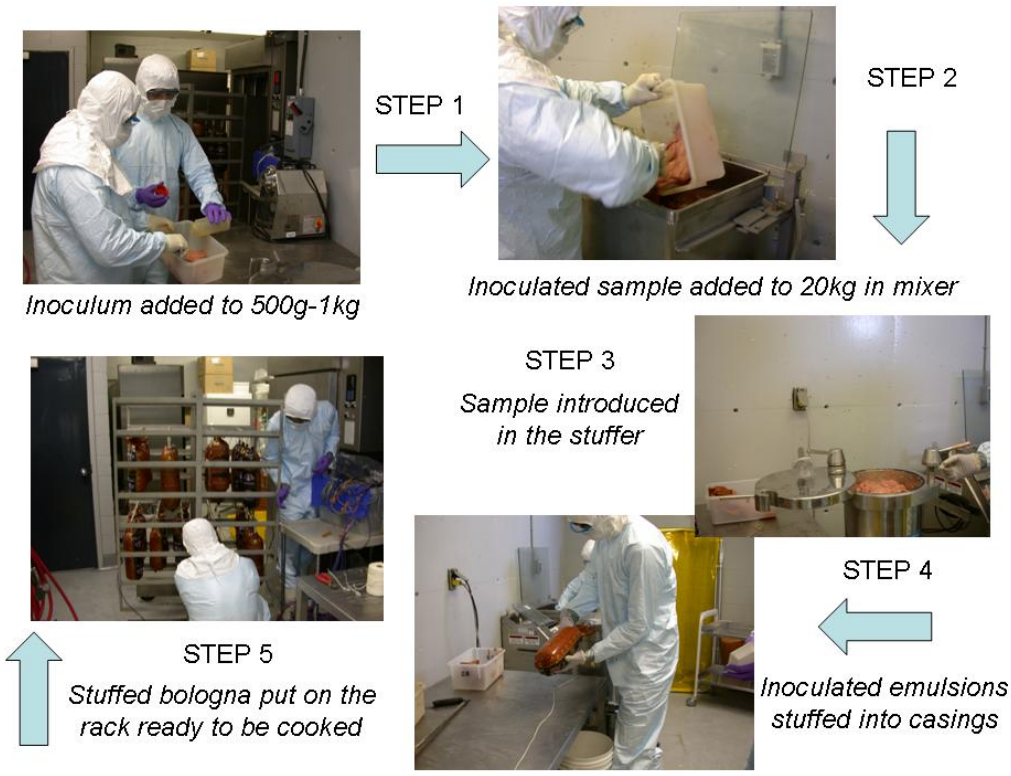


Fig. 1 Bologna processing with pathogenic organisms in a biosafety level 2 laboratory.

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