Implementation of an approach for the fast detection of food-borne bacterial pathogens in the pig and pork chain through the creation of a Joint Technological Unit ACTIA "FASTYPERS"

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Abstract

Listeria monocytogenes and Salmonella spp. are two major pathogens at the border between humans, animals and the environment. These pathogens are known to be present throughout the entire pig and pork production chain and some strains can develop different mechanisms to survive and persist into this environment.

The Joint Technological Unit ACTIA entitled "FASTYPERS" (Fastypers 2022 - ACTIA (actia-asso.eu) has been created in France to gain a deeper insight into the contamination of the pig and pork sector, for both of these pathogens, in a One-Health approach. FASTYPERS is a 5 years project, located in Maisons-Alfort, which involves the French Agency for Food, Environmental and Occupational Health and Safety (ANSES), the National Institute for Agriculture, Food, and Environment (INRAE), the French Food Safety and Dairy Product Institute (ACTALIA) and the French Pig and Pork Institute (IFIP). The goal is to develop molecular tools that can be used routinely by the pig and pork industry to detect strains that are resistant to biocides and heavy metals, able to form biofilms and present at different stages of the supply chain. Precise detection of these specific resistant/persistent strains can lead to the control procedures that target these strains and thus assist decision-makers in the pig and pork industry in enhancing food safety.

Introduction

Listeria monocytogenes (*Lm*) and *Salmonella* spp are two major food-borne pathogens. In 2019, listeriosis caused by *Lm* was the most serious zoonosis with the highest case-fatality rate (13%) among outbreak-related illnesses. In 2019, 87,923 confirmed cases of salmonellosis in humans were reported in Europe, with a European Union notification rate of 20.0 cases per 100,000 population; *Salmonella* caused 26.6% of all food-borne outbreaks [EFSA, 2021].

In the past, the consumption of contaminated pork meat and processed pork products have been linked to several listeriosis outbreaks around the world [Félix et al., 2018; Painset et al., 2019]. In humans, several cases of salmonellosis have been related to the consumption of raw or undercooked pork and pork products [Bonardi et al., 2017]. Their ability to adapt to stress, grow at low temperatures, form biofilms and then persist in pork processing facilities for years has made these two pathogens a major challenge for the pig and pork sector. Contaminated pork products are a major microbiological burden and are particularly monitored in the food

industry. Their successful control through the production chain requires appropriate cleaning and sanitation programs.

Biocides play an essential role in limiting their dissemination. They are usually formulated at a high concentration to make sure to deliver a bactericidal effect. Previous studies have reported the emergence of adaptation and increased tolerance to biocide for food borne pathogens [Sanelisiwe et al., 2021]. For *Lm*, our team recently identified genomic elements located in the accessory genome that are responsible to an increased tolerance to biocides in *Lm* strains from different ecological niches [Palma et al., 2022]. We also demonstrated that repeated exposure to sub-lethal concentration of two quaternary amonium compounds lead to adaptive mutations responsible to the increased tolerance of the two biocides and the cross resistance to Ciprofloxacin antibiotic [Douarre et al., 2022].

Heavy metal resistance could also play a role in the adaptation of *Salmonella* in different environments. Heavy metals are heavily present in farm and breeding environment [Mustafa et al., 2021]. We recently demonstrated that the acquisition of a genetic island (SGI-4) carrying genes conferring resistance to copper, arsenic and silver in the monophasic variant of Typhimurium was the driving force of the expansion of the epidemic clone S. 4,[5],12:i:- [Cadel-Six et al., 2021].

The formation of biofilm is another adaptive response and a survival strategy for microorganisms. These three-dimensional surface-associated ecosystems contribute to the enhanced resistance to various antimicrobials. In food industry, biofilm tolerance to environmental stresses such as biocide treatment can result in the persistence of bacterial pathogens and the recurrent cross-contamination of food products [Bridier et al., 2015].

The ability to detect accurately and genotype these two pathogens rapidly is crucial. For that matter, two high-throughput real-time PCR assays have already been developed and validated in ANSES to identify the two pathogens in less than 48H from pure DNA extracts. These robust and rapid molecular assays enable the identification of (i) 30 Clonal Complexes (CCs) for *Lm* [Félix et al., 2023] and (ii) the serovars the most frequently isolated in the pig and pork sectors for *Salmonella spp.* These two genotyping assays were adapted on different conventional multiplex real-time PCR systems for an easy implementation in routine diagnostic laboratories. The assays were recently successfully used on complex DNA samples to identify *Salmonella* Bovismorbificans ST142 and monophasic *Salmonella* Typhimurium ST34 strains associated with dried pork sausages in France during a foodborne outbreak [Pardos de la Gandara, 2023].

The Actia Fastypers Joint Technological Unit has been created to gain a deeper insight into adaptation of these two pathogens enabling them to survive and potentially persist in slaughterhouses and pork processing plants. The principal objectives are to (i) identify the genomic markers linked to adaptation of these strains in different compartments, from pig farming to final pork products (ii) develop faster molecular tools to detect and characterize the strains isolated from the pig and pork processing chain.

Materials and methods
To enrich the strain collection of existing partners, strains are being isolated in different compartments of the pork and pig production chain, in particular at the level of pig farming for which only a few strains are available.
The strains will be first genotyped using the *in house* high throughput real –time PCR assays to identify the clonal complex for *Lm* and the serovar for *Salmonella*.
The strains will be characterized phenotypically to assess their resistance properties as well as their ability to form biofilm. The susceptibility of *Lm* and *Salmonella* strains to various relevant biocidal active substances will be determined through Minimal Inhibitory Concentration (MIC) using microdilution broth assays while biofilm structures will be characterized using high throughput confocal laser scanning microscopy.
Genome-wide association studies (GWAS) will then be conducted to identify genetic markers associated with the resistance and persistence phenotype.

These genetic signatures will be used as templates to develop new PCR assays which target specific genes linked to the adaptation of the two pathogens in food processing environments. These novel targets will first be tested and optimized with the high throughput PCR assay before being adapted to conventional PCR for easy implementation at the technical Institute. Finally, additional tests will be performed to optimize these assays on complex sample DNA extracts (pork product and pork processing environment) without strain isolation step. Direct analysis on complex samples will be of great help to better understand the hotly debated issue of inter-strain competition in enrichment broths.

Results
Genetic markers associated with resistance
For *Lm*, 10 genes (including *qac*, *bcr* and *erm* genes) conferring resistance / tolerance to biocides were identified. These resistant genes were carried by different mobile genetic elements (MGE) acquired by horizontal transfer like plasmid (*ermC*), Genomic Island (LGI-1, *ermE*) and transposon (*Tn6188*). Preliminary results indicated that some resistant alleles are CC-specific and associated with a particular MGE. For *Salmonella* spp, 52 genes were identified for heavy metals resistance (copper, mercury, arsenic, silver, metal, and other compounds) for 168 genomes belonging to 11 different serovars.

Biofilm protocol
Workflow to study biofilms using fluorescence microscopy is composed of the following steps: setup, labelling, imaging and analysis. The sotware "BiofilmQ" for the image analysis enables a global biofilm index through the extraction of quantitative structural parameters for both species (spatial features, morphology, local structure…).

Preliminary tests consisted in the exploration of parameters to develop a high throughput biofilm analysis protocol (temperature, age, inoculum, media, image analysis). For *Salmonella*, 20 strains belonging to the 15 most frequently isolated serovars in the agro-food sectors in France within S. 4,[5],12:i:-, Typhimurium and Derby prevalent in the pig and pork sector, were analyzed. For *Lm*, the 36 strains (16 biocide-adapted strains from 10 parental strains and 10 control strains) described in Douarre et al (2022) were analyzed. Preliminary results for *Lm* showed that more biofilms were observed at 22°C in comparison to experiments carried out at 37°C.

Biocide and heavy metal susceptibility
We developed and optimized protocols for the determination of biocide susceptibility for *Salmonella* ssp. The "test" subset of 20 *Salmonella* strains also used for biofilm analyses was tested against two biocides, Benzalkonium chloride (BC) and hydrogen peroxide (H_2O_2). Minimal inhibitory concentration (MIC) results showed identical MIC against BC while 45% exhibited a MIC observed in presence of H_2O_2 varied between 20 to 40 µg/ml (n= 11 and 9, respectively).

Out of these 20 strains, one strain belonging to the serovars *S*. 4,5,12:i:-, one *S*.Typhimurium and two *S*. Agona were tested in aerobiotic and anaerobiotic conditions against copper. This phenotypical approach aimed to characterize by MIC and growth kinetics tests, the ability of strains carrying operons *pco* and *sil*, identified as coding for copper resistance in the SGI-4, to better grow in presence of this compound. The results showed no impact of *pco* and *sil* resistance genes with no advantage and even reduced specific growth rate and biomass in aerobiosis. In contrast, in anaerobiosis, strains encoding *pco* and *sil* clusters were advantaged with both higher growth resistance rate and greater biomass production. The possible co-selection or relation between the genetic markers for heavy metal resistance such as pco and sil copper operons and biocides resistance or biofilm formation will be also explored.

Conclusion
Genetic markers associated with both biocide and heavy metal resistance and biofilm formation will be used for the development of two cutting-edge molecular assays. High throughput phenotypic assays for identification of strains resistant to biocides and able to form biofilm will also be developed. For the pig and pork sector, these molecular and phenotypic

assays will represent key tools (i) to differentiate strains representing the most significant health risks (ii) to trace the origin of contamination during outbreak investigations and (iii) to adapt microbiological and hygiene management plans in pig and pork facilities and then to select the most appropriate control measures accordingly.

Funding

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