METAGENETIC ANALYSIS OF THE BACTERIAL FLORA DYNAMICS OF BIOPRESERVED PORK MEAT

A. Bozec[1,*] A. Le Roux[1], C. Feurer[1]

[1] IFIP- French institute for pig and pork industry, Le Rheu, France.

[*]arnaud.bozec@ifip.asso.fr

Abstract – The objective of this study was to evaluate the bacterial evolution of biopreserved pork meat, vacuum-packed and stored at a temperature of -1.5° C for 12 weeks. The use of metagenetic analysis allowed a new insight into the bacterial competition taking place. The comparison of control samples to biopreserved meat with two separate *Pediococcus acidilactici* and *Lactobacillus sakei* cultures showed three different scenarios. *Pediococcus acidilactici* survived well for a period of 5 weeks. Subsequently however, it was then supplanted by the bacterial flora naturally present in the product, which was, in this case, *Lactobacillus sakei, C. divergens* and *Leuconostoc gelidum*. Alternatively, the protective culture *Lactobacillus sakei* showed the ability to maintain the microbiological quality in the vacuum-packed meat at an acceptable level for 12 weeks.

Key Words – Biopreservation, metagenetic analysis, pork meat, shelf-life.

I. INTRODUCTION

This study aims to investigate the dynamics of the bacterial flora of biopreserved and vacuum packed pork meat stored for 12 weeks at -1.5°C. The purpose of this experiment is to extend the shelf life which is currently limited to 6 weeks at -1.5°C [1]. This can be done by controlling the microbial flora changes during the shelf-life, whilst maintaining the sanitary quality of the meat. Microbial evolution of the pork meat biopreserved with two separate cultures and compared to a control test sample will be investigated by mean of metagenetic and conventional microbiology throughout the 12 weeks of storage.

II. MATERIALS AND METHODS

120 carcasses were selected during the slaughtering process according to weight (kg) and carcass composition (TMP) with the aim of ensuring microbiological variability. After cooling, cutting and deboning, each pork loin was cut into three pieces and bioprotective treatment was applied (culture 1 and culture 2). A control sample without any added culture was also included in the experiment. Approximately 2.75 g of culture was sprayed to 1.1 kg of meat before packaging into vacuum shrink bags (BB3 055 B, Sealed Air). The vacuum packed meat was then cooled in a cryogenic cooling cell to liquid nitrogen. The Vacuum packagings were stored in the dark at -1.5°C for 12 weeks in a cold room.
Protective cultures 1 and 2 respectively belonged to the species *Pediococcus acidilactici* and *Lactobacillus sakei*. Regarding their qualified presumption of safety, these two species are referenced in the publication of Bourdichon et al. [2] where they are considered as microorganisms with beneficial technological use.

The study of the microbial ecosystem of porkmeat was analyzed using the targeted approach of metagenetics: this process was conducted by Quality Partner (Belgium) [3]. This analysis was achieved by using high-throughput sequencing technology (device GS Jr - Roche). After extraction, the DNA was amplified by PCR with primers targeting a hypervariable region of 16S rDNA. The generated sequences are then checked and filtered by bioinformatics analysis and manual corrections.

The microbiological evolutions of the total flora (ISO 4833-1), the lactic acid bacteria (ISO 15214), *Enterobacteriacae* (NF V08-154*)* were monitored. At each stage of the analysis (carried out over 12 weeks) bacterial counts were obtained from five pooled samples for each treatment. Statistical analysis were conducted with 9.2 SAS software version (SAS Institute, USA) using the khi-2 and FISHER tests.

III. RESULTS AND DISCUSSION

CONVENTIONAL MICROBIOLOGY

Microbiological monitoring of the total flora highlighted three distinct scenarios for the conditions tested (Figure 1). The control sample had an initial contamination level of 4 LOG CFU/cm² which is consistent with levels observed within the slaughter industry in France. The biopreserved pork meat showed levels of total flora ranging from 7 to 8 LOG CFU/cm². Lactic acid bacteria levels ranged from 7 to 9 LOG CFU/cm², showing that the total flora was mainly composed of lactic acid bacteria. This confirmed the correct colonization of the protective cultures during experiments. The addition of both protective cultures prevented the development of *Enterobacteria* until the fourth week of storage. However, bio-preservation in itself does not always guarantee this; results are dependent on the culture used which must be adapted to the food matrix to ensure an effectiveness.

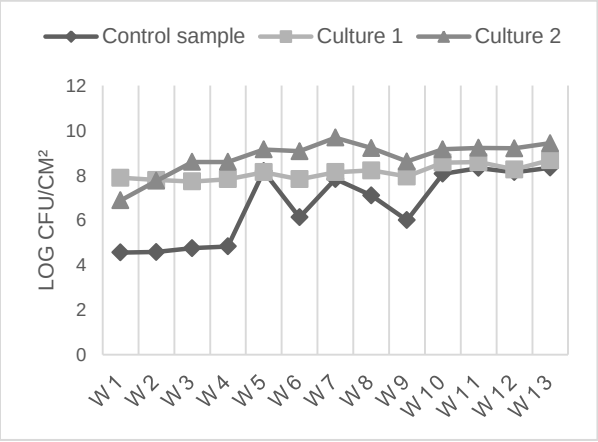

Figure 1. Evolution of the total flora

Using statistics, the effectiveness of culture 2 (*L. sakei*) against the growth of *Enterobacteria* has been demonstrated (Fisher test, p<0,005) at week 8 (W8) to W13 in the context of applying a three classes plan (n=5, C1=2, C2=0, m=10M) with $m=10^5$ CFU/cm². Over the course of the experiment, it can be concluded that a proper implementation of culture 2 prevented the *Enterobacteriaceae* flora to develop even at the very end of the storage period.

METAGENETICS

Control samples

At W1, the bacterial composition was very diverse and included more than 54 bacterial taxa identified at the genus or species level. It was dominated by the genus *Ralstonia* which represented nearly 23% of the identified sequences followed by *Pseudomonas sp*. or *P. syncyanea* (14.6%). *Ralstonia* is a soil bacterium and is an environmental contaminant on meat. *Lactobacillus sakei* which is often identified in pork meanwhile represented 2.5% of the identified sequences. At W2 the bacterial composition was similar to W1. W3 was characterized by the detection of sequences corresponding to *Carnobacterium maltaromaticum* species (13%) and *C. divergens* (9.6%) at the expense of *Ralstonia sp.* and *Pseudomonas*. *L. sakei* then represented 11.4% of the identified sequences. At W4, lactic acid bacteria (*Lactobacillus sakei* and *Carnobacterium sp.)* were scarcely detected as *Ralstonia sp* again represented nearly 20% of the identified sequences. At W5, two species known as *Carnobacterium divergens* and *C. maltaromaticum [4]* emerged with 77% of the sequences identified, highlighting a lower bacterial diversity of the sample. *Lactococcus piscium* was detected for the first time in up to 12% of the sequences. Between W1 and W5, 6, 7 10% of the bacterial composition corresponded to currently unknown species. In W6, 7, and 8, the control sample was either dominated by *C. divergens* or *C. maltaromaticum*. *L. piscium* was still detected but only slightly (from 0.7% to 3.5% in the sequences). *L. sakei* represented 11% of the sequences at W7 and W8. At W9, *L. piscium* became dominant with 46.8% of the sequences along with *C. maltaromaticum* (32.6%). At W10, the emergence of *Leuconostoc gelidum* was observed (31%), which had not been detected previously.

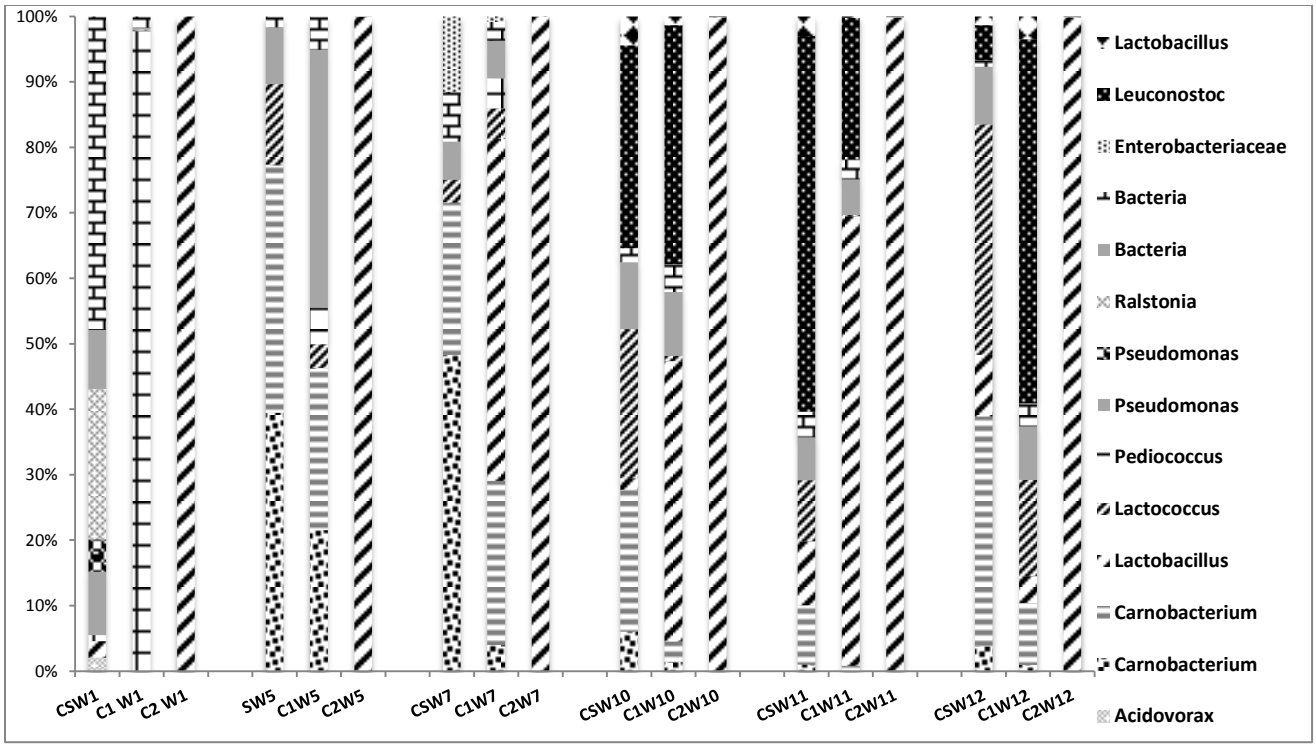

Figure 2. Proportion (%) of bacterial species in samples week 1-12

Together with *C. divergens* (21.6%) and *L. piscium* (22.8%), *Leuconostoc gelidum* was one of the most dominant species. *L. gelidum* is able to grow at refrigeration temperatures and below. This species was involved in the spoilage of vacuum packaged meat products (such as sausage and sliced ham). It is notorious for producing gas, stickiness, and a vinegar-like smell in meat. *L. piscium* is a bacterium found in food matrix such as vacuum-packed fresh meat. Moreover, its effect on pork under MAP and stored at 6°C has been demonstrated. It was described as a spoiler of ground beef packaged under modified atmosphere at 8°C [5]. *C. divergens* is an anaerobic bacterium, weakly aero-tolerant, heterofermentative bacteria and is frequently isolated from dairy products, meat, fish and shrimps. It was involved in the spoilage of fresh meat. In seafood [6], some strains can be used as bioprotective cultures. At W11, *L. gelidum* became dominant, with 60% of detected sequences. *C. divergens L. sakei* and *L. piscium* individually represented on average 10% of detected sequences. At W12 and 13, *L. piscium* and *C. divergens* became dominant again, and together accounted for 70.3% (W12) to 81.8% (W13) of the identified sequences.

Samples biopreserved with culture 1 (*P. acidilactici*)

From W1 to W4, culture 1 was dominant, representing 94.5% to 98.6% of the identified sequences in the sample. In W5, the two species *C. maltaromaticum* and *C. divergens* emerged. These findings were similar to those observed in the control sample. During this week, *L. piscium* was detected with 3.6% of the sequences. From W7 and until W9, *L. sakei* which previously represented less than 0.5% of the sequences became dominant and represented between 52.2% and 79.2% of the sequences at W9. Between W7 and W8, this dominance was shared with *C. divergens* (≈ 25%). Between W7 and W9, *L. piscium* was detected in up to 4.7% to 6.4% of the identified sequences. At W10, as in the control experiment, the emergence of *Leuconostoc gelidum* was observed (36.5%), which had never before been detected. This species, together with *L. sakei* (42.8%), accounted for the two dominant species identified in pork meat, whereas *C. divergens* only represented 3.1% of the detected sequences. W11, the scenario was similar with a higher prevalence of *L. sakei* (68.7%).

At W12, *L. sakei* became subdominant (4.1%) for the benefit of *L. piscium*. *L. gelidum* remained predominant with 55.6% of the detected sequences. At W13, as seen in the control sample, *C. divergens* and *L. piscium* were both dominant species with 36.4% and 23.7% of the identified sequences respectively. *L. sakei* was the third dominant species with almost 13% of the identified sequences. *L. gelidum* accounted then for 6.8% of the sequences. From W5, the evolution of the bacterial composition of the samples inoculated with culture 1 (*P. acidilactici*) was similar to that identified in the control sample. This demonstrates that, in the conditions of our experiment, culture 1 (*P. acidilactici*) was not competitive and was supplanted by the bacterial flora naturally present in pork meat.

Samples biopreserved with culture 2 (*L. sakei*)

Regardless of the stage in the analysis, the *L. sakei* strain of culture 2 was always largely dominant in samples with 99.8% to 100% of the identified sequences. This protective culture was therefore particularly adapted to grow in this pork meat matrice. If inoculated up to 6 LOG CFU/cm², this culture was efficient enough to compete with the naturally occurring bacterial flora in pork meat.

Comparing conventional microbiology and metagenetics results

Both methods were complementary. Conventional microbiology detected what can be cultivated in samples based on the available agar media. On the other hand however, metagenetics could only identify the dominant microbial flora in a sample, irrespective of whether they can be cultivated or not. In this study, the results were relatively well correlated with regards to the detection of the lactic acid flora and that of *Enterobacteria.* Although, in some samples, a concentration of 7 LOG CFU/cm² in *Enterobacteria* at week 6 was not detected by metagenetics, while this predominant flora should have been. This raised the issue of selectivity using the VRBG medium for the enumeration of *Enterobacteria* as well as the relevance of confirming the concentration of *Enterobacteria* recorded according to the NF V08-054 method. The use of classical microbiology in parallel with metagenetics is entirely appropriate to have an estimate of the evolution of the concentration of the total flora present in the sample and that of the lactic flora, often predominant on fresh meat.

IV. CONCLUSION

The results of this study are very encouraging. The metagenetic analysis of the dynamic of the bacterial flora of the meat samples followed for 12 weeks has given us a new insight in bacterial competitions taking place during storage. Biopreservation represents a new breakthrough in the long-term conservation of pork cuts. However, the choice of a protective culture which is adapted to the food matrix is paramount so that the strain used is not overridden by naturally occurring microbial flora.

ACKNOWLEDGEMENTS

The authors of this paper would like to thank all the partners of the project. This work was supported by the French Ministry of Agriculture (FranceAgrimer).

REFERENCES

1. Bozec A., Minvielle B., Vautier A., Le Roux A., 2005. long pork conservation vacuum. Techniporc, vol 28, n°1.
2. Bourdichon, F., et al. 2012. Food fermentations: Microorganisms with technological beneficial use. Int J Food Microbiology. 154 : 87-97
3. Taminiau B., Nezer C., Adolphe Y., Delhalle L., Poullet J.-B., Clinquart A., Daude G., 2012, Validation of the quality of minced pork with targeted metagenomic approach, University of Liège
4. Imazaki P.H, Tahiri A., Rodrigues A., Taminiau B., Nezer C., Daude G.,Clinquart A, 2011, Long shelf-life beef packaged under vacuum Isolation and Characterization Carnobacterium strains. University of Liège
5. S. Christieans, et al 2014. Which bacteria cause beef burger deterioriation? A new approach. Food Micro conference, Nantes, France
6. Chaillou et al., 2014. Origin and ecological selection of core and food-specific bacterial communities associated with meat and seafood

spoilage. The ISME Journal, 10: 1-14. doi: 10.1038/ismej.2014.202