ANTIMICROBIAL ACTIVITY OF POTENTIAL STARTER CULTURES TOWARDS FOOD PATHOGENS FOR THE PRODUCTION OF FERMENTED MEATS WITHOUT NITRATE OR NITRITE

M. Sánchez Mainar[1], R. Xafheri[2], S. Samapundo[2], F. Devlieghere[2], L. De Vuyst[1] and F. Leroy[1*]

[1]Research Group of Industrial Microbiology and Food Biotechnology (IMDO), Faculty of Sciences and Bioengineering Sciences, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium
[2]Laboratory of Food Microbiology and Food Preservation, Department of Food Safety and Food Quality, Faculty of Bioscience Engineering, Universiteit Gent, Coupure Links 653, B-9000 Ghent, Belgium.

[*] fleroy@vub.ac.be

Abstract – The ability of two coagulase-negative staphylococci (*Staphylococcus sciuri* I20-1 and *Staphylococcus sciuri* VT-S8-8) to inhibit food-borne pathogens in view of their application as functional starter cultures for the production of clean-label fermented meats was investigated. Both strains were active towards *Clostridium botulinum* and *Staphylococcus aureus*, two pathogens that may represent a risk in fermented meats prepared without added nitrate and nitrite. The inhibitory compounds were heat-stable, proteinaceous, and displayed primary metabolite kinetics, indicating a bacteriocin-like nature. During fermentation in meat models, the competitiveness of *S. sciuri* I20-1 and VT-S8-8 and production of their bacteriocins within the meat was confirmed. In meat models that also contained a rifampicin-resistant *S. aureus* strain, an immediate two-log reduction of the latter pathogen was found upon the inoculation of *S. sciuri* I20-1. This was probably due to an instant desorption of bacteriocin molecules from the added producer cells. Yet, a bacteriocin-resistant sub-population was found that grew out to the same final levels as in the control experiment, underlining the need to verify resistance phenomena when applying starter cultures that produce bacteriocin molecules.

Key Words – Antimicrobial activity, *Clostridium botulinum*, *Staphylococcus aureus*, bacteriocin.

I. INTRODUCTION

In fermented meats, the growth of meat-borne pathogens is usually suppressed by a combination of different hurdles, including the presence of lactic acid and a low pH, curing salts (nitrate and nitrite) and a decrease of water activity [1]. Yet, health concerns related to the consumption of cured meats containing nitrate and nitrite are leading to a search for alternatives for curing salts that can take over the antimicrobial activity [2]. This is particularly relevant in view of the potential presence of the pathogens *Clostridium botulinum* and *Staphylococcus aureus* in mildly acidified sausages. Whereas inhibition of *Listeria monocytogenes* by functional meat starter cultures has often been successfully carried out using bacteriocinogenic lactobacilli [3], *C. botulinum* and *S. aureus* have not received similar attention or only on a very preliminary basis [4]. The aim of this study was to investigate potential inhibitory effects towards the latter two pathogens by candidate meat starter culture strains, in view of the production of clean-label fermented meats without nitrate/nitrite.

II. MATERIALS AND METHODS

The inhibitory activities of two meat isolates, *S. sciuri* I20-1 and *S. sciuri* VTS8-8, were explored against fifty indicator strains, including strains from 17 different *Staphylococcus* species, 4 lactic acid bacteria species, 2 *Listeria* species, and against strains of *Streptococcus dysgalactiae* and proteolytic and non-proteolytic *Clostridium botulinum* strains (both vegetative cells and spores) (Table 1). For the screening experiments, the meat isolates were grown in brain heart infusion (BHI) medium (Oxoid, Basingstoke, UK). Their antibacterial activity was measured using an agar spot test and expressed in arbitrary units (AU) per ml [5].

The heat stability of the antimicrobial compounds was tested at different temperatures (30, 60, and 100 °C up to 60 min and 121 °C for 20 min), by comparing the antimicrobial activity in arbitrary units (AU)/ml before and after heat treatment. The activity of the antimicrobial compounds was also evaluated before and after incubation with proteinase K (10 mg/ml) at 37 °C for 1 h. To analyze the production kinetics of the antimicrobial compound produced by *S. sciuri* I20-1, the strain was cultivated in BHI medium at 30 °C. Growth [measured on mannitol salt agar (MSA; Oxoid)] and the activity of its antimicrobial compound [measured with the agar spot test against strains of *S. aureus* and *C. botulinum* as indicators] were followed over time for 50 h.

The antimicrobial production kinetics and the competitiveness of the two producing strains were also investigated in meat models, acidified by *Lactobacillus sakei* CTC 494 and without nitrate/nitrite. The meat models contained fresh minced pork with 30 g/kg of sodium chloride, 0.2 g/kg of ascorbate, 0.2 g/kg of manganese sulphate and either 1.0 or 3.0 g/kg of glucose. The meat was fermented at 21 °C for three days. Bacterial growth was followed on MSA (coagulase-negative staphylococci) and MRS agars (*Lb. sakei*) and by identification of isolates through $(GTG)_5$-PCR fingerprinting of genomic DNA and sequencing of the *rpoB* gene [6]. The *in situ* production of the inhibitory compounds was confirmed by placing fermented meat samples on agar layers containing indicator strains (*i.e.*, two *S. aureus* strains, E002 and G111, and five *C. botulinum* strains, 1-5), and verifying for inhibition zones.

Finally, the inhibitory activity by *S. sciuri* I20-1 towards the rifampicin-resistant *S. aureus* ST 398 (C26) strain was also tested in a co-culture experiment using the same fermented meat models as described above. This was compared with the effect of a non-inhibitory control strain (*S. sciuri* G160).

III. RESULTS AND DISCUSSION

Wide inhibition spectra were found for the two candidate strains investigated (Table 1). In total, *S. sciuri* I20-1 and *S. sciuri* VT-S8-8 inhibited 32 and 25 out of the 50 strains tested, respectively. Both potential starter cultures were able to inhibit both the vegetative cells and spores of all *C. botulinum* strains tested. Strains of *S. aureus* were often inhibited, with *S. sciuri* I20-1 inhibiting eight out of nine indicators tested. A wide inhibition spectrum was also present towards other coagulase-negative staphylococci for both *S. sciuri* strains. In contrast, no activity towards *Listeria* spp. was found. Antilisterial activity is a property that has often been described for bacteriocin-producing lactobacilli in fermented meats [1, 3], whereas bacteriocin-like activity by coagulase-negative staphylococci remains underinvestigated [4], in particular against *S. aureus* and *C. botulinum*.

Incubation with proteinase K removed all inhibitory activity from the cell-free culture supernatants of both *S. sciuri* I20-1 and *S. sciuri* VT-S8-8, indicating a proteinaceous nature of the compounds. Both antimicrobials were also heat-stable (even after 20 min at 121°C, they showed inhibition against the *S. aureus* and *C. botulinum* indicator strains of up to 200 AU/ml).

Table 1. Inhibition of different strains by *S. sciuri* I20-1 and VT-S8-8 (number of strains inhibited / total number of strains studied per species).

Indicator strains	Producer strains	
	S. sciuri I20-1	*S. sciuri* VTS-8-8
Clostridium botulinum (vegetative cells)	(8/8)	(8/8)
Clostridium botulinum (spores)	(5/5)	(5/5)
Lactobacillus sakei	(1/2)	(2/2)
Listeria innocua	(0/8)	(0/8)
Listeria ivanovii	(0/1)	(0/1)
Pediococcus acidilactici	(0/1)	(0/1)
Pediococcus pentosaceus	(0/2)	(0/2)
Staphylococcus arlettae	(0/1)	(0/1)
Staphylococcus aureus	(8/9)	(4/9)
Staphylococcus auricularis	(0/1)	(0/1)
Staphylococcus capitis	(1/1)	(1/1)
Staphylococcus carnosus	(1/1)	(0/1)
Staphylococcus chromogenes	(1/1)	(0/1)
Staphylococcus cohnii	(1/1)	(1/1)
Staphylococcus devriesei	(1/1)	(0/1)
Staphylococcus epidermidis	(1/1)	(0/1)
Staphylococcus equorum	(1/1)	(1/1)
Staphylococcus fleuretti	(1/1)	(1/1)
Staphylococcus haemolyticus	(0/1)	(1/1)
Staphylococcus pasteuri	(1/1)	(1/1)
Staphylococcus saprophyticus	(2/2)	(2/2)
Staphylococcus sciuri	(1/1)	(1/1)
Staphylococcus succinus	(0/1)	(1/1)
Staphylococcus warneri	(1/1)	(0/1)
Staphylococcus xylosus	(1/1)	(0/1)
Streptococcus dysgalactiae	(1/1)	(1/1)

Antimicrobial production of *S. sciuri* I20-1 followed primary metabolite kinetics, starting at the mid-exponential growth phase after 7 h (log 7.1 cfu/ml), reaching a maximum at the stationary phase after 11 h, and subsequently remaining constant (Table 2). The antimicrobial activity of the cell-free culture supernatant amounted up to 200 AU/ml when tested against two strains of *S. aureus* (E002 and G111) and up to 200-400 AU/ml when tested against four strains of *C. botulinum* (2-5). Taken together with the heat-stable and proteinaceous character of the compounds, these results indicate a bacteriocin-like nature of the inhibitory activity [7].

Both *S. sciuri* I20-1 and VT-S8-8 displayed satisfactory competitiveness during fermentation in meat models, as the counts on MSA and subsequent $(GTG)_5$-PCR fingerprinting of genomic DNA from isolates confirmed that they were able to survive the fermentation process (data not shown). From 20 h on, the *in situ* production in meat of inhibitory compounds by *S. sciuri* I20-1 and VT-S8-8 was demonstrated based on activity towards the indicator strains *S. aureus* E002 and G111 and, somewhat less clear, against the different strains of *C. botulinum* (Table 3).

Table 2. Growth in log (cfu/ml) as a function of time of *Staphylococcus sciuri* I20-1 in BHI medium and antimicrobial activity of the cell-free culture supernatant toward six indicator strains (AU/ml).

S. sciuri I20-1		Antimicrobial activity (AU/ml) towards indicator strains					
		S. aureus		*C. botulinum*			
Time (h)	Log (cfu/ml)	E002	G111	2	3	4	5
0	5.0	0	0	0	0	0	0
4	6.5	0	0	0	0	0	0
7	7.1	0	0	0	0	0	0
9	7.3	200	200	100	200	0	100
11	7.8	200	200	200	400	0	100
14	8.3	200	200	400	400	100	200
26	8.7	200	200	400	400	200	200
32	8.4	200	200	400	400	200	200
50	8.5	200	200	400	400	200	200

During co-culture experiments in fermented meat models, the inhibitory activity of *S. sciuri* I20-1 reduced the counts of *S. aureus* ST 398 (C26) by two logs (Figure 1). This inhibition was found at the very start of the experiment, probably due to desorption of the inhibitory compounds from the cell surface of *S. sciuri* I20-1 upon exposure to the lower pH of the meat.

Table 3. Production of inhibitory compounds by *S. sciuri* I20-1 and VT-S8-8 during fermentation in meat models, as measured against indicator strains of *S. aureus* and *C. botulinum*. Antimicrobial activity was expressed as '-' not sensitive (no inhibition zone), '+/-' weakly sensitive (hazy inhibition zone), '+' sensitive (clear inhibition zone), or 'ng' no growth of the indicator strain.

		Antimicrobial activity (inhibition zones)						
		S. aureus		*C. botulinum*				
Producing strains	Time (h)	E002	G111	1	2	3	4	5
S. sciuri I20-1	0	-	-	-	-	-	-	-
	20	+	+	+/-	+	+	-	+
	28	+	+	-	+	-	+	+
	48	+	+	n.g.	+	-	-	+
S. sciuri VT-S8-8	0	-	-	-	-	-	-	-
	20	+	+	+	+	+	+	+
	28	+	+	+	-	+	+	+
	48	+	+	-	-	-	-	-

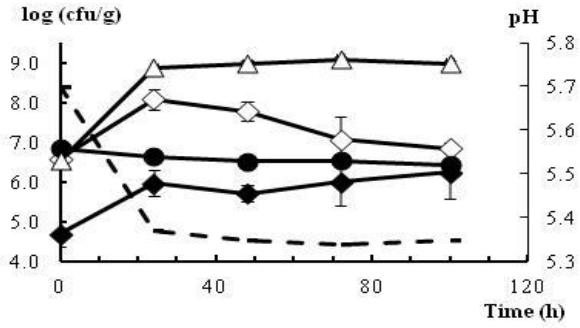

Figure 1. Co-culture of *S. aureus* ST 398 (C 26) (♦, counts on MSA supplemented with 12.5 mg/L rifampicin) and *S. sciuri* I20-1 (♢, counts on MSA), compared to a co-culture of *S. aureus* ST 398 (C 26) (●, counts on MSA supplemented with 12.5 mg/L rifampicin) and the non-inhibitory control strain *S. sciuri* G160 (counts not shown but similar to *S. sciuri* I20-1), in fermented meat models. In both co-cultures, *L. sakei* CTC 494 (Δ, counts on MRS agar) was used as an acidifier (**–**, pH decrease).

Since these inhibitory compounds generally displayed bacteriocin-like properties, the initial drop in *S. aureus* counts meets the fact that bacteriocin molecules are normally released from the cell surface of the producer cells when the pH decreases [8]. The results also showed that resistant *S. aureus* cells could grow, despite the presence of the inhibitory compound, and reach similar levels as in the control experiment. It needs to be evaluated if more realistic *S. aureus* contamination levels, *i.e.* below 3 log (cfu/g), would lead to resistance or not.

IV. CONCLUSION

In nitrate- and nitrite-free slowly acidified fermented meat models, selected *S. sciuri* strains were able to produce inhibitory compounds that were active towards the pathogens *S. aureus* and *C. botulinum*. These compounds were of a bacteriocin-like nature, considering their specific inhibition spectrum, their proteinaceous nature and heat stability, and their primary metabolite kinetics. Since studies on bacteriocin production towards the latter two pathogens in fermented meats are lacking (in contrast to the general focus on *L. monocytogenes*), the present investigation offers potential for novel functional starter culture development. Yet, further optimization is needed in view of the observed atypical inhibition kinetics and the potential emergence of resistant cells.

ACKNOWLEDGEMENTS

The authors acknowledge their financial support of the Research Council of the Vrije Universiteit Brussel (OZR, SRP, IRP, HOA, and IOF projects), the Hercules Foundation (project UABR 09/004), the Research Foundation-Flanders (project FWOAL632), and Flanders' FOOD (project NITRILOW).

REFERENCES

[1] Leroy, F., Verluyten, J. & De Vuyst, L. (2006). Functional meat starter cultures for improved sausage fermentation. International Journal of Food Microbiology 106:270-285

[2] Corpet, D. (2011). Red meat and colon cancer: should we become vegetarians, or can we make meat safer? Meat Science 89:310-316.

[3] Ravyts, F., Barbuti, S., Frustoli, M.A., Parolari, G., Saccani, G., De Vuyst, L. & Leroy, F. (2008). Competitiveness and antibacterial potential of bacteriocin-producing starter cultures in different types of fermented sausages. Journal of Food Protection 71:1817-1827.

[4] Lauková, A., Simonová, M. & Strompfová, V. (2010). *Staphylococcus xylosus* S03/1M/1/2, bacteriocin-producing meat starter culture or additive. Food Control 21:970-973.

[5] Schillinger, U. & Lücke, F.K.. (1989). Antibacterial activity of *Lactobacillus sake* isolated from meat. Applied and Environmental Microbiology 55:1901-1906.

[6] Braem, G., De Vliegher, S., Supré, K., Haesebrouck, F., Leroy, F. & De Vuyst, L. (2011). $(GTG)_5$-PCR fingerprinting for the classification and identification of coagulase-negative *Staphylococcus* species from bovine milk and teat apices: a comparison of type strains and field isolates. Veterinary Microbiology 147:67-74.

[7] Braem, G., Stijlemans, B., Van Haken, W., De Vliegher, S., De Vuyst, L. & Leroy, F. (2014). Antibacterial activities of coagulase-negative staphylococci from bovine teat apex skin and their inhibitory effect on mastitis-related pathogens. Journal of Applied Microbiology 116:1084-1093.

[8] De Vuyst, L., Callewaert, R. & Crabbé, K. (1996). Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. Microbiology 142:817-827.