Salting by vacuum brine impregnation in nitrite-free *lonza*: effect on Enterobacteriaceae

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Abstract

Lonza is a traditional Italian meat product made from whole pork muscles, which is typically cured by dry salting. In this work, we study the effects of vacuum brine impregnation (VBI) as an alternative salting method on the survival of Enterobacteriaceae, in presence and in absence of nitrites. In comparison with the traditional brining process, VBI contributed to reducing the Enterobacteriaceae population on product surface but induced contamination of the inner muscle tissues. Our results suggest that the species isolated became adapted to processing conditions, and salt tolerance was species- or strain-dependent. This result is of particular importance for future applications of VBI in *lonza* manufacturing.

Introduction

Sodium nitrite is traditionally used in meat products for multiple purposes, and particularly for its antimicrobial, antioxidant and colour formation properties (Berardo *et al*., 2016). However, in the last decades, nitrite has been scrutinised by media, consumers and scientific community for its role in the formation of carcinogenic nitrosamines (Bouvard *et al*., 2015). Food products labelled as natural, organic, or preservative-free are showing sales success in the marketplace year after year (Sindelar and Houser, 2009), and for this reason nitrite reduction or elimination is of paramount importance for the food industry.

In manufacturing of whole muscles dry-cured meat products, salting, post-salting and ripening are sequentially applied. In traditional salting, nitrates are normally added to the curing salt mixture to delay spoilage and control microbial activity during storage. They also act as a source of nitrites if nitrate-reducing bacteria are present (Skovgaard, 1992) but also thanks to endogenous enzyme activity at low pH in a reducing environment. In the last years, vacuum brine impregnation (VBI) has been proposed as an alternative method for meat salting (Corzo and Bracho, 2007; Wang *et al*., 2016). This procedure can be considerably accelerated by tumbling in concentrated solutions, *i.e*. alternating immersion phases under vacuum and atmospheric pressure conditions (Deumier *et al*., 2003a, 2003b).

In addition to the biochemical changes induced by the endogenous enzymes, some microbial enzymes from a consortium of microorganims contribute to the ripening process, in particular from catalase negative cocci (CNC), yeasts and moulds. Many intrinsic and extrinsic factors influence the evolution of these microbial groups during meat ripening, with a strong selective pressure induced by salt penetration. Inadequate curing may cause the survival of different microbial groups that can be undesirable such as enterobacteria that are considered as quality-related organisms in cured meat products (Belletti *et al*., 2013). In fact, even though the environmental conditions do not favour Enterobacteriaceae, in particular *Serratia* spp. and *Proteus* spp. can grow throughout curing (Marin *et al*., 1996; Garcia *et al*., 2000). The survival of Enterobacteriaceae during the production of cured meats is not only relevant for product shelf-life, but also for food safety, as this family includes pathogens and biogenic amines producers (Suzzi and Gardini, 2003; Chaves-López *et al*., 2006). The aim of this work was to study Enterobacteriaceae population both in presence and in absence of nitrites during VBI salting of Italian *lonza*, a traditional meat product made from whole pork muscles that is typically cured by dry salting.

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Materials and Methods

Sampling treatment

The deboned meat used for *lonza* manufacturing was obtained from pigs slaughtered 2 weeks before. Immediately after purchase, the pork loins (*Longissimus dorsi*) were frozen separately in an air-blast quick freezer at -50°C for 4 h and then kept in a freezing chamber at -18°C until use. Before treatment, whole loins were thawed at 4°C for 24 h. Loins weight ranged from 2 to 2.3 kg for all tests. The brine was a water solution containing 20% NaCl, 2% dextrose, 1% saccharose, 0.1% ascorbate, and 0.1% mixed spices. When added, nitrates were used at 0.085% concentration. The brine was added in ratio of 2 kg/kg (brine/meat).

Pork loins (30 kg per batch) were subjected to VBI treatment in trials with nitrite-added brine (NB) and without sodium nitrite (B) at 4°C for 20 min, by using a pulsed vacuum tumbler (VM, Reggio Emilia, Italy). After tumbling, each batch was kept in a stainless steel wagon at 4°C for 16 h to favour salt diffusion. In the samples subjected to the traditional process, dry-curing was carried out at 5°C for 21 days. Both types of samples were stuffed into cellulose casing and subjected to drying (8 days, 15 to 17°C, 70 to 90% humidity ratio) and curing (60 days, 14-15°C, 65 to 85% humidity ratio). Three batches for each treatment were analysed.

Sampling

Sampling of three production batches was carried out at different process times (days): 0 (raw pork loin), 1 (salting), 15, 21 (end of salting), 21 (stuffing into casing), 36 (end of drying), 66 (ripening), 96 and 116 days.

Microbiological analyses

Analyses were performed after careful removal of surface moulds. First, the cellulose casing was washed with ethanol, then cut with a sterile knife, and removed by

using sterile forceps. After that, surface (1.5 cm) and core samples (3.5 cm diameter) were taken by means of a sterile knife.

Core and surface samples (10 g) were homogenized in a Stomacher Lab-Blender 400 (Seward Medical, London, UK) in 90 mL sterile saline solution. Decimal dilutions of the suspension were prepared in physiological solution, and Enterobacteriaceae were isolated and counted in Violet Red Bile Glucose Agar (Oxoid, Basingstoke, UK) at 37°C for 24 h. Enterobacteriaceae isolates were identified with API 20 E (bioMérieux, Mercy L'Etoile, France).

Growth response to sodium chloride

To evaluate the isolates capability of growing under different concentrations of the additives used in *lonza* production, growth in Brain Heart Infusion (BHI, Oxoid) modified with different concentrations of NaCl (Oxoid) (0, 3, 5, 7, and 10%) was tested. First the experiment was performed in micro-scale: overnight cultures were centrifuged and washed twice in sterile saline solution, then 20 µL were inoculated into 200 µL of medium and optical density at 600 nm (OD_{600}) was measured by means of Bioscreen C System (Labsystems Oy, Helsinki, Finland) during 48 h. The data obtained were modelled by means of Gompertz equation, modified by Zwietering *et al*. (1991). The experiment was repeated three times. To verify if the data obtained by Bioscreen reflected the behaviour of the strains in macro-scale, for some selected strains the previous experiments were performed in flasks containing 100 mL of modified medium, maintaining the same proportion of inoculum and time of incubation.

Chemical and physical analyses

pH values were measured by using a Mettler Toledo MP 220 pHmeter (Mettler, Toledo, Spain) on aqueous dispersions (1:10) of the samples. Water activity (a_w) was measured by using a dew-point hygrometer Aqualab CX2 (Decagon Devices, Pullman, PA, USA).

Statistical analysis

One-way analysis of variance and least significant difference were used to analyse differences in mean values at 95 and 99% accuracy level.

Results

Chemical and physical analyses

Regarding pH, no statistically significant differences among the samples were observed (data not shown). VBI treatments with or without nitrates were not significantly different as regards a_w, while significantly lower values were observed with respect to control (traditional process).

Microbiological characteristics

As evidenced in Table 1, in *lonza* samples subjected to the traditional dry-curing, the presence of Enterobacteriaceae was evident only on surface samples. In particular, the count was 2.9 Log colony forming unit (CFU)/g at day 0, decreased up to 1.70 Log CFU/g at the end of salting (day 21), increased again up to 2.9 Log CFU/g after casing, and were below limit of detection in the following sampling times.

Pulsed VBI caused a count reduction between 1.0 and 1.9 Log CFU/g on the product surface after one day of treatment for NB and B samples, respectively. During salting, NB samples showed a further reduction of 1 Log CFU/g up to day 15, and after that the count increased again and reached 2.7 Log CFU/g. On the contrary, in B samples, after an initial reduction of about 1.9 Log CFU/g, the count increased regularly up to 2.6 Log CFU/g on product surface at day 21 and reached 3.2 Log CFU/g at day 21 in core samples.

For all treatments, the stuffing into casing increased the Enterobacteriaceae count on product surface. Starting from day 36 in traditional curing, and day 60 in both types of VBI samples (NB and B), Enterobacteriaceae were below limit of detection.

Identification and characterisation of Enterobacteriaceae strains isolated throughout *lonza* manufacturing

To investigate the survival potential of Enterobacteriaceae during VBI treatment, a representative number of isolates was identified and characterized for the tolerance to different concentrations of NaCl. A total of 40 strains was isolated from VBI treated samples. Table 2 shows the number of isolates of the different species identified, with a predominance of *Enterobacter cloacae*, followed by *Serratia liquefaciens*, *Citrobacter* spp., *Serratia odorifera*, and *Escherichia coli.* Other species such as *Enterobacter aerogenes, Klebsiella ornithinolytica*, *Serratia marcescens* and *Proteus vulgaris* were found at lower percentages. The latter species was isolated only after stuffing, while *E. coli, Ser. odorifera, Ser. liquefaciens* and *Ent. aerogenes* strains were isolated up to day 36 in the samples.

$O.D._{600}$ data, corresponding to the Enterobacteriaceae strains growth in presence of each salt concentration, were analysed according the modified Gompertz equation. The predicted curves fitted well with the experimental points, and the regression coefficients of the curves obtained ranged from 0.95 to 0.98.

Overall, the increase of NaCl levels influenced the A_{max} and lag phase duration of the majority of the strains. As evidenced in Table 3, all the strains were able to grow at high NaCl percentages. In particular, levels of 7% NaCl slightly increased the growth of *Ser. liquefaciens* and *Ser. odorifera* strains. Moreover, in presence of 10% NaCl all the strains of *E. coli* grew, reaching OD_{600} values up to 0.72, while two *Ser. marcescens* and one *S. liquefaciens* strain reached OD_{600} values of 0.64 and 0.58

Table 1. Enterobacteriaceae (Log colony forming unit/g) in *lonza* samples at different times.

	Days	Traditional dry-curing		Pulsed VBI (20 min) NB		Pulsed VBI (20 min) B	
		Surface	Core	Surface	Core	Surface	Core
Salting	0	2.9±0.3	nd	3.3±0.2	nd	3.2±0.3	nd
	1	2.9±0.1	nd	2.3±0.2	1.4±0.4	1.3±0.2	0.3±0.1
	15	2.7±0.2	nd	1.1±0.1	1.2±0.3	2.0±0.2	0.4±0.1
	21	1.7±0.2	nd	2.7±0.1	1.3±0.2	2.6±0.2	3.2±0.2
Drying	21	2.9±0.1	nd	3.0±0.1	1.3±0.3	2.6±0.1	3.2±0.1
	36	nd	nd	1.3±0.3	2.1±0.1	2.0±0.2	2.6±0.1
Curing and ripening	60	nd	nd	nd	nd	nd	nd
	90	nd	nd	nd	nd	nd	nd
	120	nd	nd	nd	nd	nd	nd

VBI, vacuum brine impregnation; NB, with nitrite-added brine; B, without sodium nitrite; nd, non-detectable (below limits of detection of the method employed).

respectively, after 24 h. *Ser. odorifera* showed the lower lag phase duration at 10% NaCl, while the most sensitive strains were those belonging to the species *Ent. cloacae* which reached OD_{600} values of 0.50.

The most NaCl tolerant strains, belonging to *Ser. liquefaciens, Ser. marcescens* and *E. coli* species, were also evaluated in flasks at low (0%, 3%) and high (7%, 10%) NaCl percentages (data not shown). Also in this case, differences were observed among the strains especially in the lag phase, probably due to a late stationary phase reached in the pre-culture or to specific strain behaviours. In particular, the cells grew vigorously in control and in the medium containing 3% NaCl with a maximum growth value ranging from 0.61 to 0.84 OD_{600} after a lag phase ranging from 0.45 to 1.30 h. At high NaCl concentrations, the strains grew slowly at 7% after a lag phase ranging from 1.47 to 3.03 h and a maximum growth value of 0.4 OD_{600}. At 10%, only the strain C26S4 belonging to *Ser. liquefaciens* species, showed a maximum growth value of 0.75 OD_{600} after a lag phase of 4.21 h, while the others had a maximum growth value between 0.28 and 0.31 OD_{600}.

Discussion

Salting is one of the most ancient and efficient preservation methods, especially for meats (Pittia and Paparella, 2015). Not only is the addition of salt important to reduce the a_w below a tolerable level for spoilage microorganisms (Feiner, 2006), but it also regulates the activity of the endogenous enzymes involved in the development of the sensory properties of the final product (*i.e.* flavour, texture) (Martuscelli *et al*., 2015). In fact, salting increases lipid emulsification and protein dissolution in the meat, and gives unique sensory characteristics to cured meat products (Chiralt *et al*., 2001). In this work, the effect of pulsed VBI on the Enterobacteriacaeae population during *lonza* production was studied.

Among the cured meat microbiota, Enterobacteriaceae are particularly sensitive to salt and normally grow starting from

Table 2. Identification of the strains isolated from vacuum brine impregnation-treated samples.

Time of isolation (days)	Number of strains	Species
0 (raw meat)	1	*Ent. cloacae*
	1	*Ser. liquefaciens*
	1	*Ser. odorifera*
	1	*E. coli*
1 (salting)	1	*Ent. cloacae*
	1	*Ent. aerogenes*
	1	*Ser. marcescens*
	1	*Ser. liquefaciens*
	2	*Citrobacter* spp.
	2	*E. coli*
15 (middle salting)	2	*Ent. cloacae*
	1	*Ser. odorifera*
	2	*Citrobacter* spp.
21 (end of salting)	1	*Ent. cloacae*
	1	*Ent. aerogenes*
	2	*Ser. marcescens*
	2	*Ser. odorifera*
	1	*K. ornithinolytica*
21 (after stuffing)	3	*Ent. cloacae*
	1	*Ser. liquefaciens*
	2	*Ser. odorifera*
	1	*K. ornithinolytica*
	2	*Citrobacter* spp.
	1	*E. coli*
	1	*Proteus vulgaris*
36 (drying)	1	*Ent. aerogenes*
	2	*Ser. liquefaciens*
	1	*Ser. odorifera*
	1	*E. coli*
66 days (ripening)	nd	-
96 days (ripening)	nd	-
116 days (ripening)	nd	-

Ent., Enterobacter; Ser., Serratia; E., Escherichia; K., Klebsiella. nd, non-detectable (below limits of detection of the method employed).

Table 3. Growth parameters for the forty Enterobacteriaceae strains isolated from *lonza* samples, in presence of different sodium chloride concentrations (%) in Bioscreen C System (Labsystems Oy, Helsinki, Finland).

Species	Strains (n)	0%		3%		5%		7%		10%	
		A_{max} (OD_{600})	λ (hours)	A_{max} (OD_{600})	λ (hours)	A_{max} (OD_{600})	λ (hours)	A_{max} (OD_{600})	λ (hours)	A_{max} (OD_{600})	λ (hours)
Ent. cloacae	8	0.94±0.18	0.94±0.20	0.76±0.08	1.10±0.18	0.79±0.13	1.15±0.17	0.80±0.16	1.20±0.12	0.50±0.10	2.45±0.45
Ent. aerogenes	3	0.96±0.03	1.00±0.30	0.58±0.25	1.00±0.31	0.75±0.07	1.26±0.40	0.75±0.09	1.28±0.38	0.56±0.04	2.00±0.20
Ser. marcescens	3	1.10±0.08	0.55±0.08	0.83±0.05	1.29±0.06	0.84±0.07	1.26±0.31	0.81±0.03	1.18±0.2	0.64±0.09	2.15±0.3
Ser. liquefaciens	6	1.04±0.05	1.58±0.45	0.79±0.08	2.31±1.15	0.83±0.04	2.45±1.1	0.92±0.06	2.00±0.45	0.58±0.09	3.45±1.00
Ser. odorifera	5	0.93±0.19	1.49±0.12	0.75±0.07	1.29±0.19	0.82±0.12	1.41±0.25	0.87±0.15	1.20±0.30	0.60±0.06	1.44±0.62
K.ornithinolytica	3	0.97±0.03	0.45±0.12	0.70±0.27	0.42±0.25	0.73±0.04	0.56±0.33	0.73±0.05	0.48±0.22	0.55±0.02	1.46±0.41
Citrobacter spp.	6	1.03±0.02	1.24±0.19	0.79±0.12	1.43±0.26	0.81±0.04	1.45±0.59	0.82±0.10	1.55±0.39	0.65±0.03	3.32±1.23
E. coli	5	1.09±0.06	1.29±0.31	0.91±0.03	1.41±0.51	0.85±0.05	2.33 ±0.26	0.80±0.12	1.12±0.47	0.72±0.08	2.43±1.05
Proteus vulgaris	1	1.27	0.50	1.33	1.19	1.03	1.12	0.65	1.59	0.69	2.23

Ent., Enterobacter; Ser., Serratia; K., Klebsiella; E., Escherichia; OD_{600}, optical density at 600 nm; A_{max}, maximum growth value reached; λ, lag phase. Results are expressed as the mean and standard deviation of three replicates.

a_w value of 0.93 (Vilar *et al*., 2000). Mesophilic genera of Enterobacteriaceae such as *Salmonella* spp., *Shigella* spp. and certain *Escherichia coli* may cause severe diarrhoea, as well as the psychrotrophic *Yersinia enterocolitica*. In addition, other Enterobacteriaceae that are able to grow in meat products and cause spoilage, such as *Serratia marcescens* and *Enterobacter cloacae*, may also be of clinical concern (Lindberg *et al*., 1998).

In this study, a reduction of Enterobacteriaceae count after traditional salting was observed, although this microbial group was still able to grow on *lonza* surface up to day 30. Marin *et al*. (1996) reported the persistency of Enterobacteriaceae throughout the industrial manufacturing of dry-cured ham with a final a_w value of 0.88. These authors found that *Leclercia adecarboxylata, K. pneumoniae* and *Ent. aerogenes* were still present at the end of the process, whereas *Ser. marcescens, Citr. freundii, Erwinia* spp., *Hafnia alvei, Edwarsiella* spp., and *Serratia* spp. were detectable at the end of the salting process (40-45 days). As evidenced by our results, in comparison with the traditional dry-curing, pulsed VBI contributed to the reduction of the *Enterobacteriaceae* population on the product surface but was responsible of internal contamination. In fact, VBI induces mass transfer that involves external diffusion mechanisms and non-diffusional infiltration of solution into meat spaces occupied by occluded and/or dissolved gases prior to vacuum processing (Deumier *et al*., 2003a). Thus, it is possible that enterobacteria were infiltrated during the VBI treatment together with the brine, and then remained trapped inside the inner muscle tissues.

In our VBI treated samples, the Enterobacteriaceae were isolated only up to end of drying (day 36), with *Ser. liquefaciens, Ser. odorifera, Ent. aerogenes*, and *E. coli* being the dominant species at this stage of production and showing the best ability to grow at high NaCl concentrations. Although *Ser. marcescens* showed high NaCl tolerance, it was not isolated during the drying step, probably because product conditions, other than NaCl concentration, were determinant for their survival and/or growth in agar plates.

Jordan and Davies (2001) suggested that the addition of sodium chloride, rather than contributing to inhibition can reverse the inhibitory effect of lactate and enable growth under conditions of low pH and high lactate in *E. coli* O157:H7. Our results suggest that the species we isolated became adapted to processing conditions, showing salt tolerance that can be species- or strain-dependent. *Ser. liquefaciens* is a psychrotrophic proteolytic enterobacterium that can survive under adverse conditions and can be isolated from several cured meats even in the last period of ripening (Castaño *et al*., 2002; Lyhs *et al*., 1998).

From the results obtained in the present study, it may be concluded that many Enterobacteriaceae strains isolated from Italian *lonza* can show high tolerance to sodium chloride.

Conclusions

The survival of the Enterobacteriaceae species during meat salting is a health risk of particular importance, because this family includes pathogens and species capable of producing biogenic amines. In our study, in contrast with what might be assumed on the basis of a_w decrease before drying, VBI salting of nitrite-free *lonza* was able to induce micro-ecological changes that favoured the survival of Enterobacteriaceae, although these microorganisms were never isolated from final products. This result is of particular importance in the perspective of a possible industrialisation of the VBI process for manufacturing *nitrite-free* meat products. Further studies will be needed to investigate solutions for mitigating the risk of enterobacteria survival during VBI processing.

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