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EFFECT OF NITRITE AND ASCORBATE ON THE GENERATION OF N-NITROSO COMPOUNDS, VOLATILE N-NITROSAMINES AND RESIDUES OF NITRITES IN BRESAOLA

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I. INTRODUCTION

Bresaola is a processed meat produced in Valtellina, an area in the Northen Italy, whose processing methods, as well as the raw meat characteristics, require adequate ingoing amounts of nitrite and nitrate to ensure both safety and uniform red color development in final product [1]. From a regulatory point of view, bresaola falls within the Category 08.3.4.2 "Traditional dry cured products" of Regulation (EU) 2023/2018, the constraint of which is that at the end of processing the maximum residues of nitrite and nitrate are 65 and 150 mg/kg, respectively. Despite the technological advantage, the addition of nitrites causes the generation of a wide range of N-nitroso compounds (NOCs), that may include volatile N-nitrosamines (VNAs), known to be dangerous to consumer health [2]. The aim of this work was to evaluate the effect of increasing nitrite ingoing amounts in Bresaola on nitrites residues, on the generation of NOCs and VNAs, and the effectiveness of sodium ascorbate in mitigating their formation.

II. MATERIALS AND METHODS

Experimental design – Thawed bovine *semimembranous* muscles were trimmed, weighed and divided into three homogeneous batches based on weight (average weight 4.8 kg). The batches were added with the same amount of salt (2.8%), spices (0.185%), dextrose (0.5%) and nitrate ion (73 mg/kg), while the addition of nitrite ion was 123, 252 and 378 mg/kg, respectively. Each batch was then divided into two parts, one supplemented with 500 mg/kg of ascorbate (A+) and one without (A-), obtaining 6 batches in total. The meat pieces were tumbled 2 min. (2 round x min.) at the beginning of the salting phase and every 48 hours for 2 weeks. After 14 days, each piece was stuffed into a collagenic casing (Ø 130 mm), placed in an elastic net and hung in a drying room. The drying phase lasted 7 days decreasing from 25 ± 1 °C to 19 ± 1 °C (60-90% RH), while the aging phase was carried out at 15 ± 2 °C (45-75% RH) until 38 ± 2% final weight loss. At the end of processing, the pieces were washed, dried, peeled and vacuum-packed. Each trial was replicated twice. *Sampling and analysis* – Two 2.5 cm thick slices were taken from three bresaola randomly selected within each batch, finely chopped with a knife, mixed to obtain a homogeneous sample, packed in plastic bags under vacuum and stored at -20 °C until analysis. *Total N-nitroso compounds (total NOCs)* – Total NOCs were quantified according to the method of Joosen et al. [3] with some modifications. Briefly, 50 mg of freeze-dried sample, weighted in a 3 ml syringe and diluted with 450 ml of water Milli-Q™, were incubated for 2 min. at room temperature with 100 µl of 50mM N-Ethylmaleimide (NEM) and 100 µl of 100 µM diethylenetriaminepentaacetic acid (DETP). Then, 500 µl of 5% (w/v) sulfamic acid solution was added to remove residual nitrite and after 3 min. the sample was injected into a purge vessel kept at 60 °C and filled with a standard tri-iodide reagent solution (38 mg of I_2 were added to a solution of 108 mg of KI in 1 ml water and dissolving it in 13,5 ml of glacial acetic acid). The nitric oxide (NO) released from the samples was detected by using an Eco Medics CLD 88 Exhalyzer (Eco Medics AG, Duernten, Switzerland) and quantified by a nitrite standard calibration curve in the range 10-100 µM. The results were expressed as nmol of NO per g of bresaola, taking into account sample and lyophilizate moisture in the calculation. *Volatile N-nitrosamines (VNAs)* - Nine VNAs (NDMA, NMEA, NDEA, NPYR, NDPrA, NMOR, NPIP, NDBA and NDPhA) were analyzed according to the method of Lehotay et al. [4] with slight modifications. Briefly, 5 g of minced bresaola were weighted and extracted with 10 ml H_2O/ACN 1:1 v/v and 4 g of ammonium formate; then, the mixture was shaken for 60 minutes at 210 rpm and centrifuged for 3 min. at 4000 rpm. Two ml of the upper layer of the extract was transferred to 15 ml d-SPE tubes (900 mg $MgSO_4$, 150 mg of PSA, 50 mg C18) and 2 ml of hexane was added to the tubes for lipid removal. The tubes were shaken and centrifuged for 3 min. at 4000 rpm. The upper hexane layer was wasted and 0.2 ml of the extracts was analyzed by GC-HRMS Orbitrap using the column TG-WAX (30m x 0.25 mm x 0.25µm) in Full Scan and SIM acquisition mode. Quantification was performed using as internal standards the isotopically labelled analogues of the target analytes. The limits of quantitation (LOQs) ranged from 0.05 to 0.1 µg/kg and relative standard deviations (RSD) ranged from 5 to 11%. The linearity of the method was assessed on 7 calibration levels for each VNA (ranging from LOQ to 20 µg/kg). Recoveries at two concentration levels (0.5 and 2.5 µg/kg) were between 70 and 116%. *Nitrite and nitrate* - Residual nitrite and nitrate ions were determined by high-pressure ion chromatography [5]. A Thermo Scientific Dionex ICS-6000 system with an IonPac AS27 column and AG27 pre-column (30 °C) was used. Ten grams of meat samples were extracted in 100 ml water with 1 ml 1% NaOH (pH 8) in an ultrasonic bath for 45 min., then deproteinized by Vivaspin® 15 (5 kDa, 18 °C, 6000×g, 30 min). LOQs were 2 mg/kg for nitrites and 9 mg/kg for nitrates. *Statistical analysis* - Data were analyzed by using the General Linear Model (GLM) procedure of SPSS statistic software

(version 22.0.0, IBM, NY, USA). The ingoing level of nitrite (N) and sodium ascorbate (A) were assigned as fixed effects and their interaction (N × A) was included. Estimated Marginal Means (EMMs) and the standard error of the mean (SEM) were reported for fixed effects. The statistical differences were assessed using Tukey' test, *P-value* < 0.05 for the significance.

III. RESULTS AND DISCUSSION

The results of this study are reported in Table 1. Total NOCs is a nitrosylation marker representing the amount of nitric oxide (NO), originating from nitrite and bound to the meat matrix during processing. NOCs concentration increased significantly as the ingoing amount of nitrite increased. As to the VNAs, only four of them were detected: their concentration depended significantly on the ingoing amount of nitrite. NDBA is an exception because of the different trend observed in the replicates. The sum of VNAs was also significantly affected by the ingoing amount of nitrite. Residual nitrite detected in the final product suggests that nitrite was almost completely consumed during the process, although differences were observed between the batches. Residual nitrate is the balance between added nitrate, reduction of nitrate to nitrite, and nitrate derived from nitrite oxidation; it was significantly influenced by the ingoing amount of nitrite. The addition of ascorbate contributed to reduce the concentration of Total NOCs, NPYR (a tendency for NPIP too), residues of nitrite and nitrate. Besides, the significant interaction N x A observed for total NOCs and NPYR was explained by the effectiveness of ascorbate to lower the formation of these makers when a great ingoing amount of nitrite was added (Figure 1). The positive effect in limiting the formation of NAs and reducing the residues of nitrites is well documented in literature [6,7].

Table 1 – Effect of nitrite increasing ingoing and ascorbate presence (A+) or absence (A-) on the nitrosylation markers of Bresaola.

	Nitrite (N)				*P-value*	Sodium ascorbate (A)			*P-value*	P-value
	Batch 1	Batch 2	Batch 3	SEM		A-	A+	SEM		N x A
Total NOCs (nmol/g)	385 ^{a}	700 ^{a}	1117 ^{b}	105	<0.001	1027	440	85	<0.001	0.007
NDMA (μg/kg)	0.30^{a}	0.34^{a}	0.42^{b}	0.02	<0.001	0.36	0.35	0.02	0.642	0.634
NDBA (μg/kg)	0.15	0.16	0.40	0.10	0.129	0.24	0.23	0.08	0.950	0.539
NPIP (μg/kg)	0.13^{a}	0.20^{b}	0.38^{c}	0.02	<0.001	0.22	0.26	0.02	0.073	0.876
NPYR (μg/kg)	0.01^{a}	0.03^{b}	0.09^{c}	0.01	<0.001	0.06	0.02	0.01	<0.001	0.000
Sum of VNAs (μg/kg)	0.59 ^{a}	0.73 ^{a}	1.29 ^{b}	0.11	<0.001	0.87	0.86	0.09	0.946	0.365
NO_2^- (mg/Kg)	2.56 ^{a}	3.23 ^{a}	4.13 ^{b}	0.20	<0.001	3.72	2.89	0.16	0.001	0.162
NO_3^- (mg/Kg)	102.9 ^{a}	118.7 ^{a,b}	135.5 ^{b}	6.9	0.009	129.5	108.6	5.7	0.014	0.172

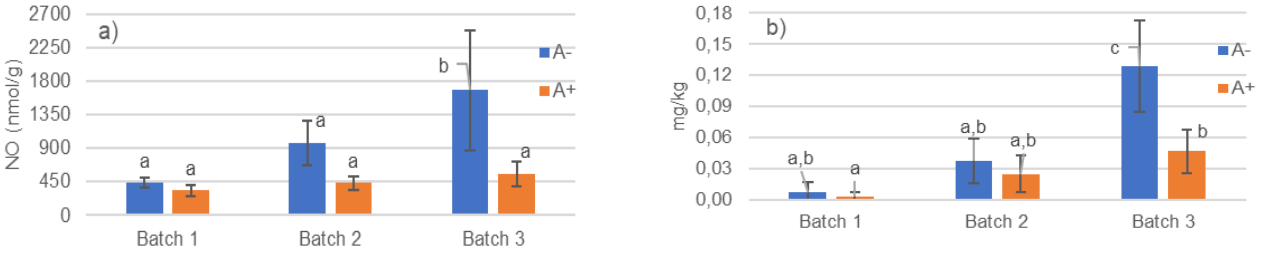


Figure 1 – Total NOCs (a) and NPYR (b) in samples of the three batches prepared with (A+) and without (A-) sodium ascorbate.

IV. CONCLUSION

An increasing ingoing amount of nitrite in bresaola product promotes the formation of N-nitroso compounds, including volatile N-nitrosamines, and rises the risk of exceeding the legal limit for nitrate ion established for this product. Although European legislation does not currently impose any legal limits in foods [2], the VNAs values measured in all samples were far below the limits imposed by some non-European countries [6].

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