# Protective cultures against foodborne pathogens in a nitrite reduced fermented meat product

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**Abstract**

In the present work, a combined hurdle approach for fermented meat preservation was investigated. Challenge tests were performed in *Chorizo* sausage model using the maximum allowed $NaNO_2$ amount (150mg/kg), a reduced amount (75 mg/kg) and no nitrite, with and without protective cultures inoculation. Cocktail strains of *L. monocytogenes* and *Salmonella* spp. were used as indicator strains. In a nitrite reduced sausage model, *L. monocytogenes* growing trend did not significantly change ($p>0.05$) when compared with that containing higher nitrite concentration (150 mg/kg $NaNO_2$). The addition of *L. plantarum* PSC20 significantly lowered *L. monocytogenes* growth when compared with control batches without PCS20 ($p<0.05$), obtaining 3.84 log cfu/g and 2.62 log cfu/g lower counts in the batches with 150mg/kg $NaNO_2$ and 75mg/kg $NaNO_2$ respectively. None of the protective cultures demonstrated *in situ* antagonistic activity against *Salmonella* spp.

This work pointed out that the reduction of nitrites with the combined use of a protective culture could be a feasible approach to control *L. monocytogenes* growth in fermented meat foods.

**Keywords:** Protective cultures; nitrite reduction; *Listeria monocytogenes*; *Salmonella* spp.; fermented pork meat

## 1. Introduction

In the era where demand for ready to eat and preservative free products is constantly growing, the microbiological food safety has to be guaranteed, proportionally with this ongoing trend. In the recently published European Food Safety Authority (EFSA) foodborne outbreak report, referred to 2016, *Salmonella* spp. human infections had the same high level of the previous year (94.530 confirmed cases), whereas human listeriosis, caused mainly by *Listeria monocytogenes*, showed a 9.3% increase (2.536 confirmed cases) (EFSA, 2017). Despite the relatively low incidence of listeriosis, compared with the number of campylobacteriosis and salmonellosis cases, its importance is due to the severity of the disease and the higher case-fatality rate (Baffoni et al. 2017; D'Ostuni et al., 2016; EFSA, 2017).

Curing with nitrite is the most used approach to control foodborne pathogens in the meat (Honikel, 2008). Nitrites have additional functions in the meat, as they help to prevent lipid oxidation and rancidity, guarantee a bright red color and a typical "cured" flavor (Sebranek & Bacus, 2007). Although nitrites are widely used in the meat industry, they are classified by International Agency for Cancer Research as potentially carcinogenic agents (IARC, 2010), due to their ability to react with amines in the gastrointestinal tract, resulting in N-nitrosamines formation. Nitrites, hitherto, are the most effective solution against *C. botulinum* growth in meat products (EFSA 2003; Hospital, Hierro & Fernández, 2014; Hospital, Hierro, Stringer & Fernández, 2016). Therefore, 150 mg/kg $NaNO_2$ and 300 mg/kg $NaNO_3$ were authorized as maximum added levels in meat in Europe until May 2018 (EFSA, 2003; European Commission, 2011). Starting from May 2018, a new regulation, proposed by the Danish authorities in 2015, was approved and the maximum accepted nitrite level in fermented salami is now 100 mg/kg (European Commission, 2018). Additionally, the EC Regulation N° 889/2008 for organic meat products, establishes 80 mg/kg for added nitrite and

50 mg/kg for residual nitrite (European Commission, 2008). The U.S. FDA accepts a maximum level of 200 mg/kg $NaNO_2$ and 500 mg/kg $NaNO_3$ in meat finished products (CFR, 2018). Although outbreaks regarding food poisoning by nitrite derived from meat products are not described in the literature, unintentional poisoning has been reported upon eating homemade sausages (Cvetković, Živković, Lukić, & Nikolić, 2018).

Therefore, meat industries are challenged to employ healthier and safer approaches for meat preservation. In the attempt of finding alternatives to nitrites for fermented food preservation, several authors suggested the use of lower nitrite levels in combination with other compounds or processing technologies, in a way that antimicrobial properties against the common foodborne pathogens could be guaranteed without alteration of sensory qualities (Alahakoon, Jayasena, Ramachandra & Jo, 2015; Cavalheiro et al., 2015). Lactic acid bacteria (LAB) with demonstrated *in vitro* antimicrobial activity against a wide spectrum of foodborne pathogens (Leroy, Geyzen, Janssens, De Vuyst & Scholliers, 2013) as well as the addition of natural extracts or phytochemicals are the mostly studied approaches for the development of innovative processed meat products (Alahakoon et al., 2015; Gaggia, Di Gioia, Baffoni & Biavati, 2011; Oliveira, Ferreira, Magalhães & Teixeira, 2018). However, several natural extracts may contain even more than the allowed nitrate amount, thus the nitrosamine formation is questioned (Bedale, Sindelar, & Milkowski, 2016). LAB strains with demonstrated sensorial or health promoting properties are approved by FDA as Generally Recognized as Safe (GRAS) and by EFSA with the Qualified Presumption of Safety (QPS) status (EFSA, 2018; FDA, 2018).

In the present work, we studied the effectiveness of a combined hurdle approach, *i.e.* a 50% reduction of nitrites plus the addition of previously characterized *Lactobacillus* strains (*Lactobacillus plantarum* PCS20 or *Lactobacillus delbrueckii* DSM 20074), against common foodborne pathogens in *Chorizo*, a dry fermented sausage produced in Spain.

## 2. Material and methods

### *2.1 Bacterial strains*

*L. plantarum* PCS20 (MSCL P977) and *L. delbrueckii* DSM 20074 were used as protective cultures for their demonstrated anti-microbial activity against several pathogens (Di Gioia et al., 2016; Savino et al., 2011). They were grown in de Man Rogosa Sharpe medium (MRS, Oxoid Ltd., Basingstoke, England) in anaerobic conditions (Anaerogen, AN0025A, Oxoid), at 37 °C for 48 h.

A cocktail of *Listeria monocytogenes* strains has been used: *L. monocytogenes* CECT 5366 (serovar 4b, source: human), CECT 934 (serovar 4a, sourse: brain of sheep with circling disease), CECT 4032 (serovar 4b, source: associated with case of meningitis after eating soft cheese) and LTA0020 (isolated from poultry minced meat in Burgos, Spain), already used in similar studies (Melero, Diez, Rajkovic, Jaime, & Rovira, 2012; Melero, Vinuesa, Diez, Jaime, & Rovira, 2013). The strains were grown at 37°C in Brain Heart Infusion Broth (BHI, Oxoid). For evaluation of viable cell population Chromogenic *Listeria* agar (Oxoid) supplemented with OCLA (ISO) Selective Supplement (SR 0226E, Oxoid) and Brillance *Listeria* Differential Supplement (SR 0228E, Oxoid) was used.

Four *Salmonella* strains were also employed in the challenge tests. All strains were isolated from meat and cheese products in Burgos. Bacterial strains were grown at 37°C in BHI. Brilliance *Salmonella* agar (Oxoid) supplemented with *Salmonella* Selective Supplement (SR 0194, Oxoid) was used for the evaluation of viable cell population.

### *2.2 Study design*

Two Challenge tests in sausage prototypes were designed, referred to as 1 and 2. Challenge test 1 aimed at studying the effect of *L. plantarum* PCS20 against *L. monocytogenes* and *Salmonella* spp. in fermented sausages, both without nitrite addition and with 150 mg/kg of nitrite. Challenge test 2 was focused on the effects of two protective cultures, *L. plantarum*

PCS20 and *L. delbrueckii* DSM 20074, against *L. monocytogenes* strains in pork meat batters treated with 75 mg/kg and 150 mg/kg of nitrite. Challenge test protocols are detailed below (2.3 and 2.4).

*2.3 Inocula preparation*

*2.3.1 Pathogen strains*

Each *L. monocytogenes* and *Salmonella* spp. strain was grown at 37°C overnight in BHI broth up to 9 log cfu/ml. Cells were washed and suspended in sterile Ringer solution (Oxoid). For Challenge test 1, dilutions were performed in order to obtain a final concentration of 4.5 log cfu/g in the meat batter (Figure 1), whereas for Challenge test 2, meat batter was inoculated with *L. monocytogenes* cocktail strains in order to obtain the final concentration of 3 log cfu/g (Figure 2).

*2.3.2 Protective cultures*

*L. plantarum* PCS20 and *L. delbrueckii* DSM 20074 were grown at 37°C overnight in MRS broth up to 9.5-10 log cfu/ml. Cells were washed and suspended to a final concentration of 6-7 log cfu/g (Figure 2).

*2.4 Challenge tests*

The batter was composed of ground pork meat and fat (70% and 30%, respectively) supplied by a meat processing company in Burgos (Spain). Spices were not used not to interfere with the results obtained.

For Challenge test 1, the ground meat (4 kg) was divided in 2 trays, each containing 2 kg. In one tray, 2% NaCl was added whereas, in the other tray, meat was supplemented with 2% NaCl plus 150 mg/kg $NaNO_2$ (Figure 1). After homogenization in a vacuum mixer, each 2 kg portion was splitted in two: 1 kg was inoculated with *L. plantarum* PCS20 whereas the other kg was not inoculated with any protective culture. Subsequently, each kg was divided in 3 batches (333 g), one inoculated with the cocktail of *Salmonella* strains, the second one with

the *L. monocytogenes* strains and the last one was not inoculated with any pathogen (control). The 12 treatments and the relative acronyms are shown in Fig. 1.

For Challenge test 2, the ground meat (4 kg) was divided in 2 trays of 2 kg meat each. 2 kg were amended with 2% of NaCl, 0.5% dextrose and 75 mg/kg $NaNO_2$ and 2 kg with 2% of NaCl, 0.5% dextrose and 150 mg/kg $NaNO_2$. Each tray was divided in two (1 kg each): one kg was inoculated with *L. monocytogenes* and the other kg was not inoculated with *L. monocytogenes*. Then each kg of meat was divided in three batches (333 g each) and submitted to different treatments: inoculated with PCS 20, with DSM 20074 and not inoculated with protective cultures. The 12 treatments and the relative acronyms are shown in Fig. 2. Each batch containing 333 g of meat batter was used to produce two sausages (two replicates per treatment). Sausages were then stuffed in collagen casings (45 mm diameter) (Viscofan, Navarra, Spain). For Challenge test 1, the fermentation was performed for 2 days at 23°C, 95% humidity, followed by a short ripening of 6 days at 15°C and lower humidity (80-75%). pH evaluation and microbiological analyses were performed at the following days: D0, D1, D2, D4, D6 and D8. Differently, for Challenge test 2, the fermentation was studied for 2 days followed by 5 days of short ripening in the same conditions as for the Challenge test 1. pH evaluation and microbiological analyses were performed at the following days: D0, D3, D5 and D7.

*2.5 pH analysis*

pH was measured with a pin electrode of a pHmeter (micropH2001, Crison, Barcelona, Spain) inserted directly 3 times into the sample.

*2.6 Microbiological Analysis*

Meat samples (10 g per sampling point) were aseptically removed from each *Chorizo* (two sausages per treatment) and homogenized in 90 ml of Buffered Peptone Water (BPW; AES Laboratoire, Combourg, France) for 2 min in a sterile plastic bag using a Smasher (AES

Laboratoire). For cell counts, decimal dilutions (1:10 in BPW) of the meat homogenate were prepared and aliquot of 100 µl were inoculated onto selective solid agar plates for, both, lactic acid bacteria and for pathogens growth. The counts were performed in triplicate. Lactic acid bacteria were counted on MRS agar plates, incubated anaerobically for 48 h at 37°C. Randomly picked colonies were subjected to morphological and PCR analysis with LAB specific primers (data not shown). Previously described selective solid medias were used for *L. monocytogenes* and *Salmonella* spp. counts determination. Then, plates were incubated for 24h and for 48h, respectively, at 37°C.

ISO protocols were used for the detection of natural contamination in not artificially inoculated batches: ISO 11290–1:1996 (ISO, 1996) and ISO 6579:2002 (ISO, 2002) for *L. monocytogenes* and *Salmonella* spp., respectively.

*2.7 Statistical analysis*

The results of microbiological analysis, for each sampling point, were obtained from two chorizo replicates per treatment; for each replicate counts were performed in triplicate. Data were subjected to one-way ANOVA analysis. Differences among means were tested by Duncan's multiple range test (significance $p<0.05$). All the analyses obtained from the Challenge tests were performed using the Statistica 8.0 (StatSoftInc., USA). Results of statistical analysis are presented as mean value ± standard deviation.

## 3. Results

*3.1 Challenge test 1*

*3.1.1 pH analysis*

No differences in pH were observed during the fermentation and short ripening process (data not shown). Considering the slight decrease of pH observed, 0.5% of dextrose was added in

186 pork meat batter in Challenge test 2 with the aim of stimulating the *Lactobacillus* growth and
187 acidification.
188 *3.1.2 Microbiological analysis*
189 The growing trend of *L. monocytogenes* and *Salmonella* spp. in Challenge test 1 is shown in
190 Figure 3. Both pathogens demonstrated ability to survive and colonize the pork meat in the
191 sausage model.
192 Regarding *L. monocytogenes* growth, a significantly lower counts ($p<0.05$) of 0.95 and 2.78
193 log cfu/g, were observed at day 4 and 6, respectively, in the batch with 150 mg/kg $NaNO_2$
194 and PCS20 (NLP) when compared with the batch containing nitrite but without PCS20 (NL)
195 (Figure 3A). Moreover, considering the initial inoculum, in the NL batch, an increase of 3.55
196 log cfu/g of *L. monocytogenes* counts was observed, whereas this increase was of 1.96 log
197 cfu/g in the NL+ batch P (Figure 3A) at the last sampling time (D8). Comparing control
198 batches without nitrate addition, P+L and L, significantly ($p<0.05$) lower *L. monocytogenes*
199 counts of 0.60 and 0.52 log cfu/g, were observed at day 4 and 6, respectively, whereas no
200 significant differences were observed at D8.
201 Lower *L. monocytogenes* growth was observed in batches where $NaNO_2$ was added
202 (NL/NL+P) in comparison with batches without additives (L/P+L). At the last sampling day
203 (D8), significant ($p<0.05$) decrease of *L. monocytogenes* counts of 2.37 log cfu/g was
204 observed when comparing NL+P and P+L batches, whereas significant ($p<0.05$) decrease of
205 0.58 log cfu/g was observed when comparing batches NL Ctr and L.
206 *Salmonella* spp. counts within the study period are shown in Figure 3B. *L. plantarum* PCS20
207 did not show antimicrobial activity against *Salmonella* spp. growth. However, nitrites
208 demonstrated a significant decrease ($p<0.05$) of *Salmonella* spp. growth (1.23 log cfu/g) in
209 N+S batch in comparison with batch S at D8.

Initial counts of LAB in the meat without protective culture were between 3-4.5 log cfu/g. The level of PCS20 inoculum was 5.6-5.9 log cfu/g. After 3 days, when the fermentation conditions were settled, LAB counts increased in all batches of 2.5-3.5 log cfu/g, reaching values in the range 7-9 log cfu/g in batches with protective culture and 7-8 log cfu/g in uninoculated batches, at the end of the study (data not shown).

*3.2 Challenge test 2*

*3.2.1 pH analysis*

pH trend in the meat subjected to different treatments is shown in Table 1. As expected, the addition of 0.5% dextrose caused a significant pH reduction at D7 (from 5.80 to 5.05; $p<0.05$), in all batches where *L. plantarum* PCS20 was inoculated. Differently, the addition of *L. delbrueckii* DSM 20074 did not lead to a significant pH reduction ($p>0.05$).

*3.2.2 Microbiological analysis*

Figure 4 shows the trend of *L. monocytogenes* inoculated at 3 log cfu/g in all batches. Comparing batches containing 75 mg/kg $NaNO_2$, with and without PSC20 (batches ½NL+P and ½NL Ctr, respectively, Fig. 4A), a significantly lower counts ($p<0.05$) of 2.20 and 2.62 log cfu/g of the inoculated *L. monocytogenes* were observed at day 3 and 5, respectively, in the batch where PCS20 was inoculated (½NL+P); this reduction was maintained until D7. Interestingly, considering the initial inoculum, the pathogen counts increase of only 1.61 log cfu/g in the batch ½NL+P compared with a 3.99 log cfu/g increase in the batch ½NL Ctr, at D7. On the other hand, in batches with higher nitrites concentration a significantly lower counts of *L. monocytogenes* of 3.93 log cfu/g were observed at D5, in batch containing PCS20 as protective culture (NL+P) in comparison with batch without PCS20 (NL Ctr), with a final decrease of *L. monocytogenes* of 3.84 log cfu/g at D7. In summary, at the end of the study, the pathogen growing trend was not statistically different ($p>0.05$) when compared batches with 75 or 150 mg/kg of nitrites (½NL Ctr and NL Ctr), while, in batches with

PCS20, *L. monocytogenes* counts were higher in ½NL+P compared with NL+P (difference of 1.49 log cfu/g).

Figure 4B shows the *L. monocytogenes* growth in pork meat batter with 150 mg/kg or 75 mg/kg $NaNO_2$ with or without *L. delbrueckii* DSM 20074 inoculum. At the end of the study, no significant differences in *L. monocytogenes* growth were observed among batches.

Counts of LAB growth were under the detection limit (<2 log cfu/g) in the control batches without protective culture inoculum at D0; whereas LAB counts were in the range 6-7 log cfu/g in the batches inoculated with PCS20 at D0 (Table 2). At the end of the study, LAB counts reached 7-8 log cfu/g in batches without PCS20, and 8-9.2 log cfu/g in batches with PCS20. Batches inoculated with DSM 20074 did not reach the same LAB count level as PCS20. In particular, 5.89 log cfu/g were obtained in the control batch with 150 mg/kg $NaNO_2$ and 6.36 log cfu/g in that with 75mg/kg $NaNO_2$, at D7. These counts are almost 3 log lower than those obtained for PCS20.

Similarly to the previous experiment, significant differences ($p<0.05$) were observed between D1 and D3, i.e. in the final part of the fermentation period (3$^{rd}$ day). At the end of the short ripening period, LAB reached counts in the range 7-9 log cfu/g.

## 4. Discussion

The aim of the present work was to evaluate the possibility of using protective cultures to eliminate or reduce nitrite amount in fermented meat products. For this purpose, the biopreservative activity of previously characterized LAB strains, *L. plantarum* PCS20 and *L. delbrueckii* DSM 20074, was studied against *L. monocytogenes* and *Salmonella* spp. in a dry fermented sausage model without nitrite, with half (75 mg/kg) and maximum (150 mg/kg) allowed nitrite amount considering the maximum amounts allowed in Europe Until May 2018.

The results showed that the addition of *L. plantarum* PCS20 as protective culture in nitrite-free sausages, artificially contaminated with pathogen, is capable of significantly reducing the pathogen load after 4 and 6 days from the beginning of the fermentation, although the same effect was not observed at D8. On the contrary, the antimicrobial activity of PCS20 was not observed against the cocktail of *Salmonella* strains, whereas their growth was significantly ($p<0.05$) reduced in the presence of 150 mg/kg nitrites. Interestingly, Hospital et al. (2014) obtained complete *Salmonella* inactivation using a halved nitrite amount (75 mg/kg) in fermented sausages at the end of the storage period. Other works showed the ineffectiveness of commercial protective cultures, as well as of meat-isolated *Lactobacillus* strains, against *Salmonella* spp., when inoculated in different meat models (Dias, Duarte, Ramos, Martins Santos & Schwan, 2013; Kotzekidou & Bloukas, 1998). The outcomes of this study support the Hugas (1998) consideration on the hurdle effect strategy.

Our study also shows that it is possible to reduce *Listeria* counts by inoculating the meat with *L. plantarum* PCS20 and a halved amount of nitrite (75 mg/kg). This result is particularly important considering the EC decision of adopting more stringent criteria for potential carcinogenic additives. Therefore, the combination of a protective culture with a reduced nitrite amount is an effective hurdle approach in fermented sausage production that may allow both to reduce pathogen load and to have the known positive effects of nitrites, such as the bright color.

The anti-*Listeria* activity observed is in agreement with a recent work (Giello, La Storia, De Filippis, Ercolini & Villani, 2018) that showed the effectiveness of the bacteriocin-producing *Lactobacillus curvatus* 54M16 strain in fermented sausages. Several authors pointed out that bacteriocin action can be hindered *in carnis* by bacteriocin binding to food matrixes or degradation by proteases or their production can be prevented by nitrites (Galvez, Abriouel, Lopez, & Ben, 2007; Kouakou et al., 2009). Therefore, non-bacteriocin producing strains

showing anti-listerial activity can be of great importance in fermented meat production, in particular in the presence of nitrites. This is the case of *L. plantarum* PCS20 strain, that does not produce bacteriocins (Cho, G.S., Huch, M., Hanak, A., Holzapfel, W.H., & Franz, C.M.A.P. 2010) and exerts anti-microbial activity in the presence of a reduced amount of nitrites. Its anti-microbial activity against *L. monocytogenes* can be attributed to cell-to-cell contact mechanisms or the production of organic acidic metabolites. An additional strength of our study is the use of four different *L. monocytogenes* strains, belonging to different serovars (Lianou & Koutsoumanis 2013; Scott et al. 2005).

Moreover, our work confirmed that dextrose is an important pH lowering agent, allowing to reach pH values between 4.5 and 5.5, a range in which nitrite is mainly in the undissociated state, possessing the greatest antibacterial activity. Moreover, a rapid pH drop below 5.1 is considered as a desirable acidification rate for protective cultures in fermented meat products (Ammor and Mayo 2007). On the other hand, the inability of *L. delbrueckii* DSM 20074 strain to demonstrate a significant pH lowering, resulted in an antagonistic failure against *L. monocytogenes* at the end of the study, even when 150 mg/kg of $NaNO_2$ were added.

Our study supports the outcomes of a recent survey (Hung et al. 2016), in which meat industry stakeholders expressed interest in the development of innovative and healthier processed meat products but asked the scientific community to provide additional evidences of the microbiological safety of developed approaches. Consumers are important players in industrial innovation shaping, thus the taste and the microbiological safety are the most important criteria for the novel food formulations (Bedale et al. 2016, Hung et al. 2016).

## 5. Conclusions

This work pointed out that a combined approach based on half of the allowed nitrite amount and of protective culture may be effective in a dry-fermented meat product (*chorizo*) to

reduce the growth of *L. monocytogenes*, a pathogen with high case fatality incidence and causing severe diseases. This study has also shown that the effectiveness of nitrites against this pathogen is not related to their amount; the inoculation with lactic acid bacteria contributing to pH lowering and to reach the effective dissociation state of nitrite is probably a crucial factor for their effectiveness. However, further studies aimed at better elucidating the anti-microbial mechanisms against pathogens in food matrix need to be pursued.

In conclusion, the results obtained from this study will provide additional scientific evidence in the evaluation of microbiological and preservative risks/benefits in fermented meat products. The proposed combined hurdle approach (a reduced amount of nitrite plus the inoculation of a protective culture) is promising for innovative fermented meat products development.

## Conflict of interest

The authors declare that they have no conflict of interest.


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464 **Figure Captions**

465

466 **Figure 1 Study design of the 12 treatments related to** Challenge test 1. Legend: **Ctr**=meat batter;
467 **Ctr-N**=150 mg/kg $NaNO_2$ added; **Ctr-P**=PCS20 added; **Ctr-NP**=PCS20+150 mg/kg $NaNO_2$ added;
468 **L**=*L. monocytogenes* added; **N+L**=150 mg/kg $NaNO_2$+ *L. monocytogenes* added; **P+L**=PCS20+*L.*
469 *monocytogenes* added; **NP+L**=150 mg/kg $NaNO_2$+ PCS20+*L. monocytogenes* added; **S**=*Salmonella*
470 spp. added; **N+S**=150 mg/kg $NaNO_2$ + *Salmonella* spp. added; **P+S**=PCS20+*Salmonella* spp. added;
471 **NP+S**=150 mg/kg $NaNO_2$ +PCS20+*Salmonella* spp. added. For each condition two sausages were
472 prepared and processed.

473

474 **Figure 2 Study design** Challenge test 2. Legend: **N Ctr**=meat batter added with 150mg/kg $NaNO_2$;
475 **½N Ctr**=75 mg/kg $NaNO_2$ added; **N+P**=150mg/kg $NaNO_2$+PCS20 added; **½N+P**=75mg/kg
476 $NaNO_2$+PCS20 added; **N+D**=150mg/kg $NaNO_2$+DSM 20074 added; **½N+D**=75mg/kg $NaNO_2$+DSM
477 20074 added; **NL Ctr**=150mg/kg $NaNO_2$+*L. monocytogenes* added; **½NL Ctr**=75mg/kg $NaNO_2$+*L.*
478 *monocytogenes* added; **NL+P**=150mg/kg $NaNO_2$+*L. monocytogenes*+PCS20 added; **NL+P**=75mg/kg
479 $NaNO_2$+*L. monocytogenes*+ PCS20 added; **NL+D**=150mg/kg $NaNO_2$+*L. monocytogenes*+DSM
480 20074 added; **½NL+D**=75mg/kg $NaNO_2$+DSM 20074 added. For each condition two sausages were
481 prepared and processed.

482

483 **Figure 3** Antimicrobial activity of *L. plantarum* PCS20 against *L. monocytogenes* and *Salmonella*
484 spp. in dry fermented sausage with and without 150 mg/kg $NaNO_2$.
485 A) *L. monocytogenes* counts within the ripening period. **L**=*L. monocytogenes* added; **N+L**=150 mg/kg
486 $NaNO_2$+ *L. monocytogenes* added; **P+L**=PCS20+*L. monocytogenes* added; **NP+L**=150 mg/kg
487 $NaNO_2$+ PCS20+*L. monocytogenes* added; B) *Salmonella* spp. counts within the ripening period.
488 **S**=*Salmonella* spp. added; **N+S**=150 mg/kg $NaNO_2$ + *Salmonella* spp. added;
489 **P+S**=PCS20+*Salmonella* spp. added; **NP+S**=150 mg/kg $NaNO_2$ +PCS20+*Salmonella* spp. added.
490

**Figure 4** Antimicrobial activity of *L. plantarum* PCS20 (A) and *L. delbrueckii* DSM20074 (B) against *L. monocytogenes* in dry fermented sausage added with 75 or 150 mg/kg $NaNO_2$. A) *L. monocytogenes* counts in batches inoculated with or without *L. plantarum* PCS20. Legend: **NL Ctr**=150mg/kg $NaNO_2$+*L. monocytogenes* added; **½NL Ctr**=75mg/kg $NaNO_2$+*L. monocytogenes* added; **NL+P**=150mg/kg $NaNO_2$+*L. monocytogenes*+PCS20 added; **½NL +P**=75mg/kg $NaNO_2$+*L. monocytogenes*+PCS20 added. B) *L. monocytogenes* counts in batches inoculated with *L. delbrueckii* DSM 20074. Legend: **N+D**=150mg/kg $NaNO_2$+DSM 20074 added; **½N+D**=75mg/kg $NaNO_2$+DSM 20074 added; **NL Ctr**=150mg/kg $NaNO_2$+*L. monocytogenes* added; **½NL Ctr**=75mg/kg $NaNO_2$+*L. monocytogenes* added; **NL+P**=150mg/kg $NaNO_2$+*L. monocytogenes*+PCS20 added; **NL+P**=75mg/kg $NaNO_2$+*L. monocytogenes*+PCS20 added; **NL+D**=150mg/kg $NaNO_2$+*L. monocytogenes* +DSM 20074 added; **½NL+D**=75mg/kg $NaNO_2$+*L. monocytogenes*+DSM 20074 added.

**Table 1. Challenge test 2.** The trend of pH during the fermentation and ripening period

| Batches** | Days * | | | |
|---|---|---|---|---|
| | **0** | **3** | **5** | **7** |
| **N Ctr** | 5.96 ±0.03$^{B}$ | 5.78 ±0.03$^{C}$ | 6.12 ±0.06$^{A}$ | 6.03 ±0.06$^{B}$ |
| **½N Ctr** | 5.90 ±0.09$^{B}$ | 5.99 ±0.03$^{B}$ | 6.11 ±0.04$^{A}$ | 5.90 ±0.01$^{B}$ |
| **½NL Ctr** | 5.77 ±0.08$^{B}$ | 5.89 ±0.02$^{A}$ | 5.92 ±0.02$^{A}$ | 5.91 ±0.02$^{A}$ |
| **NL Ctr** | 5.85 ±0.03$^{B}$ | 5.99 ±0.01$^{A}$ | 6.10 ±0.04$^{A}$ | 5.85 ±0.02$^{B}$ |
| **N+P** | 5.86 ±0.06$^{A}$ | 5.44 ±0.02$^{B}$ | 5.23 ±0.02$^{C}$ | 5.02 ±0.04$^{D}$ |
| **½N+P** | 5.85 ±0.07$^{A}$ | 5.28 ±0.06$^{B}$ | 5.21 ±0.02$^{B}$ | 5.05 ±0.02$^{C}$ |
| **NL+P** | 5.77 ±0.06$^{A}$ | 5.31 ±0.01$^{B}$ | 5.14 ±0.04$^{C}$ | 5.09 ±0.04$^{C}$ |
| **½NL+P** | 5.83 ±0.01$^{A}$ | 5.30 ±0.01$^{B}$ | 5.16 ±0.01$^{C}$ | 5.02 ±0.01$^{D}$ |
| **N+D** | 5.87 ±0.03$^{A}$ | 5.93 ±0.04$^{A}$ | 5.93 ±0.05$^{A}$ | 5.89 ±0.04$^{A}$ |
| **½N+D** | 5.80 ±0.04$^{B}$ | 5.93 ±0.02$^{A}$ | 5.93 ±0.02$^{A}$ | 5.89 ±0.05$^{A}$ |
| **NL+D** | 6.05 ±0.01$^{A}$ | 5.94 ±0.03$^{B}$ | 6.04 ±0.05$^{A}$ | 5.97 ±0.03$^{B}$ |
| **½NL+D** | 6.17 ±0.04$^{A}$ | 5.91 ±0.01$^{B}$ | 5.94 ±0.04$^{B}$ | 5.80 ±0.04$^{C}$ |

* Data are expressed as mean of n=3 measurements.

** Batch : **N Ctr**=meat batter added with 150mg/kg $NaNO_2$; **½N Ctr**=75 mg/kg $NaNO_2$ added; **½NL Ctr**=75mg/kg $NaNO_2$+*L.monocytogenes* added; **NL Ctr**=150mg/kg $NaNO_2$+*L.monocytogenes* added; **N+P**=150mg/kg $NaNO_2$+PCS20 added; **½N+P**=75mg/kg $NaNO_2$+PCS20 added; **NL+P**=150mg/kg $NaNO_2$+*L.monocytogenes*+PCS20 added; **½NL+P**=75mg/kg $NaNO_2$+*L.monocytogenes*+ PCS20 added; **N+D**=150mg/kg $NaNO_2$+DSM 20074 added; **½N+D**=75mg/kg $NaNO_2$+DSM 20074 added; **NL+D**=150mg/kg $NaNO_2$+*L.monocytogenes*+DSM 20074 added; **½NL+D**=75mg/kg $NaNO_2$+DSM 20074 added.

***$^{A,B,C}$: Mean values in the same row (corresponding to the same batch) differ significantly ($p < 0.05$).

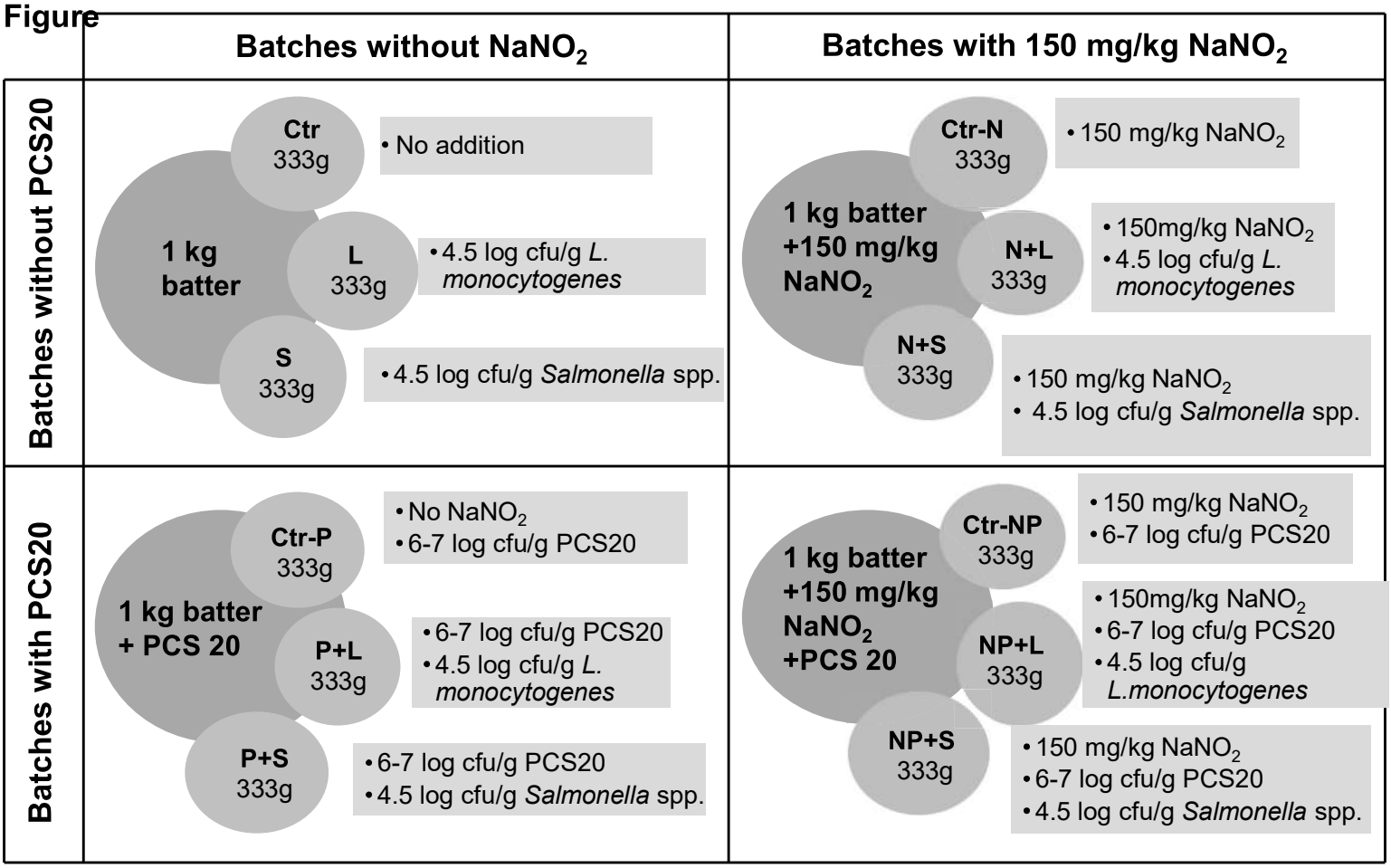
Table

**Table 2. Challenge test 2.** LAB counts (log cfu/g) within the 7 days of fermentation and ripening period

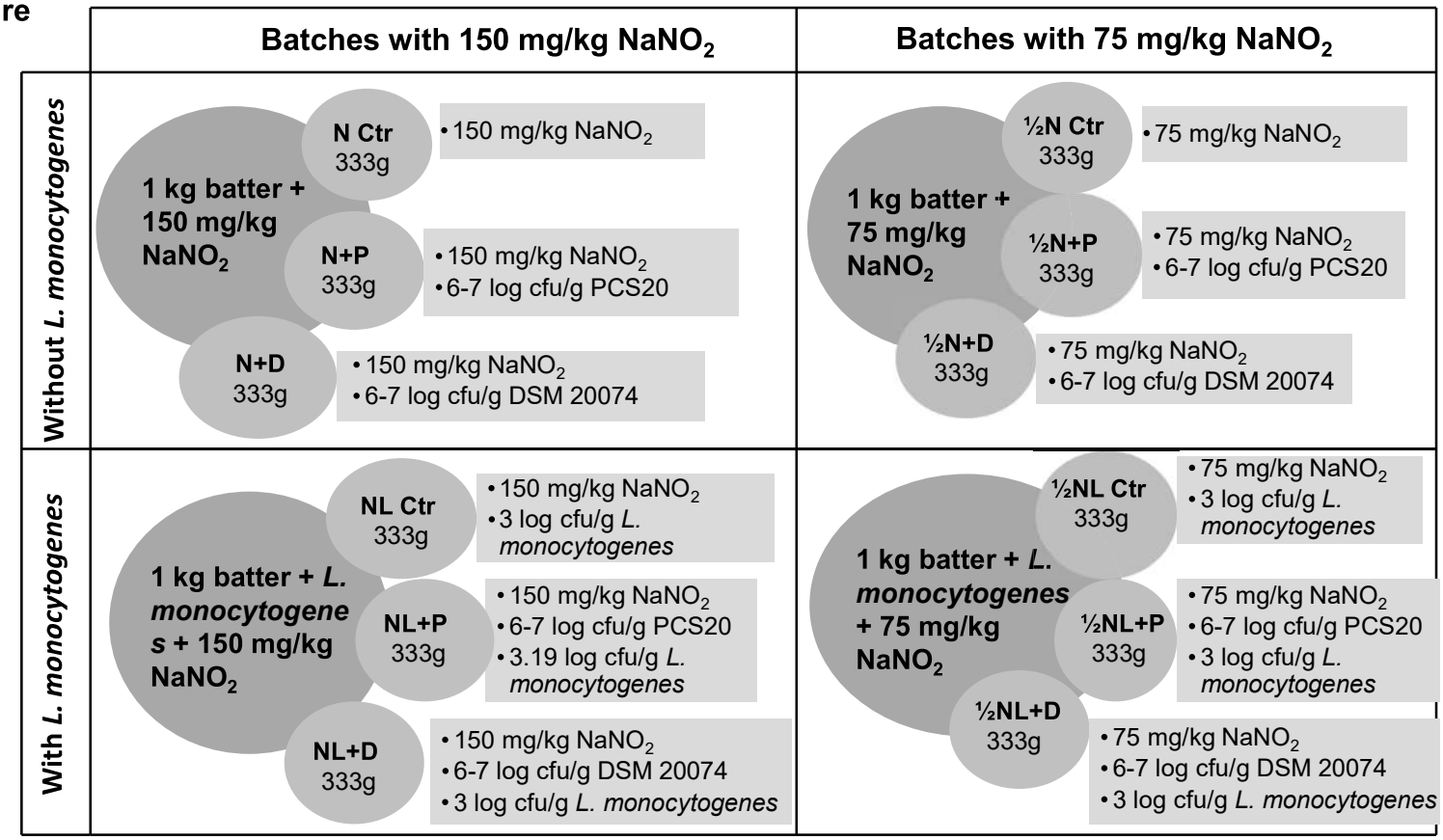
| Batches** | Days * | | | |
|---|---|---|---|---|
| | **0** | **3** | **5** | **7** |
| **N Ctr** | <2 ±0.00$^{D}$ | 6.63 ±0.25$^{C}$ | 7.82 ±0.12$^{B}$ | 7.89 ±0.12$^{A}$ |
| **½N Ctr** | <2 ±0.00$^{B}$ | 7.18 ±0.46$^{A}$ | 7.60 ±0.17$^{A}$ | 7.09 ±0.34$^{A}$ |
| **NL Ctr** | <2 ±0.00$^{D}$ | 6.59 ±0.28$^{C}$ | 8.15 ±0.07$^{A}$ | 7.38 ±0.29$^{B}$ |
| **½NL Ctr** | <2 ±0.00$^{B}$ | 6.89 ±0.27$^{A}$ | 7.15 ±0.51$^{A}$ | 7.43 ±0.28$^{A}$ |
| **N+P** | 6.34 ±0.15$^{C}$ | 8.87 ±0.20$^{B}$ | 9.09 ±0.12$^{AB}$ | 9.13 ±0.10$^{A}$ |
| **½N+P** | 6.34 ±0.13$^{D}$ | 9.06 ±0.13$^{B}$ | 9.26 ±0.06$^{A}$ | 8.03 ±0.13$^{C}$ |
| **NL+P** | 6.59 ±0.17$^{C}$ | 8.86 ±0.12$^{B}$ | 9.10 ±0.07$^{A}$ | 9.14 ±0.09$^{A}$ |
| **½NL+P** | 6.61 ±0.13$^{B}$ | 9,04 ±0.07$^{A}$ | 9.04 ±0.08$^{A}$ | 9.17 ±0.08$^{A}$ |
| **N+D** | 5.71 ±0.26$^{C}$ | 6.49 ±0.22$^{B}$ | 7.29 ±0.38$^{A}$ | 5.89 ±0.37$^{C}$ |
| **½N+D** | 5.84 ±0.11$^{D}$ | 6.98 ±0.19$^{B}$ | 7.44 ±0.15$^{A}$ | 6.36 ±0.13$^{C}$ |
| **NL+D** | 5.98 ±0.11$^{D}$ | 6.52 ±0.12$^{C}$ | 7.69 ±0.28$^{A}$ | 7.19 ±0.08$^{B}$ |
| **½NL+D** | 6.09 ±0.15$^{C}$ | 6.35 ±0.04$^{B}$ | 7.44 ±0.15$^{A}$ | 7.58 ±0.13$^{A}$ |

* Data are expressed as mean of n=3 measurements.
** Batch **N Ctr**=meat batter added with 150mg/kg $NaNO_2$; **½N Ctr**=75 mg/kg $NaNO_2$ added; **N+P**=150mg/kg $NaNO_2$+PCS20 added; **½N+P**=75mg/kg $NaNO_2$+PCS20 added; **N+D**=150mg/kg $NaNO_2$+DSM 20074 added; **½N+D**=75mg/kg $NaNO_2$+DSM 20074 added; **NL Ctr**=150mg/kg $NaNO_2$+*L.monocytogenes* added; **½NL Ctr**=75mg/kg $NaNO_2$+*L.monocytogenes* added; **NL+P**=150mg/kg $NaNO_2$+*L.monocytogenes*+PCS20 added; **NL+P**=75mg/kg $NaNO_2$+*L.monocytogenes*+ PCS20 added; **NL+D**=150mg/kg $NaNO_2$+*L.monocytogenes*+DSM 20074 added; **½NL+D**=75mg/kg $NaNO_2$+DSM 20074 added.
***$^{A,B,C}$: Mean values in the same row (corresponding to the same batch) differ significantly ($p < 0.05$).

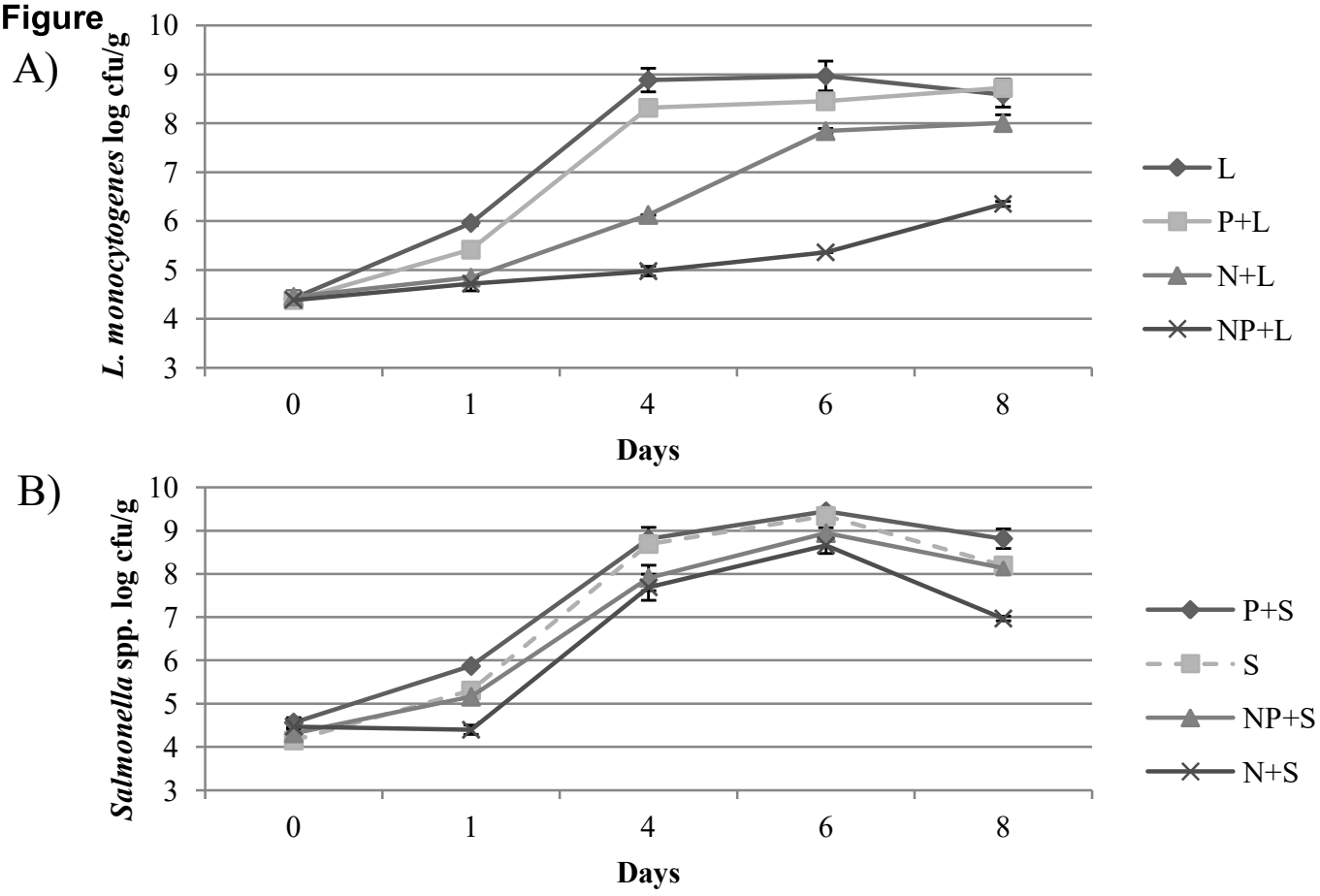
Figure


Batches without $NaNO_2$
Batches with 150 mg/kg $NaNO_2$
Batches without PCS20
Batches with PCS20
1 kg batter
Ctr 333g
• No addition
L 333g
• 4.5 log cfu/g *L. monocytogenes*
S 333g
• 4.5 log cfu/g *Salmonella* spp.
1 kg batter +150 mg/kg $NaNO_2$
Ctr-N 333g
• 150 mg/kg $NaNO_2$
N+L 333g
• 150mg/kg $NaNO_2$
• 4.5 log cfu/g *L. monocytogenes*
N+S 333g
• 150 mg/kg $NaNO_2$
• 4.5 log cfu/g *Salmonella* spp.
1 kg batter + PCS 20
Ctr-P 333g
• No $NaNO_2$
• 6-7 log cfu/g PCS20
P+L 333g
• 6-7 log cfu/g PCS20
• 4.5 log cfu/g *L. monocytogenes*
P+S 333g
• 6-7 log cfu/g PCS20
• 4.5 log cfu/g *Salmonella* spp.
1 kg batter +150 mg/kg $NaNO_2$ +PCS 20
Ctr-NP 333g
• 150 mg/kg $NaNO_2$
• 6-7 log cfu/g PCS20
NP+L 333g
• 150mg/kg $NaNO_2$
• 6-7 log cfu/g PCS20
• 4.5 log cfu/g *L.monocytogenes*
NP+S 333g
• 150 mg/kg $NaNO_2$
• 6-7 log cfu/g PCS20
• 4.5 log cfu/g *Salmonella* spp.

Figure

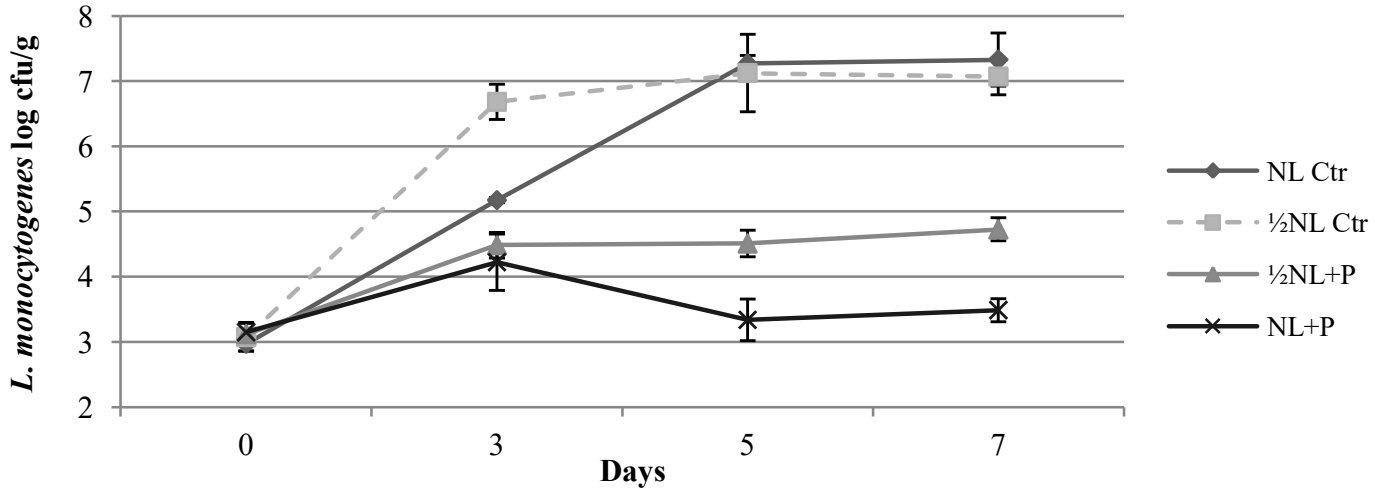

Batches with 150 mg/kg $NaNO_2$
Batches with 75 mg/kg $NaNO_2$
Without *L. monocytogenes*
With *L. monocytogenes*
1 kg batter + 150 mg/kg $NaNO_2$
N Ctr 333g
• 150 mg/kg $NaNO_2$
N+P 333g
• 150 mg/kg $NaNO_2$
• 6-7 log cfu/g PCS20
N+D 333g
• 150 mg/kg $NaNO_2$
• 6-7 log cfu/g DSM 20074
1 kg batter + 75 mg/kg $NaNO_2$
½N Ctr 333g
• 75 mg/kg $NaNO_2$
½N+P 333g
• 75 mg/kg $NaNO_2$
• 6-7 log cfu/g PCS20
½N+D 333g
• 75 mg/kg $NaNO_2$
• 6-7 log cfu/g DSM 20074
1 kg batter + *L. monocytogenes* + 150 mg/kg $NaNO_2$
NL Ctr 333g
• 150 mg/kg $NaNO_2$
• 3 log cfu/g *L. monocytogenes*
NL+P 333g
• 150 mg/kg $NaNO_2$
• 6-7 log cfu/g PCS20
• 3.19 log cfu/g *L. monocytogenes*
NL+D 333g
• 150 mg/kg $NaNO_2$
• 6-7 log cfu/g DSM 20074
• 3 log cfu/g *L. monocytogenes*
1 kg batter + *L. monocytogenes* + 75 mg/kg $NaNO_2$
½NL Ctr 333g
• 75 mg/kg $NaNO_2$
• 3 log cfu/g *L. monocytogenes*
½NL+P 333g
• 75 mg/kg $NaNO_2$
• 6-7 log cfu/g PCS20
• 3 log cfu/g *L. monocytogenes*
½NL+D 333g
• 75 mg/kg $NaNO_2$
• 6-7 log cfu/g DSM 20074
• 3 log cfu/g *L. monocytogenes*

**Figure**

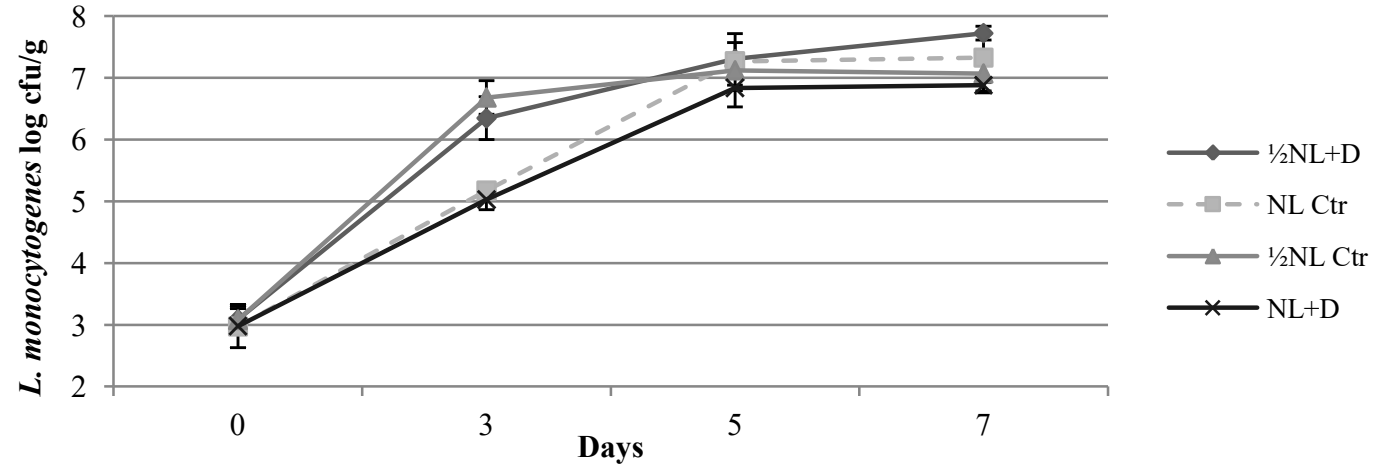

A)
L. monocytogenes log cfu/g
10
9
8
7
6
5
4
3
0
1
4
6
8
Days
L
P+L
N+L
NP+L
B)
Salmonella spp. log cfu/g
10
9
8
7
6
5
4
3
0
1
4
6
8
Days
P+S
S
NP+S
N+S

**Figure**

A)


L. monocytogenes log cfu/g
8
7
6
5
4
3
2
0
3
5
7
Days
NL Ctr
½NL Ctr
½NL+P
NL+P

B)


L. monocytogenes log cfu/g
8
7
6
5
4
3
2
0
3
5
7
Days
½NL+D
NL Ctr
½NL Ctr
NL+D