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# The potential of bacterial cultures to degrade the mutagen 2-methyl-1,4-dinitro-pyrrole in a processed meat model

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ABSTRACT

Processed meats are classified by the International Agency for Research on Cancer as category 1 because their consumption increase the incidence of colorectal and stomach cancers. Meat processing widely employs nitrite and sorbate as preservatives. When these preservatives are concomitantly used in non-compliant processes, they may react and produce the mutagen 2-methyl-1,4-dinitro-pyrrole (DNMP). This study aimed to evaluate the ability of different bacteria isolated from food matrices to biodegrade DNMP in *in vitro* reactions and in a processed meat model. A possible mechanism of biodegradation was also tested. *In vitro* experiments were performed in two steps. In the first one, only one strain out of 13 different species did not interact with DNMP. In the following step, an empirical conversion factor was calculated to assess the conversion of DNMP to 4-amino-2-methyl-1-nitro-pyrrole by the strains. The most efficient strains were *Staphylococcus xylosus* LYOCARNI SXH-01, *Lactobacillus fermentum* LB-UFSC 0017, and *Lactobacillus casei* LB-UFSC 0019, which yielded conversion factors of 0.62, 0.60, and 0.43, respectively. Thus, such strains were individually added to the processed meat model and completely degraded the DNMP. Moreover, *S. xylosus* degraded DNMP in less than 30 min. The enzymatic mechanism was evaluated using its cell-free extract. It showed that, in the aerobic system, reduction rates were 30.321 and 22.411 nmol/mg of protein/min using NADH and NADPH, respectively. A DNMP reductase was assigned to the extract and a potential presence of an oxygen insensitive nitroreductase type I B was considered. Thus, biotechnological processes may be an efficient strategy to eliminate the DNMP from meat products and to increase food safety.

## 1. Introduction

Food safety usually requires several laboratory measures in order to guarantee food quality, thus avoiding the potential spread of diseases that may generate problems for individuals, for industry, and the government. The use of preservatives, technological treatments (*e.g.* thermal processing), and regulatory limits should be met by the industry to avoid transmitting diseases or forming harmful compounds, which are potentially deleterious to human health. When preservatives are correctly used, they ensure that microbiological reactions will be slowed down or even inhibited. Besides, they increase the product's market viability and consequently help to reduce economic losses. They also reduce the hazard from the multiplication of potentially pathogenic microbes to consumers' health.

The main preservatives used in meat processing are organic acids, bacteriocins, nitrite, and sorbic acid (Lee & Paik, 2016). The reckless and/or abuse of preservatives and additives associated or not with processing conditions may generate significant concentrations of toxic chemical contaminants (IARC Working Group on the evaluation of carcinogenic risks to Humans (2018) (2018), 2018; Molognoni, Daguer, Motta, Merlo, & De Dea Lindner, 2019). Although the simultaneous use of nitrite and sorbic acid is not allowed for most categories of meat products, cases of non-compliant samples of meat products in which these preservatives were used together are still frequent (Molognoni et al., 2018).

In the 1970s, the potential formation of DNA damaging compounds

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by reaction of sorbic acid and nitrite was studied, and thus the 2-methyl-1,4-dinitro-pyrrole (DNMP) compound was identified (Kito, Namiki, & Tsuji, 1978). DNMP is formed by nitration or nitrosation followed by decarboxylation of sorbic acid under acidic conditions, heat treatment above 60 °C and molar ratio of sodium nitrite and sorbic acid of 8:1 (Namiki, Osawa, Ishibashi, Namiki, & Tsuji, 1981; Pérez-Prior et al., 2008; Sofos, 1981). The compound strong mutagenic activity was demonstrated by the *Salmonella* reverse mutation assay (Ames assay) and by the rec-assay using *Bacillus subtilis* (Namiki, Udaka, Osawa, Tsuji, & Kada, 1980).

In 2015, the European Food Safety Authority (EFSA) called on the scientific community to provide more toxicological information on the occurrence of DNMP in real conditions of meat processing (European Food Safety Authority, 2015). Processed meats (*e.g.* bacon, ham, and sausage) were classified as class I carcinogen by the International Agency for Research on Cancer (IARC) of the World Health Organization. This classification was based on evidence that the consumption of processed meat increases the incidence of colorectal and stomach cancer (IARC, 2018). In 2018, by means of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), our research group evaluated different cooked meat products sold in Brazil. For the first time, the occurrence of DNMP was reported in several samples (Molognoni et al., 2018).

Lactic acid bacteria (LAB) and coagulase-negative staphylococci (CNS), such as *Staphylococcus xylosus*, are generally recognized as safe (GRAS status) because they have a long history of application to food processing (Władyka & Bonar, 2018). They can be used by the industry (individually or in combination) as starter cultures in fermented products for their ability to improve texture, flavor, and nutrient availability (Hospital et al., 2015). Furthermore, some strains can be used as biocontrol agents since they can inhibit spoilage and growth of pathogenic microorganisms (Ben Slima et al., 2017). Moreover, some microorganisms are able to enzymatically metabolize toxic compounds such as heterocyclic aromatic amines (HAAs), polycyclic aromatic hydrocarbons (PAHs), and biogenic amines, using them as a source of carbon and nitrogen (Bartkiene et al., 2017; Nowak & Libudzisz, 2009; Stidl, Sontag, Koller, & Knasmüller, 2008).

Nitroreductases are enzymes present in several species of bacteria, fungi, protozoa, and mammals that play a major role in the metabolization of nitro compounds. They have received great attention from the scientific community, due to their ability to mediate the toxicity of these chemical species. Thus, several biotechnological and clinical applications with nitroreductases have been proposed. Nitroreductases may catalyze the reduction of the nitro group in the presence or absence of oxygen (type I) or only in the absence of oxygen (type II). These enzymes can use flavin mononucleotide (FMN) or a flavin adenine dinucleotide (FAD) as a prosthetic group and nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) as reducing agent. The type I nitroreductases can be differentiated in A and B. Type I A uses NADPH as an electron source, whilst type I B uses both NADH and NADPH sources (De Oliveira, Bonatto, Antonio, & Henriques, 2010).

Because DNMP may be present in processed meats, methods for its remediation must be studied and employed to mitigate DNMP hazard and, thus, to increase food safety. Therefore, the main goal of this study was to evaluate the ability of different GRAS bacteria to biodegrade the mutagenic compound DNMP *in vitro* and in a processed meat model, as well as to assess the efficiency of DNMP elimination from meat products by bacterial nitroreductases.

## 2. Materials and methods

### 2.1. Reagents, standards, samples, and microorganisms

Ultrapure water was produced by the Integral 10 Milli-Q system (Millipore SAS, Molsheim, France). Reagents and solvents were of analytical and chromatographic grades, respectively; standards were at least 98% pure. Sodium nitrite, potassium sorbate, 1-methylimidazole (1-MEI), ascorbic acid, methanol, acetonitrile, 2,2′,2″,2‴-(1,2-ethanediyldinitrilo)tetraacetic acid (EDTA), NADH, NADPH, potassium phosphate monobasic ($KH_2PO_4$), dipotassium phosphate ($K_2HPO_4$), dimethyl sulfoxide (DMSO), 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), and bovine serum albumin (BSA) were supplied by Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The Man, Rogosa, and Sharpe (MRS) broth, brain-heart infusion (BHI) broth, and lactose were supplied by Merck KGaA (Darmstadt, Germany).

The synthesis of DNMP was conducted according to Kito et al. (1978) and Namiki et al. (1981) and reproduced in detail by our research group (Molognoni et al., 2018). 4-amino-2-methyl-1-nitro-pyrrole (NMAP) was obtained by the method described by Osawa, Ishibashi, Namiki, Kada, and Tsuji (1986). The DNMP fortification solution (0.01 and 1 mg/mL) was prepared in DMSO. As a control, the viability of the strains, after contact with DMSO, was tested verifying the growth in culture medium.

The samples of meat model were prepared in a pilot plant under specific processing conditions, aiming to obtain endogenous DNMP, according to Molognoni et al. (2018). Sodium nitrite and potassium sorbate were used in the ratio of 8:1, at concentrations of 0.3 and 0.038%, respectively. Food grade phosphoric acid (0.25%) was used to lead the pH of the meat emulsion to 5.0 ± 0.2. The experimental formulation of meat model consisted of mechanically deboned chicken meat (60%), pork (26%), cassava starch (5.5%), textured soy protein (3.5%), sodium chloride (3.5%), sucrose (0.6%), and sodium pyrophosphate (0.3%).

The bacterial strains were provided by Laboratory of Bioprocess from Federal University of Santa Catarina (LB-UFSC), Sacco Company and American Type Collection Culture (ATCC). All the employed strains were potential cultures for meat fermentation. Stock cultures were maintained on MRS (supplemented or not with lactose) or BHI broth with 20% (w/v) glycerol at −80 °C. *Lactobacillus helveticus* ATCC 12046, *Lactobacillus plantarum* ATCC 8014, *Lactobacillus rhamnosus* ATCC 7469, *Lactococcus lactis* subsp. *lactis* ATCC 19435, *Pediococcus pentosaceus* isolated from the LYOCARNI RHM-33, *S. xylosus* isolated from the LYOCARNI SXH-01, *Lactobacillus paracasei* subsp. *paracasei* LB-UFSC 0014, *Lactobacillus fermentum* LB-UFSC 0017, *Lactobacillus acidophilus* LB-UFSC 0018, *Lactobacillus casei* LB-UFSC 0019, *Lactobacillus sakei* LB-UFSC 0022, *Streptococcus thermophilus* LB-UFSC 0025, and *Weissella minor* LB-UFSC 4458 were used in the screening to determine their ability to biodegrade DNMP.

### 2.2. Biodegradation of DNMP in vitro and in the processed meat model

The biodegradation of DNMP by the action of the microorganisms was evaluated using the methodology described by Shu, Kingston, Van Tassell, Wilkins, and Rosenkranz (1991), with adaptations. All cultures were reactivated overnight in specific culture broth medium (BHI, MRS or MRS with lactose) at 37 ± 1 °C. A density of $10^7$ cells/mL was established to perform the experiments. After incubation, the cultures were centrifuged at 3,800-*g* for 10 min at 4 °C. The resultant pellet was washed with 10 mL of 0.1 mol/L HEPES buffer solution (pH = 7.6). For the *in vitro* reactions, each culture was resuspended in a final volume of 10 mL of HEPES and an aliquot of that suspension (1 mL) was used for inoculum with 100 μg/L of DNMP. For the meat model, 2.0 ± 0.1 g of formulated meat was inoculated with 1 mL of culture resuspended in HEPES and homogenized to form a paste. This procedure was repeated adding 100 μg/L of DNMP. Blank and analyte recovery controls were prepared without the microbial inoculum and subjected to the same procedures. Finally, the test samples were incubated at 37 ± 1 °C and the reaction was stopped on an ice bath and submitted to LC-MS/MS analysis (item 2.4).

### 2.3. Screening of microorganisms

The bacterial screening was performed in two steps with the aim of identifying the highest capacity for biodegradation of DNMP among the tested strains. First, the selection of the assessed microorganisms considered their ability to decrease the DNMP concentration, according to the Eq. (1):

$$\%\ \text{Residual of DNMP} = \left(\frac{\text{Total DNMP}_2}{\text{Total DNMP}_1}\right) \times 100 \quad (1)$$

where: Total $DNMP_1$: amount of DNMP fortified in the experiment in μg/L; Total $DNMP_2$: amount of DNMP after incubation with the microorganism in μg/L. DNMP values were corrected by the recovery error.

In the second step, a more specific and selective criterion was adopted. Based on the analytical signals (in cps) of DNMP and NMAP chromatographic peaks yielded by LC-MS/MS measurements, an empirical conversion factor was calculated to describe the microorganisms' ability to biodegrade DNMP. Each microorganism selected in the first step was submitted to a concentration gradient of DNMP in the range of 50 to 5000 μg/L and incubated as described in the section above. Comparatively, a solution of ascorbic acid (1%) was submitted to the same procedure. The linearization of the graph obtained from the NMAP signal (in cps) for each fortified concentration (μg/L of DNMP) and for each microorganism was performed with the Analyst software version 1.6.2 from Sciex (Framingham, USA). The purity of each chromatographic peak was considered. The derivative of the functional relationship calculated for each microorganism was divided by the reference derivative obtained from the linear model in the x-axis (μg/L of DNMP) *versus* y-axis (cps of DNMP). Thus, the empirical conversion factor was calculated by the Eq. (2):

$$\text{Empirical conversion factor} = \frac{f'(x1) = \lim_{h=0} \frac{f(x1+h) - f(x1)}{h}}{f'(x2) = \lim_{h=0} \frac{f(x2+h) - f(x2)}{h}} \quad (2)$$

where: f′(x1): the derivative of the functional ratio of concentration in μg/L of DNMP *versus* cps of NMAP, (in cps L/μg units); f′(x2): the derivative of the functional ratio of DNMP concentration (in μg/L) *versus* DNMP cps, also in cps L/μg.

### 2.4. Instrumentation and analytical methods

The quantitation of DNMP and NMAP concentrations was performed by means of LC-MS/MS analysis. With this purpose, the 5500 QTRAP hybrid triple quadrupole-linear ion trap-mass spectrometer (Sciex, Framingham, USA) was used. It was equipped with electrospray ionization (ESI) source and coupled to a 1290 Infinity high-performance liquid chromatography (HPLC) from Agilent Technologies (Deutschland GmbH, Waldbronn, Germany). Chromatographic separation was performed in reversed-phase with the Zorbax 300SB-CN column (150 mm x 4.6 mm i.d., 5 μm particle size, 300 Å), supplied by Agilent Technologies (Santa Clara, USA).

DNMP and NMAP analyzes were performed according to the validated methods previously described by Mologni et al. (2018) and Mologni, Motta, Daguer, and De Dea Lindner (2020), respectively. For this purpose, the molecular detection was optimized by infusing the compounds at ± 50 μg/L in a flow rate of 10 μL/min in the mass spectrometer. Ionization was performed by electrospray ionization (ESI) in positive mode. The detection was performed by multiple reaction monitoring (MRM) and the ratio between fragmentation ions and enhanced product ion in the QTRAP device of the third quadrupole.

Analytes were extracted from the processed meat model in accordance to Mologni et al. (2020). For the samples submitted to the *in vitro* study, the solutions of HEPES and biomass were filtered using PTFE filter membranes with 0.22 μm pore size. Aliquots of 200 μL were directly transferred to glass vials with inserts and injected into the LC-MS/MS system. Details about LC-MS/MS analytical performance and experimental uncertainties are shown in Supplementary material.

### 2.5. Extraction of the bacterial intracellular material

The cell-free extraction method was performed according to Levine, Gregorio, Jewett, Watts, and Oza (2019), with adaptations. After overnight growth of the strain that presented the highest capacity to convert DNMP to NMAP, the cells were harvested by centrifugation at 4000-*g* at 4 °C for 10 min. For the preparation of the bacterial cell-free extract, the cell pellet was washed twice with 10 mL of 20 mmol/L HEPES buffer solution (pH = 7.4) and resuspended in 1 mL of the same solution. The bacterial cell wall was disrupted using sonic dismembrator with a probe model 100 (Fisher Scientific, Hampton, USA) for 30 s, in the intensity four, alternated with 30 s without sonication on an ice bath. This process was repeated tenfold. Cell debris were removed by centrifugation at 20000-*g* at 4 °C for 20 min, and the supernatant was used as the cell-free extract.

### 2.6. Nitroreductase analysis

The protein content of the cell extract was evaluated by the Bradford method (Bradford, 1976) using serum albumin as reference. The nitroreductase assay was performed in accordance to Shu et al. (1991) in a 96-well microtiter plate reader (Infinite 200pro, Tecan, Männedorf, Switzerland) in a media containing potassium phosphate (100 mmol/L), EDTA (0.1 mmol/L), NADH (0.5 mmol/L) or NADPH (0.5 mmol/L), and DNMP (0.5 mmol/L). The nitroreductase activity was evaluated by the rate of absorbance decrease at 340 nm ($\varepsilon$ = 6220 mol/L cm) within 10–15 min of reaction, at 35 °C. The assay was performed in triplicate and the results were expressed as means ± standard deviations.

### 2.7. Statistical analysis

All experiments were performed at least in triplicates and the results were expressed as mean ± standard deviation. Statistical analysis of the data was performed using the STATISTICA software version 10.0 (StatSoft, Tulsa, OK, USA). To assess significant differences ($p < 0.05$) between the analyses, the variances were compared using the Tukey test (ANOVA).

## 3. Results and discussion

### 3.1. Biodegradation of DNMP in vitro

The potential DNMP biodegradation was evaluated *in vitro* using different GRAS bacterial strains. In this screening, all strains were of genera and species commonly employed in the fermentation of meat products and reduction of nitro and nitroaromatic compounds, such as *Lactobacillus* sp., *Lactococcus* sp., *St. thermophilus*, *P. pentosaceus*, *W. minor*, and *S. xylosus* (De Dea Lindner, 2016; Guillén, Curiel, Landete, Muñoz, & Herraiz, 2009; Zarour et al., 2017). The use of the empirical conversion factor was due to the unavailability of a NMAP analytical standard. The empirical conversion factor selected the strains satisfactorily and to our knowledge, this approach was unprecedented in a biotechnological chemical screening. The success of this conversion factor was due to three theoretical and experimental conditions: (i) same reference and detection criteria (same fragment ions in MS/MS); (ii) characteristic precursor ion ratios; and (iii) analytical selectivity of the employed chromatographic mode, with proper retention of the analytes by the reversed-phase column (which led the compounds to present different retention times) (Fig. 1). All these criteria were in accordance with Mologni et al. (2020).

If the empirical conversion factor is one (1), it means that a perfect

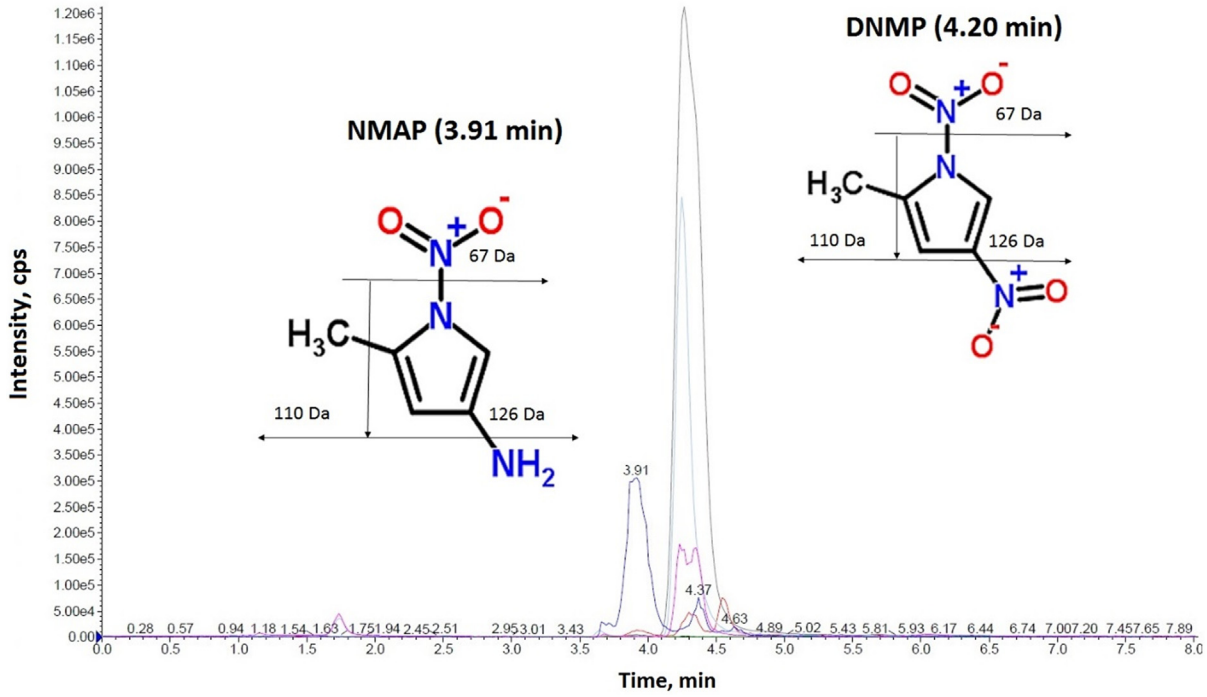


**Fig. 1.** Experimental conditions that guarantee the effectiveness of the DNMP and NMAP detection model: equal fragmentation profile in MS/MS and selectivity in reversed phase-liquid chromatography with cyanopropyl.

result was achieved. However, it would rarely happen, even under ideal conditions of molecular conversion, because the substances are not 100% pure and the chemical reactions are not totally complete. Besides, other products are generated, and the mathematical model propagates experimental and theoretical uncertainties from equipment and analysts. According to Eq. (1), the most efficient bacterial strain in the first experimental step was *L. acidophilus* that left a residual of 0.37% of DNMP, followed by *L. fermentum* (0.49%), *Lc. lactis* (0.49%), *P. pentosaceus* (0.59%), *S. xylosus* (0.65%), *L. paracasei* (0.66%), *L. casei* (0.81%), *L. rhamnosus* (1.36%), *L. plantarum* (1.55%), *L. sakei* (2.02%), *St. thermophilus* (22.23%), *L. helveticus* (28.67%), and *W. minor* (100%).

The results of the first experimental step led us to infer the interaction capacity of microorganisms with DNMP. In fact, 92.31% of the assessed microorganisms reacted with the mutagen compound. For this reason, it was determined that bacteria with a reducing capacity of less than 98% would be discarded for the following tests. Thus, from 13 bacteria used, 10 went on to the next step. As a control, all strains tested in the presence of DNMP grew in the culture medium (data not shown).

In the second experimental step, it was noted that bacterial strains were effective to convert DNMP to NMAP *in vitro*. The most efficient strain was *S. xylosus* LYOCARNI SXH-01 that presented an empirical conversion factor of 0.62 in less than 30 min (Fig. 2).

According to Eq. (2), the bacterial strains with lower conversion factors were *L. fermentum* (0.60), *L. casei* (0.43), *P. pentosaceus* (0.40), *L. rhamnosus* (0.34), *L. acidophilus* (0.31), *L. sakei* (0.30), *L. plantarum* (0.29), *L. paracasei* (0.08), and *Lc. Lactis* (0.06). Comparatively, the ascorbic acid yielded an empirical conversion factor of 0.02 (Fig. 3). Hereafter, only the strains that presented a conversion factor higher than 0.40 were applied in the processed meat model.

The conversion factor of the ascorbic acid was 31 times lower than the factor of *S. xylosus*. This comparison was based on the work of Binstok, Campos, Varela, and Gerschenson (1998), in which the antioxidant was able to decrease the concentration of DNMP to undetectable levels. However, their limit of detection was 500 μg/kg, which was quite high concentration. Recently, it has been demonstrated that DNMP can be found in processed meat products in the mean concentration of 100 μg/kg (Mologni et al., 2018).

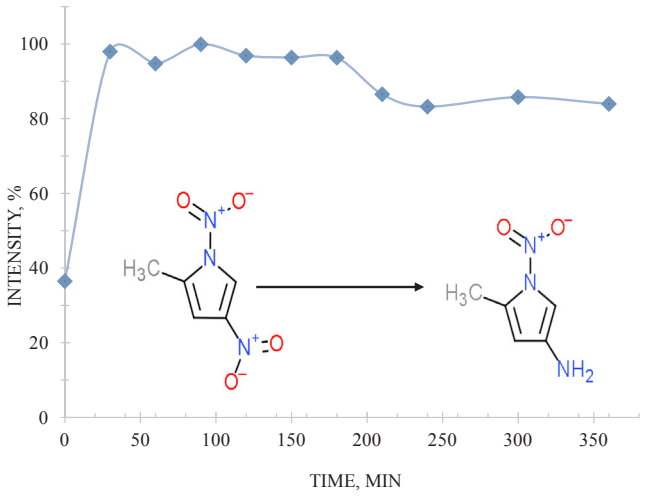


**Fig. 2.** Conversion of DNMP to NMAP by *Staphylococcus xylosus* LYOCARNI SXH-01.

Differently from DNMP, NMAP did not show mutagenicity in the Ames test, with or without S-9 microsomal fraction, as demonstrated by Osawa et al. (1986), probably because the *C*-nitro group was converted to an amino group. This conversion can be assumed as it would cause inversion in the electron flow (Mologni et al., 2020).

### 3.2. Biodegradation of DNMP in the processed meat model

Three different bacterial strains (*S. xylosus*, *L. fermentum*, and *L. casei*) that presented the highest *in vitro* conversion factors of DNMP to NMAP were separately applied in the processed meat model. The three species converted DNMP in the substrate as demonstrated in Fig. 4.

When starter cultures are used to ferment meat products, there are changes in their sensory characteristics. In addition, they improve nutrient availability due to reactions such as nitrate reduction, proteolysis, and lipolysis (Władyka & Bonar, 2018). Furthermore, these bacteria can

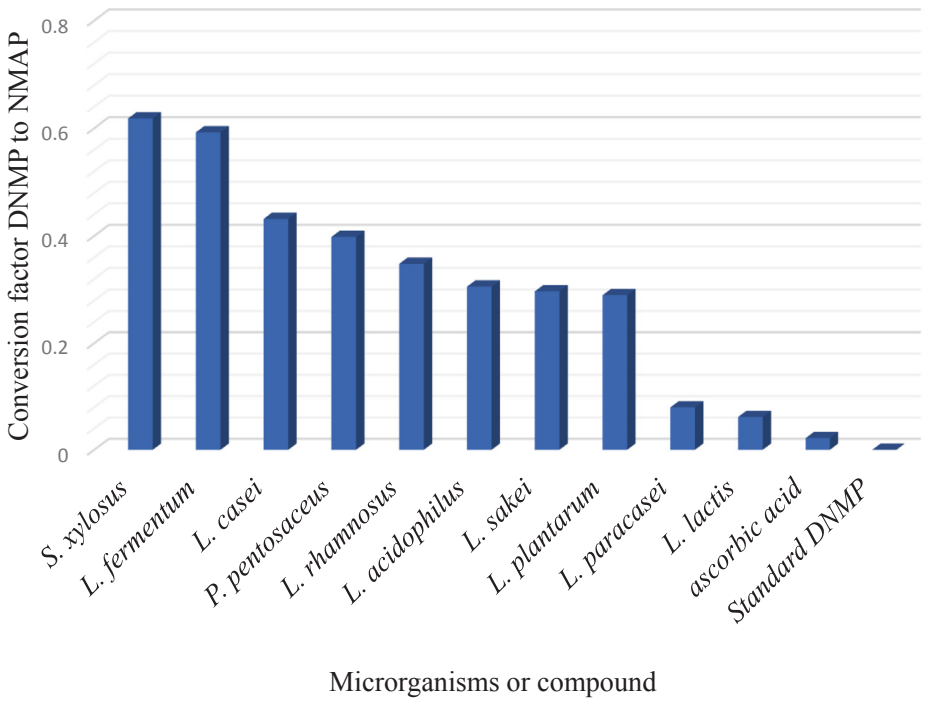


**Fig. 3.** Conversion factors obtained by the biodegradation of DNMP by *Lactobacillus plantarum* ATCC 8014, *Lactobacillus rhamnosus* ATCC 7469, *Lactococcus lactis* subsp. *lactis* ATCC 19435, *Pediococcus pentosaceus* LYOCARNI RHM-33, *Staphylococcus xylosus* LYOCARNI SXH-01, *Lactobacillus paracasei* subsp. *paracasei* LB-UFSC 0014, *Lactobacillus fermentum* LB-UFSC 0017, *Lactobacillus acidophilus* LB-UFSC 0018, *Lactobacillus casei* LB-UFSC 0019, and *Lactobacillus sakei* LB-UFSC 0022 or ascorbic acid.

be used for food preservation due to the production of antimicrobial metabolites and competition with undesirable bacteria (Jameson effect) (Cornu, Billoir, Bergis, Beaufort, & Zuliani, 2011; Di Gioia, 2016; Pilevar & Hosseini, 2017). In addition, some strains of *P. pentosaceus*, *Lactobacillus curvatus*, and *Pediococcus acidilactici* are resistant to heat treatment. Thus, they are suitable for the inoculation of thermally processed meat products (Pérez-Chabela, Totosaus, & Guerrero, 2008).

One of the fundamental criteria to choose a technological starter culture is the cell viability robustness during food processing and storage (Ammor & Mayo, 2007). The microbial cells can develop molecular and enzymatic mechanisms against oxidizing agents from the food matrix, in order to protect their macromolecules, to avoid the cellular aggregation, and to save energy (Papadimitriou et al., 2016). Thus, bacterial protection mechanisms can be indirectly used to biodegrade compounds that are toxic to consumers of fermented foods (Mukherjee & Rokita, 2015).

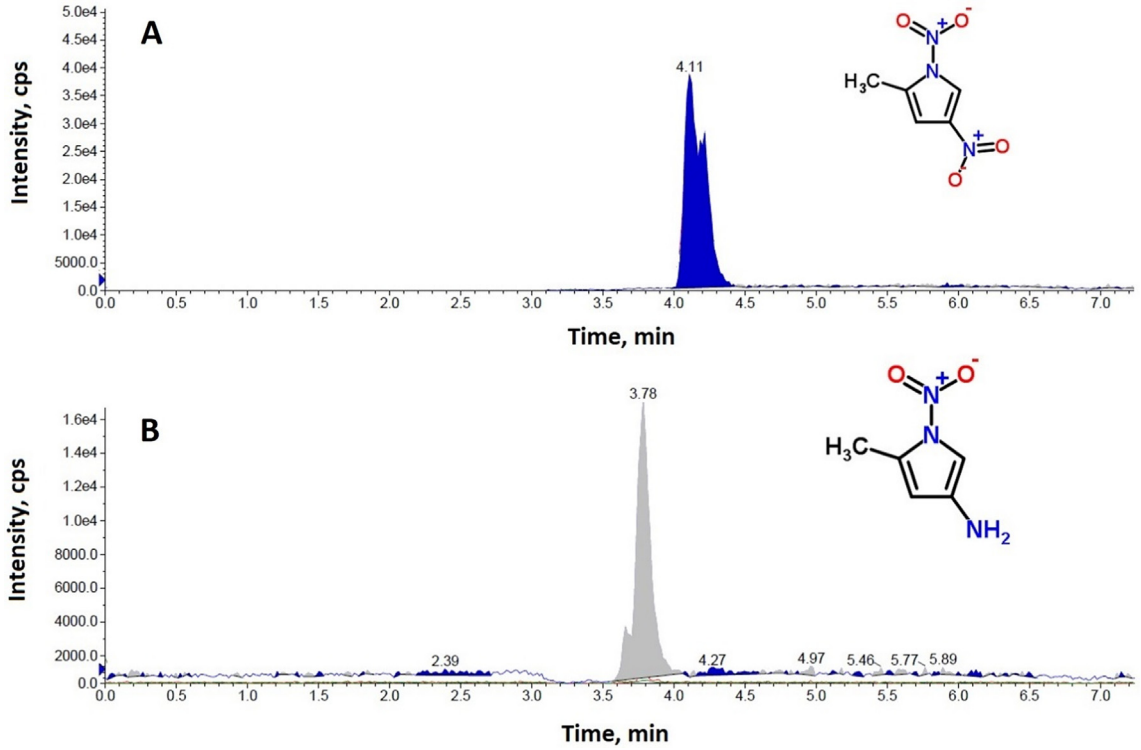


**Fig. 4.** Conversion of DNMP (part A) into NMAP (part B) in processed meat model inoculated with *Staphylococcus xylosus* LYOCARNI SXH-01 which led to analytical signals of the same order of magnitude.

The bacterial defense mechanism against oxidizing agents can be observed in a wide range of studies. Guillén et al. (2009) reported that *L. plantarum* excreted the enzyme PnbA reductase in the presence of oxidizing compounds such as 4-benzoate and 2-,4-dinitrobenzoate. This protein, which is also present in other starter cultures, reacts with different oxidizing agents, avoiding the formation of free radicals and other chemical species that may interact with DNA and other macromolecules. Pei-Ren, Roch-Chuiyu, Cheng-Chun, and Ya-Hui (2002) demonstrated that *Bifidobacterium lactis* and *Bifidobacterium longum* have activity against the toxic compound benzo[*a*]pyrene. Orrhage, Sillerström, Gustafsson, Nord, and Rafter (1994) showed that some bacteria such as *Lc. lactis*, *Lactobacillus cremoris*, *Clostridium perfringens*, *L. acidophilus*, *L. fermentum*, *Bifidobacterium longum*, *Bacteroides fragilis*, and *Escherichia coli* have binding capacities for mutagenic heterocyclic amines. Bartkiene et al. (2017) observed that *L. sakei*, *P. acidilactici*, and *P. pentosaceus*, when applied on the surface of sausages, were able to reduce the contamination by benzo[*a*]pyrene, chrysene, cadaverine, spermidine, and putrescine.

Regarding DNMP, to the best of our knowledge, no other study has evaluated its biodegradation by bacterial strains in foodstuffs before. Shu et al. (1991) evaluated some intestinal bacteria to reduce DNMP. The highest conversion was observed in anaerobiosis with values of about 20%. This means that intestinal bacteria, which play an important role in protecting the organism against mutagenic metabolites, have a low conversion capacity against DNMP. In fact, intestinal bacteria seem to be ineffective against colorectal and stomach carcinogenesis, corroborating the IARC classification for processed meats (IARC Working Group on the evaluation of carcinogenic risks to Humans (2018) (2018), 2018).

### 3.3. Nitroreductase analysis

The absorbance of NADH or NADPH with DNMP did not change in the absence of cell-free *S. xylosus* LYOCARNI SXH-01 extract. A linear decrease in the absorbance values was observed when the cell extracts were analyzed in the presence of NADH or NADPH and DNMP (Fig. 5).

When either of both reducing agents were used, the reaction occurred, which led us to categorize the enzyme as a potential nitroreductase type I B. In the cell-free extract, the average amount of protein was 0.95 ± 0.05 mg/mL. The reduction rate of DNMP was performed monitoring the decrease (oxidation) of NADH or NADPH and expressed as nmol/mg of protein/min (Table 1).

The investigation of a nitroreductase enzyme was motivated by the presence of an amino group in DNMP's reduction product (NMAP). Nitroreductases may reduce the nitro group in aerobic (type I) and

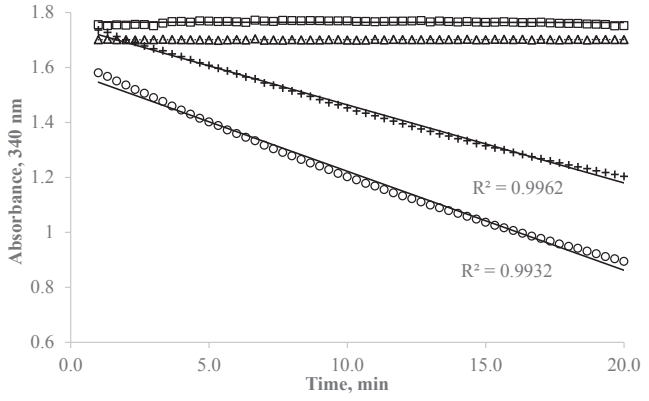


**Fig. 5.** Decrease of NADH (circles) and NADPH (crosses) absorbance in the presence of cell-free extracts of *Staphylococcus xylosus* LYOCARNI SXH-01 and DNMP. Insignificant decrease in absorbance of NADH (triangles) and NADPH (squares) was observed in absence of cell-free extract. The cell-free extract in the presence of NADH and NAPDH produced minor change in the absorbance.

**Table 1**
Reduction rate of DNMP in aerobic condition by cell-free extract of *Staphylococcus xylosus* LYOCARNI SXH-01 in the presence or absence of co-factors[α].

| Cofactor | DNMP reduction rate (nmol/mg of protein/min) |
|---|---|
| None | 0 |
| NADH (0.5 mmol/L) | 30.321 ± 3.186[a] |
| NADPH (0.5 mmol/L) | 22.411 ± 1.684[b] |

[α] Values are means ± standard deviations. Different letters in the column mean significant variance ($p < 0.05$).

anaerobic (type II) reactions. In the first system, two electrons are transferred from NADH or NADPH to the nitro group, resulting in the amine group of NMAP. In the type II system, bacteria promote the transference of two electrons anaerobically. Otherwise, just one electron is transferred to DNMP's nitro group, forming a superoxide radical that reacts with oxygen and regenerates the original nitro compound (De Oliveira et al., 2010). Oxygen-insensitive nitroreductases usually contain flavin mononucleotide (FMN) at the active site and use NADH or NADPH as a reducing substrate (De Oliveira et al., 2010). The cofactor preferences (use of NADH or/and NADPH) allow the classification of the oxygen insensitive nitroreductase in two classes (A and B). Even with this phylogenic differentiation, their mechanisms of action and the physiological use of substrates are still unclear (De Oliveira et al., 2010).

In general, type I nitroreductases catalyze two-electron transfers using a double-displacement kinetic mechanism (Koder, Haynes, Rodgers, Rodgers, & Miller, 2002). Therefore, for the reaction to happen, the enzyme alternates the prosthetic group in oxidized and reduced states, however, between these states occurs the formation of a semiquinone molecule (Miller, Park, Ferguson, Pitsawong, & Bommarius, 2018). The mandatory reduction of two electrons of aromatic nitro compounds by nitroreductases can be attributed to an extreme instability of the redox state of the prosthetic group (Koder et al., 2002). Therefore, the broad specificity of the nitroreductase substrate may allow to completely reduce a large number of compounds that, in some way, could contribute to generate oxidative stress by reducing the electron of the prosthetic group (Haynes, Koder, Miller, & Rodgers, 2002; Koder & Miller, 1998; Pitsawong, Hoben, & Miller, 2014).

Shu et al. (1991) demonstrated that when the cell-free extract of *Bacteroides thetaiotaomicron* VPI-5482 was used without the addition of cofactor, no reaction occurred, which was also observed in this work. They observed that the addition of NADH or NADPH led to reduction rates of 2.209 and 1.945 (nmol/min/mg), respectively. In anaerobic conditions, their activity values were more than 10-fold lower than those presented in Table 1. On the other hand, under aerobic experimental conditions, the enzymatic activity was 96.97% lower (Shu et al., 1991).

The cell-free extract of *S. xylosus* LYOCARNI SXH-01 used NADH and NADPH as electrons donors, with increased activity to the first. Despite the difference regarding the cofactors assessed in this research, the enzyme classification was not changed. Thus, it can be inferred that this extract contains a DNMP reductase, which potentially seems to be a type I B nitroreductase.

## 4. Conclusions

This work provided some answers to the questions raised by the IARC and EFSA about the conversion of the mutagen DNMP in processed meats (treated with sorbic acid and sodium nitrite) by GRAS bacteria commonly used for fermentation. It was shown in the *in vitro* experiments that only one of 13 microorganisms did not interact with the DNMP. The empirical conversion factor was calculated to evaluate the conversion of DNMP to the non-mutagenic compound NMAP. The three most efficient strains were *S. xylosus* LYOCARNI SXH-01, *L.*

*fermentum* LB-UFSC 0017, and *L. casei* LB-UFSC 0019, which yielded factors of 0.62, 0.60, and 0.43, respectively. Those strains were individually applied in a processed meat model and the complete degradation of the mutagen compound was observed. Furthermore, the *S. xylosus* LYOCARNI SXH-01 strain degraded the DNMP in less than 30 min. The cell-free extract of *S. xylosus* LYOCARNI SXH-01 showed, in the aerobic system, reduction rates of 30.321 and 22.411 nmol/mg of protein/min using NADH and NADPH, respectively, due to the potential presence of an oxygen insensitive type I B nitroreductase.

Future studies shall be performed in order to clarify if the strains without reducing capacity in the aerobic system behave in the same way in anaerobic environment. In addition, the isolation and purification of the enzyme(s) must be done to better understand the nitro-reduction mechanisms. Nevertheless, the application of biotechnological industrial processes may be a potential strategy to mitigate the DNMP hazard in processed meats and thus increase food safety.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2020.109441.

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