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Isolation and Characterization of Nitrate Reducing Bacteria for Conversion of Vegetable-Derived Nitrate to 'Natural Nitrite'

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Abstract: In the US, sodium nitrate is used as a preservative and curing agent in processed meats and is therefore a regulated ingredient. Nitrate reducing bacteria (NRB) can convert vegetable nitrate into nitrite allowing green/clean label status in the US as per the USDA-FSIS definition of 'natural nitrite'. The current 'in-liquid' test tube assay for detecting nitrite is not suitable for screening mixtures of bacteria nor is commercial nitrate broth suitable for growth of many Gram (+) bacteria. M17 broth was therefore used to develop M17-nitrate broth to be inclusive of Gram (+) bacteria. An 'on-agar' colony-screening assay was developed to detect the conversion of nitrate to nitrite on agar plates and could detect one NRB+ colony among ~300–500 colonies on a single plate. Samples that might have NRB were spread-plated on M17 agar plates, sandwiched with nitrate agar, and after incubation followed with sequential agar overlays containing the reagents used in the nitrate reduction assay; the appearance of red color zones above colonies indicated the presence of nitrite. NRB derived from various samples were confirmed for nitrate conversion and both nitrate and nitrite were quantified by C_8 reversed-phase (RP) ion-pairing high performance liquid chromatography (HPLC) analysis (1 ppm limit of detection). *Staphylococcus carnosus*, a strain commonly used for nitrate reduction, was able to convert 1100 ppm M17-nitrate broth to 917 ppm nitrite. *Staphylococcus caprae* and *Panteoa agglomerans*, NRB isolated using the M17-nitrate agar assay, were also able to ferment the same broth to 916 ppm and 867 ppm nitrite, respectively. This is the first report of an on-agar colony screening assay for the detection and isolation of nitrite reducing bacteria allowing NRB to be readily isolated. This may allow for the identification of new bacteria that may have a more efficient process to generate nitrite, and possibly concomitant with production of additional natural antimicrobials, as vegetable nitrite becomes more widely used to prevent spore germination.

Keywords: nitrate; nitrite; vegetable nitrate; natural nitrite; HPLC; fermentation; agar screening

1. Introduction

Nitrate and nitrite are an important part of the environmental nitrogen cycle that impacts regulations on environmental use of nitrate as fertilizers, which impacts the amount that is taken up by vegetables, and that which may end up in drinking water affecting human health, and that which is added to foods as food preservatives [1,2].

Currently, most worldwide guidelines recognize the importance of nitrate and nitrite as food preservatives and strictly regulate the use of sodium/potassium nitrate or sodium/potassium nitrite as ingredients in cured meat [3,4]. It has been noted that the consumptive uptake of nitrate/nitrite from food additives is less than 5% of total exposure from other sources and that the contribution from food additives does not exceed the acceptable daily intake (ADI) established for nitrate, however, considering all sources of nitrate, this would well exceed the (European) established ADI [4]. The concern is that nitrate that is consumed gets converted to the more reactive form (nitrite) by oral bacteria and even more so by those in the intestinal tract [2,3,5]. Since vegetables contain among

the highest levels of nitrate contributed by consumed foods, it is likely one of the reasons why the European Commission regulates the maximum levels of nitrate in spinach and lettuce while other individual countries in Europe set their own limits for nitrate in other vegetables [6]. In the US, however, there is growing optimism that higher levels of nitrate in the diet can be helpful with "dietary approaches to stop hypertension" (i.e., the DASH diet) and reduce hypertension and coronary heart disease [2].

Despite some common ground on sodium/potassium nitrate and nitrite as food additives, there are still major differences how different parts of the world (i.e., Europe vs. US) deals with alternative (natural) forms of nitrate/nitrite. These distinctions in regulation occur with respect to what is considered and accepted as "natural". In regards to nitrite (and nitrate), European regulations require that they be either sodium or potassium nitrite (or nitrate) and of specific purity and consider natural as a marketing term, while in the US, "natural" connotates a specific product identity which impacts the type of ingredients that may be used. In the US, regulations are less clear for meat products cured with natural sources of nitrate or nitrite, such as vegetable juices or powdered vegetable extracts [7,8]. According to the United States Department of Agriculture, Food Safety Inspection Service (USDA-FSIS), labeling standards for meat and poultry products labeled "natural" are not permitted to contain any artificial flavoring, color, chemical preservatives, or synthetic ingredients for producing and labeling "natural" foods [5]. Chemically-derived sodium nitrite and nitrate are not acceptable for use in natural products. For natural meat products, meats can be processed without the addition of sodium nitrate/nitrite and replaced with natural sources of nitrate (vegetable extracts) or nitrite (fermented vegetable extracts) that maintain similar characteristics to conventionally cured products [7,9]. Pre-converted (and standardized) vegetable-derived nitrite may provide more consistent levels of nitrite than depending on natural chemical conversion of nitrate to nitrite. However, USDA-FSIS regulations necessitate these products be labeled as "uncured" and "no nitrate or nitrite added except those naturally occurring in added ingredients" [10]. Natural sources of nitrate such as celery juice concentrate, celery juice powder (CJP), and evaporated sugar cane juice, may be fermented to nitrite and replace synthetic nitrite in naturally cured meat products. Vegetables have an excess of nitrate, such as celery (3151 ppm), turnip greens (9040 ppm), beets (3288 ppm), spinach (2470 ppm), and melon (4932 ppm) [2,11–13]. Currently, celery powder is used extensively as a vegetable nitrite source in natural or organic meat processing due to its subtle flavor and minimal color effect on products [14].

In the US, "clean-label" foods are not constrained to "natural" or "organic" definitions but have simple ingredients that are familiar to consumers and perceived as being derived from a nonchemical source, such as vinegar, flavorings, cultured sugars or dairy ingredients, or ingredients derived from plant material [5]. Thus, vegetable-derived nitrite allows the use of a clean label advantage to the meat industry than if obtained from a chemical origin and addresses a broader category of foods than those simply labelled as "natural" [15].

Nitrate is not a very reactive compound, thus, reduction of vegetable nitrate to natural nitrite is essential for curing reactions. The natural curing process using naturally obtained nitrate is carried out with an additional step of bacterial reduction of vegetable nitrate into nitrite by specific nitrate-reducing bacteria. Celery juice, or its dried powders, are known to have high nitrate content and have been used in combination with lactic acid starter culture in the production of naturally cured meat. Currently, pre-generated nitrite (i.e., natural nitrate that is converted to nitrite by microorganisms) is utilized to ease and speed up curing time [11]. Nitrate to nitrite reduction can be achieved with a bacterial starter culture, such as *Staphylococcus xylosus or Staphylococcus carnosus,* possessing a specific nitrate-reducing ability [16].

Nitrate reducing bacteria (NRB) are defined by their ability to reduce nitrate into nitrite or other nitrogenous compounds. This reduction is facilitated by the nitrate reductase enzyme produced by these bacteria. Nitrate reducing bacteria comprise of membrane-

bound nitrate reductases with an active site in the cytoplasm. *Staphylococcus carnosus* is a popular nitrate-reducing bacteria used in the industry. Non-pathogenic coagulase-negative staphylococci (CNS) are commonly found as natural flora in fermented meat products. The role of non-pathogenic CNS is significant in the improvement of sensory properties of fermented sausages, by reduction of nitrates to nitrite and then to nitrous oxide, by preventing rancidity through peroxide decomposition, and by producing flavor and aroma compounds through proteolysis and lipolysis [17].

Nitrate reducing bacteria (NRB) might be expected to be found where nitrate is plentiful (i.e., vegetables) and/or where nitrate-reducing bacteria might be found (intestinal tract, rumen), and therefore some samples used in this study were derived from these sources. We also describe our efforts to develop a differential agar to isolate NRB from these sources, identify and characterize them, and use high performance liquid chromatography (HPLC) to quantify nitrate and nitrite in liquid fermentates. Hopefully, the on-agar assay used in this work will help further the isolation of new NRB from mixed bacterial samples that may be used in research with cured and processed meats to demonstrate equivalency of vegetable-derived nitrite with sodium nitrite.

2. Materials and Methods

2.1. Bacterial Cultures and Media

Active cultures were grown in tryptic soy broth (TSB, BD Bacto, Franklin Lakes, NJ, USA) in 9-mL tubes at 37 °C. Cultures were maintained for storage by centrifugation (6000 × g, 5 °C) of 9 mL of fresh overnight cultures and cell pellets were resuspended in 2–3 mL of fresh sterile TSB containing 10% glycerol. Cell suspensions were placed into glass vials and stored in an ultra-low freezer (−80 °C). Frozen stocks were revived by transferring 100 µL of the thawed cell suspension into 9 mL of TSB, incubating overnight at 37 °C, and subcultured twice before use. Cultures screened for nitrate reducing activity included 500+ cultures from in-house culture collections and bacteria screened from 20+ samples from retail foods and animals harvested at the Robert M. Kerr Food and Agricultural Products Center (i.e., FAPC) slaughter facility.

2.2. The Nitrate Reduction Test (in Broth)

The nitrate reduction test is a qualitative procedure for determining the ability of bacteria to reduce nitrate into nitrite [18]. Bacteria were cultured overnight in nitrate broth (HiMedia Labs, Mumbai, India) at 37 °C and then tested for the presence of nitrite. M17 broth (BD, Difco brand, Franklin Lakes, NJ, USA), to which we added 0.1% potassium nitrate (Fisher Chemical, Fisher Scientific, Atlanta, GA, USA), was also used for conducting the nitrate reduction test in broth when it became obvious that many of the Gram (+) cultures in our culture collection were not growing well in commercial nitrate broth. In our experience, M17 broth allows for growth of lactic acid bacteria (LAB) and the absence of Tween 80 surfactant, that is a component of MRS broth, allows more non-LAB to grow than would otherwise not grow in MRS broth.

Nitrite detection is a two-step process whereby the reduction of nitrate to nitrite is determined by the addition of nitrate reagent A (sulfanilic acid) followed by reagent B (alpha-napthylamine). If a red color develops, this then confirms the presence of nitrite. If there is no color change, then either nitrate remains unreduced and is determined by the addition of zinc powder which is a strong reducer that will reduce nitrate to nitrite to turn the culture red, confirming there was unreduced nitrate in the tube. If there is still no color change with the addition of zinc powder, then the only explanation is that nitrate was reduced to nitrite, and then further reduced to other nitrogen compounds (Figure 1):

    a.   Nitrate reagent A: sulfanilic acid (Fisher); 1 g in 200 mL of 5 N acetic acid,

    b.   Nitrate reagent B: alpha-napthylamine (Fisher); 2 g in 250 mL of 5 N acetic acid,
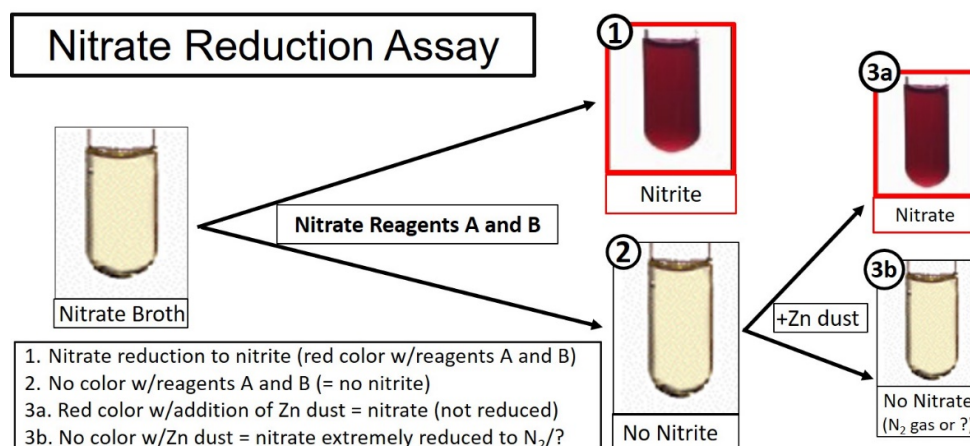
    c.   Reagent C: Zinc powder (50 mg; Fisher).

Figure 1. Representation of the nitrate reduction test assay performed in liquid with nitrate broth culture samples by addition of reagents A and B. Development of red color with addition of reagents A and B indicates nitrite. Absence of nitrite may suggest no reduction, which can be demonstrated by addition of zinc powder to produce a red color with nitrate. The absence of color with reagents A and B as well as with zinc powder indicates nitrate was reduce beyond nitrite to other forms of nitrogen.

2.3. The Nitrate Reduction Assay (on Agar)

The nitrate reduction assay using individual broth cultures was slow and laborious for screening potential NRB from food/animal samples. The nitrate reduction colony assay method for use on agar plates was developed in order to facilitate the screening of colonies from various food and animal samples. The agar plate nitrate reduction assay uses a similar principle of the culture broth (tube) nitrate reduction test but is performed directly on colonies on petri plates. A dilution series of test samples were surface plated on pre-poured M17 agar plates, allowed to dry (adsorb), and then overlaid with 10–12 mL nitrate agar (HiMedia) to entrap the plated colonies in a sandwich overlay technique. The overlaid plates were allowed to incubate overnight at 37 °C, and then plates with a range of 25–250 colonies were selected to be overlaid with reagent agar layers tempered at 46–48 °C before use. A plain agar layer containing addition of nitrate reagent A (2 mL reagent A is added in 50 mL 0.5% soft agar) was mixed and ~6–8 mL is overlaid onto the colony-sandwiched plate. After 5–10 min, another 6–8 mL plain agar layer containing nitrate reagent B (2 mL reagent B is added in 50 mL 0.5% soft agar) was overlaid on top of the nitrate reagent A layer and then tilted to facilitate the soft agar in running to the other side of the plate. The chemicals in the two separate overlays diffuse to the lower levels, reacting with nitrite in the order of addition as in the liquid nitrite test, and zones of red color observed around colonies indicated the presence of nitrite and nitrate-reducing bacteria. The various overlays are similar to those used for other purposes including sandwich overlays for detection of bacteriocin-producing colonies [19,20] and thin agar overlays for recovery of injured bacteria [21,22].

2.4. Isolation and Identification of Nitrate Reducing Bacteria

Colonies with red color zones were isolated from the on-agar nitrate reduction assay plates, cultured, and confirmed for nitrate reducing activity in nitrate broth. Identification was obtained by PCR amplification of DNA corresponding to the 16S rRNA sequences using universal 16S rRNA primers designated 8-Forward (5′-AGAGTTT-GATCCTGGCTCAG-3′) and 1541-Reverse (5′-AAGGAGGTGATCCAGCCGCA-3′) to provide amplification of a 1533-bp stretch of 16S rRNA sequence for bacterial identification [23,24]. Both primers have a melting temperature (T_m) of 55 °C. Thermal cycling was performed using an Opticon 2 Thermal Cycler (MJ Research/Bio-Rad Laboratories, Hercules, CA, USA): initial denaturation at 95 °C for 4 min, followed by 40 cycles of 94 °C for

1 min (denaturation), 60 °C for 45 s (annealing), 72 °C for 1 min (extension), followed by a final extension cycle at 72 °C for 4 min, and a final hold at 4 °C. Amplified products were cleaned up using the GenCatch™ PCR Cleanup Kit (Epoch Life Sciences, Sugarland, TX, USA) to purify amplimers after PCR from enzymes, dNTPs, and salts, and then submitted to the Oklahoma State University DNA Sequencing Core Facility for DNA sequencing. The identification of bacteria was determined using the National Center for Biotechnology Information (NCBI) BLAST program. Isolates of similar bacteria were compared using the MEGA-X software tool for sequence alignment [25] to determine if sequenced information was identical (i.e., re-isolation of the same strain) or showed sequence differences, thus more likely to be different strains.

2.5. Vegetable Juice Extraction and Nitrate Detection

A retail vegetable "juicer" (Black and Decker, Towson, MD, USA) was used to extract juice from celery, white cabbage, and iceberg lettuce obtained from a local market as a potential source of vegetable nitrate. Extracted juices were centrifuged at 10,000 rpm for 10 min (5 °C) to remove residual solids and the liquid extract was decanted and autoclaved to prevent microbial growth.

2.6. Fermentation Using Nitrate-Reducing Bacterial Isolates

Vegetable juice extracts (celery, lettuce, cabbage) were used as nitrate sources and inoculated with NRB to test their fermentation ability to reduce nitrate into nitrite. Vegetable juice samples (9 mL) in test tubes were inoculated with 1 mL of bacterial culture (10^8 –10^9 cfu/mL) and incubated overnight at 37 °C. The presence of nitrate was confirmed with zinc powder and the ability to reduce nitrate to nitrite post-fermentation was initially determined qualitatively using the nitrate reduction test. Subsequent fermentations were performed in both celery extract and M17-nitrite broth (in triplicate) with strains designated as generating the most nitrite. During microbial culture of vegetable extracts, samples were retrieved periodically over 36–72 h, and nitrite was determined by HPLC analysis.

2.7. Quantitation of Nitrate and Nitrite using High Performance Liquid Chromatography (HPLC)

Octylamine orthophosphate has been used as an ion-pairing agent during HPLC analyses of nitrate and nitrite [26–30]. Aqueous methanol (10% *v/v*) mobile phase was used in combination with a reversed-phase (RP) C_8 Phenomenex Luna™ 250 mm HPLC column (Phenomenex, Torrance, CA, USA) as the stationary phase for the simultaneous quantification of nitrite and nitrate [29]. The HP 1050 HPLC system included a diode array detector for signal detection of nitrate and nitrite at 210 nm. Standards for nitrate (1000 ppm; Ricca Chemical Co., Arlington, TX, USA) and nitrite (1000 ppm; Ricca Chem. Co.) were used to derive varying concentrations (10, 50, 100, 200, 300, 600, 800 and 1000 ppm for standard curves and calibration. Additional HPLC system components included a solvent degasser, a quaternary pump capable of pumping 4 different solvents, an automatic sampler, and a computer workstation running HP Chemstation™ software (Agilent, Santa Clara, CA, USA) was used for analyses.

The HPLC mobile phase was prepared using octylamine orthophosphate and phosphoric acid (Fisher Scientific, Atlanta, GA, USA). Octylamine orthophosphate (1.65 mL) was added into 800 mL of HPLC-grade water (this solution is cloudy). The pH was then adjusted to pH = 7.0 with the addition of ~450 μL of phosphoric acid (this solution became clear). The volume was then adjusted to 900 mL and 100 mL of methanol was added to result in a 10% (*v/v*) methanol mobile phase. The prepared mobile phase was then vacuum-filtered (0.22 μ) and held under vacuum for 10 min while sitting in a Branson™ ultrasonic bath (Emerson, St. Louis, MO, USA) to facilitate degassing the solution of dissolved gases [31].

HPLC sample preparation was a modification of Chou et al. [28] and Hongsibsong et al. [32]. Samples were prepared by adding 2 mL of sample (media broth or fermented vegetable juice) to 28 mL preheated deionized water (80 °C) and holding in an 80 °C water bath for 30 min. After heating, 600 µL of each sample was filtered through 0.2 µ polytetrafluoroethylene (PTFE) Whatman syringeless filter vials (Whatman PLC, Kent, England, UK). During HPLC analysis, a 20 µL sample volume was injected into the column with an isocratic solvent flow rate of 0.6 mL/min.

2.8. Statistical Analysis

Microbial fermentation trials and subsequent HPLC analyses were performed in triplicate replication. Replications were performed as autonomous and separate experiments. Data are presented as the mean of multiple replications with standard deviation of the mean represented by error bars. Statistical analysis was done using one way analysis of variance (ANOVA) provided by the statistical capabilities within Sigma Plot ver. 13 (Systat Software, San Jose, CA, USA) to determine significant differences in nitrite present at given timelines of fermentation. The Holm–Sidak test for pairwise multiple comparisons was then used to determine significant differences ($p < 0.05$). Data bars with different letters are significantly different ($p < 0.05$) and data bars with the same letter are not significantly different ($p > 0.05$).

3. Results and Discussion

3.1. Nitrate Reduction Assay (Liquid) using Modified M17-Nitrate Broth

During the screening of ~500 bacterial cultures from two culture collections, it was observed that Gram (-) bacteria grew better than Gram (+) bacteria in commercial nitrate broth in which growth was noticeably sparse. A modified M17-nitrate broth (i.e., M17 broth + 0.1 % potassium nitrate) was used in which Gram (+) bacteria grew more luxuriously and Gram (+) bacteria demonstrating nitrate reducing ability were detected. The use of this modified M17-nitrate broth proved successful in screening nitrate reduction among the Gram (+) bacteria in these collections. Through use of M17-nitrate broth, six (6) new isolates were identified as nitrate reducers as confirmed subsequently using the nitrate reduction broth test in liquid after the addition of nitrate reagents A and B (Figure 2).
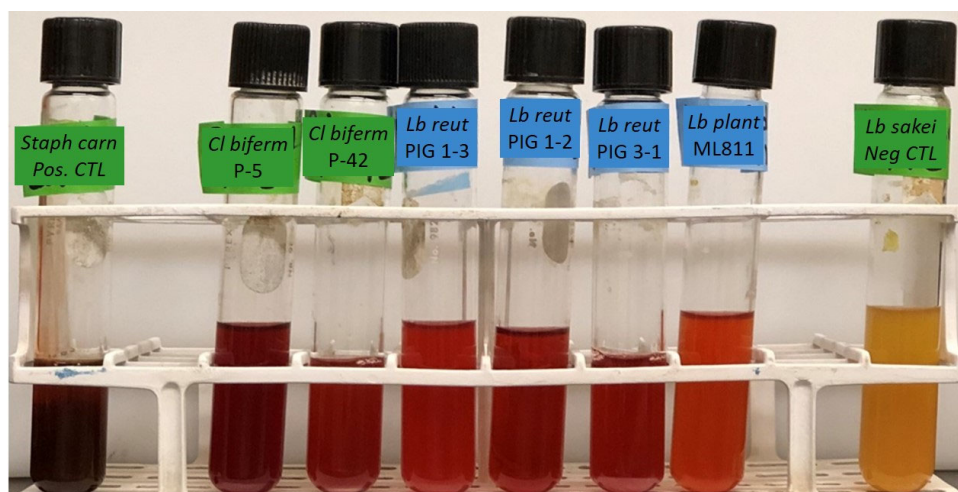

Figure 2. Nitrate reduction test performed on M17-nitrate broth culture broths of various strains from culture collections including: *Staphylococcus carnosus* (positive control), *Clostridium bifermentums* P-5, *Clostridium bifermentums* P-42, *Lactobacillus reuterii* PIG 1-3, *L.actobacillus reuterii* PIG 1-2, *Lactobacillus reuterii* PIG 3-1, *Lactobacillus planturum* ML811, and *Lactobacillus sakei* (negative control). The red color generated after addition of sulfanilic acid and alpha-napthylamine signifies the presence of nitrite (reduced from nitrate). The bacterial identities were determined by 16S rRNA sequence analysis.

3.2. The Nitrite Assay Modified for On-Agar Use to Screen Bacterial Samples from Foods and Animals

The traditional nitrate reduction test was easy to perform for individual cultures because they had to be grown from frozen/lyophilized stocks and they already existed as individual strains against which the test could be readily applied. Although sophisticated real-time PCR assays can identify NRB within a mixed bacterial sample [33], the NRB would still need to be isolated from the other bacteria. An agar-based method would allow both identification of nitrate reducing activity and recovery of the isolates. Identifying NRB from food or animal intestinal samples was laborious using the culture tube method considering the mixture of bacteria that might be present in a given sample, and each at different levels. The only literature reference to an on-agar nitrate test was an old report using nitrate–blood agar whereby nitrate-reducing bacteria would cause oxidation in red blood cells changing hemoglobin to methemoglobin [34]. The on-agar nitrate reduction assay developed in this study was a modification of the in-broth nitrate reduction test. After various optimizations, we were able to conveniently screen mixed bacteria simultaneously from various source samples for nitrate reduction giving clear indication of nitrate reduction (Figure 3). Color reactions occur within 5–10 min. Colonies surrounded by zones of red color (Figure 3) were then recovered either directly through the agar or by an inverted agar isolation method [19], isolated by streak isolation, and pure culture isolates were confirmed by the nitrate reduction test (Figures 1,2).
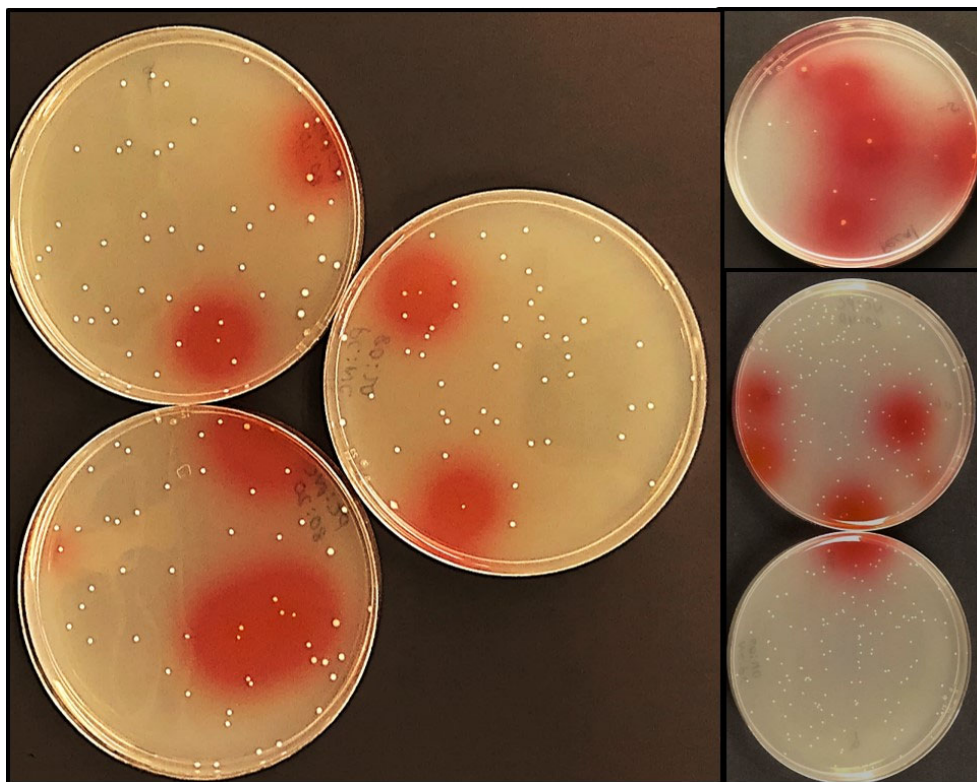

Figure 3. Nitrate reducing bacterial colonies showing red color zones after sandwich overlays with soft agar containing nitrate reagents A and B. Colonies with surrounding red color were isolated using the inverted agar method and streak plated on new plates and confirmed for nitrate reduction in nitrate broth (the plates above are shown from the bottom side of the petri plate).

Other investigators have used different and more elaborate methods to isolate nitrate reducing bacteria. Ogilvie et al. (1997) used low temperature anaerobic chemostats maintained at 5 °C to isolate nitrate reducing bacteria from estuarine sediments [35]. Still other investigators working in the oil field industry used anaerobic conditions and chemically defined media for enrichment of

biosurfactant-producing nitrate reducing bacteria that could be useful in controlling H₂S in the off-shore oil and gas industry [36]. For our purposes, the on-agar nitrate reduction assay was easy to perform with minimal hardware. However, one feature that we could improve upon in the future is to examine plates after anaerobic incubation in comparison with aerobically-incubated plates.

3.3. Identification of Nitrate Reducing Bacteria by 16S rRNA DNA Sequencing

Bacteria identified as NRB by the on-agar (plated sample isolates) or in-liquid nitrate reduction test (stored cultures) were subjected to 16S rRNA PCR, sequenced, and DNA sequences were queried on the NCBI database using BLAST [19]. The detection and identification of nitrate reducing bacteria demonstrated a variety of bacteria involved in nitrate reduction and the use of M17-nitrate broth allowed improved detection of Gram (+) bacteria, some of which may be useful in generating vegetable-derived nitrite for food processing applications (Table 1).

Table 1. Nitrate reducing bacteria detected in culture collections or food/animal samples.

Isolates	Source
Clostridium bifermentums P-5	FAPC culture collection
Clostridium bifermentums P-42	FAPC culture collection
Escherichia coli 309-7	Hog small intestinal sample
Escherichia coli 69	Hog small intestinal sample
Escherichia coli NCYU-26-73	Hog small intestinal sample
Escherichia fergusonii Z6	Hog small intestinal sample
Escherichia coli PL-AGW6	Hog small intestinal sample
Escherichia coli F9792	Hog small intestinal sample
Lactobacillus plantarum ML811	FAPC culture collection
Lactobacillus reuteri PIG1-2	FAPC culture collection
Lactobacillus reuteri PIG1-3	FAPC culture collection
Lactobacillus reuteri PIG3-1	FAPC culture collection
Pantoea agglomerans Lett1	Food sample (iceberg lettuce)
Shigella flexneri SFL1520	Hog small intestinal sample
Staphylococcus caprae Cab1	Food sample (white cabbage)
Streptococcus hyointestinalis 1336	Hog small intestine sample
Streptococcus hyointestinalis 1340	Hog small intestine sample
Staphylococcus carnosum	Control strain (FAPC culture collection)

Some of the NRB detected in this study could be foodborne pathogens (*E. coli*, *Shigella*). *Pantoea agglomerans* is a known plant pathogen that is known to cause opportunistic human infections [37], yet some suggest that it may still be beneficial as a feed additive for domestic animals [38]. However, only cell free clarified culture supernatant is needed as there is no need for the live bacterium subsequent to the conversion process and extracts may be pasteurized before/during drying to powder form. *Streptococcus hyointestinalis* is a bacterium commonly isolated from swine intestines and is also known to produce nisin H, a variant of a antimicrobial bacteriocin that has been allowed as a food preservative [39]. Many bacteriocins have also been identified within strains of *Lactobacillus plantarum* [20,40] and *Lactobacillus reuteri* [41] also found to be NRB in this study (Table 1). Additional antimicrobial activities, such as bacteriocins and lactic acid, could complement the role of nitrite in processed meats to prevent spore germination if nitrite, bacteriocin, and other antimicrobials could be produced simultaneously from these organisms.

3.4. HPLC Analysis of Nitrate and Nitrite in Vegetable Juices, Nitrate Broth, and after Bacterial Fermentation

HPLC analysis allowed us to quantify nitrate and nitrite in liquid media, in vegetable extracts, and after fermentation using either an industry standard bacterial strain or our bacterial isolates (Figure 4). Celery juice is often used as a nitrate source for nitrite because celery extracts do not contribute much flavor to manufactured food products [11]. Reversed phase HPLC analysis of nitrate and nitrite using a C_8 column and 10% MeOH mobile phase containing octylamine orthophosphate as the ion-pairing agent demonstrated similar peak distribution and excellent linearity ($r^2 \geq 0.999$) as observed by Chou et al. [28] (Figure 4). HPLC analysis of nitrate and nitrite was examined at various wavelengths using the diode array detector and demonstrated excellent peak distributions. Peaks for nitrite and nitrate at 210 nm had the best combination of sharp peaks and separated chromatographic distance of the four wavelengths examined (254, 214, 210, and 204 nm; data not shown) and the use of isocratic solvent parameters kept the baseline level. The selectivity of different HPLC column packings (C_{18}, C_8) were both conditionally acceptable and provided for either a shorter or longer chromatographic run (data not shown). The suitability of any of these would be dependent on additional peaks that might interfere with quantitation when more complex extracts are obtained from vegetables and/or processed meat applications.
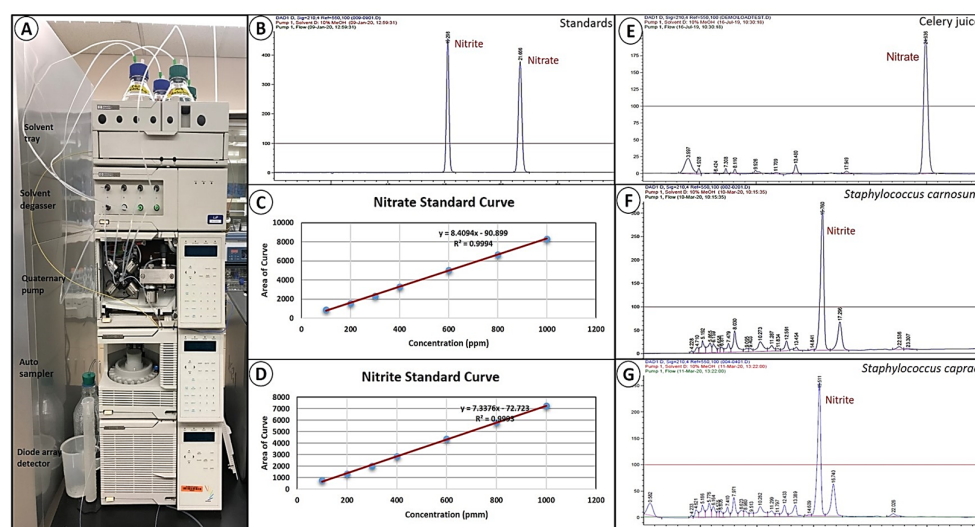

Figure 4. Analysis of nitrate and nitrite using C_8-RP ion-pairing high performance liquid chromatography (HPLC). Panel **A:** HPLC system. Panel **B:** chromatogram of nitrite and nitrate standards. Panel **C:** standard curve of nitrate standards. Panel **D:** standard curve of nitrite standards. Panel **E:** celery juice extract. Panel **F:** celery juice extract fermented with industry standard strain, *Staphylococcus carnosum*. Panel **G:** celery juice extract fermented with bacterial isolate, *Staphylococcus caprae*.

Because of the biochemistry of nitrate-to-nitrite conversion, it was thought to best perform periodic sampling during the time course of fermentation rather than end-point analysis (Figure 5). This would be important if one were to design a fermentation for an optimum recovery of nitrite from nitrate reduction. Although it would be expedient if maximum levels of nitrite could be maintained for a longer period instead of short term peak periods (Figure 5). Magrinya et al. [42] performed nitrate-to-nitrite fermentative conversion directly in sausages fermented for 6, 12, or 24 h and found different meat curing efficiencies. Similarly, we found that *S. caprae* reduced nitrate into nitrite poorly when fermented in celery juice compared to *S. carnosus* and *P. agglomerans* (Figure 5). However, *S. carnosum* (917 ppm), *S. caprae* (916 ppm), and *P. agglomerans* (867 ppm) produced higher and nearly similar levels of nitrite when grown in M17-nitrite broth suggesting that

medium optimization could lead to improved yields of nitrite with select strains (Figure 5). This type of inconsistency is the main reason that vegetable extracts have shifted from direct, in-product fermentation to being pre-converted (fermented) to nitrite and standardized by food ingredient suppliers so that consistent results and product quality could be obtained when used.

Bacterial species produce nitrate reductase enzyme to enable them to reduce nitrate into nitrite. This activity can be readily confirmed using a simple biochemical nitrate reduction tube assay to detect nitrite, nitrate, or absence of both. Some bacteria can reduce nitrate to nitrite and then further, to nitrogen gas. Commercial 'nitrate broth' was not suitable for many Gram (+) or lactic acid bacteria, so we adapted M17 broth as a more suitable medium (M17-nitrate broth) to test these organisms (0.1% potassium nitrate as the nitrate source). The modified agar plate colony screening assay increased our chances of screening nitrate reducing bacteria from a mixture of bacteria plated onto agar plates and we readily found such bacteria from animal and food sources.
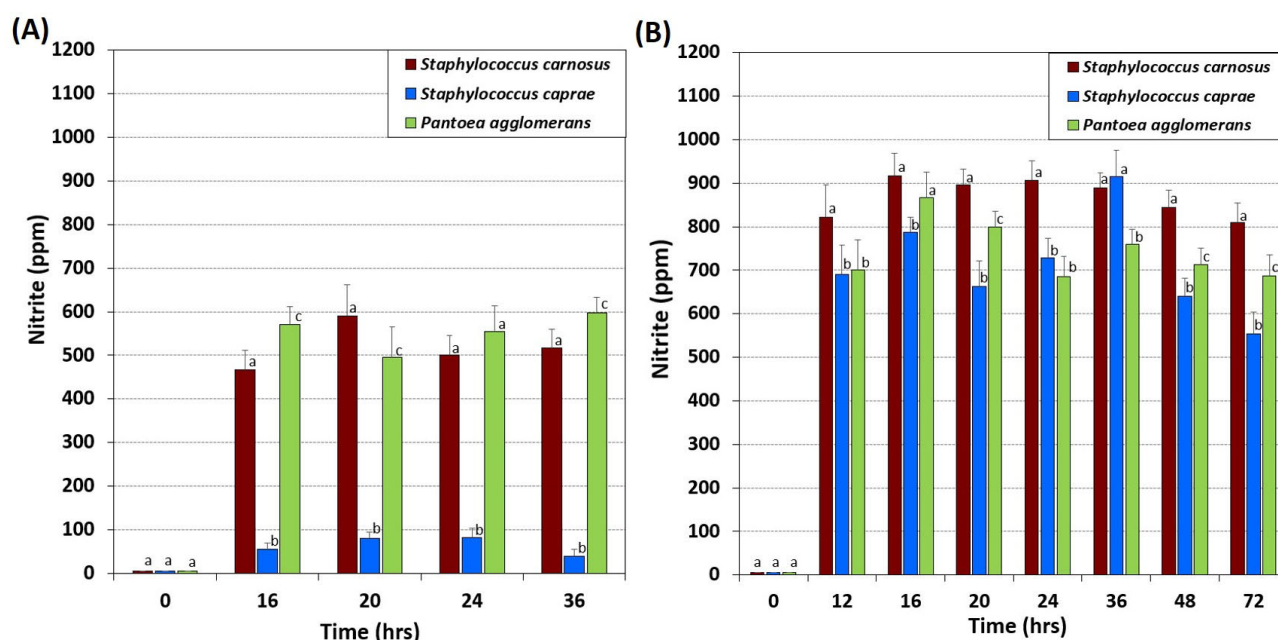

Figure 5. The fermentation time course of reduction of nitrate to generate nitrite by *Staphylococcus carnosus, Staphylococcus caprae,* and *Pantoea agglomerans.* Analysis of nitrite was performed using C₈-RP ion-pairing HPLC chromatography. Panel (**A**)**:** fermentation in celery juice; Panel (**B**)**:** fermentation in M17-nitrate broth. All data are derived from triplicate fermentations and presented as the mean of triplicate samples analyzed for nitrite at each time point and error bars represent the standard deviation from the means. Statistical analysis was performed using one way analysis of variance (ANOVA) to compare data within a given timeline. Data bars with the same letter are not significantly different ($p > 0.05$) and data bars with different letters are significantly different ($p < 0.05$).

The qualitative analysis of nitrate in vegetable juices was confirmed using the simple nitrate reduction test. Celery, cabbage, and lettuce used in this study are well-known sources of nitrate [2,12]. As well, HPLC quantification of nitrate content in vegetable juice extracts proves the presence of high nitrate content. Nitrate present in vegetable extracts or juice can be fermented using NRB resulting in "natural nitrite". The vegetable sourced nitrite is considered "natural nitrite" and allows a "green/clean label" tag in processed meats or for use in "all natural" designated products. New sources of NRB may provide more efficient bacteria to generate nitrite.

Staphylococcus carnosus is a common NRB strain used in processed meats to produce vegetable-based nitrite [43]. As observed it has converted nitrate into nitrite efficiently.

Similarly, *Staphylococcus caprae* was able to efficiently reduce nitrate broth into nitrite. Both strains are coagulase-negative staphylococcus. The food samples used for isolating bacteria were cabbage and lettuce (Table 1). As cabbage and lettuce are high in nitrate content, it is plausible to recover indigenous nitrate-reducing bacteria. The consequence of this association could be considered beneficial since most foods that are in contact with soils, such as vegetables, are considered readily contaminated with bacterial spores (i.e., *Clostridium* spp.) in which spore germination is prevented by nitrite. The animal samples were derived from intestinal contents of animals (pigs) harvested in our abattoir, which are often fed vegetable waste that is rich in nitrate, resulting in the detection and isolation of nitrate reducing bacteria.

4. Conclusions.

The data presented herein demonstrates that a variety of bacteria can reduce nitrate to nitrite. Some of our data with direct fermentation of vegetable (celery) extracts did not lead to as high a conversion rate to nitrite as obtained with nitrate broth, perhaps because of the nutrient content of vegetable extracts alone is not as bacteriologically conducive to luxurious growth required to demonstrate the high levels of reductase activity observed with nitrate broth. HPLC analysis with C_8 RP columns using ion-pairing mobile phase lends itself towards quantification of nitrate and nitrate in vegetable extracts and fermentation broths. Since vegetable-derived nitrite is considered "natural nitrite", it is not subject to the same labelling restrictions as chemically-derived sodium nitrite [5]. This presents a desirable transition for many companies in the USA allowing them to state that their products "have no added preservatives; no added nitrites (i.e., except that which may be found in vegetable extracts)". The optimization of the fermentation process for nitrate reduction with new isolates may make production of vegetable-derived nitrite more efficient.

The modified nitrate broth using M17 broth (with 0.1% KNO_3 added) facilitates the detection of Gram (+) bacteria, and facilitating the detection and identity of new NRB. Further studies defining the optimization of conditions for generating nitrite, and their application in generating natural nitrite is needed to enhance and optimize the conversion of vegetable extracts for food applications targeting the inhibition of spore germination in *Clostridium spp.* The combination of nitrate reduction and possible production of other antimicrobials (bacteriocins, lactic acid) often produced by Gram (+) bacteria may synergistically inhibit spore-forming bacteria and other foodborne pathogens.

References

1. Brkić, D.; Bošnir, J.; Bevardi, M.; Bošković, A.G.; Miloš, S.; Lasić, D.; Krivohlavek, A.; Racz, A.; Ćuić, A.M.; Trstenjak, N.U. Nitrate in leafy green vegetables and estimated intake. *Afr. J. Tradit. Complement. Altern. Med.* **2017**, *14*, 31–41.
2. Hord, N.G.; Tang, Y.; Bryan, N.S. Food sources of nitrates and nitrites: the physiologic context for potential health benefits. *Am. J. Clin. Nutr.* **2009**, *90*, 1–10.
3. Mortensen, A.; Aguilar, F.; Crebelli, R.; Di Domenico, A.; Dusemund, B.; Frutos, M.J.; Galtier, P.; Gott, D.; Gundert-Remy, U.; Lambré, C.; Leblanc, J.-C.; Lindtner, O.; Moldeus, P.; Mosesso, P.; Oskarsson, A.; Parent-Massin, D.; Stankovic, I.; Waalkens-Berendsen, I.; Woutersen, R.A.; Wright, M.; Van Den Brandt, P.; Fortes, C.; Merino, L.; Toldrà, F.; Arcella, D.; Christodoulidou, A.; Cortinas Abrahantes, J.; Barrucci, F.; Garcia, A.; Pizzo, F.; Battacchi, D.; Younes, M. EFSA panel on food additives nutrient sources added to food: Re-evaluation of potassium nitrite (E 249) and sodium nitrite (E 250) as food additives. *EFSA Jour.* **2017**, *15*, e04786.
4. Mortensen, A.; Aguilar, F.; Crebelli, R.; Di Domenico, A.; Dusemund, B.; Frutos, M.J.; Galtier, P.; Gott, D.; Gundert-Remy, U.; Lambré, C.; Leblanc, J.-C.; Lindtner, O.; Moldeus, P.; Mosesso, P.; Oskarsson, A.; Parent-Massin, D.; Stankovic, I.; Waalkens-Berendsen, I.; Woutersen, R.A.; Wright, M.; Van Den Brandt, P.; Fortes, C.; Merino, L.; Toldrà, F.; Arcella, D.; Christodoulidou, A.; Barrucci, F.; Garcia, A.; Pizzo, F.; Battacchi, D.; Younes, M. EFSA panel on food additives nutrient sources added to food: Re-evaluation of sodium nitrate (E 251) and potassium nitrate (E 252) as food additives. *EFSA Jour.* **2017**, *15*, e04787.
5. Flores, M.; Toldrá, F. Chemistry, safety, and regulatory considerations in the use of nitrite and nitrate from natural origin in meat products - Invited review. *Meat Sci.* **2021**, *171*, 108272.
6. Santamaria, P. Nitrate in vegetables: toxicity, content, intake and EC regulation. *J. Sci. Food Agric.* **2006**, *86*, 10–17.
7. Sebranek, J.G.; Bacus, J.N. Cured meat products without direct addition of nitrate or nitrite: what are the issues? *Meat Sci.* **2007**, *77*, 136–147.
8. Jo, K.; Lee, S.; Yong, H.I.; Choi, Y.-S.; Jung, S. Nitrite sources for cured meat products. *LWT* **2020**, *129*, 109583.
9. Sebranek, J.G.; Jackson-Davis, A.L.; Myers, K.L.; Lavieri, N.A. Beyond celery and starter culture: Advances in natural/organic curing processes in the United States. *Meat Sci.* **2012**, *92*, 267–273.
10. Sullivan, G.A.; Jackson-Davis, A.L.; Niebuhr, S.E.; Xi, Y.; Schrader, K.D.; Sebranek, J.G.; Dickson, J.S. Inhibition of *Listeria monocytogenes* using natural antimicrobials in no-nitrate-or-nitrite-added ham. *J. Food Prot.* **2012**, *75*, 1071–1076.
11. Djeri, N.; Williams, S.K. Celery juice powder used as nitrite substitute in sliced vacuum-packaged turkey bologna stored at 4C for 10 weeks under retail display light. *J. Food Qual.* **2014**, *37*, 361–370.
12. Bahadoran, Z.; Mirmiran, P.; Jeddi, S.; Azizi, F.; Ghasemi, A.; Hadaegh, F. Nitrate and nitrite content of vegetables, fruits, grains, legumes, dairy products, meats and processed meats. *J. Food Compos. Anal.* **2016**, *51*, 93–105.
13. Chung, S.Y.; Kim, J.S.; Kim, M.; Hong, M.K.; Lee, J.O.; Kim, C.M.; Song, I.S. Survey of nitrate and nitrite contents of vegetables grown in Korea. *Food Additive Contamination* **2003**, *20*, 621–628.
14. Ko, Y.M.; Park, J.H.; Yoon, K.S. Nitrite formation from vegetable sources and its use as a preservative in cooked sausage. *J. Sci. Food Agric.* **2017**, *97*, 1774–1783.
15. Mcdonnell, L.M.; Glass, K.A.; Sindelar, J.J. Identifying ingredients that delay outgrowth of *Listeria monocytogenes* in natural, organic, and clean-label ready-to-eat meat and poultry products. *J. Food Prot.* **2013**, *76*, 1366–1376.
16. Hwang, K.E.; Kim, T.K.; Kim, H.W.; Seo, D.H.; Kim, Y.B.; Jeon, K.H.; Choi, Y.S. Effect of natural pre-converted nitrite sources on color development in raw and cooked pork sausage. *Asian-Australas J. Anim. Sci.* **2018**, *31*, 1358–1365.
17. Casaburi, A.; Blaiotta, G.; Mauriello, G.; Pepe, O.; Villani, F. Technological activities of *Staphylococcus carnosus* and *Staphylococcus simulans* strains isolated from fermented sausages. *Meat Sci.* **2005**, *71*, 643–650.
18. Buxton, R. Nitrate and nitrite reduction test protocols. In *Laboratory Protocols*, American Society for Microbiology: Washington, D.C., 2011; pp https://www.asmscience.org/content/education/protocol/protocol.3660 (last accessed 05 March 3664, 2021).
19. Henning, C.; Vijayakumar, P.; Adhikari, R.; Jagannathan, B.; Gautam, D.; Muriana, P.M. Isolation and taxonomic identity of bacteriocin-producing lactic acid bacteria from retail foods and animal sources. *Microorganisms* **2015**, *3*, 80–93.
20. Garver, K.I.; Muriana, P.M. Detection, identification and characterization of bacteriocin-producing lactic acid bacteria from retail food products. *Int. J. Food Microbiol.* **1993**, *20*, 241–258.
21. Kang, D.H.; Fung, D.Y.C. Application of thin agar layer method for recovery of injured *Salmonella* Typhimurium. *Int. J. Food Microbiol.* **2000**, *54*, 127–132.
22. Wu, V.C.H.; Fung, D.Y.C.; Kang, D.H.; Thompson, L.K. Evaluation of thin agar layer method for recovery of acid-injured food-borne pathogens. *J. Food Prot.* **2001**, *64*, 1067–1071.
23. Pitulle, C.; Citron, D.M.; Bochner, B.; Barbers, R.; Appleman, M.D. Novel bacterium isolated from a lung transplant patient with cystic fibrosis. *J. Clin. Microbiol.* **1999**, *37*, 3851–3855.
24. Luna, R.A.; Fasciano, L.R.; Jones, S.C.; Boyanton, B.L.; Ton, T.T.; Versalovic, J. DNA pyrosequencing-based bacterial pathogen identification in a pediatric hospital setting. *J. Clin. Microbiol.* **2007**, *45*, 2985.
25. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549.
26. Gennaro, M.C.; Bertolo, P.L. Simultaneous determination of nitrites, nitrates and amines by ion-interaction reversed-phase high-performance liquid chromatography: Application to lagoon water. *J. Chromatogr. A* **1990**, *509*, 147–156.

27. Ferreira, I.M.P.L.V.O.; Silva, S. Quantification of residual nitrite and nitrate in ham by reverse-phase high performance liquid chromatography/diode array detector. *Talanta* **2008**, *74*, 1598–1602.

28. Chou, S.S.; Chung, J.C.; Hwang, D.F. A high performance liquid chromatography method for determining nitrate and nitrite levels in vegetables *J Food Drug Anal* **2003**, *11*, 233–238.

29. Cheng, C.F.; Tsang, C.W. Simultaneous determination of nitrite, nitrate and ascorbic acid in canned vegetable juices by reverse-phase ion-interaction HPLC. *Food Addit Contam* **1998**, *15*, 753–758.

30. Najdenkoska, A. Development of HPLC method for analysis of nitrite and nitrate in vegetable. *JAFES* **2016**, *67*, 33–39.

31. Brown, J.N.; Hewins, M.; Van Der Linden, J.H.M.; Lynch, R.J. Solvent degassing and other factors affecting liquid chromatographic detector stability. *J. Chromatogr. A* **1981**, *204*, 115–122.

32. Hongsibsong, S.; Poliem, W.; Narksen, W.; Kerdnoi, T.; Prapamontol, T. Determination of nitrate in the edible part of vegetables from markets around Chiang Mai City, northern Thailand by using high performance liquid chromatography. *Asian J. Agricult. Res.* **2014**, *8*, 7.

33. López-Gutiérrez, J.C.; Henry, S.; Hallet, S.; Martin-Laurent, F.; Catroux, G.; Philippot, L. Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. *J. Microbiol. Methods* **2004**, *57*, 399–407.

34. Cook, G.T. A plate test for nitrate reduction. *J. Clin. Pathol.* **1950**, *3*, 359–362.

35. Ogilvie, B.G.; Rutter, M.; Nedwell, D.B. Selection by temperature of nitrate-reducing bacteria from estuarine sediments: species composition and competition for nitrate. *FEMS Microbiol. Ecol.* **1997**, *23*, 11–22.

36. Fan, F.; Zhang, B.; Morrill, Penny, L.; Husain, T. Isolation of nitrate-reducing bacteria from an offshore reservoir and the associated biosurfactant production. *RSC Adv.* **2018**, *8*, 26596–26609.

37. Morin, A. Pantoea. *Encyclopedia of Food Microbiology (Second Edition)*, Batt, C.A.; Tortorello, M.L., Eds. Academic Press: Oxford, UK, 2014; pp 1028–1032.

38. Morin, A.; Parveen, Z. Pantoea. *Encyclopedia of Food Microbiology*, Robinson, R.K., Ed. Elsevier: Oxford, UK, 1999; pp 1623–1630.

39. Connor, P.M.; Shea, E.F.; Guinane, C.M.; Sullivan, O.; Cotter, P.D.; Ross, R.P.; Hill, C. Nisin H is a new nisin variant produced by the gut-derived strain *Streptococcus hyointestinalis* DPC6484. *Appl. Environ. Microbiol.* **2015**, *81*, 3953.

40. Kavitha, S.; Harikrishnan, A.; Jeevaratnam, K. Characterization and evaluation of antibacterial efficacy of a novel antibiotic-type compound from a probiotic strain *Lactobacillus plantarum* KJB23 against foodborne pathogens. *LWT* **2020**, *118*, 108759.

41. Kuleaşan, H.; Çakmakçı, M.L. Effect of reuterin, produced by *Lactobacillus reuteri* on the surface of sausages to inhibit the growth of *Listeria monocytogenes* and *Salmonella* spp. *Food / Nahrung* **2002**, *46*, 408–410.

42. Magrinyà, N.; Bou, R.; Rius, N.; Codony, R.; Guardiola, F. Effect of fermentation time and vegetable concentrate addition on quality parameters of organic botifarra catalana, a cured–cooked sausage. *J. Agric. Food Chem.* **2012**, *60*, 6882–6890.

43. Neubauer, H.; Götz, F. Physiology and interaction of nitrate and nitrite reduction in *Staphylococcus carnosus*. *J. Bacteriol.* **1996**, *178*, 2005–2009.