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Food waste to new food: Risk assessment and microbial community analysis of anaerobic digestate as a nutrient source in hydroponic production of vegetables

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ABSTRACT

In this study, the microbiological food safety of using anaerobic digestate as a fertilizer in hydroponic production of vegetables was evaluated. The used anaerobic digestate was a liquid residue obtained from the digestion of food waste in the production of biogas. Replacing the customary inorganic fertilizer used in hydroponic production with this recycled fertilizer (biofertilizer) could allow for sustainable urban food production close to retailers and consumers. However, in striving for circular food production, it is vital that the food safety of utilizing recycled resources is ensured. Especially in the application of hydroponic farming, where the nutrient loop is shorter than on arable land, a microbiological food safety risk assessment is crucial when adopting new and recycled fertilizers. The biofertilizer based on anaerobic digestate was therefore studied with regard to its microbial community (16S rRNA gene amplicon sequencing) during production of vegetables in a hydroponic system. The biofertilizer was also challenge tested with food borne pathogens (*Salmonella enterica*, *Listeria monocytogenes* and *Bacillus cereus*). Furthermore, the microbial activity of the biofertilizer was studied using isothermal calorimetry. The results showed that the microbial community of the biofertilizer changed distinctly through a necessary initial nitrification process, and that the most abundant genus was *Mycobacterium*. Deliberate contaminations with 5 $\log_{10}$ CFU mL^{-1} of either *S. enterica* or *L. monocytogenes* in the nitrified biofertilizer were no longer detectable with selective plating after 48 h of incubation at 20 °C. Selective plating for *B. cereus* revealed that the biofertilizer contained low levels (~10 CFU mL^{-1}) of the bacterium, and an inoculation of 5 $\log_{10}$ CFU mL^{-1} *B. cereus* decreased to these levels within 24 h of incubation at 20 °C. Analysis of the microbial activity of the biofertilizer indicated that the biofertilizer does not seem to support microbial activity without the addition of an external nutrient source that contains an accessible carbon source and trace elements. The type of biofertilizer investigated in this study is thus regarded as microbiologically safe for use in hydroponic cultivation. The constant presence of viable *B. cereus*, however, emphasizes the fundamental importance of continuous risk assessment in case of any modifications or supplementations of the biofertilizer, since it clearly can act as a reservoir for bacterial endospores.

1. Introduction

The societal and political interest in more sustainable and circular food production systems is increasing and in parallel to this development, the focus in waste treatment is being directed towards increased resource recovery. It is imperative to reduce food loss to increase food security, however, this is complex as losses occur in the whole production and supply chain. Food waste from household consumption contributes largely to food loss as demonstrated in the study of Garcia-Herrero et al. (2018). Besides measures for reducing production of food waste, methods for its valorisation is important.

Urban farming is an example of alternative food production systems

Abbreviations: CFU, Colony Forming Units; VC, Viable Count; FC, Flow Cytometry; IC, Isothermal Calorimetry.

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that provides locally produced food, thereby contributing to a reduction in long-distance transportation of foodstuffs into cities. However, for effective food production in cities, where possibilities for cultivation in soil are scarce, alternative production systems with low area requirements are needed. One solution to this constraint is to turn to hydroponic farming, where the food crop is grown directly in a nutrient solution (Bergstrand, 2010), minimizing the space needed for cultivation. These production systems enable the possibility of farming in varying system designs such as horizontal, vertical or in several layers, and in diverse locations such as in basements, on rooftops and in containers, are thus attractive and promising systems to explore further in an urban context. Hydroponics have been used in traditional large-scale production of vegetables over the last three to four decades, however the nutrient supply is almost exclusively based on industrially manufactured, mineral (inorganic) fertilizers that challenge the pursuit for sustainable and renewable nutrient and resource loops (Benke and Tomkins, 2017; Kozai, 2013).

Various valorisation strategies can be considered for food waste as discussed by Otles and Kartal (2018). In Sweden, selected organic wastes, including food waste, are largely used for biogas production, with a nutrient-rich liquid digestate remaining as the by-product after the anaerobic digestion process. Using this anaerobic digestate as the nutrient solution in hydroponic cultivation systems could pave the way for a circular urban food production system as well as valorising food waste. Evidently, the use of recycled and biobased fertilizers constitutes an advantage from an environmental perspective compared to the mineral fertilizers used in conventional hydroponic production. One major point of attention is however the close contact between the crop and the nutrient solution in hydroponic production systems. It is therefore paramount to primarily investigate and establish whether the anaerobic digestate is microbiologically safe to use for food production (Turner et al., 2020).

In Sweden, anaerobic digestate based on selected waste originating from the food and/or feed chain can be certified as biofertilizer according to SPCR 120 (Avfall Sverige, 2020), a Swedish national regulation that needs to meet the criteria of the EU-regulation EC No. 1069/2009 regarding the treatment of biowaste (European Parliament and of the Council, 2009). In order to fulfil the requirements for this certification, the feedstock used in the biogas process is initially hygienized by heat treatment (Avfall Sverige, 2020). Previous studies have concluded that the combination of thermal pre-treatment followed by anaerobic digestion is successful in reducing *Salmonella*, *Enterococci* and *Escherichia coli* to acceptable/non-detectable levels as required by EU-regulation (Bagge, 2009; Seruga et al., 2020). However, while the presence and survival of these specific bacteria have been closely investigated, more in-depth studies into the overall biosecurity and pathogen content in anaerobic digestate from biowaste are encouraged (Tampio, 2016; Zhao and Liu, 2019). Regarding extended utilization in shorter nutrient cycles such as a hydroponic setup, which omits the natural processes occurring in contact with organic compartments such as soil, a thorough risk assessment becomes even more relevant.

In a pilot study preceding the present work, three different, geographically distributed biogas plants in Sweden were sampled and the microbiological quality of the biofertilizer studied. In addition to the requirements in the certification, control of spore-forming species and presence of antibiotic resistance were conducted. The results confirmed that all formal criteria were met, however, biofertilizer from all plants had unsanitary levels (EFSA, 2005) of the food-borne spore-forming pathogen *Bacillus cereus* (unpublished data). This is in agreement with a previous study conducted on the hygiene aspects of biofertilizers where high levels of *Bacillus* spp. were detected (Bagge, 2009), and it was deduced that neither the hygienization treatment nor the following anaerobic digestion affected the number of *Bacillus* spp.

The overall scope of this study was to assess microbial risks related to the use of SPCR120 certified anaerobic digestate as a nutrient source in the hydroponic production of vegetables. The microbial viability and activity in the biofertilizer, before use in a hydroponic system, was initially studied over time with cultivation-based viable count, and cultivation-independent isothermal calorimetry. Challenge testing with the three major food-borne pathogens *B. cereus*, *Salmonella enterica* ser. Typhimurium, and *Listeria monocytogenes* was also performed to investigate the biofertilizer's susceptibility to contamination and ability to support microbial survival and growth. For assessment in hydroponic production settings, samples of circulating nutrient solution, based on either biofertilizer or inorganic fertilizer, were collected during a growth cycle in a greenhouse experiment and 16S rRNA gene amplicon sequencing was used to study the bacterial community composition over time.

2. Materials and methods

2.1. Fertilizers

Two types of fertilizer products have been included in this study: nitrified biofertilizer (*i.e.* anaerobic digestate) and inorganic (mineral) fertilizer. The biofertilizer is the remaining residue, aside from biogas, that is produced from the anaerobic digestion of organic waste. For this study, anaerobic digestate certified according to SPCR 120 (Avfall Sverige, 2020) was obtained from a local Swedish biogas plant. The initial composition of the feedstock supplied to the biogas reactor was: organic household waste 37%, manure 31%, slaughter residues 19%, other organic food waste 13%, and iron chloride 0.03% that was added as a process enhancer in the anaerobic fermentation. In the resulting nutrient-rich anaerobic digestate, most of the mineralized nitrogen is in the form of ammonium (NH_4^+), which can be phytotoxic in high concentrations (Britto and Kronzucker, 2002). Before use in hydroponic production systems, the biofertilizer was therefore diluted and nitrified as described in Asp et al. (2020) and Bergstrand et al. (2020). The process together with experimental set-up is presented schematically in Fig. 1. The inorganic fertilizer used in this study is a standard solution for hydroponic production systems and was composed of 1 + 1 g L^{-1} respectively of the solid nutrient mixes Kristalon Indigo and Calcinit (Yara, Oslo, Norway).

2.2. Accelerated microbial activity assessment of nitrified biofertilizer

Microbial viability and activity in the nitrified biofertilizer (n-bf) was studied in parallel over time through cultivation-based viable count (VC), flow cytometry (FC) and cultivation-independent isothermal calorimetry (IC) measurements (described below). The experimental setup is described in Fig. 1 and process schemes of the individual methods are provided as supplementary material in Appendix A Figures A1-A3. Two identical sets of samples were prepared. For all samples, 10 mL of nitrified biofertilizer was transferred into 20 mL polyethylene vials (Sarstedt, Nümbrecht, Germany). Each set included the following samples: for control (n-bf:blank), for measuring the effect of aeration (n-bf:aerated), and for measuring the effect of supplementation with either BHI broth (a complex, rich medium) or glucose (a simple carbon source) (n-bf:supplemented). One set of samples was placed in an incubator at 37 °C (Termaks, Bergen, Norway) and the other set of samples was placed in an isothermal calorimeter (described further below). At time point zero (start of experiment) for both sets of n-bf:supplemented samples, 0.5 mL of 2X and 4X BHI broth (BD Difco, USA) was added to the one set of vials, and 0.5 mL of 20 g L^{-1} and 200 g L^{-1} glucose (VWR International) was added to the other set of vials. Additional supplements with BHI or glucose in the same sets of vials were repeated after 2, 4, and 6 days. At each time point of supplementation and for each set, the n-bf:aerated samples were opened to mimic the procedure of adding air into the biofertilizer but without adding any extra nutrients. The n-bf:blank samples, in both sets, were left undisturbed and never opened throughout the entire experimental period. The effect of each of the different supplements/treatments was analysed

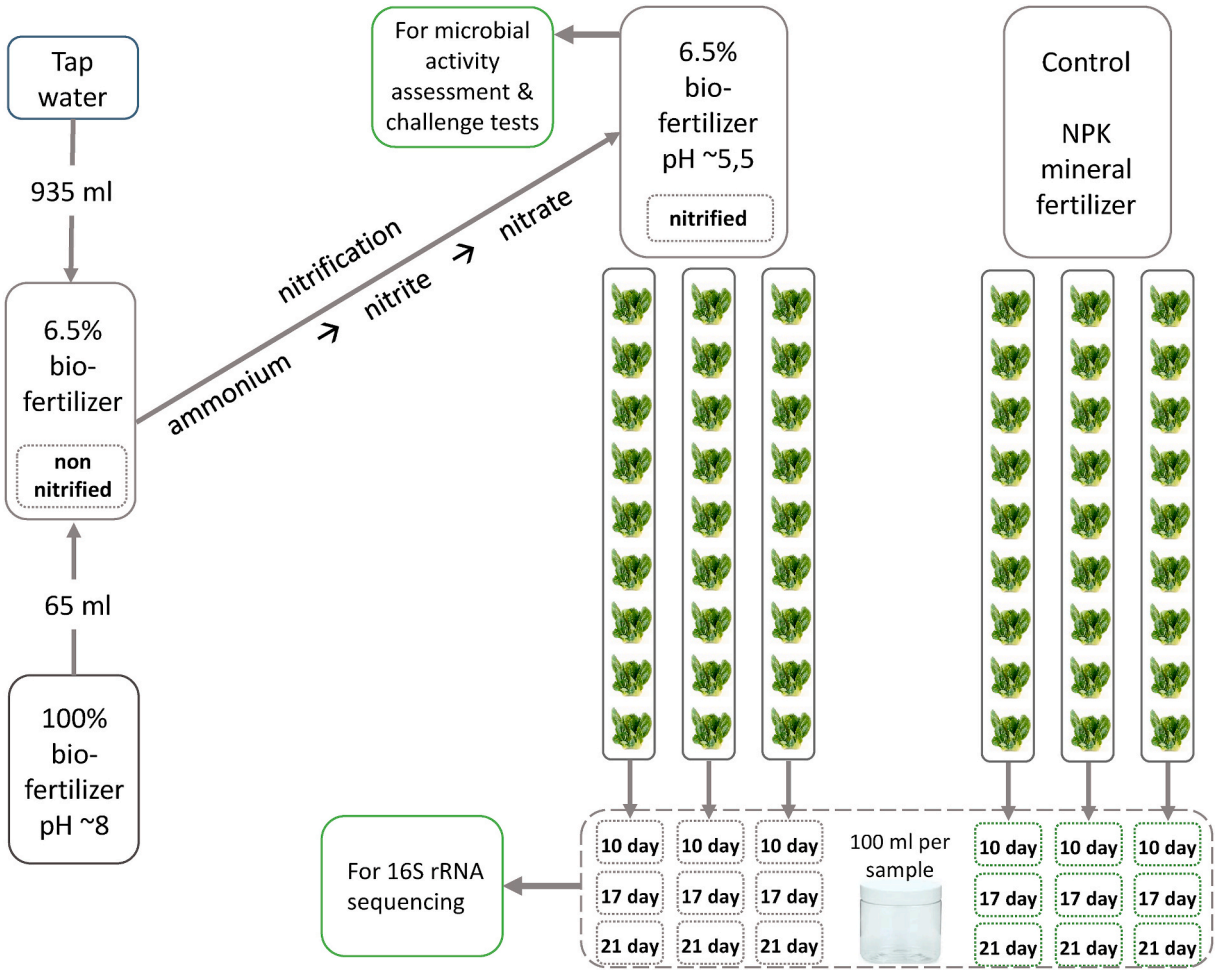

Fig. 1. Schematic illustration of the treatment of the biofertilizer obtained from a biogas production plant before use in hydroponic cultivation, including experimental setup and sampling procedures. Boxes in green annotate the different analyses performed in this study.

continuously and in real-time with IC, and after 24 h with VC and FC. The reason for choosing an accelerated assessment at 37 °C and 6 days instead of mimicking the 21-day growth cycle at 20 °C in the hydroponic system was due to the emergence of mould over time in samples supplemented with either BHI or glucose during the initial pre-studies to set up the experiment.

2.2.1. Viable count (VC)

The following samples, from the 37 °C incubator, were analysed in three replicates ($n = 3$) using VC: n-bf:blank, n-bf:aerated, and n-bf: supplemented with 2X or 4X BHI broth (BD Difco, USA). Samples supplemented with glucose were not analysed by VC (since the online IC measurements showed no generation of heat, *i.e.,* no microbial activity). Samples were collected and analysed by appropriate dilutions using 0.9% sterile NaCl solution (Merck KGaA, Darmstadt, Germany), and plated with three technical replicates per dilution on Brain Heart Infusion (BHI) agar (BD Difco, USA). After 24 h incubation at 37 °C, the number of colonies on the plates were counted and mean values were used to calculate the $\log_{10}$ colony forming units (CFU) per mL, with respect to the dilution factor used.

2.2.2. Flow cytometry (FC)

Microbial cell count was measured in parallel with FC to account for the anaerobic bacteria whose growth are not supported in the VC analysis described above. FC was carried out on a BD Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) on the same samples that were plated (except for the zero samples). Samples were diluted 1:100000 with 0.9% NaCl and filtered through a 40 μm cell strainer sieve. Samples were stained using a mixture of SYBR Green/propidium iodide. To each sample, 6 μl of staining solution was added, the samples were vortexed and then incubated in the dark for 15 min at 37 °C. After incubation the samples were vortexed again and FC was carried out on the BD Accuri C6 plus flow cytometer with the fast fluid speed (flow rate of 66 μl/min), a sample volume of 50 μl and using a FL1-H and FL3-H threshold of 1000. Tubes with milli-Q-water were run between samples to rinse the system. The blue 488 nm laser was used with the optical filters FL1 (533 ± 30 nm) and FL3 (>670 nm). The data were collected and analysed using the BD Accuri C6 Plus software. A log scale density plot of FL1-H vs FL3-H was obtained to visualize the fluorescence of the dye. Instrument settings and electronic gates were kept the same for all samples to achieve comparable data.

2.2.3. Isothermal calorimetry (IC)

Microbial viability and activity were studied in parallel over time using cultivation-independent IC measurements. An IC measures thermal power (the rate of heat production) from, e.g., microbial processes. It is a non-invasive technique that gives continuous results, making it possible to follow the kinetics of a process. The thermal power is proportional to the rate of a process and to the enthalpy of the process (how much heat that is produced per unit reaction). If the thermal power is integrated, heat is obtained, which is proportional to the extent of a process. In the case of microbial activity, aerobic and anaerobic processes differ in that aerobic processes always have an enthalpy close to -455 kJ $mol^{-1}(O_2)$ (Gnaiger and Kemp, 1990; Hansen et al., 2004),

while anaerobic processes can have widely different enthalpies depending on which end products are produced. With the vial head-space of the conditions of the present experiment, aerobic activity would produce about 50 J before all oxygen was depleted.

The IC instrument used in this project was a prototype of a Flex/Ultra-instrument (Calmetrix Inc, USA) with 20 mL polyethylene vials. The instrument contains eight calorimeters that all measure at the same constant temperature. For these experiments, the calorimeters were set to 37 °C. Each calorimeter had a reference with a similar heat capacity as the 10 mL samples, to minimize external disturbances. Baselines were measured with samples of inert material and calibrations were made with electric heaters in calibration vials. The following samples were analysed in three replicates ($n = 3$): n-bf:blank, n-bf:aerated, n-bf:supplemented with 2X or 4X BHI broth, and n-bf:supplemented with 0.5 mL of 20 g L^{-1} and 200 g L^{-1} glucose. The samples were treated and supplemented as described in Section 2.2. An additional supplementation after 7 days was included in the IC measurements, where samples previously supplemented with BHI broth were now supplemented with 20 g L^{-1} glucose, and samples previously supplemented with glucose were now supplemented with 2X BHI broth. At each timepoint the vials were taken out from the calorimeter for respective treatments, except the n-bf:blank samples that were left inside the calorimeter throughout the experiment.

2.3. Challenge tests

2.3.1. Bacterial strains

Three food-borne pathogens were used for the challenge tests conducted on the biofertilizer: *Bacillus cereus* (strain F2085), a gift from SVA, Swedish National Veterinary Institute (Fricker et al., 2011), *Salmonella enterica* ser. Typhimurium (strain CCUG-98112-08), and *Listeria monocytogenes* (strain LM052), a model strain obtained from EU reference laboratories (Guiller, 2013). All strains were stored as glycerol stocks at −80 °C and resuscitated at 37 °C overnight by streaking on Brain Heart Infusion (BHI) agar (BD Difco, USA) for *B. cereus* and *L. monocytogenes,* and Luria Bertani agar (BD Difco, USA) for *Salmonella* prior to inoculation of pre-cultures preceding the challenge tests.

2.3.2. Pre-cultures for inoculation into nitrified biofertilizer

The three bacterial strains were collected from glycerol stocks stored at −80 °C and resuscitated on agar plates as described above. The inverted plates were incubated overnight for approximately 15 h; *B. cereus* at 30 °C, *L. monocytogenes* and *S. enterica* ser. Typhimurium at 37 °C. One discrete colony for each strain was selected and transferred with a sterile plastic loop (VWR International), and inoculated into a Falcon tube (Sarstedt, Nümbrecht, Germany) with 50 mL of nutrient broth: BHI broth (BD Difco, USA) for *B. cereus* and *L. monocytogenes* and LB broth (BD Difco, USA) for *S. enterica* ser. Typhimurium. The tubes were placed on a rocking table and incubated overnight for approximately 15 h at the same temperatures as above. The concentrations of the overnight (O/N) cultures were measured using FC (BD Accuri C6, BD Biosciences, San Jose CA, USA) with optical density at 620 nm (Ultrospec 2100, Amersham Biosciences Corp., USA).

2.3.3. Set-up, performance and monitoring of challenge tests

The experiments were performed in three independent biological replicates ($n = 3$) for each strain to account for potential biological variance within the biofertilizer and respective strain. The initial targeted starting concentration of each bacterial strain was 3 $\log_{10}$ CFU (Colony Forming Units) mL^{-1} in the nitrified biofertilizer, but based on the outcome of the first replicate of challenge tests, the starting concentration was increased to 5 $\log_{10}$ CFU mL^{-1} for the following two replicates. For each set of replicates, three 1000 mL sterile baffled shake flasks containing 200 mL of nitrified biofertilizer were prepared and inoculated with one bacterial strain each, to reach the starting concentrations stated above. After inoculation, the flasks were incubated (New Brunswick Innova 40/40R, Eppendorf International) under aerobic conditions at 20 °C and 100 rpm. One flask containing only 200 mL of nitrified biofertilizer (no bacterial inoculation) was included in each round of replicates as a control. The establishment and survival of the pathogens in the nitrified biofertilizer was monitored through plating on selective agar according to the following NMKL procedures (Nordic Committee on Food Analysis): for *B. cereus* (NMKL 67) MYP (Mannitol egg Yolk Polymyxin) agar (Merck KGaA, Darmstadt, Germany) was used, for *Salmonella* (NMKL 71) XLD (Xylose Lysine Deoxycholate) agar (Merck KGaA, Darmstadt, Germany), and for *L. monocytogenes* (NMKL 136) ALOA (Agar Listeria according to Ottaviani and Agosti) (Merck KGaA, Darmstadt, Germany) was used. Samples were collected at the following timepoints during incubation: 0 h, 4 h, 8 h, 24 h, 48 h, 10 days, 17 days and 21 days. Samples were diluted by a stepwise 10x dilution series to appropriate dilutions with sterile 0.9% NaCl (Merck KGaA, Darmstadt, Germany) and two succeeding dilutions were plated on three agar plates (100 µl/plate). After 24 h incubation at 37 °C, the number of CFU were counted and mean values were used to calculate the logarithmic CFU per mL, with respect to the dilution factor.

In parallel, corresponding samples were prepared and analysed using IC. Volumes of 10 mL of nitrified biofertilizer, inoculated with each strain to a starting concentration of 3 $\log_{10}$ CFU mL^{-1} or 5 $\log_{10}$ CFU mL^{-1}, together with control samples, were monitored in duplicates at 20 °C for 21 days and heat development was registered as described in Section 2.2.3.

2.4. Microbial community analysis of fertilizers in hydroponic production settings using 16S rRNA gene amplicon sequencing

Hydroponic systems were assembled as described in Bergstrand et al. (2020) and fertilized with either nitrified biofertilizer ($n = 3$) or inorganic fertilizer ($n = 3$) (Fig. 1). Each growing unit consisted of an individual loop in which the nutrient solution was circulated. New solution and water were added to account for the plant uptake but no solution was lost through drainage. Each circulating nutrient solution was sampled over time at 0, 10, 17 and 21 days; at the final sampling the plants were fully grown (complete growth cycle). All samples (100 mL) were immediately frozen (−20 °C) after sampling.

2.4.1. Sample treatment, DNA extraction and 16S rRNA gene amplicon sequencing

Prior to filtration, the samples were thawed slowly, to avoid rupturing of the bacterial cells, in a refrigerator (5–8 °C). Pre-filtration to remove particles was performed with glass fibre filters, pore size 2.0 µm (AP2004700, Merck, Darmstadt, Germany) followed by collection of the microflora on a 0.22 µm filter (GTTP04700, Merck, Darmstadt, Germany). Filtrations were performed under sterile conditions. DNA extractions were performed with FastDNA SPIN Kit for Soil (MP Biomedicals, USA). The 0.22 µm filters containing the bacterial cells of the samples were cut into thin shreds with a sterilized knife, inserted into the lysing tubes (one filter per tube), and extraction was performed according to the manual. Quality control of all DNA extractions was performed using quantitative PCR (LightCycler Nano, Roche Diagnostics, Basel, Switzerland) and CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, USA). The PCR reactions were performed with a General Bacteria Assay, in which each well contained a mixture of 9.1 µl SuperQ water, 2 µl ImmoBuffer (10X) (Roche Diagnostics, Basel, Switzerland), 2 µl dNTP (2 mM) (Roche Diagnostics, Basel, Switzerland), 3 µl $MgCl_2$ (25 mg μl^{-1}) (Roche Diagnostics, Basel, Switzerland), 0.6 µl Primer bact F (5′-TCCTACGGGAGGCAGCAGT-3′) (10 µM) (Roche Diagnostics, Basel, Switzerland), 0.6 µl Primer bact R (5′-GGACTACCAGGGTATCTAATCCTGTT-3′) (10 µM) (Roche Diagnostics, Basel, Switzerland), 1 µl EVAGREEN (20X) (Roche Diagnostics, Basel, Switzerland), 0.5 µl BSA (10 mg mL^{-1}) (Roche Diagnostics, Basel, Switzerland), IMMOLASE™ DNA Polymerase (5 U μL^{-1}) (Roche Diagnostics, Basel, Switzerland),

and 2 μl of sample to be analysed. All the concentrations in parentheses are stock concentrations. The PCR program consisted of an initial denaturation at 95 °C for 10 min, followed by the cycling of denaturation at 95 °C for 10 s, annealing at 60 °C for 6 min, and extension at 72 °C for 30 s, in 45 cycles.

The company DNASense (https://dnasense.com/) performed and analysed the 16S-rRNA gene amplicon sequencing. Primers chosen for the sequencing of V4 variable region were the primer pair 515FB (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806RB (5′-GGACTACNVGGGTWTCTAAT-3′).

3. Results and discussion

To assess the microbiological food safety of using anaerobic digestate as a biofertilizer in hydroponic vegetable production, the nitrified biofertilizer was investigated from three perspectives; (1) the activity and viability of the natural microflora present in the nitrified biofertilizer was assessed with VC, FC and IC, (2) the susceptibility of the biofertilizer for food-borne pathogens *S. enterica, L. monocytogenes* and *B. cereus* to grow and establish themselves was investigated with challenge testing, and (3) the microbial community of the non-nitrified and nitrified biofertilizer, as well mineral fertilizer, was analysed with 16S rRNA gene amplicon sequencing.

3.1. Microbial activity assessment in the original nitrified biofertilizer

IC was utilized to measure the heat developed over time in samples with (n-bf:supplemented) or without supplementation (n-bf:blank and n-bf:aerated) of nutrients. The heat developed is a result of metabolic activity of the organisms in the sample, and it was thus utilized as a cultivation-independent method of investigating microbiological viability and/or growth, an asset when assessing complex samples that may contain viable but not culturable cells. It also has the advantage of monitoring microbiological viability and growth without the introduction of bias that the agar plates selected in traditional standard plate count may account for, and the calorimetric measurement also gives an on-line and continuous output. At the same time, VC analysis was performed. Obtained VC results of samples without supplementation indeed pointed towards an actively growing microflora present, since a rich number of colonies was obtained on the plates at each sampling point. Contradictory to these results, there was no heat generation detected within the same samples (without supplementation) when utilizing IC. The IC thus provided a presentation of the microbiological state of the biofertilizer without the bias that the introduction of nutrients from an agar plate may introduce.

Fig. 2A and B shows the analyses from VC and FC after supplementation of BHI broth, and from IC after supplementation of BHI broth and glucose to the biofertilizer during the full duration of the accelerated microbial activity assessment experiment. As can be seen in Fig. 2B, the supplementation of glucose generates no metabolic activity. Regarding the supplementation of BHI broth, the first supplementation generates a heat production of around 100 J, with the following two supplementations generating a heat production of around 50 J, and the last two supplementations generating around 25 J. If it is assumed that the headspace of the vials is filled with air each time a vial is opened, the first supplementation generates more than the 50 J that aerobic metabolism can give, so this part does include anaerobic processes, but the lower heats indicate that the processes may be mainly aerobic. Since the last two supplementations do not reach 50 J of heat produced, it is hypothesized that maximum growth capacity in the matrix has been reached, possibly due to restrictions in water activity or antagonistic behavior within the microbial community. The corresponding VC (Fig. 2A) shows a 2.5 log increase in CFU mL^{-1} after the first supplementation, and the following supplements induce no substantial increase in growth with either VC or FC (Fig. 2A). When performing microbial food safety risk assessments, not only presence/absence but also levels or concentrations of microorganisms, are valuable pieces of information needed to be able to evaluate food safety risks. From these results it appears however that the heat produced from metabolic activity is challenging to correlate with the number of CFU mL^{-1} and cell count mL^{-1}. According to a review by Braissant et al., the heat production of creating a cell should be rather constant, and it is also stated that if cell lysis takes place, this will cause a discrepancy between the heat generated and cell count (Braissant et al., 2013). The fact that the sample contains a complex, mixed microbial community complicates the interpretation of the heat flow and heat generated after multiple supplementations, as the metabolism of different bacteria will generate different heat flows, and also there might be a succession of bacteria or a decline in some species caused by metabolites produced by the predecessor. This hypothesis was further hinted at when the visual inspection of the agar plates from the VC indeed revealed varying colony morphology and appearance after the different supplementations, and

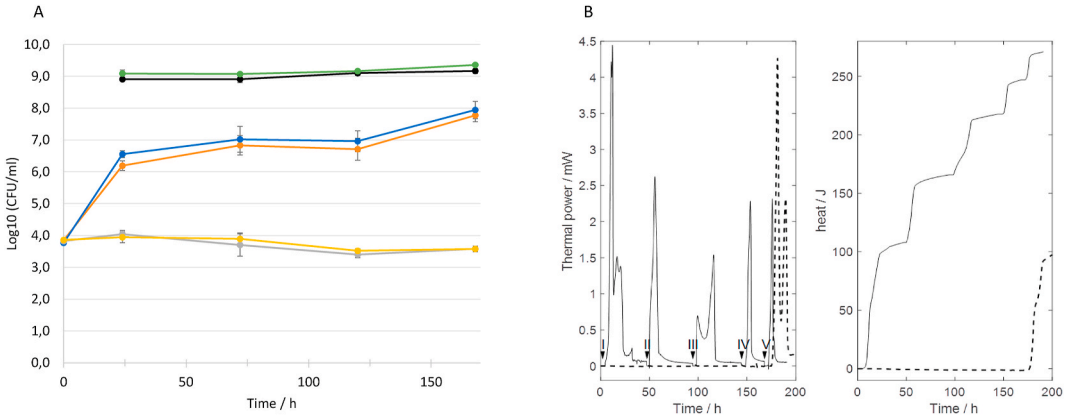

Fig. 2. Accelerated microbial activity assessment in nitrified biofertilizer at 37 °C over seven days with repeated supplementations of BHI broth or glucose. Microbial activity and growth were measured using flow cytometry, viable count and isothermal calorimetry. (A) Flow cytometry (FC) (log cells mL^{-1}) and viable count (VC) (log cfu mL^{-1}) with supplementations of 2X and 4X BHI broth at t = 0, 48, 96, 144 h and sampling at t = 24, 72, 120, 168 h. FC: BHI 2X = black, BHI 4X = green; VC: BHI 2X = orange, BHI 4X = blue. Controls including no supplementation with aeration (yellow) and without aeration (grey). Average values with the standard deviation of three biological replicates are presented (n = 3). (B) Isothermal calorimetry measurement (one representative replicate) showing thermal power (mW) and heat (J) generated after supplementation of BHI broth (black) and glucose (dashed) to the biofertilizer at t = 0, 48, 96, 144 h, denoted in roman numerals. To the left, thermal power in mW generated from each supplementation. At V, the supplementations were switched so that samples previously supplemented with BHI were now supplemented with glucose (and vice versa). To the right, the heat in J generated from each supplementation.

also revealed some colonies exerting antimicrobial behaviour on their surroundings (data not shown).

This study has highlighted the difficulties of correlating VC with calorimetric data, which has already been observed in studies of other complex natural matrices (Alklint et al., 2004). However, it can be stated regarding both the calorimetric data and the VC that while bacteria are indeed present in the biofertilizer, no metabolic activity is generated until the supplementation of an external complex nutrient source. As seen in Fig. 2A, the supplementation of glucose did not lead to subsequent metabolic activity while BHI broth did, indicating that an accessible source of carbon was not the (sole) limiting factor for microbiological activity, but potentially a combination of a carbon source and necessary trace elements that the BHI broth provides. In terms of the microbiological safety of utilizing this biofertilizer for hydroponic vegetable production, it is thus essential to avoid the addition of a nutrient source that can allow for the establishment of pathogenic bacteria.

Although cultivation-based VC is a commonplace method of evaluating food safety in a matrix, it may allow for the introduction of false negative results when viable but not culturable cells remain undetected. As presented in this study, it might also produce false positive results when the agar plates provide the nutrients necessary to allow for microbial growth, while the biofertilizer on its own does not provide the necessary factors for growth, rendering its natural microflora dormant. Studying the microbial community of complex natural matrices is in general difficult as several parameters of the matrix and its microbial processes are unknown. As pointed out by Wadsö in a work using IC for studying the microbial activity in soil (Wadsö, 2009), IC is useful due to heat measurements being non-specific, and might thus be a preferable tool when investigating the total sum of complex microbial activities within a natural sample. Although IC is insufficient on its own for determining the microflora, separating the processes of one microorganism from the other, or separating microbial metabolic activity from microbial growth, IC and VC are excellent complementary tools when investigating the presence, viability and activity of complex microbial communities in their natural matrix.

3.2. Challenge tests of the nitrified biofertilizer with B. cereus, S. enterica and L. monocytogenes

Microbial contamination of biofertilizer in hydroponics could cause serious consequences as the biofertilizer is recirculated and the plants are exposed to it during their entire growth cycle. A previous review, assessing the internalization ability of bacteria present in nutrient solution in hydroponic setups, concluded that present pathogenic bacteria and viruses internalize readily and more frequently compared to soil-based systems (Riggio et al., 2019). This endorses the need for a deeper and more systematic understanding of how pathogenic bacteria would behave in the biofertilizer, in case of a contamination scenario, to assess the risks of using biofertilizers from anaerobic digestate for food production in hydroponic systems. As *Bacillus cereus* occurs naturally in the biofertilizer, and *Salmonella* and *Listeria* are able to internalize into growing crops (Golberg et al., 2011; Shenoy, 2015), a challenge test experimental setup was performed to simulate contamination with the food-borne pathogens *S. enterica* serovar Typhimurium, *L. monocytogenes* and *B. cereus,* and assess their establishment, survival and growth in the nitrified biofertilizer over time.

Fig. 3 shows the outcome of the inoculation of the food-borne pathogens *B. cereus*, *S. enterica* and *L. monocytogenes* in the biofertilizer. In the two biological replicates performed, *S. enterica* and *L. monocytogenes* were no longer detectable through selective plating within 48 h of incubation. Throughout the two biological replicates, *B. cereus* was steadily present in the control of non-inoculated biofertilizer and estimated at 1 $\log_{10}$ CFU mL^{-1}. The biofertilizer inoculated with *B. cereus* decreased to these levels within 24 h after incubation. Previous microbiological controls at three Swedish biogas production

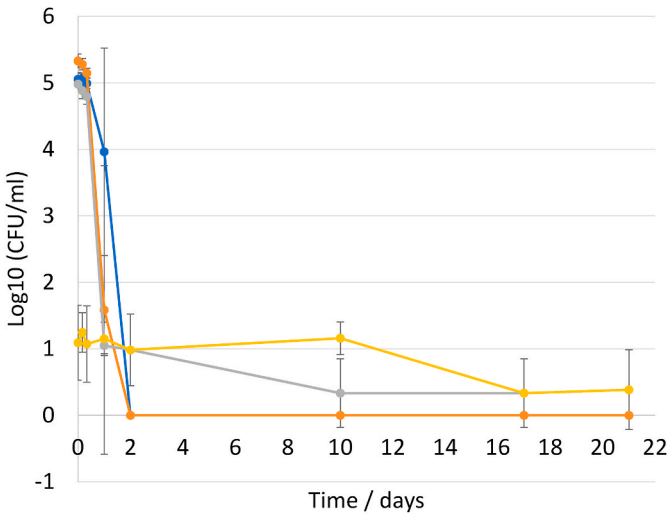

Fig. 3. Challenge test of nitrified biofertilizer with the food-borne pathogens *S. enterica*, *L. monocytogenes* and *B. cereus*. Viable count (log cfu mL^{-1}) using selective plating of *S. enterica* (blue), L. monocytogenes (orange) and B. cereus (grey) over 21 days after initial inoculation of respective pathogen in concentration 5 log cfu mL^{-1} biofertilizer. Non-inoculated biofertilizer plated on MYP agar selective for B. cereus (yellow). Average values with the standard deviation of two biological replicates are presented (n = 2).

plants had shown that the biofertilizer product after hygienization and anaerobic digestion contained up to 4.3 $\log_{10}$ CFU *B. cereus* per gram of biofertilizer (unpublished data). This level should be given attention, since the majority of food-borne outbreaks caused by *B. cereus* have been implicated with concentrations of 5–8 $\log_{10}$ CFU g^{-1} of food of emetic toxin producing *B. cereus*. Occasional outbreaks of both emetic and diarrhoeal *B. cereus* illnesses with even lower levels have also been reported. The maximum acceptable levels of *B. cereus* in food vary slightly between countries, but in general concentrations between 3 and 5 $\log_{10}$ CFU g^{-1} are considered satisfactory and above 5 $\log_{10}$ CFU g^{-1} unsatisfactory (Allende et al., 2016). Another important factor to consider/include in our study is that after the nitrification process, preceding the introduction of the biofertilizer in the hydroponic growth system, the levels of *B. cereus* was monitored to 1 $\log_{10}$ CFU/mL indicating that this process might lower the initial high concentration to acceptable levels. The continuous presence of low levels of *B. cereus* throughout the challenge tests however indicates that the biofertilizer has a capacity to act as a reservoir for *B. cereus* spores and this is a critical factor to consider in each risk assessment for this product matrix. The bacteria's ability to form spores (Bottone, 2010) provides an explanation as to how it can be present after hygienization and anaerobic digestion of the biofertilizer, and also to how it can be steadily present in the biofertilizer in the challenge test experiments despite a large amount of the inoculation dying off after a very short time after incubation. The fact that *S. enterica* and *L. monocytogenes* do not establish themselves, even seemingly dying off within 48 h after incubation in the biofertilizer, implies that the biofertilizer constitutes a highly inhospitable environment for these food-borne pathogens. In the case of still having viable but non culturable cells, a calorimetric measurement where the biofertilizer was inoculated with the pathogenic bacteria was performed in parallel with the selective plating. As well as the non-supplemented control samples in Section 3.1, these samples showed no signs of microbial activity (data not shown), which corresponds to the results from the selective plating. The apparent lack of nutrients supporting microbial growth in the biofertilizer (seemingly an accessible carbon source and trace elements) could be hindering the establishment of these pathogenic bacteria. This is supported by the findings in the accelerated microbial activity assessments in Section 3.1 where growth was only obtained after supplementation of BHI. This is also in line with a recent

study by Fernández-Domínguez et al. (2021) who reported that non-biodegradable compounds increased largely after anaerobic digestion. Besides the lack of available nutrients, the chemical composition of the biofertilizer could exert an additional inhibitory effect on the establishment and survival of the food-borne pathogens. The pH of the biofertilizer was measured initially and was between 5.8 and 6.1, thus the pH of the biofertilizer should not be hindering the establishment of the bacteria. A previous chemical analysis of the presence of PPCP's (Pharmaceuticals and Personal Care Products) in the biofertilizer (pilot study, unpublished data), shows that the samples collected from local Swedish biogas production plants (from where this biofertilizer is collected) may contain considerable levels, exceeding 100 ng g^{-1} of fenbendazole (a compound used to treat roundworm in animals (Zamanian et al., 2018)), however this compound is not reported to have any antimicrobial activity (EMEA, 2004). Antimicrobial agent sulfaclozine (used for treatment of various poultry diseases (Sentepe and Eraslan, 2010)) was detected in low concentration, which might very well have an impact on the establishment of the pathogens. Theobromine, an antimicrobial bitter compound (Lakshmi et al., 2019) found in cocoa was detected in levels of μg g^{-1}, and caffeine (which also possesses an antimicrobial activity (Pruthviraj et al., 2011)) was found in similar levels, which also might have an effect in hindering the establishment of the pathogens. Fungicides propiconazole, fludioxonil and imazalil were detected in considerable amounts, ranging from 100 to 900 ng g^{-1}. It is possible the presence of these compounds and/or other inhibitory compounds produced by methanogens during anaerobic digestion in the biogas production plants, in combination with the apparent lack of nutrients, make the biofertilizer a non-growth-supporting environment for the food-borne pathogens to survive.

3.3. Microbial community analysis in hydroponic production settings utilizing 16S-rRNA gene amplicon sequencing

Previous studies on the microbial community of anaerobic digestates reveal that the results vary widely depending on the composition and treatment of ingoing substrate, conditions of digestion, and variable region chosen to be sequenced, however, most studies conclude the most dominant phyla to be Firmicutes, Bacteroidetes, and Proteobacteria (Sun et al., 2015; Treu et al., 2016; Tuan et al., 2014). The general focus in sequencing of biofertilizers in previous studies has been on plant-growth promoting microbes, with less focus on risks regarding human health in the utilization of biofertilizer for food production. The purpose of this study was thus to consider the information from the microbial community analysis from a food safety perspective.

The library preparation of the 16S rRNA gene amplicon sequencing yielded low amounts of DNA (<1 ng μl^{-1}) for all samples except for the samples of non-nitrified biofertilizer (>10 ng μl^{-1}) and the starting sample of nitrified biofertilizer (before addition to the hydroponic channels) (>10 ng μl^{-1}). A negative control was also included in the library preparation. The 25 most abundant genera across all samples can be observed in Fig. 4A. If no genus level classification could be obtained, the lowest assigned taxonomic classification was given. In addition, the phylum level classification was given (Proteobacteria at class level). The microbial communities of the samples were also analysed with multivariate statistical analysis (principal component analysis), demonstrating the similarity in microbial community between the samples, as can be observed in Fig. 4B.

The 16S rRNA gene amplicon sequencing revealed that the most abundant genus within all the samples of nitrified biofertilizer from the hydroponic channels was *Mycobacterium* (belonging to phylum Actinobacteria). This genus could not be detected in the samples of non-nitrified biofertilizer or the inorganic fertilizer. Since it is also present in the Day 0 sample (*i.e.*, before circulation in the hydroponic production system), a transfer or contamination from the plant roots to the biofertilizer can be ruled out. Seeing that the nitrified and non-nitrified biofertilizer differ so drastically in the microbial community composition, as can be observed in the principle component analysis, it is apparent that the nitrification process, including lowering of the pH from 8 to 5.5, aeration, and changes in the composition of nitrogen compounds, affects the microflora present. *Mycobacterium* are known to be hardy bacteria that have acidic tolerance and resistance to disinfection, and can survive and grow in a low organic carbon environment (water) (Primm et al., 2004). Since it is not detected in the non-nitrified biofertilizer it indicates that it emerges in the biofertilizer in some step after nitrification, however a count of 0 in relative abundance might not mean that the genus is absent, but that it is below the limit of detection (Hugerth and Andersson, 2017). Regardless, finding *Mycobacterium* in a sample such as the biofertilizer is not unreasonable as they have been found to be common in cattle manure and in water distribution systems, and can survive there for long periods of time (Hruska and Kaevska, 2012). It is also not such a surprising result to find this genus in the nitrified biofertilizer considering that species within the genus are denitrifying bacteria, and can rely on nitrate as an energy source during anaerobic conditions (Khan and Sarkar, 2012; Philippot, 2005). While *Mycobacterium tuberculosis* and *Mycobacterium leprae* are well-known pathogenic mycobacteria, several species of environmental mycobacteria can also be opportunistic human pathogens (Primm et al., 2004), and a further investigation of species level would be interesting in the

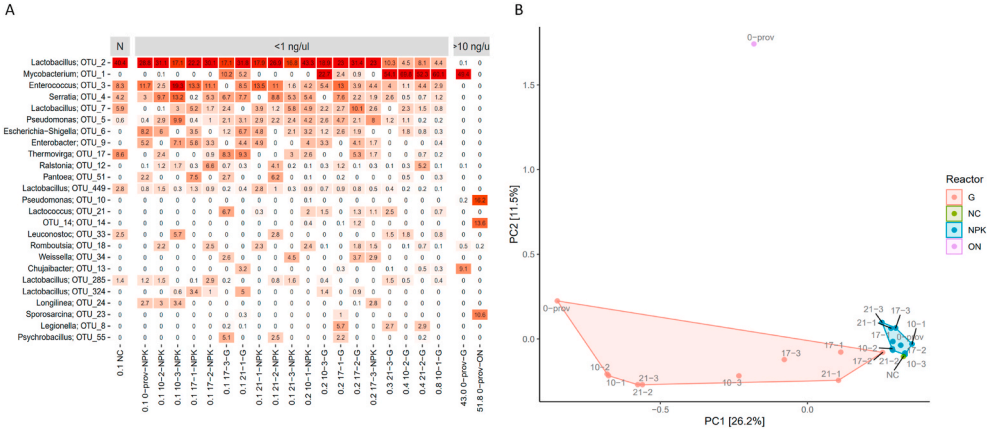

Fig. 4. Microbial community analysis with 16S rRNA gene amplicon sequencing of nitrified, non-nitrified and mineral fertilizer samples collected from hydroponic cultivation experiments. (A) Heatmap of the 25 most abundant genera in the fertilizers. The intensity of red represents the relative abundance, with a darker red being a higher relative abundance. Furthest to the left is the negative control (containing sequenced background from the PCR reagents), with samples containing <1 ng μl^{-1} of DNA in the middle, and samples containing >10 ng μl^{-1} of DNA furthest to the right. Samples are named with the first number denoting its DNA-concentration, followed by day of sampling, number of biological replicate and type of sample with NPK = mineral fertilizer, G = nitrified biofertilizer and ON = non-nitrified biofertilizer. (B) Principal component analysis illustrating the phylogenetic relationship between the samples. Samples of nitrified biofertilizer (G) are marked in red, samples of mineral fertilizer (NPK) in blue, with non-nitrified biofertilizer (ON/0-prov) in purple and negative control (NC) in green.

aspect of ensuring microbiological safety in the utilization of this biofertilizer for production of food.

In the non-nitrified biofertilizer the most abundant genera from the16S rRNA gene amplicon sequencing were *Pseudomonas*, *Leuconostoc* and *Sporosarcina*. *Pseudomonas* and *Sporosarcina* are naturally found in soil (Mercado-Blanco and Bakker, 2007; Pregerson, 1973), and *Leuconostoc* is normally found widespread throughout the environment, in fermented foods and in plant matter (Holland and Liu, 2011). The microflora of the nitrified biofertilizer changed over time, as can be observed in the principal component analysis. The microflora of the nitrified biofertilizer also varied between the samples taken at the same timepoint but from different channels; in comparison the samples of the inorganic fertilizer are much more clustered and vary less between samples. This behaviour can be connected to the variance shown between samples from the viable count analysis in Section 3.1, where the microflora differed in different replicates of the same kind of sample, and also from the discovery that some members of the microbial community exert antagonistic behaviour towards others.

In the inorganic fertilizer, the most abundant genera were *Lactobacillus*, *Enterococcus*, *Serratia*, and *Pseudomonas*. The high relative abundance of OTUs detected in the negative control in the samples is a result of the low DNA yield of the samples (mainly in the samples from hydroponic channels with the inorganic fertilizer), rendering the sequenced background more prominent. It was concluded that these genera cannot be distinguished to originate from the sample or the sequenced background. The genera can originate in the sequenced background as the ingredients of the PCR reaction of the sequencing may contain bacterial DNA, which is a common occurrence. (Bech Lukassen, DNASense, 2020, personal communication). It was furthermore not expected to have high yields of DNA in the inorganic fertilizer. In the inorganic fertilizer the most abundant genera were *Lactobacillus*, although this was also the most abundant in the negative control and is believed to be sequenced background, *Enterococcus*, also present in negative control but in generally lower abundances, *Serratia*, also present in negative control, and *Pseudomonas* present in negative control but in very low relative abundances.

The low DNA yield from the biofertilizer samples was a quite unanticipated result as the biofertilizer was expected to have a rich microflora as a result of the anaerobic digestion. If the nitrification process that the biofertilizer undergoes was the culprit for the reduction in natural microflora, it would at least have been expected to find some genera of nitrifying bacteria in the 16S rRNA gene amplicon analysis. As this was not the case one explanation is that the apparent lack of factors for growth, as is fortified by the findings in the microbial activity assessment of the biofertilizer in Section 3.1, has simply reduced the types of microorganisms that can survive to very hardy bacteria such as *Mycobacterium* or spore formers that can endure in the low-carbon environment that the biofertilizer constitutes. In a study of the microbial community of soil, it is found that the low DNA yield is in fact a result of poor growth rather than an inadequate DNA extraction (Kleyer et al., 2019). It is also reported that low DNA content might introduce bias in 16S rRNA gene amplicon sequencing analysis, which is an important parameter to keep in mind when drawing conclusions regarding the composition of the microbial community (Chandler et al., 1997).

4. Conclusions

Based on microbial activity studies, challenge tests and microbial community analysis, this study has generated insights important for the food safety risk assessment of a biofertilizer used for food production in a hydroponic cultivation system. The biofertilizer was based on anaerobically digested food waste, thus offering a possibility for valorisation of food waste. The microbiological community of the biofertilizer changes distinctly through a necessary initial nitrification process, and the nitrified biofertilizer does not provide a favourable environment for the food-borne pathogens *B. cereus*, *S. enterica* and *L. monocytogenes* to either grow or establish. The following conclusions were made based on the results obtained in this study: The most abundant genus of bacteria in the nitrified biofertilizer was *Mycobacterium*. Low levels of *B. cereus* (~10 CFU mL^{-1}) were naturally present in the nitrified biofertilizer. Four log_{10} reductions of *B. cereus* were observed after its inoculation in the nitrified biofertilizer within 24 h at 20 °C. An inoculation of 5 log_{10} CFU mL^{-1} of *S. enterica* and *L. mono*cytogenes in the nitrified biofertilizer was no longer detectable with selective plating after 48 h of incubation at 20 °C. To conclude, the nitrified biofertilizer analysed in this study does not seem to support microbial proliferation unless it is supplemented with rich substrates containing a carbon source and other trace elements. These are promising results for implementing future potential upcycling of food waste as a resource to produce new food. However, further research is essential and risk assessments should continuously be performed on this type of resource as the microbial community might vary with seasons and ingoing substrates. Additionally, any modifications and/or additional enrichment (e.g. components containing an accessible carbon source) of the biofertilizer should be preceded by a re-evaluation of the microbial risk assessment before use in order to ensure safe food production systems.

CRediT authorship contribution statement

Julia Södergren: Conceptualization, Methodology, Investigation, Writing – original draft. **Christer U. Larsson:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Lars Wadsö:** Resources, Data curation, Visualization, Writing – review & editing. **Karl-Johan Bergstrand:** Resources, Writing – review & editing. **Håkan Asp:** Resources, Writing – review & editing. **Malin Hultberg:** Conceptualization, Funding acquisition, Methodology, Resources, Writing – review & editing. **Jenny Schelin:** Supervision, Conceptualization, Methodology, Writing – original draft.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2021.130239.

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