Novi *et al. Plant Methods* (2024) 20:119
https://doi.org/10.1186/s13007-024-01254-8

Plant Methods

METHODOLOGY Open Access

A rapid LAMP assay for the diagnosis of oak wilt with the naked eye

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Abstract

Background Oak wilt disease, caused by *Bretziella fagacearum* is a significant threat to oak (*Quercus* spp.) tree health in the United States and Eastern Canada. The disease may cause dramatic damage to natural and urban ecosystems without management. Early and accurate diagnosis followed by timely treatment increases the level of disease control success.

Results A rapid assay based on loop mediated isothermal amplification (LAMP) was first developed with fluorescence detection of *B. fagacearum* after 30-minute reaction time. Six different primers were designed to specifically bind and amplify the pathogen's DNA. To simplify the use of this assay in the field, gold nanoparticles (AuNPs) were designed to bind to the DNA amplicon obtained from the LAMP reaction. Upon inducing precipitation, the AuNP-amplicons settle as a red pellet visible to the naked eye, indicative of pathogen presence. Both infected and healthy red oak samples were tested using this visualization method. The assay was found to have high diagnostic sensitivity and specificity for the *B. fagacearum* isolate studied. Moreover, the developed assay was able to detect the pathogen in crude DNA extracts of diseased oak wood samples, which further reduced the time required to process samples.

Conclusions In summary, the LAMP assay coupled with oligonucleotide-conjugated gold nanoparticle visualization is a promising method for accurate and rapid molecular-based diagnosis of *B. fagacearum* in field settings. The new method can be adapted to other forest and plant diseases by simply designing new primers.

Keywords Oak wilt, Red oak, LAMP assay, Gold nanoparticles, *Bretziella Fagacearum*

Introduction

Oak trees (*Quercus* spp., Fagaceae) are widely distributed across the United States being found in both natural, planted, and urban forest ecosystems [1]. They are estimated to account for 11% of all US trees. In the Eastern US, oaks are most abundant in the oak-hickory forest type group [2]. Besides their economic importance as a source of high-quality lumber [3], oaks are ecologically important for wildlife (e.g., habitat, food) and provide many ecosystem services (e.g., reducing soil erosion and air pollution) [4]. They are also valued for their aesthetics and for cultural purposes [1].

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Oak wilt, caused by the fungus *Bretziella fagacearum*, is a destructive vascular wilt disease of *Quercus* spp. in the Eastern USA and was recently found in Southern Ontario, Canada. The red oak species (Section Lobatae) are highly susceptible to oak wilt compared to the moderate to low susceptibility of the white oak species (Section Quercus) [2]. The pathogen spreads from diseased trees to healthy ones below-ground through connected roots and above-ground via insect vectors [5]. Although *B. fagacearum* cannot be eradicated from a tree once it is infected, treatments such as girdling, and herbicide spraying are available to prevent or reduce the probability of spread to healthy trees. Early detection and timely treatment of single or small clusters of infected trees offer the best chance of controlling oak wilt [6].

Unfortunately, damage to oaks caused by other biotic agents and abiotic factors may be confused with foliar symptoms of oak wilt. For example, drought stress coupled with two lined chestnut borer (*Agrilus bilineatus*) infestations cause symptoms in red oaks that can be hard to distinguish from those caused by oak wilt. Laboratory testing of woody samples from suspected diseased trees is advisable, particularly if treatment is warranted. Traditional fungus culture and polymerase chain reaction (nested PCR, real-time PCR, qPCR) techniques are currently used by US plant diagnostic laboratories for testing both red oak and white oak species for *B. fagacearum*. Small wood chips or drill shavings from sapwood of suspect oaks are generally tested. Culture assay requires 14 to 21 days to provide results, while results from PCR-based methods may be possible within less than one day [7]. In Canada, two real-time PCR / qPCR TaqMan® assays were developed for laboratory settings; high sensitivity and reliable results in detecting *B. fagacearum* from environmental samples were reported [8, 9]. In addition, a DNA endonuclease-targeted CRISPR trans reporter (DETECTR) assay using DNA amplified by recombinase polymerase amplification (RPA) was developed and validated using environmental samples, specifically, oak wilt fungal mats and *B. fagacearum*-infested insects [9]. The method was then optimized for cost-effective and "point of care" (i.e., field) use based on results of beta testing by multiple potential users. Research by others has yielded preliminary or potential techniques for rapid DNA assay of sapwood samples in the field [10–13].

Nucleic acid amplification tests (NAATs), such as PCR-based assays, have been considered as one of the most sensitive methods for pathogen detection due to their ability to amplify very small amounts of the pathogen's genetic material [14, 15]. However, PCR-based assays require expensive equipment to maintain multiple cycles of varying temperature for the amplification process. They also require trained laboratory personnel for operation, which makes their adaptability to field tests nearly impossible [16]. To overcome this challenge, several isothermal nucleic acid amplification methods have been developed to eliminate the necessity for sophisticated equipment and to save time and costs. Recombinase polymerase amplification (RPA) [17], helicase-dependent amplification (HDA) [18], and loop-mediated isothermal amplification (LAMP) [19] are examples of such methods that have been utilized for the diagnosis of plant diseases [20]. Among these, LAMP-based assays were found to be highly sensitive and more flexible for optimizing reactions based on the target pathogen [21]. Since this method requires at least four primers with the inclusion of loop primers, the nucleic acid amplification is highly specific to the target and occurs much faster under the right conditions [22]. For these reasons, several plant pathogen detection studies based on LAMP have been reported for rapid onsite testing [23].

In this study, we report the development, characterization, and testing of the first LAMP assay for rapid and sensitive detection of *B. fagacearum*, the causal agent of oak wilt, in *Quercus rubra* (northern red oak) samples. Furthermore, the combination of our LAMP assay with a recently developed amplicon visualization technique employing nanoparticle assembly [13] enables naked-eye detection of the oak wilt fungus.

Materials and methods

Materials

WarmStart® LAMP Kit (DNA & RNA) was purchased from New England Biolabs (Ipswich, MA), which contains Warmstart LAMP 2X Master Mix and LAMP fluorescent dye (for fluorescence detection). Primers for LAMP experiments and oligonucleotide sequences for AuNP conjugation were fabricated by Integrated DNA Technologies (Coralville, IA). All solutions were prepared using nuclease-free water purchased from Stemcell Technologies (Vancouver, Canada). Sodium chloride (NaCl), gold (III) chloride trihydrate, trisodium citrate dehydrate and Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were obtained from Sigma-Aldrich (St. Louis, MO). GelRed® Nucleic Acid Stain 10000X Water was purchased from Millipore Sigma (Burlington, MA). GeneRuler Low Range DNA Ladder, 10X UltraPure TAE Buffer and UltraPure DNase/RNase-Free Distilled Water were purchased from Thermo Fisher Scientific (USA) for gel electrophoresis visualization of amplicons. Ethanol was procured from Fisher Scientific (USA). Ethylenediaminetetraacetic acid (EDTA) was obtained from Boston Bioproducts (Milford, MA).

Fungal culture and DNA extraction

Fungal cultures were isolated from oak wilt-infected *Q. ellipsoidalis* wood samples. Wood tissues exhibiting a distinctive streaking of oak wilt infection were collected

using sterile procedures and plated on Barnett's media [24] and incubated for approximately 14 days at 25 °C. *Bretziella fagacearum* and other fungi that were frequently obtained during the procedure were then grown on half-strength potato dextrose agar (PDA) to get pure cultures. *Bretziella fagacearum* was cultured on PDA for 7 to 14 days at 25 °C. DNA from the pure fungal cultures was then extracted following manufacturer's instructions from a commercial kit (QIAamp DNA Mini Kit) with slight modifications in the buffer volumes. DNA was extracted from other fungal cultures by following manufacturer's instructions from a commercial kit (Qiagen DNeasy Plant Mini Kit). Through PCR using universal ITS1F/ITS4 fungal primers [25–27], Sanger sequencing, and BLAST searches, the other fungi were determined to be *Dicarpella* sp., *Fusarium sporotrichioides*, *Graphostroma* sp., *Querciphoma carteri*, and *Epicoccum nigrum*. These were used in the analytical specificity studies described below. An additional analytical specificity test was also conducted using a closely related fungus, *Ceratocystis fimbriata* CBS 146.53 obtained from ATCC (Manassas, VA) and cultured according to instructions provided. The DNA extraction for this organism was done using the same protocol used for *Bretziella fagacearum* pure cultures.

DNA extraction from red oak samples

Drill shavings of Northern pin oak (*Q. ellipsoidalis)* samples were obtained from various locations of known oak wilt infection centers in Central Minnesota (Anoka, Chisago and Sherburne counties) in the summer of 2022. The DNA from the wood samples were extracted using a previously developed extraction protocol. Each extraction used around 110 mg of wood shavings [11]. Each extraction yielded about 340 µL of DNA. The samples were predetermined to be healthy or infected by *B. fagacearum* using nested PCR (Fig. 1) [7]. This is the benchmark procedure to which this new method is compared. Ten

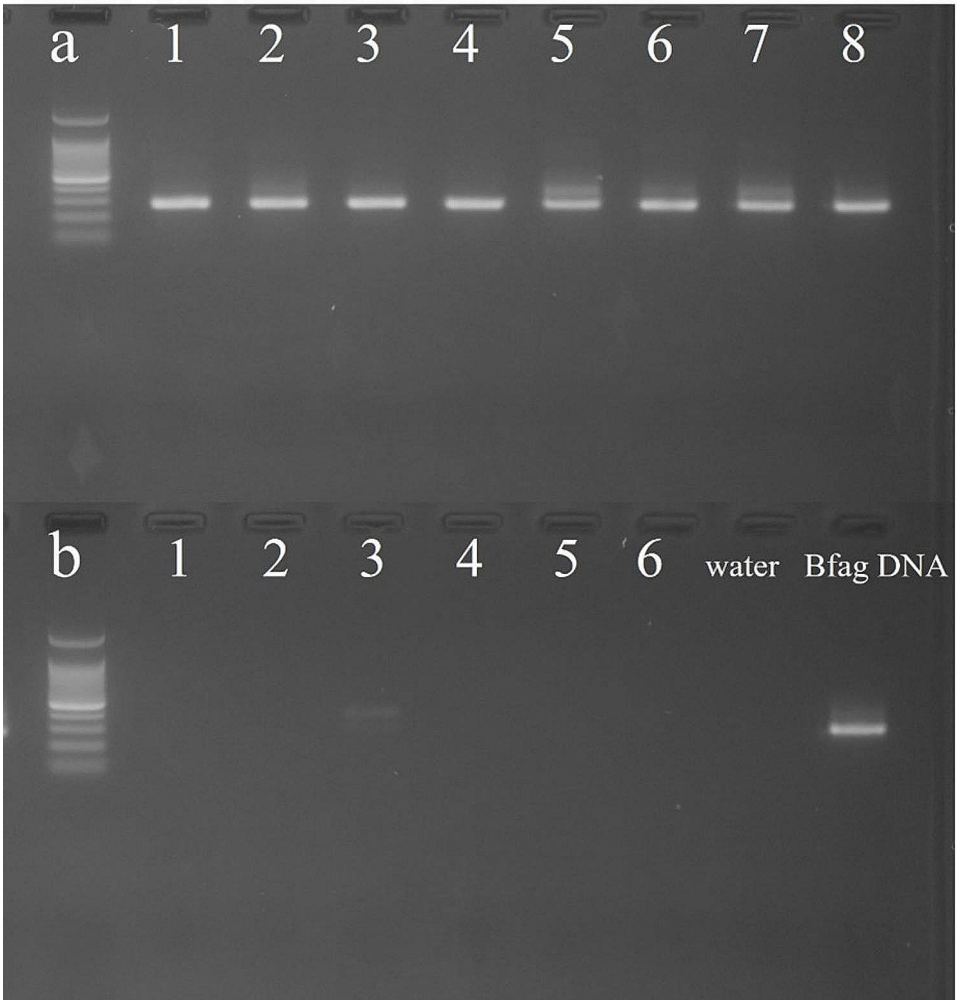

Fig. 1 Agarose gel electrophoresis of nested PCR products obtained from testing red oak samples using crude NaOH-extracted DNA. **(a)** Infected samples; **(b)** healthy samples, water control, positive control. Samples were from the same branches as those used for the LAMP assay

samples each of healthy and infected drill shavings were used for the study and DNA was extracted using the full protocol including purification steps. Additionally, five samples each of healthy and diseased wood tissues from the Plant Disease Clinic at UMN were sent to Frontline Biotechnologies Inc., for DNA extraction [11] and these were used in this study for confirmatory tests. A subset of crude extracts (without purification steps) of two samples each of healthy and diseased trees were used to test the feasibility of using a simple and less time-consuming method for obtaining DNA for the LAMP assay.

Evaluation of the LAMP assay

Three sets of LAMP primers, targeting the ribosomal DNA (rDNA) internal transcribed spacer (ITS) of *B. fagacearum*, were designed using the NEB® LAMP primer Design Tool, version 1.3.1 (New England Biolabs Inc.). The nucleotide sequence of *B. fagacearum* strain CBS130770 (GenBank accession no. MH865866) was used as a reference. Each set included 6 different primers – forward and backward inner primers (FIP & BIP), forward and backward outer primers (F3 & B3) and forward and backward loop primers (LF & LB). Based on the results of preliminary screening experiments, one set of primers was chosen to be used for the rest of the study (Table S1).

The LAMP reaction mixture was prepared according to the manufacturer's instructions for the WarmStart® LAMP Kit (DNA & RNA). Briefly, 1µL of the sample DNA was added to a mixture of 12.5 µL of the 2X LAMP master mix, 2.5 µL of 10µM primer mix, 0.5 µL of 50X fluorescent dye with the final volume made up to 25 µL using nuclease-free water. Nuclease-free water was used as negative control, where no amplification is expected due to the absence of a template DNA strand. The reaction mixture was incubated at 65 °C for 30 min to allow amplification followed by termination at 85 °C for 5 min. The fluorescence intensity of the final amplicons was measured at the end of this period. StepOnePlus™ Real-Time PCR System was used both for incubation and fluorescence measurements. The indicator used in the LAMP assay is an intercalating dye detectable via the SYBR/FAM channel.

To assess the analytical specificity of the designed primers, experiments were conducted using extracted DNA samples from other selected fungal species. The selected fungi were common species detected in sapwood of logs cut from oak wilt killed *Q. ellipsoidalis* trees. Specifically, the experiments included DNA extracted from *Dicarpella* sp., *Fusarium sporotrichioides*, *Graphostroma* sp., *Querciphoma carteri*, and *Epicoccum nigrum*.

To determine the limit of detection (LOD) of the designed LAMP assay, tests were conducted for different concentrations of *B. fagacearum* DNA using the chosen primer set. Pure culture of *B. fagacearum* was used for the DNA extraction and diluted to the required concentrations for this experiment using nuclease free water. Initial concentrations ranged from 0.15 pg/mL to 15 ng/mL in logarithmic increments to determine the range of LOD. Based on the results, further experiments were conducted with DNA concentrations of 0.015, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.15 ng/mL for accurate determination of the LOD. The DNA concentrations were confirmed using a Qubit Fluorometer. StepOnePlus™ Real-Time PCR System was used for the LAMP reaction and fluorescence detection.

Additionally, the chosen primer set was tested against an available closely related fungus, *Ceratocystis fimbriata* to check for cross reactivity. Agarose gel electrophoresis was conducted to visualize the LAMP reaction products and evaluate the results. GelRed® Nucleic Acid Stain 10000X Water agarose gel (1%) was prepared, and a low-range DNA ladder was included as a reference alongside the samples for comparison.

Application of the LAMP assay for oak wilt detection

The designed LAMP assay was conducted on healthy and infected red oak samples to test their applicability in detecting oak wilt in actual trees. The following equations were used to calculate the diagnostic sensitivity and specificity of the assay based on the obtained fluorescence results,

$$Sensitivity = \left(\frac{a}{a+c}\right) X\ 100$$

$$Specificity = \left(\frac{d}{b+d}\right) X\ 100$$

where, a is the number of true positives, b is the number of false positives, c is the number of false negatives and d is the number of true negatives [28]. All tests were conducted in triplicates. To show that LAMP can be done without a sophisticated PCR thermocycler, a set of experiments were also conducted using Thermo Scientific™ Compact Digital Dry Bath/Block Heater for incubation and Agilent-Cary Eclipse Fluorescence Spectrophotometer for fluorescence detection. Additionally, agarose gel electrophoresis of the LAMP products obtained from red oak samples was done to confirm the fluorescence test results.

Optical detection of LAMP products with AuNP-oligos

Both healthy and *B. fagacearum* infected red oak samples were tested using the designed LAMP assay and their fluorescence intensities were measured. To enable a faster identification of target amplification a rapid optical

detection was employed with laboratory synthesized nanoparticles [13]. The final appearance of the solution was used to distinguish between positive (red pellet) and negative samples (uniform pink supernatant). Non-complementary AuNP-oligonucleotides were added to the LAMP reaction products. Salt solution and ethanol were then added to induce precipitation of the gold conjugated target amplicon. Both crude and purified DNA samples from red oak were used for this test. All experiments were conducted in duplicates.

Results and discussion

Primer selection

The analytical specificity of LAMP assay relies on the selectivity of the primers targeting *B. fagacearum.* We designed and evaluated three different sets of six primers each, labelled as P42, P55 and P87. Here, DNA from different fungal species isolated from sapwood of oak wilt killed trees – *Dicarpella* sp., *Fusarium sporotrichioides*, *Graphostroma* sp., *Querciphoma carteri*, and *Epicoccum nigrum* were used as controls against *B. fagacearum* DNA to test the analytical specificities of the primer sets. With DNA amplification, the fluorescence intensity is expected to be high due to the dye binding to the double stranded DNA. Based on the results, among the three primer sets, P87 exhibited the highest selectivity for amplifying oak wilt DNA.

The fluorescence intensities measured for LAMP products obtained from P87 in the amplification reaction is shown in Fig. 2a. The control DNA samples showed similar results to nuclease-free water, while samples containing *B. fagacearum* DNA displayed the highest intensity, reaching around 1,900,000 arbitrary unit (a.u). This indicates the absence of amplification in the controls despite the presence of fungal DNA, except for the target fungus. It is to be noted that the intensity of nuclease-free water and other fungal species is just above zero, likely due to the residual fluorescence contributed by the dye and the primers present in the reaction mixture. The other primer sets P42 and P55 either failed to show specific detection of the target DNA or had cross reactions and were therefore eliminated from further studies. The results from P42 and P55 are shown in the supplementary information (Fig. S1).

Limit of detection

The assay limit of detection was studied using the P87 primer set by testing various concentrations of DNA extracted from a pure culture of *B. fagacearum*. The LOD was found to be 30 fg/µL with the fluorescence intensity in the detectable range of around 1,200,000 a.u (Fig. 2b). As the DNA concentration increased the fluorescence intensity increased before reaching a plateau at around 1,700,000 a.u. Although the LOD is a hundred times higher than the one achieved using qPCR [7], it is much lower than other oak wilt detection assays (DETECTR and nanoaggregation enhanced chemiluminescence) developed to date [9, 10]. Moreover, the assay has a high analytical sensitivity due to the LOD being significantly below the concentrations of DNA usually extracted from an infected oakwood sample. The total DNA extracted from infected wood is usually around 100 ng/mL, which includes DNA from both *B. fagacearum* and the oak tree. It is expected that the amount of *B. fagacearum* DNA in this mixture is significantly higher than 30 fg/µL in infected samples. Following optimized sampling protocol

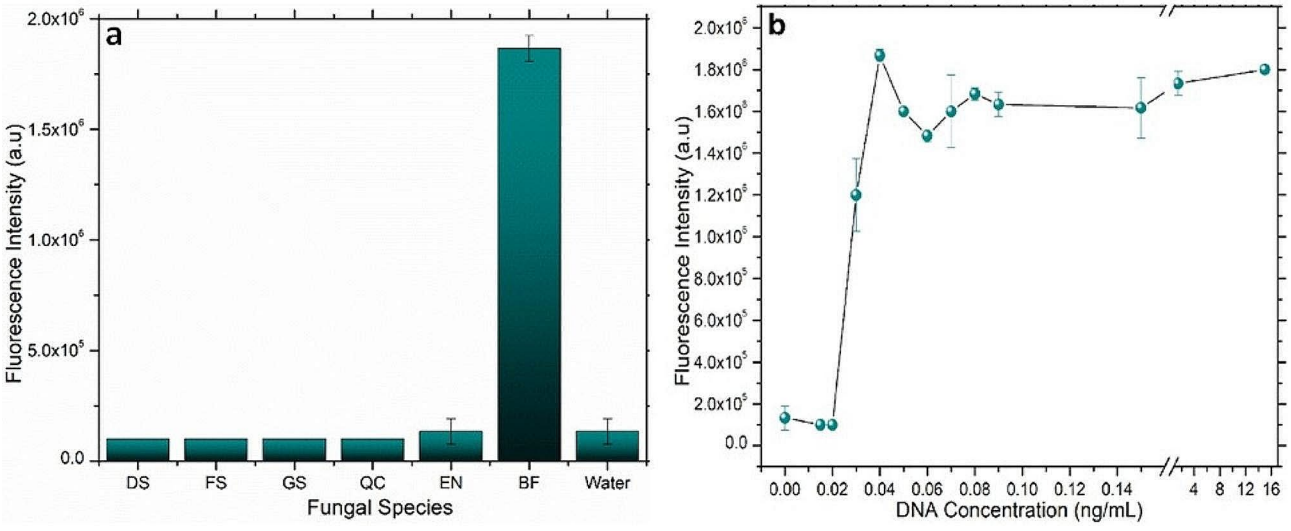

Fig. 2 Characterization of the developed LAMP assay using spectrofluorometric analysis. **(a)** Selectivity of the assay for *Bretziella fagacearum (BF).* No detection is observed for other fungi including *Dicarpella sp. (DS), Fusarium sporotrichioides (FS), Graphostroma sp. (GS), Querciphoma carteri (QC), and Epicoccum nigrum (EN).***(b)** Limit of detection of the LAMP assay (LOD: 30 fg/µl)

for infected oak trees is extremely important to achieve accurate results. Additionally, further enhancements in primer design and target DNA preparation could improve the LOD of the LAMP assay.

Cross reactivity study with *Ceratocystis fimbriata*.

Once the primer set was chosen, a preliminary assessment of cross reactivity with a closely related species was conducted. *Ceratocystis fimbriata* was selected for this purpose because it is the causal agent of wilt disease in several plants, including woody hosts, with associated economic losses. This pathogen has spread out in several countries and causes similar wilt symptoms in hosts such as sweet potato, coffee, fig, hickory, mango, *Eucalyptus*, etc. [29]. Therefore, it was essential to test whether the designed LAMP assay could distinguish oak wilt caused by *B. fagacearum* from other wilt diseases. LAMP was done using DNA extracted from *B. fagacearum* and *C. fimbriata* in triplicates and visualized using gel electrophoresis. Based on the gel images, no amplicons were generated for samples containing the *C. fimbriata* DNA (Fig. 3). This indicates that the developed LAMP assay was selective for *B. fagacearum*. Several fungi belonging to the *Ceratocystis* genus may be closely related to *B. fagacearum* and a full study is required to assess other potential cross reactivities using the designed primers. However, for the purposes of this work, the selectivity exhibited by the chosen primer set was considered sufficient for further studies.

LAMP detection of oak wilt in red oak

The efficiency of the developed LAMP assay in detecting *B. fagacearum* in real oak samples was investigated. Experiments were conducted using nucleic acids extracted from 10 healthy and 10 infected red oak drill shavings. The StepOnePlus™ Real-Time PCR System, which was used for both incubation and fluorescence measurement. Based on the results, all infected samples showed high fluorescence intensity at the end of the LAMP reaction, indicating the occurrence of DNA amplification (Fig. 4a). To ensure that only the target fungal DNA is being amplified irrespective of the presence of DNAs of the plant and any other fungal species present in the diseased samples, healthy wood DNA samples were also tested as controls. The DNA amplification did not occur in any of the healthy samples, which was indicated by the absence/very low fluorescence at the end of

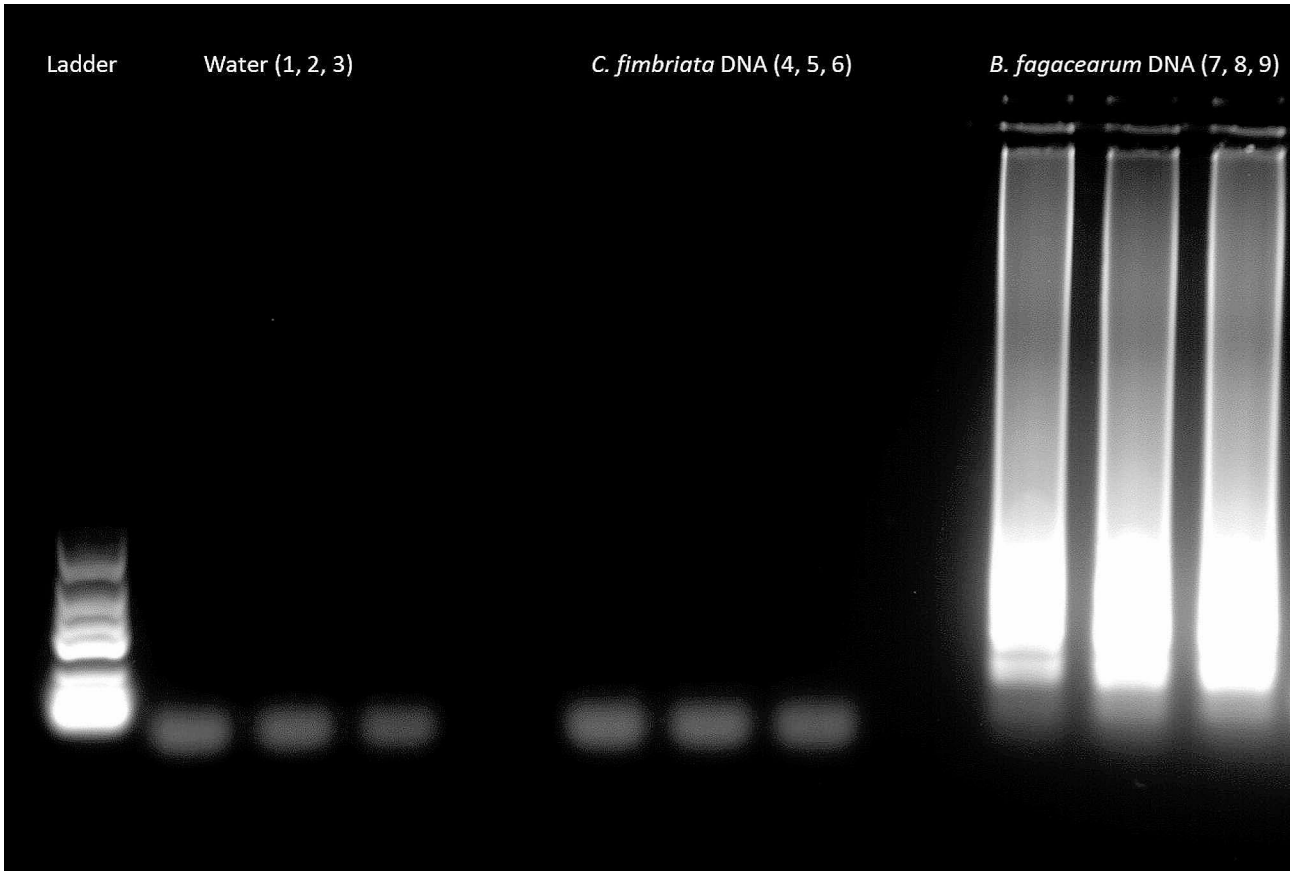

Fig. 3 Gel electrophoresis results showing the typical LAMP amplicon bands for positive controls with *B. fagacearum* DNA, and faint bands at the bottom of the gel, indicative of no amplification in negative control and the samples containing the *C. fimbriata* DNA.

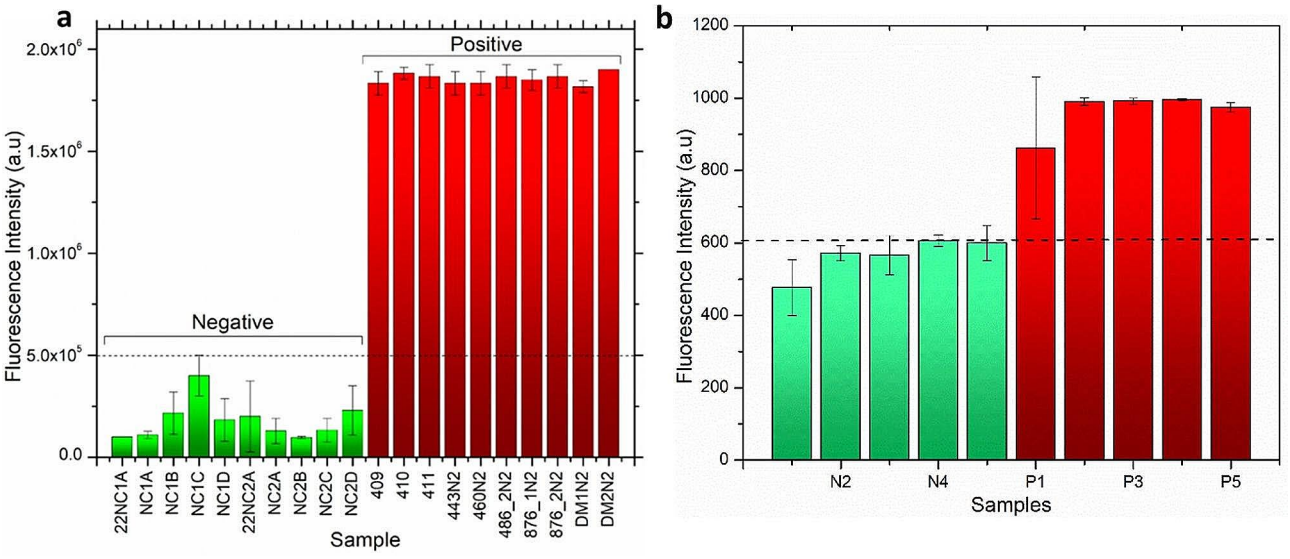

Fig. 4 Detection of the fungus *Bretziella fagacearum* in wood chip samples from healthy and infected oak trees. The wood chip samples were treated to extract and purify the fungal DNA which was then amplified using LAMP. The product of the LAMP assay was characterized using fluorospectroscopy. The results show high specificity and sensitivity. **(a)** Results from LAMP assay conducted using StepOnePlus™ Real-Time PCR System for heating and fluorescence detection. **(b)** Results from LAMP assay conducted using Thermo Scientific™ Compact Digital Dry Bath/Block Heater for heating and Agilent-Cary Eclipse Fluorescence Spectrophotometer for fluorescence detector. The difference in the fluorescence intensity ranges in each set of experiments reflects the varied sensitivities of the fluorescence devices used

the LAMP assay. Based on this, the diagnostic sensitivity and specificity of the assay were calculated to be 100% for the sample size used in the study.

Tests were also conducted on a different set of DNA samples obtained from 5 healthy and 5 infected oak tree samples using the Thermo Scientific™ Compact Digital Dry Bath/Block Heater for incubation and Agilent-Cary Eclipse Fluorescence Spectrophotometer for the final measurements. This was done to exhibit the adaptability of the developed LAMP assay in field testing, where a PCR instrument cannot be run. Both positive and negative controls were used to standardize the fluorescence measurements. The results obtained using this method correlated with the previously tested samples and are shown in Fig. 4b. The difference in the range between the fluorescence intensities from both experiments can be attributed to the different instruments used. The infected samples showed a higher fluorescence intensity compared to the healthy samples. In all cases the residual fluorescence seen in the healthy samples comes from the primer interaction with the DNA binding fluorescence dye used with the LAMP master mix.

Agarose gel electrophoresis was conducted to confirm the results obtained with fluorescence. A typical ladder-like pattern of DNA amplicons obtained from a LAMP reaction was observed in all the infected samples (Fig. 5a and b). Whereas the controls including all the healthy samples showed a single light band at the bottom of the gel, indicating the absence of amplicons (Fig. 5c and d). The ladder-like pattern for positive samples is a characteristic feature of LAMP products due to the generation of a high concentration of DNA copies of varying sizes [30].

Optical detection of LAMP products using hierarchical nanoparticle assembly

While fluorescence detection is extremely sensitive, it does require expensive instrumentation and thus is not suitable for field detection. Apart from fluorescence, amplification after a LAMP reaction can also be indicated by turbidity in the sample. This is because nucleic acid amplification causes the release of magnesium pyrophosphate as a by-product proportionally to the number of amplicons, which causes turbidity [31]. However, without a turbidimeter, distinguishing positive and negative samples with the naked eye was difficult, especially when the sample size is as small as 25 µL. Therefore, to improve the detection of this turbidity with the naked eye, a newly developed method involving hierarchical gold nanoparticle assembly was employed here [13]. The addition of AuNP-oligos gave the amplicons solution a pink color, which upon precipitation using NaCl and alcohol resulted in a red pellet in the sample tube while the remaining solution was clear.

The binding of AuNP-oligos with the DNA amplicons was confirmed using transmission electron microscopy. The precipitation of DNA that are bound to the AuNP-oligos causing them to form closely spaced globular structures are seen as the large pellet in a positive sample tube (Fig. 6a). The attached AuNP-oligos can be seen as

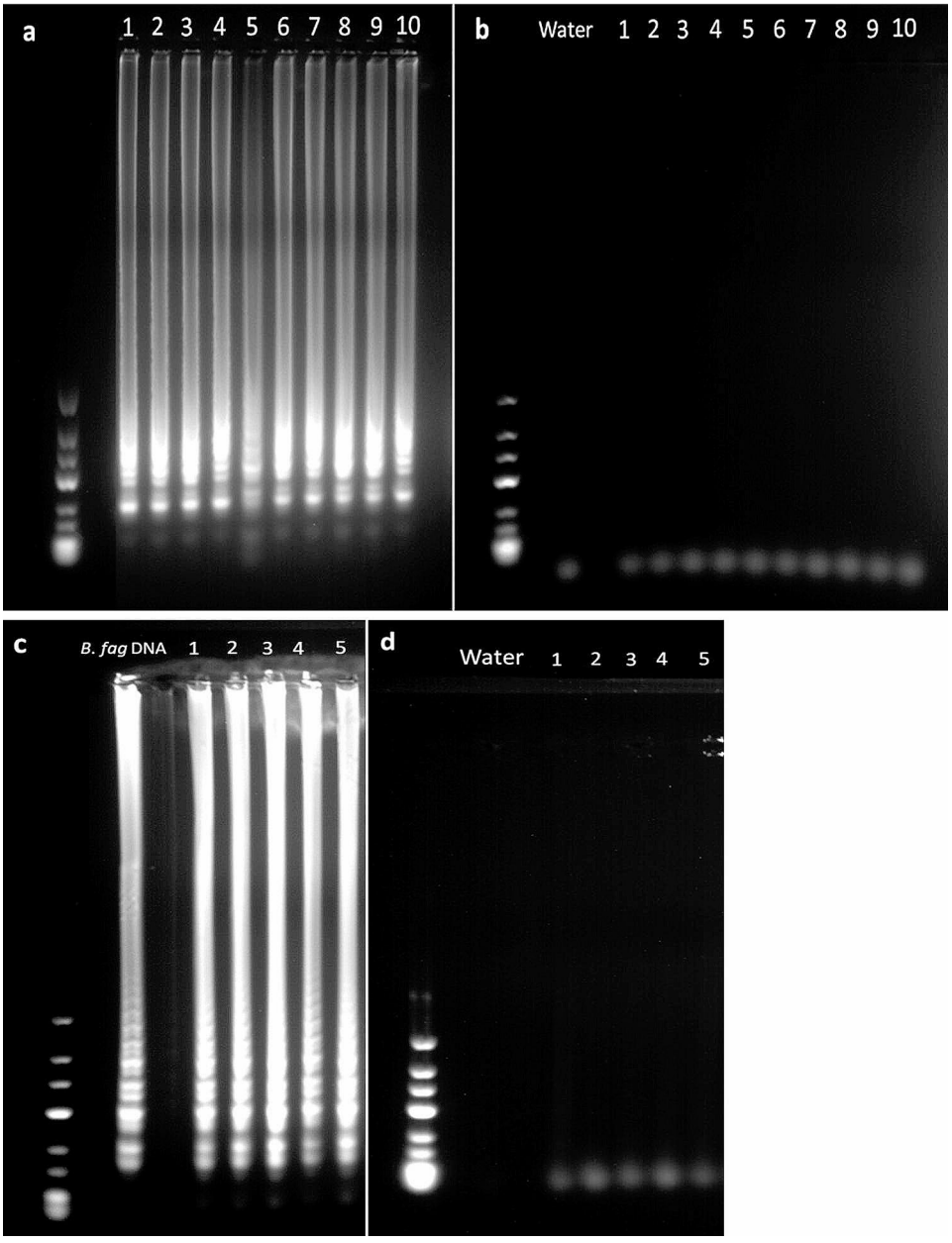

Fig. 5 Agarose gel electrophoresis of LAMP products obtained from testing red oak samples. Infected samples tested on **(a)** real-time PCR instrument, and **(b)** portable dry heat block. Healthy samples along with nuclease-free water as control tested on **(c)** the same PCR device as a), and **(d)** the same heat block as b)

tiny dots in and around the larger DNA globules (Fig. 6b-d). When there are not enough DNA copies due to lack of amplification, no precipitation occurred, and the solution remained uniformly pink. The AuNP-oligos act as amplicon labels by forming an assembly through the non-specific interaction of the short amplicon sequences with the oligonucleotides under low stringency conditions [32]. Here, the precipitation of DNA was induced by the presence of salt and alcohol, which is generally used in the nucleic acid extraction process since DNA in a salty solution cannot dissociate without a polar solvent [33]. Based on this test, all infected samples showed a red pellet after centrifugation due to the presence of the *B. fagacearum* DNA copies attached to the AuNP-oligos. On the other hand, the solution in all the healthy sample tubes remained pink even after centrifugation (Fig. 7a and b). The visualization process required less than 10 min and did not require any instrumentation.

Amplified DNA resulting from LAMP or RPA assays can be visualized using changes in turbidity, visible color, or fluorescence. Methods that require opening of sample tubes after assay reaction in order to add solutions for

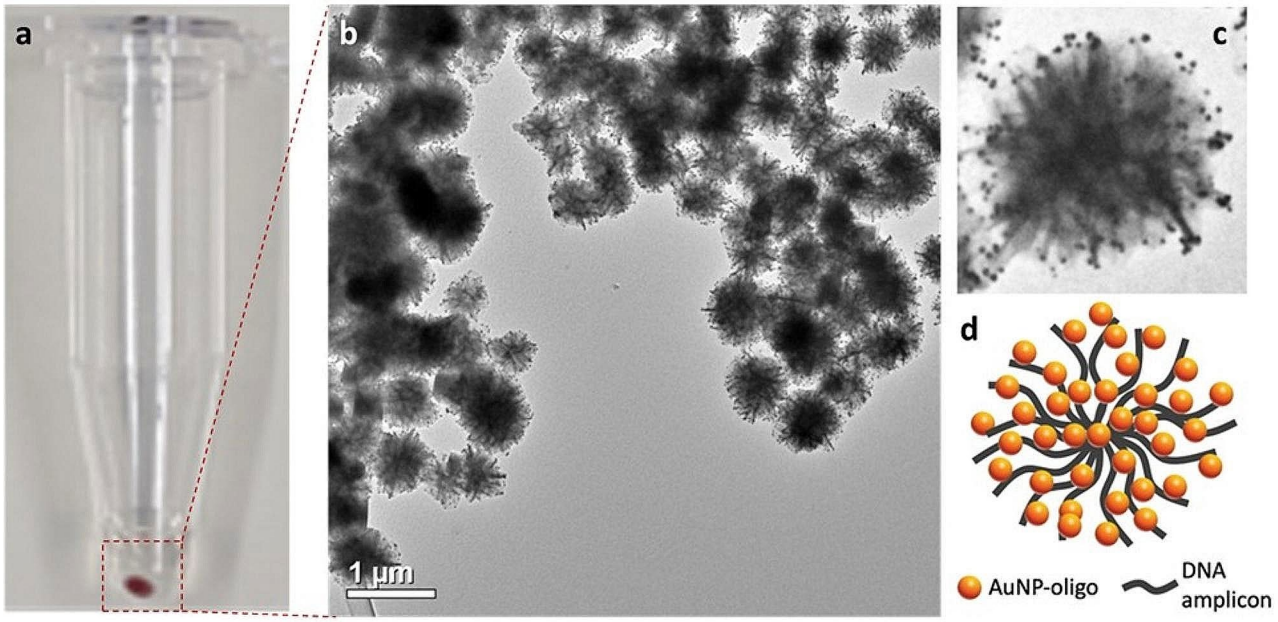

Fig. 6 Concept of amplicon visualization using hierarchical nanoparticle assembly. **(a)** The visible pellet at the bottom of the tube is formed following induced precipitation of assembled AuNP-oligo with the DNA amplicons, **(b)** and **(c)** transmission microscope image of globular nanostructures, and **(d)** conceptual diagram of one assembled globule. The assembly of hundreds of these structures yields a red pellet visible to the naked eye

visualization step may allow for cross contamination due to aerosolized products [34]. This concern is relevant to colorimetric and fluorescent-based assays as well as the AuNP-oligos assay reported here.

Rapid optical detection for crude DNA extracts

The LAMP assay and visualization can be completed in 40 min. However, the first step in any detection assay is nucleic acid extraction and purification, which can be time-consuming. We have previously confirmed that nested PCR detection of *B. fagacearum* in red oak sapwood can be successfully performed using a crude DNA extract, i.e., without any DNA purification step [11]. To evaluate the possibility of LAMP detection directly on crude samples, crude DNA extractions from two each of infected and healthy red oak wood samples were tested using the developed LAMP assay. Both the fluorescence measurements and the nanoparticle assembly tests were able to differentiate between the healthy and infected samples as depicted in Fig. 8. Given that most nucleic acid-based tests require purified samples for detection, the ability to identify infection without the need for the purification step significantly reduces the preparation time. Therefore, with the availability of a portable heating block, this assay could be conducted in the field along with rapid visual detection of oak wilt.

Conclusions

This work reports the first development and application of a LAMP assay for the detection of *Bretziella fagacearum*, the causal agent of oak wilt disease. The total time required for the LAMP reaction was 30 min, achieving a limit of detection down to 30 fg/µL. Testing with real red oak samples showed 100% diagnostic sensitivity and specificity, with the absence of false negatives and false positives. In addition, a ten-minute reaction with oligonucleotide-coated gold nanoparticles enables a naked-eye visualization of *B. fagacearum* DNA amplicons. This rapid visual differentiation of positive samples obtained at the end of the LAMP assay eliminates the need for any instrumentation. Further, the LAMP assay coupled with the rapid visualization technique was able to specifically detect *B. fagacearum* DNA from crude DNA extracts of infected red oak wood samples in a preliminary study, overcoming the need for DNA purification. Therefore, with just the use of a simple hand-held heating block, this assay holds potential for rapid field testing of oak wilt. A logical next step in method development would be to test the combined protocol on known diseased red oak in a field setting. Since a larger sample size would be a better representation of disease epidemiology, additional tests with more samples will be considered to corroborate the diagnostic sensitivity and specificity results obtained in this work. The protocol would include NaOH extraction of DNA, LAMP assay using dry heat block, and visualization with oligonucleotide-conjugated gold nanoparticles. Evaluation of the same protocol for sapwood samples

Fig. 7 Detection of the fungus *Bretziella fagacearum* in wood chip samples from healthy and infected oak trees. After DNA extraction and amplification using LAMP, visualization of the amplicon is performed using oligos-conjugated gold nanoparticles. In the presence of the target DNA, the nanoparticles bind to the DNA amplicons, and assemble into globular nanostructures that can be easily precipitated to form a visible red pellet at the bottom of the microtube. **(a)** Results from LAMP assay conducted using a real-time PCR instrument, **(b)** Results from LAMP assay conducted using a portable dry heat block

from known diseased white oak species (e.g., *Q. macrocarpa*, *Q. alba*) is also needed since NaOH extraction of DNA from these species may require a purification step.

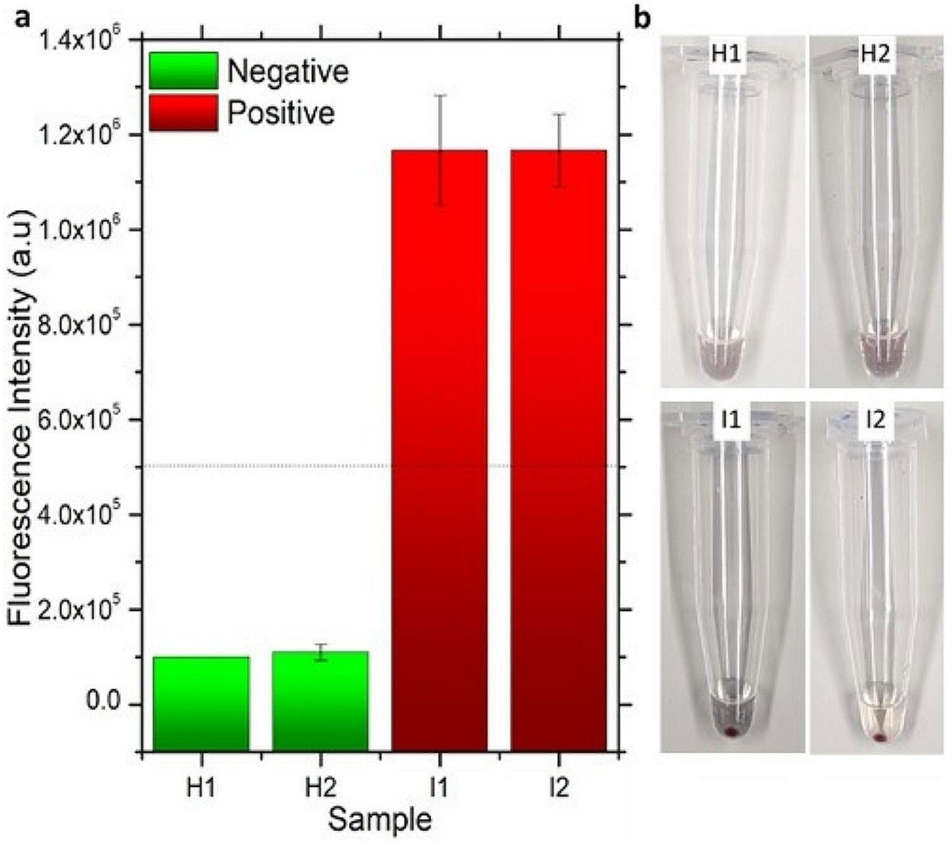

Fig. 8 LAMP detection of the fungus *Bretziella fagacearum* in crude samples, without nucleic acid purification. **(a)** Amplicon characterization using spectrofluorometry, and **(b)** Amplicon visualization using the hierarchical assembly of oligos-conjugated gold nanoparticles

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13007-024-01254-8.

Supplementary Material 1: **Table S1** Nucleic acid sequences of LAMP primers sets used for specificity tests including the chosen set; **Fig. S1** Primer specificity tests using different fungal species: Dicarpella sp. (DS), Fusarium sporotrichioides (FS), Graphostroma sp. (GS), Querciphoma carteri (QC), Epicoccum nigrum (EN), Bretziella fagacearum (BF). (a) LAMP reaction results for tests with primer set 42, (b) LAMP reaction results for tests with primer set 55

Acknowledgements
The authors thank Dr. Brett Arenz from the Plant Disease Clinic at the University of Minnesota – Twin Cities and Dr. Anil Meher from Frontline Biotechnologies Inc., Saint Paul, USA for providing oak wood samples to conduct the second set of experiments.

Author contributions
AA conceived and directed the research and acquired the funding. HA designed the LAMP primers. HA and VTN developed the initial LAMP protocol. VTN optimized the LAMP assay, conducted experiments with the oak samples and applied the developed rapid visualization test for detection. MM collected and prepared the oak samples and conducted the nested PCR experiments with direction from JJ. AZ conducted additional specificity tests. VTN and MM analyzed the data. VTN, MM and JJ wrote the manuscript with input from all authors.

Funding
This work was mainly supported by the Minnesota Environment and Natural Resource Trust Fund as recommended by the Legislative Citizen Commission on Minnesota's Resources, through the Minnesota Invasive Terrestrial Plants and Pests Center, and the USDA National Institute of Food and Agriculture, Hatch project 1006789. Parts of this work were carried out in the Characterization Facility, University of Minnesota, which receives partial support from NSF through the MRSEC program. The authors are also grateful for the Schwan Food Company Graduate Fellowship for financial support.

Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not Applicable.

Consent for publication
Not Applicable.

Competing interests
The authors declare no competing interests.

Received: 16 February 2024 / Accepted: 31 July 2024
Published online: 06 August 2024

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