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(54) Title: DETECTION AND QUANTIFICATION OF LACTIC ACID PRODUCING BACTERIA IN FOOD PRODUCTS

(57) Abstract: Methods and kits for rapidly detecting and/or quantifying lactic acid bacteria using real time PCR including a first primer, a second primer, and a probe. Each of the primers and the probe are complementary to separate regions of the 16S rRNA gene of lactic acid bacteria. The methods and kits may be used for the detection and/or quantification of lactic acid bacteria in food such as yogurt, to support product labeling, and in salsa.

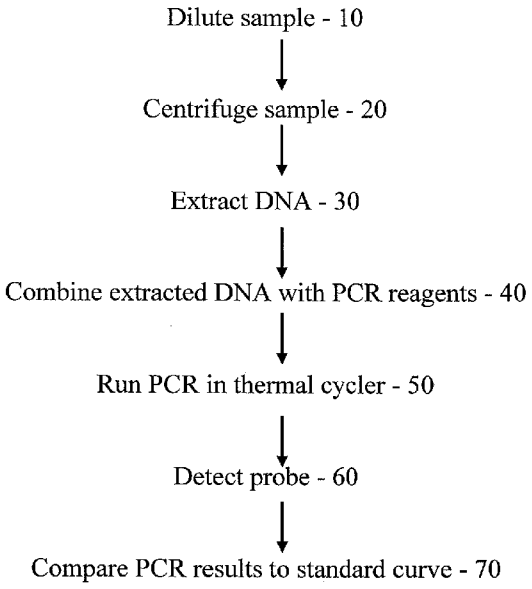

Figure 1

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DETECTION AND QUANTIFICATION OF LACTIC ACID PRODUCING BACTERIA IN FOOD PRODUCTS

Priority

This application claims priority to U.S. Provisional Patent Application Number 61/501,470 filed June 27, 2011entitled "detection and quantification of lactic acid producing bacteria in food products", the *entire* disclosure of which is hereby incorporated by reference.

Background

The group of bacteria which produce lactic acid as the end product of carbohydrate metabolism are referred to as lactic acid bacteria. These bacteria can be found in nature, such as in decomposing plants, as well as in certain food products, such as yogurt. They include bacteria in the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, *Streptococcus, Aerococcus*, *Carnobacterium*, *Enterococcus*, *Oenococcus*, *Sporolactobacillus*, *Teragenococcus*, *Vagococcus*, and *Weisella*, for example.

Lactic acid bacteria are used in the production of yogurt, in which the bacteria produce the lactic acid which contributes to the characteristic flavor of the product. Depending upon the yogurt production process, live lactic acid bacteria may be present in the final yogurt product. The presence of such live bacteria is often considered a desirable feature in yogurt. For example, the presence of live lactic acid bacteria in yogurt may be associated with certain health and digestive benefits. Lactic acid bacteria ferment lactose in milk, making yogurt easier to digest for individuals with lactose intolerance. Consumers also associate other health benefits with the presence of live and active lactic acid bacteria cultures

in yogurt. The presence of live lactic acid bacteria in yogurt is therefore and is preferred by some consumers.

The National Yogurt Association (N.Y.A.) requires that yogurt have active lactic acid bacteria cultures be above a certain concentration at the time of manufacture and that the total number of bacteria increase by one log during an activity test conducted at the end of shelf life in order to be labeled with a seal stating that the product contains live and active cultures. The Food and Drug Association (F.D.A.) has proposed regulations that would similarly require that a certain minimum number of bacteria be present in the yogurt both at the point of manufacture and at the end of shelf life in order for the product to be labeled as including live and active lactic acid bacterial cultures. It is therefore desirable to quantify the live lactic acid bacteria present in yogurt to support such labeling.

In other food products, the presence of high levels of lactic acid bacteria may not be desirable. For example, an overgrowth of lactic acid bacteria in food products such as salsa products and in deli style sliced meats can cause an unpleasant decline in product quality and can produce a slimy layer on the food. While such bacterial overgrowth is not harmful to consumers, it is unappetizing. It is therefore desirable to quantify lactic acid bacteria prior to releasing such products onto the market or prior to further processing of such product ingredients. For example, if the product ingredients, such as the vegetables used for making salsa, have unacceptably high levels of bacteria, these ingredients may be discarded such that no product is made using those ingredients. In this way, additional investments are not wasted on production and packaging of a product that will ultimately have unacceptably high levels of bacteria. Deli style meat products are hygienically handled and are packaged in a vacuum or modified atmosphere during manufacturing; such products are expected to be able to maintain good sensory quality for 2 to 4 weeks if they are stored below 10°C. However, spoilage by lactic acid bacteria sometimes occurs within the shelf-life period, requiring the

producer to make recalls. It is important to be able to detect lactic acid b
product and decrease damage and product recalls.

Traditionally, lactic acid bacteria in food products have been detected and quantified using selective medium such as acidified MRS (de Man, Rogosa and Sharpe) agar. However, such methods are time consuming, requiring days for bacterial growth to occur to a sufficient degree for the bacterial colonies to be visible on the medium plate. Although useful, this process, referred to as plating, requires a significant amount of work and delay before the results are available. During this time, food ingredients or products may be held before proceeding with further processing or release to the market. Therefore, it is desirable to develop a rapid method to quantify lactic acid bacteria in food products to reduce the delay in processing and product release.

A more rapid method uses a Bactometer to detect lactic acid bacteria. The Bactometer can be used to detect lactobacilli in food products such as salad dressing and in food ingredients such as chopped vegetables used in salsa production. The process can include incubation of the product at 30±1°C for 24 ± 1hr in module wells containing a medium selective for lactobacilli. The modules are then placed into the Bactometer system and the colony count is correlated to detection time. If any growth is detected in the module well, the material may be streaked onto agar plates, where the colonies may be further identified after growth on selective medium. However, the Bactometer quantifies bacteria by measuring the specific physicochemical changes caused by the growth of the bacteria. Because the bacteria cannot be detected until growth occurs, this process is also time-consuming. In addition, some bacteria are alive but may not grow in the culture medium that is used. These bacteria are referred to as viable but non-culturable (VBNC) bacteria and cannot be detected by either Bactometer or culture methods. Therefore it is desirable to

develop a rapid and culture-independent method to quantify total lactic a products.

One culture-independent technique used for the detection of bacterial nucleic acid is the method of polymerase chain reaction, commonly known as PCR, which is generally regarded as the most sensitive and rapid method used to detect nucleic acids in a given sample. To perform PCR, a pair of oligonucleotide sequences complementary to the nucleic acid of the bacteria are required as primers. One primer is complementary to bacterial nucleic acid at the 5' end of the target bacterial nucleic acid sequence, while the other primer is complementary to bacterial nucleic acid at the 3' end of the target nucleic acid sequence. For the technique of real time PCR (RT-PCR), a probe is used which is also complementary to the target bacteria nucleic acid sequence between the two primers, allowing detection and quantification of the target material.

Although the techniques of real time PCR are known to be useful for the detection and quantification of target nucleic acid sequences, they rely upon the identification of unique sequences to function as primers and probes and such identification can be challenging. Such sequences must not only be unique to the target material, but also should not bind to themselves to form primer dimers. The probes also should satisfy the design guidance including not having a guanine residue at the 5' end of the probe; having an appropriate Tm (melting temperature); being as short as possible but at least 13 nucleotides; not having runs of identical nucleotides; and other characteristics. It can therefore be difficult to identify first and second primer sequences and probe sequences meeting these guidelines as is necessary to increase the likelihood of success with real time PCR.

Summary

Embodiments of the invention allow for the rapid and specific detection and quantification of lactic acid bacteria using the techniques of real time PCR.

Embodiments also include kits for detection of lactic acid bacteri primer sequence, a second primer sequence, and a probe sequence, where each sequence is complementary to a separate region of the 16S rRNA gene of lactic acid producing bacteria. For example, in some embodiments each of the first and second primer sequence and the probe sequence is complementary to a separate region of SEQ. ID. No. 4.

In some embodiments, the kit for PCR detection of lactic acid bacteria including a first primer comprising SEQ. ID. No. 1, a second primer comprising SEQ. ID. No. 2, and a probe comprising SEQ. ID. No. 3.

In some embodiments, the probe includes a fluorophore, such as carboxyfluorescein. In some embodiments, the kit also includes a membrane impenetrable dye.

In other embodiments, the invention includes a method of detecting lactic acid bacteria in a sample including diluting the sample, centrifuging the sample to produce a pellet, performing bacterial DNA extraction using the pellet, combining the extracted bacterial DNA with PCR reagents comprising a first primer comprising SEQ. ID. No. 1, a second primer comprising SEQ. ID. No. 2, a probe comprising SEQ. ID. No. 3, a DNA polymerase, and deoxynucleotide triphosphates, cycling the combined extracted DNA and PCR reagents through a Thermocycler, and obtaining data corresponding to a signal detected by the thermocycler. The method may further include comparing the data corresponding to the detected signal to a standard curve to quantify the amount of lactic acid producing bacteria present in the sample. In some embodiments, the sample is a food sample such as yogurt. In some embodiments, the probe includes a fluorophore such as carboyxyfluorescein.

Figures

Figure 1 is a flow chart of a method of detecting a lactic acid bacteria according to embodiments of the invention;

Figure 2 is real time PCR results for yogurt products;

Figure 3 is a standard curve of lactic acid bacteria in yogurt products versus Ct produced using the PCR results shown in Figure 2;

Figure 4a is real time PCR results for salsa vegetables;

Figure 4b is real time PCR results for salsa vegetables;

Figure 4c is real time PCR results for salsa vegetables;

Figure 5a is a standard curve of bacteria concentration versus C_t for salsa vegetables shown in Figure 4a;

Figure 5b is a standard curve of bacteria concentration versus C_t for salsa vegetables using the PCR results shown in Figure 4b; and

Figure 5c is a standard curve of bacteria concentration versus C_t for salsa vegetables using the PCR results shown in Figure 4c.

Detailed Description

It has now been discovered that lactic acid bacteria can be identified using real time PCR. These lactic acid bacteria can be selectively multiplied using PCR techniques using a pair of primer sequences specific to these bacteria, and can then be identified using a probe which is also specific to these bacteria. Embodiments of the invention can be used to rapidly detect and quantify lactic acid producing bacteria in food products. Exemplary food products include yogurt products, vegetables such as vegetables used in salsa production and brined

cucumbers, deli style meat products, salad dressings, ketchup, dairy prod
fish products such as herring, wine, beer, lemonade, fruit juices, and nectars, for example. Embodiments of the invention can also be used for environmental testing, such as testing of food production facilities.

The lactic acid bacteria which are found in yogurt as live and active cultures include *Streptococcus* thermophilus, *Lactobacillus delbrueckii subspecies bulgaricus* and *Lactobacillus delbruickii subspecies lactis*. When the techniques of quantitative real time PCR are applied to yogurt samples, the concentration of colony forming units of these bacteria (such as CFU/g of yogurt) can be quickly and accurately ascertained. As such, methods of the invention can be used to quantify the number of live lactic acid bacteria present in the yogurt product at a particular time, such as at the completion of production, to support labeling of the yogurt product as containing live and active cultures, or at the end of shelf life.

Alternatively, embodiments of the invention may be used to quantify the level of lactic acid producing bacteria present in vegetables, such as vegetables used to produce salsa or other food products. During salsa production, for example, the vegetables are first chopped, then mixed with sugar and salt and other ingredients, then placed into containers such as cans or glass bottles, and last heated to pasteurize to kill potential pathogens. These vegetables may include tomatoes, onions, peppers, corn, cilantro, etc. One way to monitor the quality of salsa ingredients is to quantify the lactic acid bacteria present in the vegetables prior to pasteurization. If the vegetables have greater than a threshold amount of lactic acid bacteria, a sufficient number of lactic acid bacteria may not be killed during processing, such that the final product may be spoiled by lactic acid bacteria, decreasing the shelf life and product quality. Such vegetables may be discarded and may not be used for salsa production. In contrast, if the vegetables contain less than a threshold number of lactic acid bacteria, the

further processing steps may be considered sufficient such that the final s
be spoiled by lactic acid bacteria during normal shelf life. In some embodiments, the threshold value is 10^5 cfu/gram, while in other embodiments, the threshold value is 10^6 cfu/gram, in chopped vegetables for use in salsa production.

In other embodiments, the methods and kits are used to detect and quantify lactic acid bacteria in salad dressing. The basic detection steps are similar to detection quantification of vegetables, including sample preparation, DNA isolation and RT-PCR reaction.

In still other embodiments, the methods and kits are used to detect and quantify lactic acid bacteria in deli style meats. Examples of deli style meats which may be used in embodiments of the invention include turkey, ham, roast beef, salami, chicken, etc. In order to avoid this, deli meat may be tested according to embodiments of the invention to detect and quantify lactic acid bacteria, such as in the final deli meat products.

Embodiments of the invention employ a pair of oligonucleotide primers and a probe, each of which were designed to specifically target lactic acid bacteria. The pair of primers includes a forward or upstream primer complementary to a sequence that is located at the 5' end of a sequence of a target nucleic acid and a reverse or downstream primer complementary to a sequence that is located at the 3' end of the sequence of target nucleic acid target. The target nucleic acid sequence is unique to the lactic acid bacteria, and allows specific identification of the detected bacteria as lactic acid bacteria. Furthermore, the pair of primers and the probe are specific to the target nucleic acid sequence, such that they do not bind with the nucleic acid of any other bacteria.

Primers useful in embodiments of the invention include SEQ ID Nos. 1 and 2, shown below. The primer shown above as SEQ ID. No. 1 is a forward or upstream primer. The primer identified as SEQ ID. No. 2 is a reverse or downstream primer. A probe useful in

embodiments of the invention is shown below as SEQ. ID No. 3. In som
probe identified as SEQ ID No. 3 is a TaqMan® probe having a fluorophore at its 5′ end and a quencher at its 3' end.

GGTTGAACTCAAAGGAATG SEQ. ID No. 1

CGTTGCTTCGAATTAAACCA SEQ. ID No. 2

CGCACAAGCGGTGGAGCA SEQ. ID No. 3

Real time PCR probes such as TaqMan® probes may be used in embodiments of the invention. TaqMan® probes which may be used in embodiments of the invention are complementary to an internal region of the target oligonucleotide sequence, between the regions of the target sequence to which the primers bind. Prior to hybridization of the TaqMan® probe to the target sequence, the fluorophore on one end of the probe is in close physical proximity to the quencher on the opposite end of the probe. In such a state, the quencher prevents the fluorophore from producing a detectable signal. During each hybridization stage of the PCR cycle, a probe becomes hybridized to the target sequence (or the replicate of the target sequence) by the action of DNA polymerase, during which process the probe and the quencher are cleaved such that they are no longer in close proximity and the quencher no longer quenches the fluorophore. Thus, in the hybridized state, the fluorophore is detectable. As such, the level of detected fluorescence directly relates to the quantity of target nucleic acid sequence present in the sample at each cycle.

Any real time PCR fluorophore may be used in embodiments of the invention. For example, in some embodiments the fluorophore is carboxyfluorescein (FAM). Other fluorophores may be used, such as tetrachlorofluorescein (TET). Examples of quenchers that may be used in embodiments of the invention include tetramethylrhodamine (TAMRA) and dihydrocyclopyrroloindole tripeptide minor groove binder.

Other types of real time PCR probes that may be useful in embod invention would likewise include SEQ. ID. No. 3 and would be readily identifiable by those skilled in the art.

Each of Sequence ID Nos. 1, 2 and 3 is complementary to the 16S rRNA gene of lactic acid bacteria. These bacteria include Lactobacillus *delbrueckii subsp. lactis*, *Lactobacillus delbrueckii subsp. bulgaricus, Streptococcus thermophilus*, *Leuconostoc fallax*, *Leuconostoc inhae*, *Leuconostoc gelidum*, *Leuconostoc argentium*, *Leuconostoc mesenteroides ssp cremoris*, *Leuconostoc mesentoides ssp mesenteroides*, *Leuconostoc gasicomitatum*, *Leuconostoc kimchii*, *Leuconostoc cireum*, *Pediococcus acidilactici*, and *Pediococcus pentasaceus*. Furthermore, because the 16S rRNA gene target of the sequences is unique to lactic acid bacteria, each of the primer and probe sequences will bind only to these bacteria, making the sequences selective for the identification of lactic acid bacteria. Embodiments of the invention therefore include first and second primers and a probe, each of which are complementary to the 16S rRNA gene. This allows for rapid and specific detection and quantification of lactic acid bacteria, without false detection of other bacteria.

A portion of the 16S rRNA gene of lactic acid which can provide the target for primer and probe sequences is shown below as SEQ. ID. No. 4.

CTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGG
GGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC
TTACCAGGTCTTGACATCC SEQ. ID. No. 4

In some embodiments, the first and second primers and the probe are each complementary to separate regions of the 16S rRNA gene, such as separate regions of SEQ. ID. No. 4. For example, a first primer may be complementary to a first region of SEQ. ID. No. 4, a second primer may be complementary to a second region of SEQ. ID. No. 4, and the

probe may be complementary to a third region of SEQ. ID. No. 4, with n second or third regions overlapping.

A method of detecting and quantifying the number of lactic acid bacteria according to embodiments of the invention is shown in the flow chart identified as Figure 1.

The first step is preparation of the test sample 10. The test sample is a material such as a food product having an unknown amount of bacteria. For test samples such as yogurt, preparation may include dilution of the sample in a solution such as a buffer (such as buffer peptone water). Alternatively, the sample may be diluted in water or other appropriate liquid. For test samples such as salsa vegetables and deli meat, preparation may include mixing the sample with a liquid such as a buffer or water and then pipetting out the liquid and then using the liquid as the sample for further testing.

The next step is to centrifuge the sample 20. The diluted yogurt or the pipetted liquid (for salsa vegetables and deli meat, for example) is then centrifuged to separate out the bacteria from the liquid. The pellet obtained by centrifugation is then used for further testing.

The next step is DNA isolation 30 (also known as DNA extraction), in order to make the DNA accessible for replication and detection using PCR. DNA isolation 20 includes breaking down the bacterial cell wall to release genomic DNA. This may be done by physical disruption of the cell well, such as by mixing with glass beads, or by chemical or enzymatic disruption, for example. Next, the proteins and fat soluble compounds are removed, such as through the use of appropriate chemicals such as phenol or ethanol, for example. Finally, the DNA is bound, extracted, and concentrated, such as through filtration and elution of the DNA, for example, or other techniques known to those skilled in the art. There are several commercial kits available for isolation of bacterial DNA. For example, DNA kits can be purchased from Norgen Biotek Corp., Zygen, Promega, Qiagen, Mo Bio

Laboratories and others. One such kit is the PowerFood™ Microbial DN
available from Mo Bio Laboratories, Inc.

The next step is the preparation of a reaction mix 40. The reaction mix may include the primers, probe, and the extracted DNA, along with the PCR reagent. The PCR reagent may include a DNA polymerase such as Taq polymerase, $MgCl_2$, dNTPs (Adenine, Thiamine, Cytosing and Guanine), and water. Several commercialized real-time PCR reagents are available for purchase which include these components, such as kits produced by Bio-Rad, Quanta Bioscience, Qiagen, Invitrogen and others. The reaction mix can be prepared by combining the reactants in a reaction well of a RT-PCR reaction plate for processing by the PCR Thermocycler.

Next, as an optional step, the wells containing the prepared reaction mix may be sealed and centrifuged under conditions to remove any bubbles from the wells. The optional step may be preferred by some individuals, but is not necessary.

Once the reaction mix is ready, the reaction plate is inserted into a RT-PCR system for thermocycling 40. During the PCR thermocycling step, the number of copies of the target genetic material is increased to a detectable and quantifiable level. This step may be performed using any RT-PCR thermal cycler in any RT-PCR system. The RT-PCR system may include a thermal cycler, an optical component for fluorescence excitation and emission collection, and a computer with data acquisition and analysis software. Real time PCR systems are commercially available from Applied Biosystems and Bio-Rad, for example.

The next step is detection of the probe 50 which can occur after each cycle of the thermal cycler. During the PCR process, any fluorescence produced by fluorescence dye is detected by the RT-PCR system. The amount of fluorescence increases with each cycle, as the number of copies of the target sequence increases. The number of cycles required for

fluorescence to reach above the fluorescence threshold is the cycle thresl fluorescence threshold is an arbitrary level of fluorescence chosen on the basis of the baseline value which can be adjusted for each experiment so that it is in the region of exponential amplification across all plots. Samples having a greater number of bacteria will reach the fluorescence threshold more quickly and therefore have lower Ct values, while those with fewer bacteria will require more cycles to reach the fluorescence threshold and therefore have higher Ct values. The Ct value can therefore be a useful basis for determining the bacteria concentration of a test sample. However, the data obtained from the RT-PCR system, such as fluorescence data like the Ct value, must be compared against a known standard in order to correlate the PCR data to a corresponding bacterial concentration.

The next step is therefore conversion of the RT-PCR data to a corresponding value of bacteria concentration. In some embodiments, this includes comparing the RT-PCR data to a known standard such as a standard curve. In some embodiments, the RT-PCR data which is used is the Ct value and the standard curve is a curve of bacterial concentration verses Ct. Such a known standard can be prepared by performing RT-PCR on samples having known bacterial concentrations under the same conditions as the test sample as described in Examples 1 and 2, below, for example. In other embodiments, the known standard of comparison may be provided as a mathematical formula (such as the formula for the line of the standard curve), and converting the RT-PCR data to a corresponding value of bacteria concentration can include putting the RT-PCR data (such as the Ct value) of the unknown sample into the mathematical formula to obtain the value of the bacteria concentration. In still other embodiments, the known standard of comparison can be a look up table, and the RT-PCR data may be compared to the table to determine the corresponding value of bacteria concentration.

RT-PCR techniques have the ability to detect DNA present in bo
organisms. However, for many purposes, only the live lactic acid bacteria will be of interest. For example, when embodiments of the invention are used to quantify the number of lactic acid bacteria present in yogurt, it is desirable to detect only the live and active bacteria for labeling of the yogurt as containing live and active cultures. Likewise, in foods such as deli meat and salsa, in which the presence of lactic acid producing bacteria is not desirable, only the live bacteria present a concern as the overgrowth of the bacteria causes the loss of food quality, and such overgrowth can only occur if the bacteria are alive.

In alternative embodiments, the methods and systems may be used for environmental testing and monitoring. Samples may be taken from the environment by wiping machine surfaces with a sponge, swab or other type of wipe for example. In some embodiments, environmental sponge sample kits might be used to sample the environment. An enriched medium can then be added into the sponge kit for pre-enrichment and incubated overnight under appropriate conditions for growth, such as 36°C. The cells can then be concentrated by centrifuging, followed by DNA isolation and RT-PCR reaction as described herein.

In some embodiments, a particular yogurt product can be tested twice to confirm that growth is occurring. Since only live bacteria will grow, the increased numbers of bacteria that are detected must be alive. For example, the method of PCR detection of the lactic acid bacteria in a yogurt product may be performed, then may be repeated after the same product has been incubated at 43°C for 4 hours. The increase in lactic acid bacteria (such as the log increase) following incubation can be considered to represent or be due to live bacteria since only live bacteria could grow.

Alternatively, other methods may be used to ensure that all of the lactic acid bacteria detected using the embodiments described herein are alive. In some embodiments, a membrane impenetrable dye may optionally be used prior to extraction of the DNA. Such

dyes bind only to the DNA of dead cells and make such DNA unavailabl RT-PCR reaction. Examples of such membrane impenetrable dyes include ethidium monoazide (EMA) and propidium monoazide (PMA). In other embodiments, the method may first isolate RNA from the test sample rather than DNA. Unlike DNA, RNA degrades very quickly once the bacteria are dead, the amount of RNA present in the sample is directly related to the number live bacteria. The RNA can then be combined with a reverse transcriptase to transcribe the RNA into DNA. The DNA thus produced can then be used to perform RT-PCR using the primers and probes as described herein.

Embodiments of the invention include methods of detecting and quantifying lactic acid bacteria and also include kits for use in detecting and quantifying lactic acid bacteria using RT-PCR. In some embodiments, the kit includes a first and second primer and a probe, all of which are complementary to, and specific to, lactic acid bacteria. In some embodiments, the target sequence of the primers and probe is in the 16S RNA gene of the lactic acid bacterial. In some embodiments, the primers include SEQ ID Nos. 1 and 2 and the probe includes SEQ ID. No. 3. The probe may include a fluorescent label and may be a TaqMan® probe. In some embodiments, the kit may also include one or more of a DNA polymerase, dNTP's, water, and/or magnesium chloride. In some embodiments, the kit further comprises a membrane impermeable dye.

Experimental

In the following examples, PerfecCTA™ qPCR FastMix™, purchased from Quanta Bioscience, was used as the PCR reagent. MiniOpticon, purchased from Bio Rad, was used as the RT-PCR Thermocycler. SEQ. ID Nos. 1 and 2 were manufactured for use as RT-PCR primers and sequence ID No. 3 was manufactured as a probe with a 6-carboxyfluorescein label by Applied Biosystems, and these primers and probes were used in each of the examples.

Example 1.

In this example, a standard curve of the concentration of lactic acid bacteria present in a yogurt product versus Ct was produced. Twelve yogurt products were used to create the standard curve. A concentration of 10^5 cfu/gram of lactic acid bacteria were used to make the yogurt. During the 4 hour fermentation process, the yogurt was sampled once every hour from the fermentation tanks. Three fermentation tanks were sampled independently. Bacteria concentrations were determined by plating the yogurt products and counting the bacteria cultures on M17 and MRS plates as described below. In addition, the same yogurt products were tested using RT-PCR, also as further described below. The bacteria concentrations as determined by plating were then plotted against the RT-PCR results to give a standard curve which can be used for determining the bacteria concentration in an unknown test yogurt sample based on RT-PCR results for that sample.

Plating of the yogurt to determine the bacteria concentration was performed as follows. The yogurt was diluted by varying amounts based on predicted bacteria levels in order to obtain the necessary colony counts on the plates of between 25 and 255 colonies. The 12 yogurt products were found to have bacterial levels which fell into four approximate groupings: 10^5, 10^7, 10^8, and 10^9 CFU/g.

To prepare the yogurt products for testing using RT-PCR, 11 gram yogurt samples were mixed with 99 ml of buffer peptone water to form a 1 to 10 dilution. One ml sample of the diluted yogurt were added to a 2 ml tube and centrifuged for 5 minutes at 13000 g, RT to form a pellet. The supernatant was decanted once the pellet was used for further testing. Then, the bacterial DNA was extracted from each pellet product using PowerFood™ Microbial DNA Isolation Kit, purchased from MO BIO Laboratories, Inc., according to the product directions as follows. The cell pellet was resuspend in the 450ul of solution PF1. The resuspended cells were transferred to the Microbead tube and vortexed for 10 minutes,

then centrifuged in the Microbead tube for 1min at 13000 g, RT. The su
transferred to a tube with 100ul of solution PF2, and vortexed to mix, then incubated at 4°C for 5 minutes. It was then centrifuged in the PF2 tube for 1min at 13000 g, RT 900ul of solution PF3 was then mixed with the supernatant in a clean tube. 650ul of PF3 and samples mixing solution was then loaded onto a spin filter tube with vacuum and this process was repeated until all the supernatant was loaded onto the spin filter. 650ul of solution PF4 was then added, followed by 650ul of solution PF5. The tube was centrifuged for 2min at 13000 g, RT, then the spin filter basket was placed into a clean 2ml tube and 100ul of solution PF6 was added. The tube was centrifuged for 1min at 13000 g, RT, and the spin filter basket was discarded. The DNA was then ready for RT-PCR or for freezing or refrigerating and storing.

In preparation for performing RT-PCR, a Master Mix was prepared for use with each sample by combining the PCR reagent, primers, probe and water in the amounts shown in Table 1 below. A total of three replicates were planned for each of the 12 yogurt samples, for a total of 36 reactions, therefore a sufficient quantity of master mix was prepared for a total of 38 reactions (to be certain to have enough for the planned 36 reactions).

Table 1:

Reagents	Volume (µL) needed per reaction	Total volume (µL) needed for all reactions
PERFECTA QPCR FASTMIX	10	380
Primer and probe mixing	1	38
dH2O	7	266
Extracted DNA	2	76
Total	20	760

Next, for each reaction, 18 µl of Master Mix as prepared above was combined with 2 µl of the extracted DNA in a well of a RT-PCR reaction plate A total of 3 replicates were prepared for each extracted DNA sample from each of the 12 yogurt products, for a total of 36 reactions. The reaction plates were sealed and centrifuged for 2 minutes at 25000 rpm.

The reaction plates were inserted into the Thermocycler and proc

Table 2 below.

Table 2:

Temperature	Duration	Number
95°C	10 minutes	once
95°C	15 seconds	40 cycles
60°C	1 minute	40 cycles
signal readying	(after each cycle)	40 cycles

Fluorescence readings were obtained from each reaction during the PCR process. A plot of fluorescence (RFu) versus cycle is shown in Figure 2. Ct is defined as the PCR cycle number at which the reporter fluorescence is greater than the threshold, which is an arbitrary level of fluorescence chosen on the basis of the baseline variability.

The Ct values obtained for the three replicates of each yogurt product were averaged, and these average results are shown in Table 3 below, along with the corresponding bacteria count as determined by plating. Finally, the curve of Ct against bacteria concentration (log CFU/gram) was plotted for each reaction as shown in Figure 3 to create a standard curve.

Table 3

Sample	Ct	Lactic acid bacteria count log (cfu/gram)
A1	21.04	5.76
A2	21.08	5.72
A3	21.25	5.72
A4	19.31	6.89
A5	18.97	7.36
A6	19.17	7.08
A7	16.66	8.11
A8	16.18	8.36
A9	16.29	8.23
A10	14.19	9.28
A11	14.13	9.30
A12	14.26	9.30

Example 2

In this example, a standard curve of lactic acid bacteria concentration was created for salsa vegetables. Three sets of salsa vegetable (as used for salsa production) were used for preparing the standard curve. The salsa vegetable sets were spiked with three different levels of lactic acid bacteria (approximately 10^8 cfu/gram, 10^6 cfu/gram and 10^4 cfu/gram). Each test was performed in triplicate to accommodate biological variability, and the tests were then repeated a second time. As with the yogurt standard curve in Example 1, the bacteria concentration in the salsa vegetables was determined by plating on M17 and MRS. The salsa vegetables were diluted by varying amounts based on predicted bacteria levels in order to obtain the necessary colony counts on the plates of between 25 and 255 colonies. The corresponding RT-PCR data was obtained to create the standard curve for the salsa vegetables.

To prepare the salsa vegetables for RT-PCR, 11 gram samples of bacteria spiked salsa vegetables were mixed with 99ml of buffer peptone water to form a on to ten dilution. 1ml of the diluted samples were added to a 2ml tube and centrifuged for 5 minutes at 13000 g, RT. The supernatants were decanted and the remaining pellets were used for DNA isolation.

The bacterial DNA was then extracted from the salsa vegetable pellets using PowerFood™ Microbial DNA Isolation Kit, purchased from MO BIO Laboratories, Inc., according to the product directions as described in example 1.

A Master Mix was prepared for use in the RT-PCR reactions by combining PCR reagent, primers, probe, and cDNA as shown in table 4 below. It was planned that each salsa product would be tested in triplicate, therefore sufficient Master Mix was prepared for 10 reactions, so that at least 9 reactions for each of the two test runs.

Table 4

Reagents	Volume (µl) needed for each reaction	Total volume (µl) needed for all reactions
PERFECTA QPCR FASTMIX	10	100
Primer and probe mixing	1	10
Extracted DNA	9	90
Total	20	200

For each reaction, 11 µl of Master Mix was combined with 9 µl of extracted DNA in a reaction well of a RT-PCR reaction plate. For each salsa sample, the reactions were performed in triplicate. The reaction wells were then sealed and centrifuged for 2 minutes at 25000 rpm. After centrifugation, the reaction wells were inserted into the Thermocycler and RT-PCR was performed according to the parameters shown in Table 5.

Table 5

Temperature	**Duration**	**Number**
95C	10 minutes	once
95C	15 seconds	40 cycles
60C	1 minute	40 cycles
signal readying	(after each cycle)	40 cycles

Fluorescence readings were obtained throughout the RT-PCR process. The RT-PCR data for all samples is shown in Figures 4a, 4b and 4c. The Ct value for each sample was determined based upon the threshold fluorescence value. The Ct value for each set of three samples for each salsa vegetable set were averaged, and the resulting average Ct is shown in Table 6 below, with the corresponding value of bacteria concentration (log CFU/g salsa). The average Ct value was plotted against bacteria concentration (log CFU/g salsa) to create the standard curves for salsa vegetables shown in Figures 5a, 5b and 5c. It can be seen that the results of the first and second sets of tests were virtually the same confirming the validity of the testing method.

Table 6:

		Test 1		Test 2	
Samples		Ct	Log cfu/gram	Ct	Log cfu/gram
B1	Replica 1	7.09	8.82	8.15	8.72
B2	Replica 1	14.58	6.82	15.74	6.72
B3	Replica 1	21.26	4.82	22.06	4.72
B4	Replica 2	6.38	8.87	6.66	8.93
B5	Replica 2	14.23	6.87	14.94	6.93
B6	Replica 2	20.06	4.87	21.46	4.93
B7	Replica 3	6.78	8.91	6.45	8.79
B8	Replica 3	14.52	6.91	15.06	6.79
B9	Replica 3	20.46	4.91	21.57	4.79

Claims

1. A kit for PCR detection of lactic acid bacteria comprising:

a first primer comprising SEQ. ID. No. 1;

a second primer comprising SEQ. ID. No. 2; and

a probe comprising SEQ. ID. No. 3.

2. The kit of claim 1 wherein the probe further comprises a fluorophore.

3. The kit of claim 2 wherein the fluorophore comprises carboxyfluorescein.

4. The kit of claim 2 further comprising a membrane impenetrable dye.

5. A kit for detection of lactic acid producing bacteria comprising:

a first primer complementary to a first region of SEQ ID. No. 4;

a second primer complementary to a second region of SEQ. ID. No. 4;

a probe complementary to a third region of SEQ. ID. No. 4.

6. The kit of claim 5 wherein the first primer comprises SEQ. ID. No. 1.

7. The kit of claim 5 wherein the second primer comprises SEQ. ID. No. 2.

8. The kit of claim 5 wherein the probe comprises SEQ. ID. No. 3.

9. The kit of claim 8 wherein the probe further comprises a fluorophore.

10. The kit of claim 9 wherein the fluorophore comprises carboxyfluorescein.

11. A kit for PCR detection of lactic acid producing bacteria compris

a first primer complementary to a first region of a 16S rRNA gene of lactic acid bacteria;

a second primer complementary to a second region of the 16S rRNA gene of lactic acid bacteria;

a probe complementary to a third region of the 16S rRNA gene of lactic acid bacteria and labeled with a fluorophore.

12 The kit of claim 11 wherein the first primer comprises SEQ. ID. No. 1.

13. The kit of claim 11 wherein the second primer comprises SEQ. ID. No. 2.

14. The kit of claim 11 wherein the probe comprises SEQ. ID. No. 3.

15. The kit of claim 14 wherein the probe further comprises a fluorophore.

16. The kit of claim 15 wherein the fluorophore comprises carboxyfluorescein.

17. A method of detecting lactic acid bacteria in a sample comprising:

preparing the sample;

performing bacterial DNA extraction using the prepared sample to obtain extracted bacterial DNA;

combining the extracted bacterial DNA with PCR reagents comprising a first primer comprising SEQ. ID. No. 1, a second primer comprising SEQ. ID. No. 2, a probe comprising SEQ. ID. No. 3, a DNA polymerase, and deoxynucleotide triphosphates;

cycling the combined extracted DNA and PCR reagents through a thermocycler; and

obtaining data corresponding to a signal detected by the thermocycler.

18. The method of claim 17 further comprising comparing the data c
detected signal to a standard curve to quantify the amount of lactic acid producing bacteria present in the sample.

19. The method of claim 18 wherein the food sample comprises yogurt.

20. The method of claim 17 wherein the probe further comprises a fluorophore.

21. The method of claim 20 wherein the fluorophore comprises carboxyfluorescein.

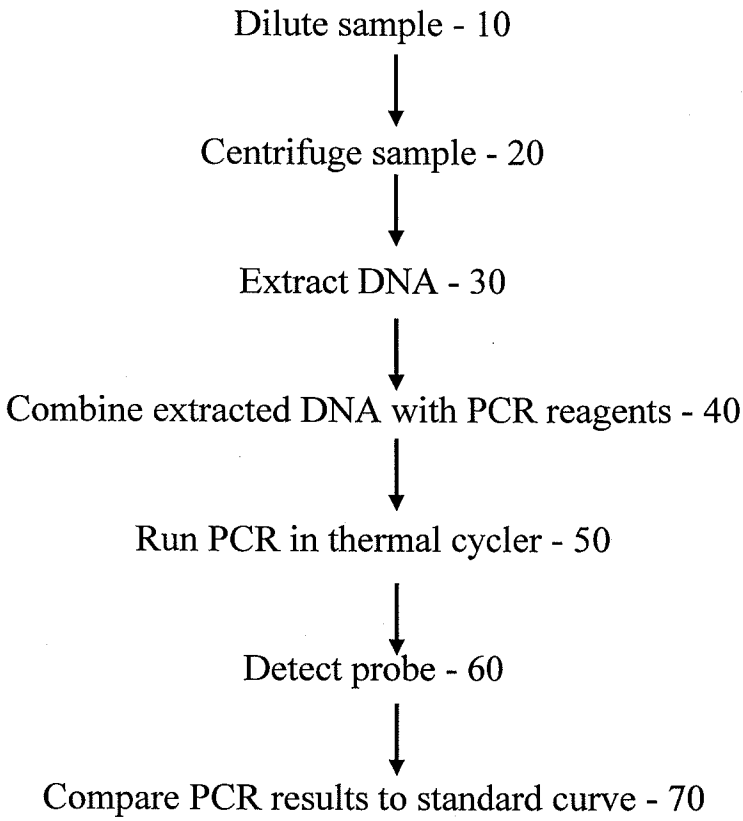
Dilute sample - 10
Centrifuge sample - 20
Extract DNA - 30
Combine extracted DNA with PCR reagents - 40
Run PCR in thermal cycler - 50
Detect probe - 60
Compare PCR results to standard curve - 70

Figure 1

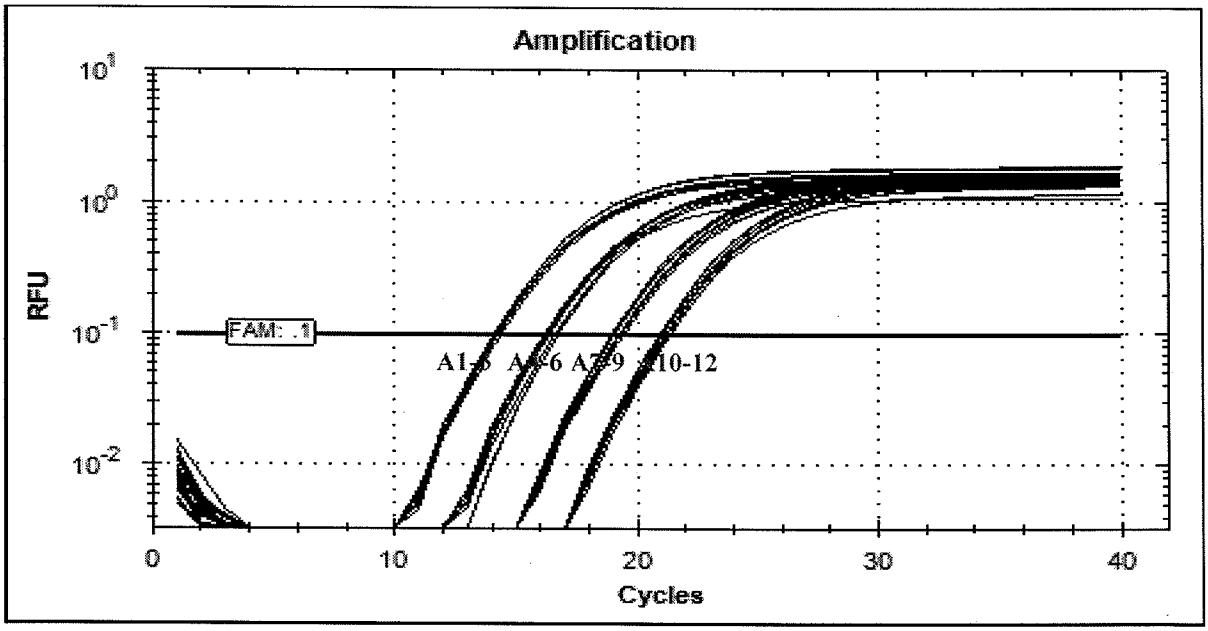

Figure 2

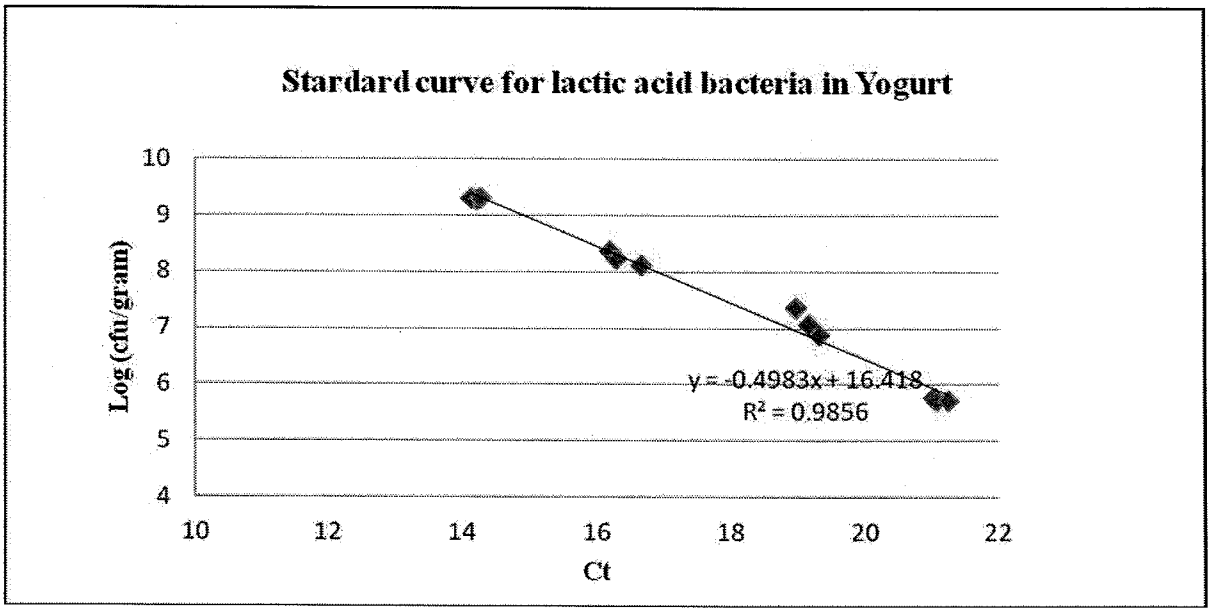

Figure 3

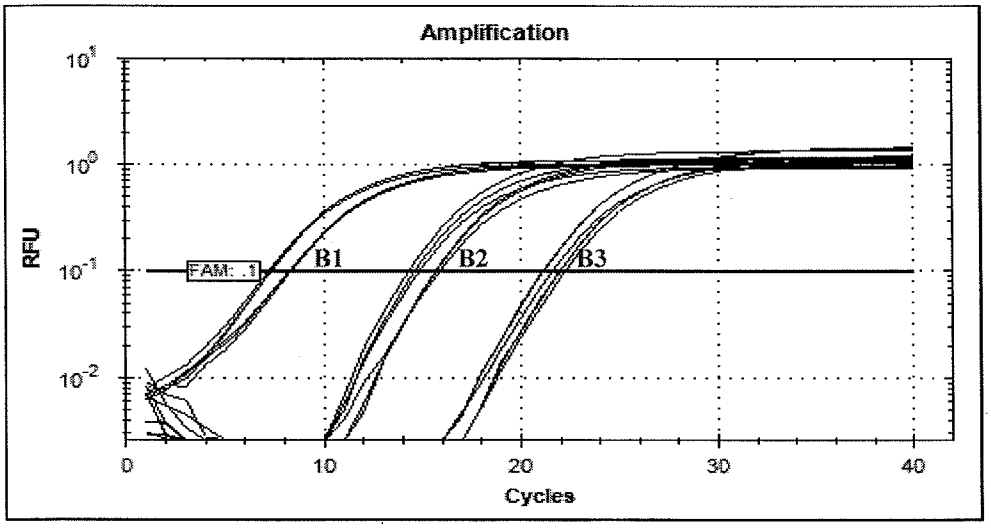

Figure 4a

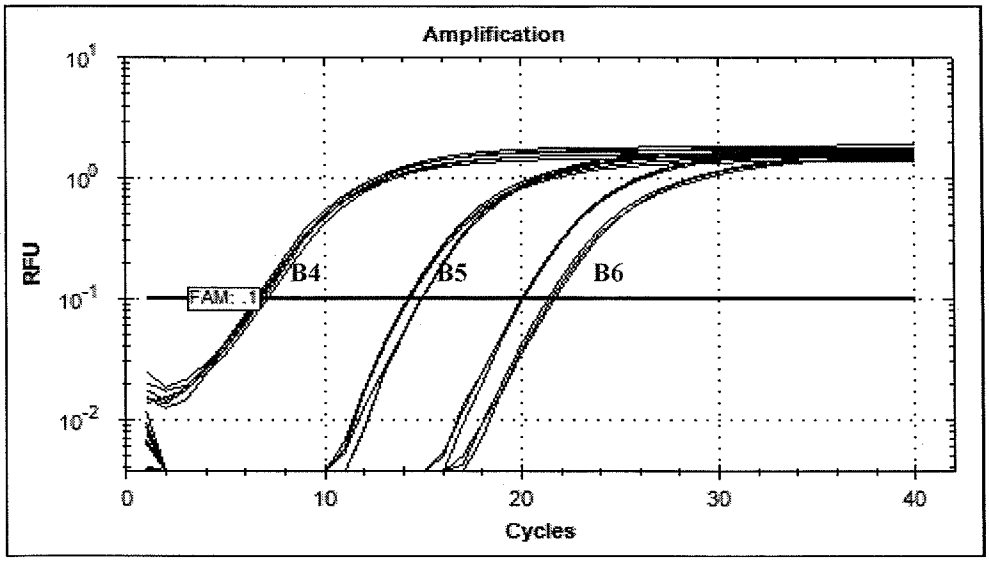

Figure 4b

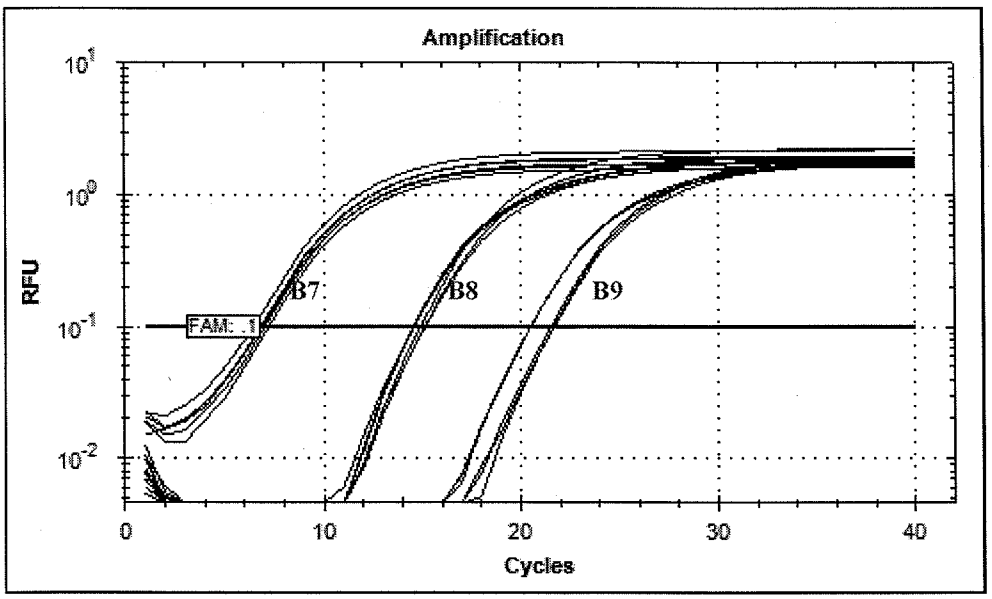

Figure 4c

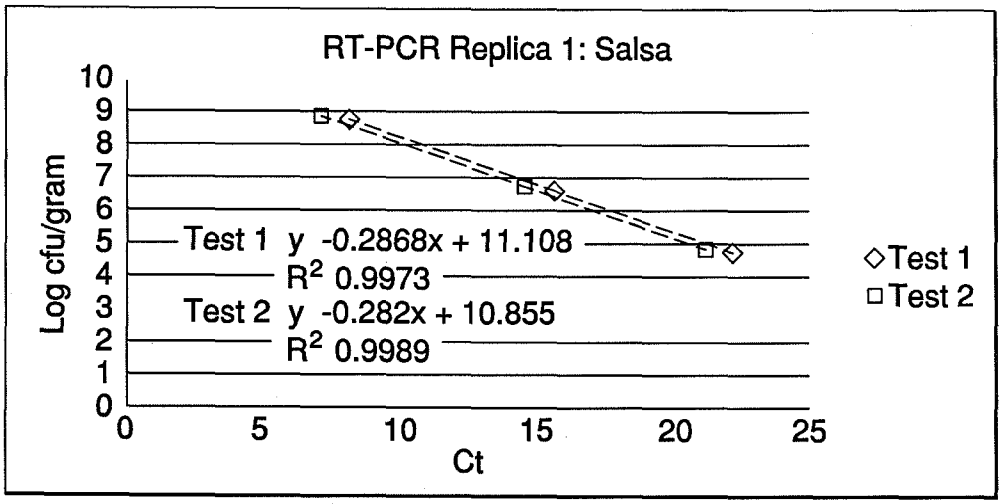

FIG. 5a

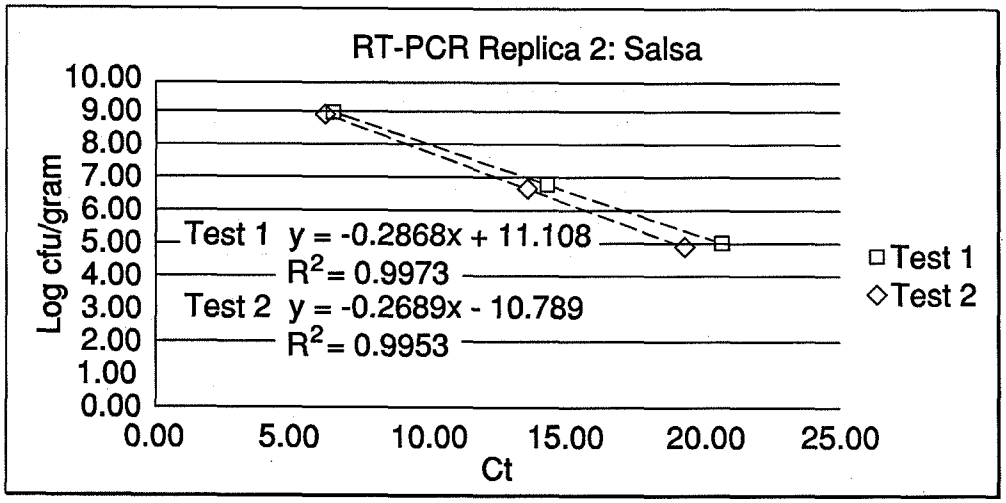

FIG. 5b

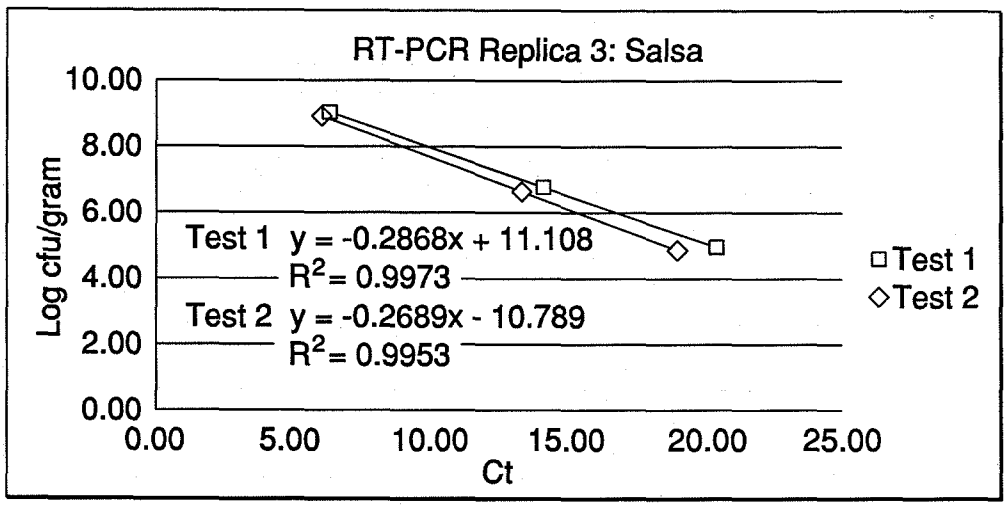

FIG. 5c

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/043958

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68 (2012.01)
USPC - 435/6.12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12Q 1/00, 1/02, 1/04, 1/10, 1/22, 1/68; C07H 21/00, 21/04; C12N 15/09, 15/10, 15/11, 15/31 (2012.01)
USPC - 204/400, 403.01; 205/775, 777.5; 435/4, 6.1, 6.11, 6.12, 6.15, 7.2, 7.32

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Patbase, Google Patent, PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2007/0292852 A1 (NAKAKITA et al) 20 December 2007 (20.12.2007) entire document	11
A	US 7,309,589 B2 (MONTAGNIER et al) 18 December 2007 (18.12.2007) entire document	11
A	US 6,103,468 A (RUSSELL et al) 15 August 2000 (15.08.2000) entire document	11
A	GAMMON et al. "Development of real-time PCR methods for the rapid detection of low concentrations of Gluconobacter and Gluconacetobacter species in an electrolyte replacement drink," Letters in Applied Microbiology, 01 March 2007 (01.03.2007), Vol. 44, No. 3, Pgs. 262-267. entire document	11
A	SELIM et al. "Development and Assessment of a Real-Time PCR Assay for Rapid and Sensitive Detection of a Novel Thermotolerant Bacterium, Lactobacillus thermotolerans, in Chicken Feces," Applied and Environmental Microbiology, 01 August 2005 (01.08.2005), Vol. 71, No. 8, Pgs. 4214-4219. entire document	11

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 September 2012	28 SEP 2012
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/043958

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☒ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

The submitted electronic sequence listing had errors and could not be entered into ISA/US search system.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2012/043958

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-10, 12-21
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The submitted electronic sequence listing had errors and could not be entered into ISA/US search system.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.