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(54) **Title:** METHODS FOR CLASSIFICATION OF MICROORGANISMS FROM FOOD PRODUCTS

(57) **Abstract:** A method comprising: (a) storing a food product; (b) removing a sample from said food product at a minimum of two time points; (c) classifying at least one microorganism from each sample by clustering or molecular analysis, or a combination thereof; (d) screening at least one classified microorganism from each sample for sensitivity to at least one antimicrobial compound; and (e) selecting an effective antimicrobial compound to be applied to a food product based on the screening.



METHODS FOR CLASSIFICATION OF MICROORGANISMS FROM FOOD PRODUCTS

FIELD OF THE INVENTION

The present invention relates to the field of food spoilage monitoring and control.

BACKGROUND OF THE INVENTION

Many food products are subject to spoilage or pathogenicity as a result of improper handling, contamination, or simply due to aging. Food spoilage is a complex process many times involving a variety of microorganisms, food preservatives, additives, and food matrices in addition to temperature, pH, and water activity. While some spoiled food products are safe to eat, changes in appearance, flavor, color, texture, pH, nutritional value, toxicity/pathogenicity, and microbial population, can reduce commercial value and prevent sale or use of the product. Spoiled food products may also be unsafe to consume, causing food-borne illnesses such as infections or intoxication. As a result, food loss from farm to fork causes considerable environmental and economic effects.

Chemical reactions that cause offensive sensory changes in foods are many times mediated by a variety of microorganisms. Lactic acid bacteria and yeast are often the predominant organisms in spoiled food, but details of which species and strains that cause the spoilage has not been extensively studied. Traditional methods for detection of spoilage have been formulated with the goals of identifying concentrations of spoilage microorganisms or volatile compounds produced by the microorganisms. However, many of these methods are considered inadequate because they are time-consuming, labor intensive, and/or do not provide consistent results.

Often, spoilage microorganisms are not originally found in the food product, but are found in water, soil, air, and animals. While good manufacturing practices with strict attention to sanitation and hygiene can prevent colonization by some microorganisms, the prevalence of microorganisms in the environment makes it difficult to avoid contamination. However, controlling contamination and growth of microorganisms in food products can be an important first step in delaying spoilage, extending shelf life, and preventing pathogenicity of food products. In order to help with this control,

antimicrobial compounds may be added to foods, packaging, or the food processing environment to prevent contamination and growth of many spoilage organisms.

There remains a need for methods to identify the key microbial members of the community that are associated with food spoilage and pathogenicity. Further, methods are also needed to accurately predict spoilage microorganisms and the corresponding effective antimicrobial compounds for use in a variety of food products in order to inhibit spoilage, extend shelf life, and prevent pathogenicity of food products.

BRIEF SUMMARY OF THE INVENTION

Methods are provided for the classification and identification of microorganisms in a food product. Methods include classifying microorganisms into spoilage groups and identifying microorganisms using molecular analysis. Various methods are also provided for inhibiting spoilage, extending the shelf life, and preventing pathogenicity of food products using antimicrobial compounds selected for effectiveness against classified microorganisms.

The present disclosure relates to a method of classifying at least one microorganism in a food product, said method comprising: (a) storing a food product; (b) removing a sample from said food product at a minimum of two time points; and (c) classifying at least one microorganism present in each sample by clustering, molecular analysis, or a combination thereof. In some aspects, clustering in step (b) comprises: (a) diluting said sample; (b) plating said diluted sample on selective and differential media; (c) incubating said plated sample for an appropriate time to allow for growth of target microorganisms; (d) selecting at least one isolate from said incubated plated sample; and (e) clustering said at least one isolate. In certain aspects, the microorganisms are spoilage microorganisms, pathogenic microorganisms, or a combination thereof.

In some aspects, an isolate is clustered by performing RAPD PCR analysis wherein said isolate is clustered with microorganisms sharing at least 70% or at least 80% banding pattern similarity. In some aspects, the method further comprises molecular typing of said at least one isolate. In some aspects, molecular analysis is performed by isolating bacterial DNA from each sample; and molecular typing said isolated bacterial DNA. In certain aspects, molecular typing comprises sequencing the 16S ribosomal RNA

gene, or a fragment thereof, and comparing said 16S ribosomal RNA gene sequence (or V4 region) to a database in order to identify at least one bacterial isolate or sequencing the Internal Transcribed Spacer region (ITS), or a fragment thereof, and comparing said ITS sequence to a database in order to identify at least one fungal isolate. In certain aspects, the 16S ribosomal RNA sequence (or V4 region) and ITS sequence are both used to identify bacterial and fungal strains.

In some aspects, storage of the food product occurs at constant temperature, such as the commercial storage temperature. In certain aspects, the product is a food product, ingredient product, or environmental product. In some aspects a sample is removed from the food product at a minimum of 3 time points during storage, such as from 3-6 time points.

In other aspects, a method of inhibiting spoilage of a food product is provided, said method comprising: (a) storing a food product; (b) removing a sample from said food product at a minimum of two time points; (c) classifying the microorganisms from each sample by clustering or molecular analysis; (d) screening at least one classified microorganism from each sample for sensitivity to at least one antimicrobial compound; (e) selecting an effective antimicrobial compound; and (f) applying said selected antimicrobial compound to a food product, wherein application of said selected antimicrobial compound inhibits spoilage of said food product compared to said food product without said antimicrobial compound. In certain aspects, the method comprises classifying said microorganisms into at least one spoilage cluster or pathogenicity cluster, such as classifying into a spoilage cluster or pathogenicity cluster using RAPD PCR analysis, such as clustering microorganisms sharing at least 70% or at least 80% banding pattern similarity. In some embodiments, the at least one classified microorganism in step (d) is selected from at least one spoilage cluster, such as wherein at least one classified microorganism in step (d) sharing at least 70% banding pattern similarity with at least one microorganism classified from said food product.

In certain aspects, a method of inhibiting spoilage of a food product comprises isolating bacterial DNA from each sample; and molecular typing said isolated bacterial DNA to identify the bacterial strains in each sample. In certain aspects, at least one classified microorganism in step (d) is a bacterial strain identified by molecular typing,

such as sequencing the 16S ribosomal RNA gene, or a fragment thereof, and comparing said 16S ribosomal RNA gene sequences to a database in order to identify bacterial strains in each sample. In some aspects, the shelf life of the food products is extended following addition of an antimicrobial compound.

In some aspects information correlating food product with the corresponding classified microorganism and the classified microorganism with the corresponding effective antimicrobial compound is stored in a database. Also provided herein is a database constructed by the methods disclosed herein and containing information correlating food products with the corresponding classified microorganism and the classified microorganism with the corresponding effective antimicrobial compound.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts an example of a plate set up for an antimicrobial screen.

Figure 2 reports the proportion of chloroplast to bacterial sequences obtained from the chicken salad samples stored at 10 °C prior to the best-before date (days -16, -9, and -6)

Figure 3 sets forth the inhibition of growth (%) of bacterial isolates associated with chicken salad by selected antimicrobial products.

Figure 4 reports the inhibition of growth (%) of yeast isolates associated with chicken salad by selected antimicrobial products.

Figure 5 shows the changes in bacterial diversity in chicken salad samples stored at 10 °C prior to the best-before date (Days -16, -9, and -6) as determined by sequencing of the 16S ribosomal RNA gene.

Figure 6 reports the proportion of chloroplast to bacterial sequences obtained from the coleslaw samples stored at 10 °C prior to the best-before date (days -17, -10, -7, and -3).

Figure 7 sets forth the inhibition of growth (%) of bacterial isolates associated with chicken salad by selected antimicrobial products.

Figure 8 reports the changes in bacterial diversity in coleslaw samples stored at 10 °C prior to the best-before date (days -16, -9, and -6) as determined by sequencing of the 16S ribosomal RNA gene.

DETAILED DESCRIPTION

The present inventions now will be described more fully hereinafter with reference to the accompanying drawings, in which some, but not all embodiments of the inventions are shown. Indeed, these inventions may be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will satisfy applicable legal requirements. Like numbers refer to like elements throughout.

Many modifications and other embodiments of the inventions set forth herein will come to mind to one skilled in the art to which these inventions pertain having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the inventions are not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

In light of the description provided below, the following numbered embodiments are provided:

Embodiment 1. A method of classifying at least one microorganism in a food product, said method comprising:

- (a) storing a food product;
- (b) removing a sample from said food product at a minimum of two time points; and
- (c) classifying at least one microorganism present in each sample by clustering, molecular analysis, or a combination thereof.

Embodiment 2. The method of embodiment 1, wherein clustering in step (c) comprises:

- (a) diluting said sample;
- (b) plating said diluted sample on selective and differential media;
- (c) incubating said plated sample for an appropriate time to allow for growth of target microorganisms ;
- (d) selecting at least one isolate from said incubated plated sample; and

(e) clustering said at least one isolate.

Embodiment 3. The method of embodiment 1 or 2, wherein said microorganisms are spoilage microorganisms pathogenic microorganisms, or a combination thereof.

Embodiment 4. The method of any one of embodiments 1-3, wherein said isolate is clustered using Randomly Amplified Polymorphic DNA (RAPD) PCR analysis.

Embodiment 5. The method of embodiment 4, wherein said isolate is clustered with microorganisms sharing at least 70% banding pattern similarity.

Embodiment 6. The method of any one of embodiments 4 or 5, wherein said isolate is clustered with microorganisms sharing at least 80% banding pattern similarity.

Embodiment 7. The method of any one of embodiments 2-6, said method further comprising molecular typing of said at least one isolate.

Embodiment 8. The method of embodiment 7, wherein said molecular typing comprises sequencing the 16S ribosomal RNA gene, or a fragment thereof, and comparing said 16S ribosomal RNA gene sequence to a database in order to identify at least one bacterial isolate.

Embodiment 9. The method of embodiment 7, wherein said molecular typing comprises sequencing the Internal Transcribed Spacer region (ITS), or a fragment thereof, and comparing said ITS sequence to a database in order to identify at least one fungal isolate.

Embodiment 10. The method of embodiment 8, wherein said molecular typing further comprises sequencing the ITS, or a fragment thereof, and comparing said ITS sequence to a database in order to identify at least one fungal isolate.

Embodiment 11. The method of any one of embodiments 1-10, wherein said molecular analysis comprises:

isolating bacterial DNA from each sample; and
molecular typing said isolated bacterial DNA.

Embodiment 12. The method of embodiment 11, wherein molecular typing comprises sequencing the 16S ribosomal RNA gene, or a fragment thereof, and comparing said 16S ribosomal RNA gene sequences to a database in order to identify bacterial strains in each sample.

Embodiment 13. The method of embodiment 12, wherein the V4 region of said 16S ribosomal RNA gene is sequenced.

Embodiment 14. The method of any one of embodiments 11-13, wherein said molecular analysis identifies bacterial strains present in said sample.

Embodiment 15. The method of any one of embodiments 1-14, wherein storing said amount of food product occurs at constant temperature.

Embodiment 16. The method of embodiment 15, wherein said constant temperature is representative of the commercial storing temperature of said product.

Embodiment 17. The method of any one of embodiments 1-16, wherein said product is a food product, ingredient product, or environmental product.

Embodiment 18. The method of any one of embodiments 1-17, wherein a sample is removed at a minimum of 3 time points.

Embodiment 19. The method of embodiment 18, wherein a sample is removed at 3-6 time points.

Embodiment 20. A method of inhibiting spoilage of a food product, said method comprising:

- (a) storing a food product;
- (b) removing a sample from said food product at a minimum of two time points;
- (c) classifying the microorganisms from each sample by clustering or molecular analysis;
- (d) screening at least one classified microorganism from each sample for sensitivity to at least one antimicrobial compound;
- (e) selecting an effective antimicrobial compound; and
- (f) applying said selected antimicrobial compound to a food product, wherein application of said selected antimicrobial compound inhibits spoilage of said food product compared to said food product without said antimicrobial compound.

Embodiment 21. The method of embodiment 20, said method further comprising classifying said microorganism into at least one spoilage cluster or at least one pathogenicity cluster.

Embodiment 22. The method of embodiment 21, wherein said microorganism is classified into a spoilage cluster or pathogenicity cluster using Randomly Amplified Polymorphic DNA (RAPD) PCR analysis.

Embodiment 23. The method of embodiment 22, wherein said microorganism is clustered with microorganisms sharing at least 70% banding pattern similarity.

Embodiment 24. The method of any one of embodiments 22 or 23, wherein said microorganism is clustered with microorganisms sharing at least 80% banding pattern similarity.

Embodiment 25. The method of any one of embodiments 20-24, wherein said at least one classified microorganisms in step (d) is selected from at least one spoilage cluster.

Embodiment 26. The method of embodiment 25 wherein at least one classified microorganisms in step (d) shares at least 70% banding pattern similarity with at least one microorganism classified from said food product.

Embodiment 27. The method of embodiment 20, wherein classifying in step (c) comprises:

isolating bacterial DNA from each sample; and

molecular typing said isolated bacterial DNA to identify the bacterial strains in each sample.

Embodiment 28. The method of embodiment 27, wherein said at least one classified microorganism in step (d) is a bacterial strain identified by said molecular typing.

Embodiment 29. The method of embodiment 27 or 28, wherein molecular typing comprises sequencing the 16S ribosomal RNA gene, or a fragment thereof, and comparing said 16S ribosomal RNA gene sequences to a database in order to identify bacterial strains in each sample.

Embodiment 30. The method of any one of embodiments 20-29, wherein the shelf life of said food product is extended following addition of said antimicrobial compound.

Embodiment 31. The method of any one of embodiments 1-30, wherein information correlating food product with the corresponding classified microorganism

and the classified microorganism with the corresponding antimicrobial compound is stored in a database.

Embodiment 32. A database constructed according to embodiment 31.

I. Overview

Methods for the classification of microorganisms and inhibition of their growth in a food product are provided. As used herein, the term “product” refers to any solid, liquid, or gaseous composition found in the production chain of a food product. For example, in some embodiments, a product can refer to a consumable food product or a food product ingredient. In some embodiments a product can refer to a product obtained from the environment in which a food product is prepared or stored, hereinafter referred to as a food product environment. A “food product” may be any product that has nutritive value and is suitable for ingestion into the gastrointestinal tract.

Methods are provided herein for classifying and identifying microorganisms in a food product or food product environment. For example, methods are provided for classifying and identifying spoilage microorganisms and pathogenic microorganisms in a food product or food product environment. As used herein, the term “spoilage microorganism” refers to any microorganism that contributes to the spoilage or fouling of a food product. A spoiled food product can be characterized by changes in appearance, flavor, color, texture, pH, nutritional value, toxicity/pathogenicity, microbial population, or any other characteristic that causes the food product to be less desirable. A spoilage microorganism may contribute to spoilage of a food product alone, or in combination with at least one other spoilage microorganism. By inhibiting the growth of food spoilage microorganisms, the shelf life of a food product may be increased. As used herein, the term “shelf life” refers to the amount of time that a food product can be stored without becoming unfit for consumption.

Examples of spoilage microorganisms, as used herein, include but are not limited to: fungi such as *Zygosaccharomyces* and related genera, *Debaryomyces hansenii*, *Saccharomyces spp.*, *Candida* and related genera, *Dekkera/Brettanomyces*, *Byssoschlammys*, *Zygomycetes* (i.e., *Mucor* and *Rhizopus*), *Penicillium*, *Aspergillus*, and *Fusarium*; and bacteria such as lactic acid bacteria (LAB), including species of

Lactobacillus, *Pediococcus*, *Leuconostoc*, and *Oenococcus*, *Pseudomonas* and related genera including *P. fluorescens*, *P. fragi*, *P. lundensis*, *P. viridiflava*, *Shewanella putrefaciens*, and *Xanthomonas campestris*, *Bacillus*, *Enterobacteriaceae* including *Salmonella* spp., *E. coli*, *Shigella*, *Yersinia*, *Obesumbacterium*, *Proteus*, *Serratia*, *Klebsiella*, *Enterobacter*, and any other bacteria or fungi including *Acinetobacter*, *Acetobacter*, *Micrococcus*, *Clostridium*, *Flavobacteriaceae*, *Alcaligenes*, *Chromobacterium*, *Coliforms*, *Halobacterium*, *Erwinea carotovera*, *Alicyclobacillus*, *Gluconobacter*, *Altemaria*, *Botrytis*, *Moraxella*, *Rhodotorula*, *Bacillus nigrificans*, *Saccharomyces*, *Torula*, *Zygosaccharomyces*, *Psychrobacter*, *Alcaligenes*, *Flavobacterium*, *Moraxella*, *Photobacterium*, and *Brochotrix*.

By "pathogenic microorganism" it is meant a microorganism that may elicit a disease response in an individual. Food-borne pathogenic microorganisms are pathogenic microorganisms that may grow in food products and/or may enter the body following ingestion of food products. For example, pathogenic microorganisms may be, *Escherichia coli*, *Escherichia coli* serotype O157:H7, *Staphylococcus aureus*, *Listeria*, *Enterococcus faecium*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium botulinum*, *Cryptosporidium parvum*, *Giardia lamblia*, *Shigella*, *Vibrio*, *Yersinia*, and *Salmolla*. As used herein, the term "pathogenic" or "pathogenesis" refers to the ability of a microorganism to elicit a disease state in a subject. Pathogenic microorganisms can cause infections wherein the microorganism is responsible for disease symptoms or intoxications, wherein the microorganism produces a toxin that ultimately causes disease symptoms. In some embodiments a microorganism can be classified both as a spoilage microorganism and a pathogenic microorganism.

II. Classifying microorganisms

Methods are provided herein to classify microorganisms from a food product or food product environment. In some embodiments, the classification of microorganisms can be used to select an antimicrobial for inhibiting spoilage and/or extending shelf life of a food product. As used herein, the term "classifying" or "classify" refers to the grouping of microorganisms having at least one similar characteristic. For example, microorganisms can be classified by similarities in metabolic profile, sugar utilization,

morphology, genotype (i.e. RAPD profile), environmental source, spoilage mechanisms, pathogenicity, or any other characteristic which can differentiate classes of microorganisms.

In order to classify at least one microorganism, a sample of the food product or food product environment can be obtained by removing a portion of the food product or food product environment in such a manner as to avoid contamination. In certain embodiments, a food product is homogenized prior to removal of a sample for classification. For example, a food product can be masticated with or without an appropriate diluent (such as peptone or water) prior to removal of a sample for classification of microorganisms therein. In certain embodiments, a sample taken directly from a food product is mixed with an appropriate diluent (such as peptone or water) and masticated prior to classification of microorganisms therein.

In order to classify a succession of at least one microorganism present in a food product or food product environment over time, a food product can be stored prior to sampling. The food product can be stored for up to 1 day, 2 days, 4 days, 6 days, 7 days, 8 days, 10 days, 12 days, 14 days, 18 days, 21 days, 28 days, 35 days, 42 days, or any number of days desired to classify a succession of at least one microorganism. Storage can occur in environmental conditions that replicate the typical storage condition of the food product, or at any environmental condition desired for classification of microorganisms.

For example, storage can occur at freezer temperatures, (including, but not limited to, -40°C, -30°C, -25°C, -24°C, -23°C, -22°C, -21°C, -20°C, -19°C, -18°C, -17°C, -16°C, -15°C, -12°C, -10°C, -8°C, -5°C, -2°C, 0°C, -23°C to -18°C, or -8°C to -3°C), refrigeration temperatures, (including, but not limited to, 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C, 12°C, 15°C, or 2°C to 8°C), or room temperature (including, but not limited to 20°C, 23°C, 25°C, 26°C, 27°C, 28°C, 30°C, or 23°C to 27°C). In some embodiments, storage can occur at a combination of freezer, refrigeration, and room temperature. For example, storage can occur at freezer temperatures prior to refrigeration temperature. In certain embodiments, freezing or thawing of the food product can occur during storage.

Storage can occur at any relative humidity desired for classification of microorganisms or storage of the food product. For example, storage can occur at about 5%, about 10%, about 12%, about 15%, about 20%, about 25%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, or at any relative humidity typical for storage of the food product.

In some embodiments, the air surrounding packaged food products can be modified during storage. For example, the food product can be stored in modified atmosphere packaging (i.e., decreased oxygen, increased nitrogen, or increased carbon dioxide) or vacuum packaging. Storage can occur in any desired light conditions. For example, storage can occur in sunlight (natural light), artificial light, partial light, or in the absence or partial absence of light.

In some embodiments, microorganisms can be classified in a succession of samples of a product in order to determine how the microbial populations of the product change during storage. For example, a sample of food product can be taken for classification at least 1 time, at least 2 times, at least 3 times, at least 4 times, at least 5 times, at least 6 times, at least 7 times, at least 8 times, at least 9 times, at least 10 times, at least 12 times, at least 14 times, at least 21 times, or any other number of sampling times in order to provide a classification of the succession of microorganisms present in a food product during storage. In particular embodiments, a sample of a food product can be taken at least 2-5 times during storage of the food product in typical storage conditions for the individual food product in order to classify the succession of microorganisms present in the food product during storage.

Classification of microorganisms, as described herein, can be accomplished by any method that groups microorganisms according to similar characteristics. In some embodiments, classifying is accomplished by clustering, molecular analysis, or a combination thereof.

As used herein, the term “clustering” or “cluster analysis” refers to a method of determining groups of related microorganisms having similar genotypes. In some embodiments, clustering is achieved by using randomly amplified polymorphic DNA (RAPD) PCR that results in a RAPD profile. In some examples, ribosomal spacer (RS) RAPD PCR may be used, for example based on techniques such as those disclosed in US

2013/0029341 A1. Microorganisms can be considered closely related if their RAPD profiles are 70% similar, 75% similar, 80% similar, 85% similar, 90% similar, 95% similar, 99% similar, or 100% similar. In some embodiments, similarity of RAPD profiles is determined by analyzing the band-based similarity coefficient, Dice, with 2% tolerance for band-matching and dendrograms constructed using UPGAMA cluster analysis with any appropriate software, such as the BioNumerics software (Applied Maths Inc., Austin, TX, available as of 2013). As used herein, a “spoilage cluster” refers to a cluster of microorganisms, wherein at least one microorganism is a spoilage microorganism. Likewise, a “pathogenicity cluster” refers to a cluster of microorganisms, wherein at least one microorganism is a pathogenicity organism.

In order to select a microorganism for clustering analysis, a sample of the food product or food product environment can be taken once or at multiple time points during storage, as described herein. In some embodiments, the sample is frozen for later classification. Such a sample can be directly frozen or frozen after mastication and diluting. A sample can be diluted to any appropriate concentration for isolation of individual populations of microorganisms (i.e. colonies). For example, a sample can be diluted by 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} , 10^{-8} , 10^{-9} , 10^{-10} , 10^{-11} , or 10^{-12} .

The sample or diluted sample can be plated on any appropriate growth media for growth of the desired microorganisms, or microorganisms expected to be in the sample. In some embodiments, the sample can be plated on selective and differential media. For example, yeasts and molds can be grown on potato dextrose agar or potato dextrose agar acidified with 18 ml/L of 10% tartaric acid solution; total aerobic bacteria can be grown aerobically on tryptic soy (TS) agar; and lactic acid bacteria can be grown anaerobically on de Man, Rogosa, and Sharpe (MRS) agar. The plated sample can then be incubated for the appropriate amount of time to grow the target microorganism. As used herein, the target microorganism for a particular media is any microorganism that grows on the media in the growth conditions selected.

Clustering analysis can be performed on an isolate grown following incubation of the plated sample. As used herein, the term “isolate” refers to an individual colony representing a pure culture of a microorganism. Accordingly, any isolate can be selected

for clustering analysis according to the procedures described elsewhere herein, such as RAPD analysis.

Isolates can be selected for clustering and/or molecular analysis in order to classify and identify the microorganisms present in a food sample. Molecular analysis can be used to identify individual microorganisms in order to provide a specific representation of the microbial population of a food product. As used herein, “molecular analysis” refers to any method that used nucleic acid-based technology to identify a microorganism. Molecular analysis can identify a microorganism at the genus, species, subspecies, serotype, or any other taxonomic level that adequately identifies the isolate. Any method can be used for molecular analysis, such as 16S ribosomal RNA sequencing, 16S ribosomal RNA V4 region sequencing, internal transcribed spacer (ITS) sequencing, *rpoB* sequencing, *gyrB* sequencing, fingerprinting (i.e. denaturing gradient gel electrophoresis), terminal restriction fragment length polymorphism (T-RFLP), fluorescent *in situ* hybridization, or any other appropriate method for identifying a microorganism. Specifically, 16S ribosomal V4 region sequencing can be used to identify bacterial strains and ITS sequencing can be used to identify fungal strains.

In some embodiments, molecular analysis can be performed on a food product sample in order to characterize the population of microorganisms therein. Conducting a molecular analysis on the food product sample could assist in identification of microorganisms that cannot be grown according to standard microbiological culturing methods. For example, in certain embodiments, total genomic DNA can be isolated directly from a food product sample. The V4 region of 16S ribosomal RNA and/or the ITS region can be amplified from the total genomic DNA in order to identify at least one microorganism present in the sample.

III. Methods of Inhibiting Spoilage or Pathogenesis of a Food Product

Methods are provided herein for inhibiting spoilage and/or pathogenesis of a food product. Using clustering or molecular analysis described elsewhere herein, a strategy for targeted inhibition of spoilage and/or pathogenic microorganisms can be developed. For example, antimicrobial compounds having activity against the classified group of microorganisms, and/or any microorganisms identified by molecular analysis can be

selected and applied to the food product in order to inhibit spoilage and/or pathogenesis. As used herein, inhibiting spoilage and/or pathogenesis refers to inhibition of the growth of spoilage or pathogenic microorganisms. In general, the term “inhibits” or “inhibiting” refers to at least 1%, at least 10%, at least 15%, at least 25%, at least 50%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, at least 99.9%, or at least 99.99 % reduction in the number of a specific microorganism or a group of microorganisms grown in a food product or growth media with an antimicrobial compound when compared to a food product or growth media without the antimicrobial compound. In some embodiments, inhibiting includes bacteriostatic and bactericidal activity, such as wherein organisms do not grow, multiply, or produce toxins. Assays to determine inhibition should be carried out under standard conditions for growth and culture of the specific microorganism or group of microorganisms, such as those conditions described elsewhere herein.

Sensitivity of a selected microorganism to an individual antimicrobial compound, or effectiveness of the antimicrobial compound, can be expressed in terms of percent (%) inhibition, which can be based on the relative relation of the area under the growth curve of a treated microorganism to its untreated control. The area under the growth curve can be selected from the beginning of growth (OD_{600} change of 0.05) until the beginning of stationary growth phase, or any other growth period, depending on specific growth conditions or detection method. Microbial growth can be measured by determining optical density at any wavelength appropriate for detection of microbial growth in the selected media and growing conditions. In specific embodiments optical density is measured at 600 nm.

In some embodiments, inhibiting spoilage of a food product results in a longer shelf life of the food product. For example, the shelf life of a product can be extended at least 1 day, at least 2 days, at least 3 days, at least 5 days, at least 7 days, at least 10 days at least 14 days, at least 21 days, at least 28 days, at least 35 days, or any other amount of time depending on the normal shelf life of the product.

Microorganisms or groups of microorganisms classified from a food product sample can be screened for sensitivity to antimicrobial compounds by any method known in the art. In some embodiments, isolates from plated samples can be grown on or in

appropriate growth media containing an antimicrobial compound to be screened for effectiveness against the isolate. As used herein, an “effective antimicrobial compound” refers to an antimicrobial compound that inhibits the growth of a chosen microorganism. A sample list of potential antimicrobial compounds and expected concentrations of effectiveness is provided in Table 1. Other antimicrobial compounds include bacteriocins, such as the R-type pyocins of *Pseudomonas aeruginosa*, as described in WO07134303 or any other bacteriocin, such as, colicin, klebicin, subtilin, epidermin, herbicolacin, brevicin, halocin, agrocine, alveicin, camocin, curvaticin, divercin, enterocin, enterolysin, erwinicin, glycinecin, lactococin, lacticin, leucococin, mesentericin, pediocin, plantaricin, sakacin, sulfolobacin, vibriocin, warnerinand, nisin or the like. Screening of antimicrobial compounds can be performed in a high-throughput manner wherein multiple microorganisms and/or multiple antibiotic compounds are tested simultaneously in separate growth media. For example, screening can be performed in a 96-well format. (Figure 1).

Table 1.

	Yeast & mold isolates	Gram-pos LAB* strains	Gram-pos	Gram- neg
Antimicrobial Singles (microbial based)				
Natamax SF	5	/	/	/
Nisaplin	/	500	400	/
Antimicrobial Singles (plant based)				
Rosemary extract (35% phenolic compounds)	150	150	150	150
Green Tea extract (75% catechin level)	150	150	150	150
Mustard essential oil (5% AITC)	500	/	1000	1000
Fermentate/Fermentate blends				
MicroGARD 100	/	/	/	12000
MicroGARD 200	12000	/	/	12000
MicroGARD 300	/	/	/	/
MicroGARD 400	12000	/	/	12000
MicroGARD 430	12000	12000	12000	12000
MicroGARD 520	/	12000	12000	/
MicroGARD CM 1-50	/	/	/	/
MicroGARD CS 1-50	/	7500	7500	/
MicroGARD 730	12000	12000	12000	/

MicroGARD 740	12000	12000	12000	12000
BioVia YM10	12000	12000	12000	12000
Antimicrobial Blends (Natural and Non-Natural)				
NovaGARD CB 1	12000	7500	7500	/
NovaGARD NR 100	/	400	250	/
NovaGARD 285	7500	/	/	2500
NovaGARD LM 100	/	2000	/	/

In some embodiments, any microorganism selected from a group of microorganisms can be screened for sensitivity to antimicrobial compounds as a representative of the group. In this manner, antimicrobial compound effective against the tested microorganism would be expected to be effective against other microorganisms of the group (i.e. spoilage cluster). Thus, an effective antimicrobial compound against a microorganism classified from a food product can be selected without screening the specific microorganism classified from the food product.

A selected antimicrobial compound can be applied to a food product or food product environment to inhibit spoilage and/or pathogenic microorganisms. The antimicrobial compound can be applied to the food product or food product environment directly, to the food product packaging, to the atmosphere of the food product, or by any other method that inhibits the growth of spoilage and/or pathogenic microorganisms.

In some embodiments, information correlating a food product with classified groups of microorganisms or identified microorganisms can be stored in a database. Such a database can also contain data associating the effectiveness of a particular antimicrobial compound against specific microorganisms classified or identified from a food product. The database could provide a reference for developing a strategy for inhibiting spoilage and/or pathogenic microorganisms without repeating the methods of classification and sensitivity testing as described herein.

All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this disclosure pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

EXPERIMENTAL

Example 1. Chicken Salad and Coleslaw Sampling for Classification of Microorganisms

The objective of this study is to identify the key microbial members of the community that are associated with food spoilage. In addition there may be uncultured bacteria that are influential in the progression of spoilage and the changes that occur in the microbiota over time need to be determined.

Two 32 ounce (907 g) Sycamore Farms Premium Chunk White Meat Chicken Salads were purchased at Sam's Club (Waukesha, WI). The tubs were packed by Suter Company (Sycamore, IL), the sell-by data was 26 April 2012 and they were numbered 14:33 and 14:34. It is known that High Pressure Pasteurization is used by this company to increase shelf-life of their products. The listed ingredients are as follows: cooked white chicken, mayonnaise (soybean oil, water, egg yolks, distilled vinegar, sugar, salt, mustard flour, calcium disodium EDTA added to protect flavor), celery, water chestnuts, sugar, bread crumbs (bleached wheat flour, sugar, yeast, salt), vinegar, glucone delta lactone, onion, salt, gum arabic.

On days 16, 9 and 6 before the sell-by date the chicken salad was opened in a sterile hood, stirred with a sterilized spoon and samples removed for microbial analyses and pH determination. Twenty-two grams of salad was weighed into a sterile whirlpak bag and masticated with 198 ml sterile peptone (0.1%) for 1 min at 120 strokes per min before further dilution in sterile buffered 0.1% peptone for microbial counts. Aliquots of the masticated sample were frozen in liquid nitrogen and stored at -20°C, with or without equal amounts of Tryptic soy (TS) broth with 20% glycerol, for community analysis and to allow further culturing of isolates respectively. Ten gram of sample was used to determine pH of the sample with and without a 1:10 dilution in water.

Two tubs of Chef Classics coleslaw were purchased at Sam's Club (Waukesha, WI). The food was described as freshly shredded cabbage, diced red peppers and bits of

carrots tossed in a creamy mayonnaise dressing. The sell-by/best-before date on the tubs was 27 April 2012. On days 17, 10, 7 and 3 before the sell-by date the coleslaw was opened in a sterile hood, stirred with a sterilized spoon and samples removed for microbial analyses and pH determination. Twenty-two grams of salad was weighed into a sterile whirlpak bag and masticated with 198 ml sterile peptone (0.1%) for 1 min at 120 strokes per min before further dilution in sterile buffered 0.1% peptone for microbial counts. Aliquots of the masticated sample were frozen in liquid nitrogen and stored at -20°C, with or without equal amounts of Tryptic soy (TS) broth with 20% glycerol, for community analysis and to allow further culturing of isolates respectively. Ten gram of sample was used to determine pH of the sample with and without a 1:10 dilution in water.

Example 2. Strain Clustering and Identification

Yeast and mold were enumerated on potato dextrose agar acidified with 18ml/L of 10% tartaric acid solution after autoclaving. Pour plates were incubated at room temperature (~22°C) for 36 hours for yeast and 5 days for molds before colony forming units (CFU) were counted. TS agar pour plates were incubated aerobically for three days at room temperature for counts of total aerobic bacteria. Pour plates with De Man, Rogosa, and Sharpe (MRS) agar with amphotericin (5 µg/ml) were incubated anaerobically at 32°C for three days to determine counts of lactic acid bacteria.

Well-spaced colonies (~16) from each media at each of the three time points were picked from the agar plates and inoculated into 1.0 ml of the appropriate medium in a 96 well matrix block. Genomic DNA from the isolates was obtained using the NucleoSpin 96 Tissue Kit (Macherey Nagel, Düren, Germany) with the following modifications to the manufacturer's protocol. For lysis of bacteria, cells were pre-incubated for 30 min with lysozyme (10mg/ml) at 37°C. For isolation of yeast, the manufacturer's support protocol for yeast was followed using 250 µl of yeast culture that was lysed overnight using zymolyase (G Biosciences, St. Louis, MO).

Randomly amplified polymorphic DNA (RAPD) PCR was performed using Primer 4 (5'- d[AAGAGCCCGT]-3') (SEQ ID NO: 1) with a Ready-to-Go RAPD Analysis Bead (GE Healthcare) and manufacturer's protocol. RAPD profiles were analyzed by the band-based similarity coefficient, Dice, with 2% tolerance for band-

matching and dendrograms constructed using UPGAMA cluster analysis with BioNumerics software (Applied Maths Inc., Austin, TX). Isolates were considered closely related if their banding patterns were 80% similar.

After extraction, bacterial gDNA was amplified for sequencing of the 16S ribosomal RNA gene using bacterial primers 8F (5'-GATCCTGGCTCAG-3'; SEQ ID NO: 2) with M13F tail (TGTAACACGACGG; SEQ ID NO: 3) and 1541R (5' – TGATCCAACCGCA; SEQ ID NO: 4) with M13R tail (GGAAACAGCTATG; SEQ ID NO: 5). A 25µl PCR reaction mixture contained: 2.5µl of 10x PCR buffer, 200 µM concentration of each dNTP, 5mM MgCl₂, 0.2 mM of each primer, 1µl of DNA template, 2.5 U of Platinum Taq and sterile deionized water. PCR was performed on a GeneAmp® PCR System 2700 thermocycler (Applied Biosystems), with the following cycling conditions: 94°C for 5min; 30 cycles of 94°C for 30s, 57°C for 30s, 72°C for 90s, and followed by 72°C for 7 min for final elongation. The PCR product was sequenced by Sanger sequencing (DuPont, Wilmington, Delaware). EzTaxon, a web-based tool for the classification of bacteria based on 16S ribosomal RNA gene sequences was used to determine the putative identification of each isolate. For identification of the fungal isolates the ITS region was amplified using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3'; SEQ ID NO: 6) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'; SEQ ID NO: 7). A 50µl PCR reaction mixture contained: 5µl of 10x PCR buffer, 200 µM concentration of each dNTP, 2mM MgCl₂, 0.2 mM of each primer, 5µl of DNA template, 2.5 U of Platinum Taq and sterile deionized water. PCR was performed on a GeneAmp® PCR System 2700 thermocycler (Applied Biosystems), with the following cycling conditions: 94°C for 5min; 30 cycles of 94°C for 30s, 55°C for 30s, 72°C for 90s, and followed by 72°C for 7 min for final elongation. The PCR product was sequenced by Sanger sequencing (DuPont, Wilmington, Delaware). A curated ITS database by University of Sydney was used to determine the putative identification of the yeast isolates.

Example 3. Microbial Community Analysis

A 1 ml portion of the masticated sample was submitted to centrifugation for 5 min at 8 000 x g to harvest the bacterial cells. Cells were washed twice by re-suspending

pellet in 1 ml T₅₀E₁₀ pH 8.0 (50 mM Tris, 10 mM EDTA) buffer and collecting the cells by centrifugation as before. The cell pellet was suspended in 300 µl T₅₀E₁₀ pH 8.0 buffer to which 10 µl lysozyme solution (100 mg/ml, Fisher Scientific, BP535-10) was added. Cells were incubated at 37°C for 30 min. An additional 300 µl of lysis buffer from Promega LEV Blood kit (Promega, Madison, WI) was added to the cells before bead-beating (0.1mm zirconium/silica beads, BioSpec, Bartlesville, OK) for 2 min. To degrade proteins, samples were incubated at 37°C for 30 min with 10 µl proteinase K (1 mg/ml, Sigma, City). Genomic DNA was isolated with the LEV Blood kit on a Maxwell® 16 (Promega, Madison, WI) as per manufacturer's instructions.

The V4 region of 16S ribosomal RNA gene was amplified with the following primers: F515 (CCTATCCCCTGTGTGCCTTGGCAGTCTCAGGTGCCAGCMGCCGCGGTAA; SEQ ID NO: 8) and bar-coded 806R (CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNNNNNNNNGGACTACVSGGGTATCTAAT; SEQ ID NO: 9) with 5PRIME HotMaster *Taq* Polymerase (Fisher Scientific, Pittsburgh, PA; includes *Taq*, dNTPs and buffer). PCR reactions were purified using the Purelink™ PCR purification kit (Invitrogen, Grand Island, NY) with Binding Buffer B2 (low cut-off). Barcoded samples were pooled together in equal amounts and gel purified (Agarose gel purification, Roche, Madison, WI). GS FLX Titanium amplicon sequencing (454 Life Sciences, Branford, CT) was done at the W.M. Keck Center for Comparative and Functional Genomics, University of Illinois, Urbana-Champaign. Data were analyzed using the QIIME pipeline (www.qiime.org). Sequences were de-multiplexed by sample, quality filtered and clustered into operational taxonomic units (OTUs) at 97% similarity. OTUs were aligned to the SILVA 16S rRNA reference database (www.arb-silva.de) and genus level taxonomy was assigned.

Example 4. Determining Sensitivity to Antimicrobial Products

All strains used for the susceptibility assay were checked for purity prior to the assay and maintained at -86°C in suitable broth containing glycerol 33-50% (v/v). To produce vegetative cells of the bacterial strains, they were inoculated into either TS broth (Oxoid, Denmark); MRS (Merck, Denmark) for bacteria or YM-broth (Sigma, Denmark)

for yeast strains and incubated overnight at 30°C and 25°C, respectively. The susceptibility assay was performed on a fully automated assessment system consisting of a 9-hole Cytowell (Thermo), a Biomek FxP pipetting robot (Beckman Coulter), an ORCA robotic arm on a 3 meter track (Beckman Coulter), 2 Synergy HT spectrophotometers (Biotek), a Cytomat 6001 incubator (Thermo) and a Cytomat 2C incubator (Thermo). All instruments are placed under a laminar flow hood.

Per test plate 12 commercially available antimicrobials (Table 1) can be tested simultaneously against a maximum of two indicator strains. The selection of antimicrobial and the test concentration were based on the expected inhibition action of the antimicrobials according to the application guides (Appendix A) for those products against 3 groups of microorganisms: Gram-positives (but not lactic acid bacteria); Gram-positive lactic acid bacteria; and yeast & mold isolates. Approximately, 1.5ml per well of a single concentration of the investigated antimicrobial prepared as a pH adjusted (pH 5.5 $\pm$ 0.2) water stock (stock preparation according to application guide, 2 fold assay concentration, Table 1) was added to a 96-deepwell plate according to the scheme set forth in Figure 1. All wells of a 96- microwell plate (MTP) were filled with 95 μ l of pH adjusted double strength cultivation broth. Using the pipetting robot, 100 μ l from the deep well plate, containing the antimicrobial solutions, were transferred (column by column) to the MTP test plate. Thus diluting the active compounds 2-fold, but creating a normal strength media. (Up to 12 identical MTP plates can be created from one deep well plate). Finally 5 μ l of a 1000 fold diluted (Ringers, Merck) overnight culture were added to the MTP test plate. A maximum of 2 spoilage isolates (strain 1: A1 to D12; strain 2 E1 to H12) were employed per MTP test plate (Figure 1).

In all experiments growth control without antimicrobial were included for each strain. The wells D1 to D12 for strain 1 and E1 to E12 for strain 2 were used as growth control, since no antimicrobial was added to column D and E. The test plates inoculated with bacterial and fungal isolates were incubated at 30°C and 25°C, respectively. The growth of the microorganism was monitored by measuring the optical density at 620 nm in a Biotek synergy reader every 60 minutes for 24 up to 48 hours, depending on the organism. The susceptibility of the spoilage isolates is expressed in % inhibition, which is based on the relative relation of the area under the growth curve of a treated spoilage

organism to its untreated control. Used for calculation of the area below the curve is the time point when the growth control starts growing (OD change of 0.05) until it reaches stationary growth phase (but no more than 16 hours onwards the growth kick off). Value presented is the average of 3 replicates.

Example 5. Results of Microbial analysis of Chicken Deli Salad

Microbial plate counts increased over the experimental period (Table 2). The majority of the lactic acid bacteria isolated from the three time points (44/47) belonged to the *Leuconostoc citreum* group and clustered into five RAPD types (Table 4) with most (35/44) clustering in one group with 80% similarity. It was initially discovered after the first plating time point that colonies growing on the TS agar medium were yeast, after which point amphotericin (5 µg/ml) was added to the medium for the remaining plating time points. In subsequent time points, colonies that grew on the TS agar were very tiny and determined to be mainly lactic acid bacteria that were able to grow poorly in aerobic conditions. This resulted in the majority of the isolates from the TS agar medium being contaminated (33/48). Thirteen of the remaining isolates were lactic acid bacteria, which were already represented by the isolates from the MRS plates. Only one isolate from the TS agar plates, identified as a *Bacillus*, was selected for AM sensitivity screening (Table 4). There were ten clusters of yeast strains (Table 5), but the majority of isolates were represented by two clusters. One group (47 isolates) was putatively identified as *Candida zelandiodes* and the other (29 isolates) as *Torulaspora delbreukii*.

Ten bacterial isolates (Fig. 3) and ten fungal isolates (Fig. 4) representing the diversity of the isolated strains were tested to determine their sensitivity to selected antimicrobial products. The antimicrobial sensitivity was expressed as % inhibition. Differences in susceptibility were observed among the isolates. For example, out of the spoilage yeast *Torulaspora* species appeared more tolerant compared to the isolated *Candida* species. Moreover, the gram positive bacteria, mainly *Leuconostoc* spp. were nearly equally suppressed by the range of MicroGARD considering the % inhibition. According to their mode of action, there was no antimicrobial that could inhibit the growth of both bacterial isolated and yeast strains. However, two component systems (antifungal e.g. BioVia YM10 and anti-Gram-positive e.g. a MicroGARD) could be

selected as effective. If a single solution were required NovaGARD CB1 could be promoted as it was able to inhibit the growth of the majority of the bacterial and yeast strains.

Over 3000 16S ribosomal RNA genes sequences were obtained per sample for the culture independent bacterial community analysis. However, when bacterial populations were low, then the majority of the sequences were identified as being chloroplast sequences (Fig. 2). The chloroplast sequences were removed from the data before further analysis. At the first sampling time (sixteen days prior to the sell-by date) the majority (>60%) of the bacterial sequences detected belonged to the *Photobacterium* genus, but as the chicken salad spoiled the *Leuconostoc* genus became predominant and the bacterial diversity decreased (Fig. 5).

Table 2. Microbial plate counts and pH changes during storage of the two chicken salads (A and B) at -10°C before the sell-by date at Day 0.

	Day -16		Day -9		Day -6	
Chicken Salad	A	B	A	B	A	B
Microbial Plate counts (log ₁₀ CFU/g)						
Lactic Acid Bacteria	2.00	2.48	5.05	5.40	6.19	5.87
Total Aerobes	2.69	3.91	>5.30 ^a	>5.30 ^a	6.35	7.68
Yeast	2.57	3.85	>5.30 ^a	>5.30 ^a	7.85	8.05
pH						
Solid	4.92	4.82	4.53	4.92	4.69	4.71
Liquid	4.72	4.75	4.76	4.72	4.89	4.88

a: colonies were too numerous to count at the highest dilution plated.

Table 3: Related clusters of bacterial strains isolated from chicken salad on MRS plates on days before the sell-by date, as determined by RAPD typing and sequencing of the 16S ribosomal RNA gene.

Cluster	# of isolates	Days	Putative identification	Representative Isolate
1	3	-16, -9, -6	<i>Leuconostoc citreum</i>	CKS2-B2-A05
2	35	-16, -9, -6	<i>Leuconostoc citreum</i>	CKS2-B2-E09; CKS2-B2-G05; CKS2-B2-H09
3	1	-16	<i>Pediococcus</i>	CKS2-B2-E02
4	1	-16	<i>Lactobacillus oligofermentans</i>	CKS2-B2-E01

5	1	-9	Possibly contaminated	
6	4	-9	<i>Leuconostoc citreum</i>	CKS2-B2-H06
7	1	-9	<i>Leuconostoc citreum</i>	CKS2-B2-D06
8	1	-9	<i>Leuconostoc citreum</i>	CKS2-B2-F06
Total	47			

Table 4: Related cluster of bacterial strains isolated from chicken salad on Tryptic Soy Agar plates on days before the sell-by date, as determined by RAPD typing and sequencing of the 16S ribosomal RNA gene.

RAPD Cluster	# of isolates	Days	Putative identification	Representative Isolate
1	2	-9	Possibly contaminated	
2	2	-9, -6	Possibly contaminated	
3	2	-9	Possibly contaminated	
4	1	-9	Possibly contaminated	
5	5	-9, -6	<i>Leuconostoc citreum</i> group	
6	1	-16	Possibly contaminated	
7	3	-6	<i>Leuconostoc gelidum</i>	
8	1	-16	<i>Leuconostoc gelidum</i>	
9	6	-16, -6	Possibly contaminated	
10	1	-6	Possibly contaminated	
11	9	-16	Possibly contaminated	
12	2	-6	<i>Bacillus subtilis</i> group	CKS2-B3-B10
13	4	-9, -6	<i>Leuconostoc citreum</i> group	
14	1	-16	Possibly contaminated	
15	1	-6	Possibly contaminated	
16	1	-9	Possibly contaminated	
17	2	-9	Possibly contaminated	
18	1	-9	Possibly contaminated	
19	1	-9	Possibly contaminated	
20	1	-9	Possibly contaminated	
21	1	-16	Possibly contaminated	
Total	48			

Table 5: Related clusters of fungal strains isolated from chicken salad on days before the sell-by date, as determined by RAPD typing and sequencing of the ITS region.

Cluster	# of isolates	Days	Putative identification	Representative Isolate
1	1	-6	<i>Candida zelanoides</i>	CKS2-B1-A12
2	1	-9	<i>Candida zelanoides</i>	CKS2-B1-B03
3	1	-9	<i>Candida zelanoides</i>	CKS2-B1-D06

4	1	-9	<i>Candida zelanoides</i>	CKS2-B1-F06
5	47	-16, -9, -6	<i>Candida zelanoides</i>	CKS2-B1-F08, CKS2-B1-G10
6	6	-16, -9, -6	<i>Candida zelanoides</i>	
7	29	-16, -9, -6	<i>Torulaspor a delbreukii</i>	CKS2-B1-B04
8	2	-16	<i>Torulaspor a delbreukii</i>	CKS2-B1-F02
9	5	-9, -6	<i>Yarrowia lipolytica</i>	CKS2-B1-A03
10	1	-6	<i>Candida zelanoides</i>	CKS2-B1-G07
Total	94			

In this study the bacteria and fungi associated with spoilage of chicken salad over time were determined by isolating the predominant organisms growing on agar plates and by sequencing of the 16S ribosomal RNA gene to compare cultured bacterial diversity with that of the total bacterial community present. The isolated strains of bacteria and yeast were grouped using RAPD profiles in order to determine the diversity of strains present in the chicken salad. RAPD profiles are capable of discriminating strains within species based on their genetic content (as a measure of phenotypic potential) and this strain discrimination is important in order to group isolates before further characterization as well as to compare organisms across time and location. In the future a database of characterized strains could allow predictions to be made on the potential of certain strains to cause food spoilage as well as the strains sensitivity to antimicrobials.

In this study, RAPD typing was used to determine the strain level diversity of the bacteria and yeast isolates and subsequent sequencing of the bacterial 16S ribosomal RNA gene and the fungal ITS region was used to assign taxonomy to the organisms. Two steps were therefore necessary to group and then select isolates for further characterization.

The key bacteria that were associated with spoilage of these two tubs of chicken salad, from the same supplier and lot, were *Leuconostoc* species belonging to the *L. citreum* group by both culture and non-culture-based methods. The predominant yeast by culture were *Candida zelanoides* and *Torulaspora delbreukii*. There were differences in the antimicrobial sensitivity (% inhibition) of the isolates associated with spoilage, but there were products that could inhibit the growth of the majority of the bacterial and yeast strains.

Example 5. Results of Microbial analysis of Coleslaw

Bacterial plate counts increased over the experimental period (Table 6), but no fungi grew on the PDA plates. At the first sampling time point, the lactic acid bacteria isolated clustered into three RAPD types and were putatively identified as *Leuconostoc carnosum*, *L. mesenteroides*, and *L. gelidum*. At the final two sampling time points for which counts and isolates were obtained the majority of isolates (24/32) grouped into one RAPD type identified as *Weissella koreensis*. Colonies that grew on the TS agar were very tiny and as with the chicken deli salad (TN 0118915) were determined to be mainly lactic acid bacteria able to grow poorly in aerobic conditions. Only six isolates from the TSA plates were not LABs (Table 8).

Nine bacterial isolates (Fig. 7) representing the diversity of the isolated strains were tested to determine their sensitivity to certain antimicrobial products. The antimicrobial sensitivity was expressed as % inhibition. The isolates appeared to be susceptible to the majority of the tested antimicrobials, with the only differences in susceptibility being to the plant-based antimicrobials.

Over 3000 16S ribosomal RNA genes sequences were obtained per sample for the culture independent bacterial community analysis. An extra sampling day was added to the coleslaw samples as the bacterial counts were not as high as for the chicken samples. As before when bacterial populations were low, then the majority of the sequences were identified as being chloroplast sequences (Fig. 6). The chloroplast sequences were removed from the data before further analysis. One sample from Day -7 was removed due to poor PCR amplification. At the first sampling time (seventeen days prior to the sell-by date) the majority (>60%) of the bacterial sequences detected belonged to the *Xanthomonas* genus. As the coleslaw spoiled *Leuconostoc* species appeared (>30%) in Tub 2, but at the last sampling time the majority of the sequences (>90%) were *Weissella* in both tubs.

Table 6. Microbial plate counts and pH changes during storage of the two coleslaws (A and B) at -10°C before the sell-by date at Day 0.

	Day -17		Day -10		Day -7	
Coleslaw	A	B	A	B	A	B
Microbial Plate counts (log₁₀ CFU/g)						

Lactic Acid Bacteria	1.54	3.39	3.68	4.29	4.79	5.28
Total Aerobes	2.88	3.00	4.01	5.33	4.89	5.52
Yeast	<2.0 ^a	<2.0 ^a	<2.0 ^a	<2.0 ^a	<2.0 ^a	<2.0 ^a
pH						
Solid	4.55	4.46	4.45	4.43	4.41	4.41
Liquid	4.75	4.78	4.63	4.59	4.69	4.70

a: colonies were too numerous to count at the highest dilution plated.

Table 7: Related clusters of bacterial strains isolated from coleslaw on MRS plates on days before the sell-by date, as determined by RAPD typing and sequencing of the 16S ribosomal RNA gene.

Cluster	# of isolates	Days	Putative identification	Representative Isolate
1	11	-17	<i>Leuconostoc carnosum</i>	CKS2-B2-D04, CKS2-B2-E03
2	5	-17, -10	<i>Leuconostoc mesenteroides</i>	CKS2-B2-F08
3	1	-7	Possibly contaminated	
4	3	-17	<i>Leuconostoc gelidum</i>	
5	24	-10, -7	<i>Weissella koreensis</i>	CKS2-B2-A11, CKS2-B2-E12
6	2	-10	Possibly contaminated	
Total Isolates	46			

Table 8: Related clusters of bacterial strains isolated from coleslaw on Tryptic Soy Agar plates on days before the sell-by date, as determined by RAPD typing and sequencing of the 16S ribosomal RNA gene.

Cluster	# of isolates	Days	Putative identification	Representative Isolate	DCS#
1	14	-17, -10, -7	<i>Leuconostoc</i> spp.		
2	1	-17	<i>Pseudomonas veronii</i> group	CKS2-B3-E03	1794
3	3	-10	Possibly contaminated		
4	1	-7	Possibly contaminated		
5	2	-10, -7	Possibly contaminated		
6	3	-10, -7	Possibly contaminated		
7	1	-17	Possibly contaminated		
8	1	-17	<i>Pseudomonas deceptionensis</i>	CKS2-B3-F03	1800
9	3	-17, -10	<i>Leuconostoc</i> spp.		

10	2	-17	<i>Leuconostoc carnosum</i>		
11	3	-17, -10, -7	<i>Leuconostoc carnosum</i>		
12	2	-17	<i>Leuconostoc carnosum</i>		
13	3	-17, -10	<i>Pseudomonas veronii</i> group	CKS2-B3-G04	1797
14	1	-7	<i>Bacillus subtilis</i> group	CKS2-B3-C12	1796
15	2	-17	Possibly contaminated		
16	1	-7	Possibly contaminated		
Total Isolates	43				

In this study the bacteria associated with spoilage of coleslaw over time were determined by isolating the predominant organisms growing on agar plates and by sequencing of the 16S ribosomal RNA gene to compare cultured bacterial diversity with that of the total bacterial community present. The culture studies indicated that the *Pseudomonas* species and *Leuconostoc* species were present at the early time points. At this time point in the non-culture based analysis, both tubs contained *Pseudomonas* species (5-10%) and over 20% of the sequences in tub 2 were *Leuconostoc*. The majority of the bacteria, however, by non-culture based methods belonged to the genus *Xanthomonas*, a genus of Gram-negative bacteria that are plant-associated, many of which are plant pathogens. These remained predominant until the final sampling time at which time the over 90% of the sequences belonged to the *Weissella* genus. This was confirmed in the plate counts, in which most of the isolates belonged to one RAPD type and were identified as *Weissella koreensis*. Although *Leuconostoc* were present in the coleslaw samples and did increase during spoilage especially in tub 2 they did not become the predominant spoilage organisms as in the chicken deli salad (TN0118915). As in the chicken deli salad samples the final bacterial communities in both tubs were very similar to each other, although there were differences in the proportions of bacteria present over time.

Although the reason for the *Weissella* to become more predominant over the *Leuconostoc* that were present at the earlier time points is not understood, all the bacterial strains tested were susceptible to a number of the tested antimicrobials.

CLAIMS

1. A method comprising:
 - (a) storing a food product;
 - (b) removing a sample from said food product at a minimum of two time points;
 - (c) classifying at least one microorganism from each sample by clustering or molecular analysis, or a combination thereof;
 - (d) screening at least one classified microorganism from each sample for sensitivity to at least one antimicrobial compound; and
 - (e) selecting an effective antimicrobial compound to be applied to a food product based on the screening.
2. The method of claim 1, wherein for each sample the clustering in step (c) comprises:
 - diluting said sample;
 - plating said diluted sample on selective and differential media;
 - incubating said plated sample for an appropriate time to allow for growth of target microorganisms;
 - selecting at least one isolate from said incubated plated sample; and
 - clustering said at least one isolate.
3. The method of any one of claims 1 or 2, said method further comprising classifying said at least one microorganism into at least one spoilage cluster or at least one pathogenicity cluster.
4. The method of claim 3, wherein said at least one microorganism is classified into a spoilage cluster or pathogenicity cluster using Randomly Amplified Polymorphic DNA (RAPD) PCR analysis.

5. The method of claim 4, wherein said Randomly Amplified Polymorphic DNA (RAPD) PCR analysis comprises a ribosomal spacer (RS) RAPD PCR analysis.
6. The method of claim 4 or 5, wherein said at least one microorganism is clustered with microorganisms sharing at least 70% banding pattern similarity or at least 80% banding pattern similarity.
7. The method of any one of claims 1 to 6, wherein said at least one classified microorganism screened in step (d) is selected from at least one spoilage cluster.
8. The method of claim 7, wherein said at least one classified microorganism screened in step (d) shares at least 70% banding pattern similarity with at least one microorganism classified from said food product.
9. The method of claim 1, wherein classifying in step (c) comprises:
isolating bacterial DNA from each sample; and
molecular typing said isolated bacterial DNA to identify the bacterial strains in each sample.
10. The method of claim 9, wherein said at least one classified microorganism screened in step (d) is a bacterial strain identified by said molecular typing.
11. The method of claim 8 or 9, wherein molecular typing comprises sequencing the 16S ribosomal RNA gene, or a fragment thereof, and comparing said 16S ribosomal RNA gene sequences to a database in order to identify bacterial strains in each sample.
12. The method of any one of claims 1 to 11, further comprising:
(f) applying said selected antimicrobial compound to a food product, wherein application of said selected antimicrobial compound inhibits spoilage of said food product compared to said food product without said antimicrobial compound.

13. The method of claim 12, wherein the shelf life of said food product is extended following applying said antimicrobial compound.
14. The method of any one of claims 1 to 13, further comprising storing information correlating food product with the corresponding classified microorganism and the classified microorganism with the corresponding antimicrobial compound in a database.
15. A database storing information correlating food product with the corresponding classified microorganism and the classified microorganism with the corresponding antimicrobial compound is stored in a database generated according to the method of claim 14.

Figure 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	AM 1/ strain1	AM 2/ strain1										AM 12/ strain1
B	AM 1/ strain1	AM 2/ strain1										AM 12/ strain1
C	AM 1/ strain1	AM 2/ strain1										AM 12/ strain1
D	Sterile water/ strain1	Sterile water/ strain1										Sterile water/ strain1
E	Sterile water/ strain2	Sterile water/ strain2										Sterile water/ strain2
F	AM 1/ strain2	AM 2/ strain2										AM 12/ strain2
G	AM 1/ strain2	AM 2/ strain2										AM 12/ strain2
H	AM 1/ strain2	AM 2/ strain2										AM 12/ strain2

Figure 2.

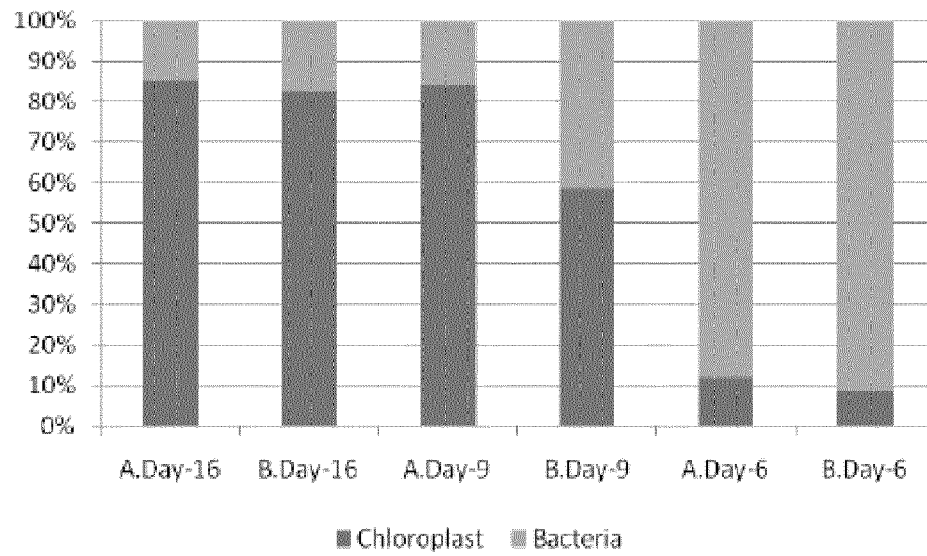


Figure 3.

Isolate	DCS	Putative ID	Nisaplin	Rosemary extract	Green Tea extract 150	Mustard oil (5% AITC)	Micro GARD 300	Micro GARD 430	Micro GARD CS 1-50	Micro GARD 730	Micro GARD 740	Nova GARD CB 1	Nova GARD NR 100	Nova GARD LM 100	BioVia YM
CKS2-B2-A05	DCS 1781	<i>Leuconstoc citreum</i> group	99.6	10.8	15.6		87.9	99.6	99.6	98.8	99.6	93.7	99.6	99.7	-3.7
CKS2-B2-E09	DCS 1783	<i>Leuconstoc citreum</i> group	99.6	15.2	3.1		52.7	99.6	99.5	91.3	99.6	77.1	76.1	98.3	3.1
CKS2-B2-G05	DCS 1784	<i>Leuconstoc citreum</i> group	99.7	15.1	1.9		80.3	99.7	99.6	98.6	99.6	92.8	99.6	99.8	9.3
CKS2-B2-H09	DCS 1785	<i>Leuconstoc citreum</i> group	97.3	28.6	5.0		49.0	99.5	99.4	96.1	99.5	68.2	97.1	99.6	-1.7
CKS2-B2-D06	DCS 1788	<i>Leuconstoc citreum</i> group	99.6	-3.9	-12.4		47.5	99.6	99.5	90.3	99.6	71.8	95.7	99.3	-7.2
CKS2-B2-H06	DCS 1789	<i>Leuconstoc citreum</i> group	99.1	2.3	-14.1		34.5	99.3	97.9	75.5	98.3	49.0	61.2	89.9	-7.8
CKS2-B2-F06	DCS 1790	<i>Leuconstoc citreum</i> group	99.6	-5.2	-16.2		99.6	99.6	99.5	99.5	99.5	99.5	99.6	99.7	-16.4
CKS2-B2-E01	DCS 1787	<i>Lactobacillus oligofermentan</i>	99.6	10.5	0.3		99.6	99.6	99.6	99.6	99.5	99.6	99.6	99.1	-14.8
CKS2-B2-E02	DCS 1786	<i>Pediococcus pentosaceus</i>	98.3	6.1	30.0		98.6	98.3	98.1	98.1	98.1	98.5	98.7	99.0	15.8
CKS2-B3-B10	DCS 1782	<i>Bacillus subtilis</i> group	99.8	84.4	68.0	-52.0	-73.6	99.9	77.3	99.8	99.8	99.8	99.7		79.0

Figure 4A.

Isolate	DCS	Putative ID	Nata max SF	Rosemary extract	Green Tea extract	Mustard essential oil	Micro GARD 200	Micro GARD 400	Micro GARD 430	Micro GARD 730	Micro GARD 740	Nova GARD CB 1	Nova GARD 285	Bio Via YM 10
CKS 2- B1- A12	DCS	<i>Candida zelandoides</i>	90.4	-0.8	25	61.5	59.1	33.8	20	32.7	45.8	52.2	99.5	98
CKS 2- B1- D06	DCS	<i>Candida zelandoides</i>	91.2	-32.5	-5.2	46	52.6	32.2	13.4	43.6	40.3	44.6	99.3	77.4
CKS 2- B1- F06	DCS	<i>Candida zelandoides</i>	84.2	-14.5	22.7	49.2	68	55.1	53.5	55.3	61.7	64.1	99.5	78.1
CKS 2- B1- G10	DCS	<i>Candida zelandoides</i>	63.3	51.4	-4.3	46.6	53.5	31.8	14	37.7	44.7	41.5	100	74.5
CKS 2- B1- B03	DCS	<i>Candida zelandoides</i>	63.8	22.1	16.1	59.2	61.2	44.2	21.9	48.5	53.7	53.9	99.7	71.8
CKS 2- B1- F08	DCS	<i>Candida zelandoides</i>	92.9	-10.9	4.3	59.8	54.7	30.1	4.4	31.9	44.9	50.2	99.4	77.8
CKS 2- B1- G07	DCS	<i>Candida zelandoides</i>	99.2	-8.6	19	53.7	66.1	50.8	43.7	57.7	60	64.7	99.6	87.3

Figure 4B.

CKS 2- B1- F02	DCS 1809	<i>Torulasp ora delbreukii</i>	99.6	-33.3	-34.8	77	-56.2	-71.9	-92.2	-73.2	-101.3	-35	99.9	77.7
CKS 2- B1- B04	DCS 1808	<i>Torulasp ora delbreukii</i>	99.9	-39.3	-1.9	63.2	27.4	-5.6	-1.3	-12.3	-20.7	-18.7	100	85.2
CKS 2- B1- A03	DC S 18 10	<i>Yarrowia lipolytica</i>	88.5	-7.9	-3.1	26.2	-36.2	20.7	43.1	-37.6	-31.7	-20.3	98.5	36.8

Figure 5

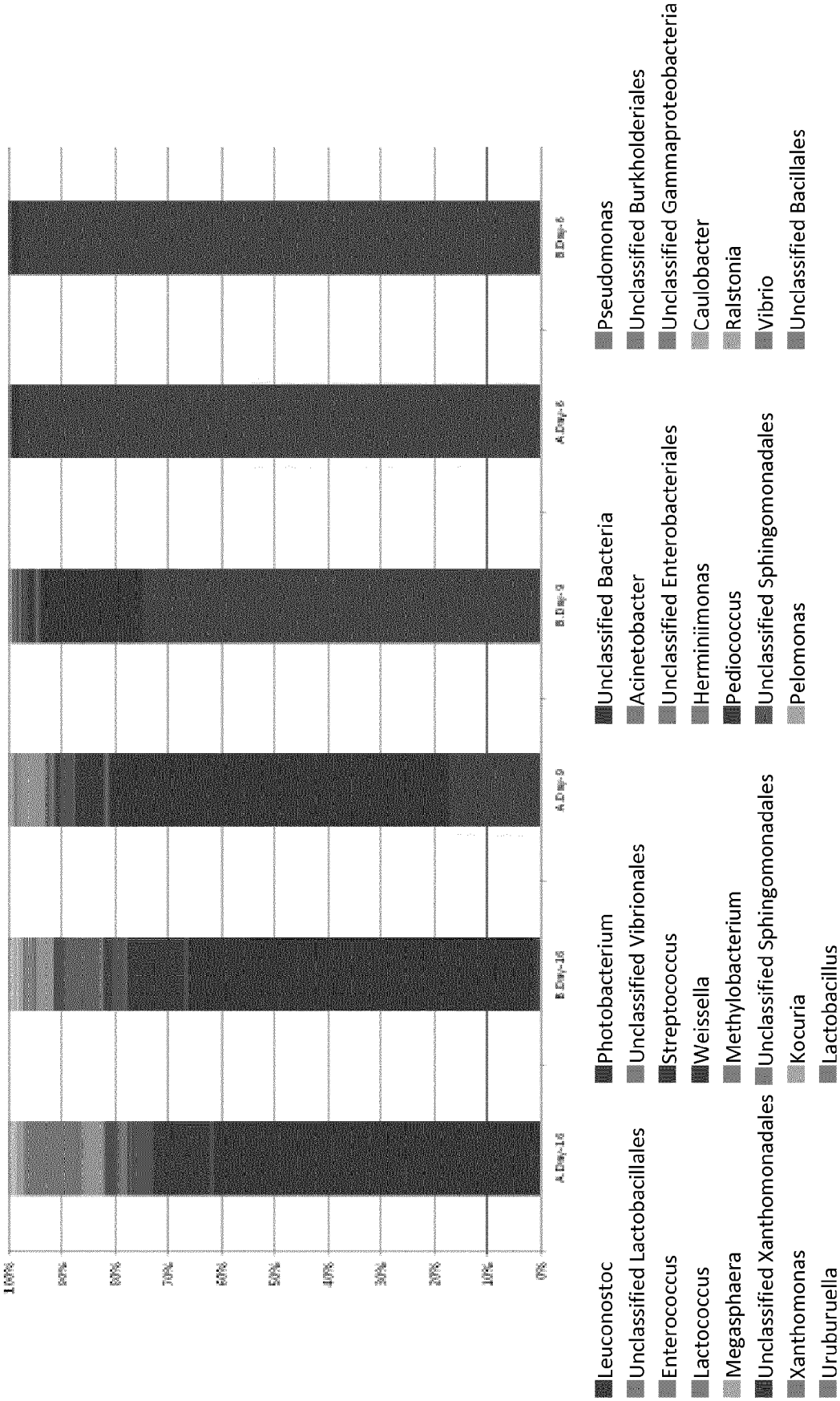


Figure 6

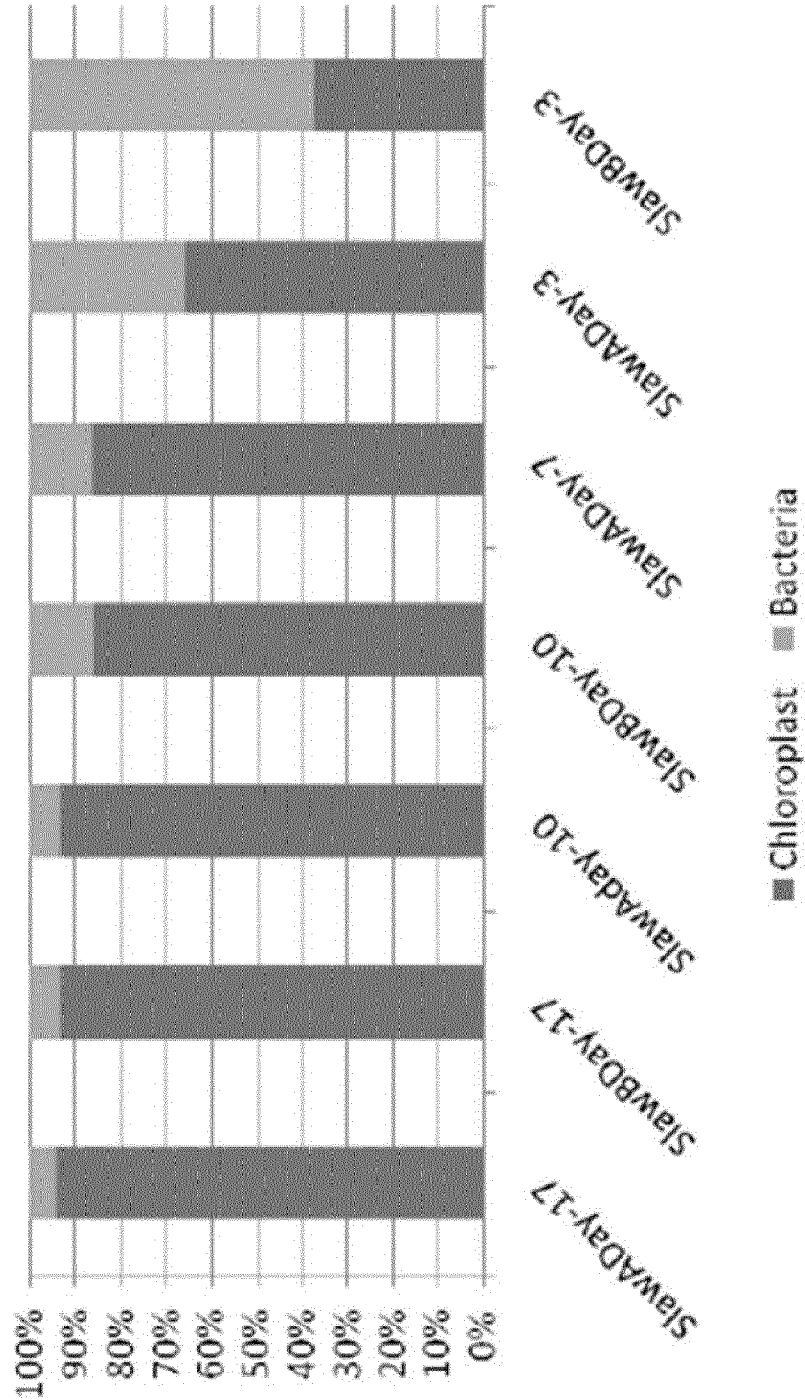
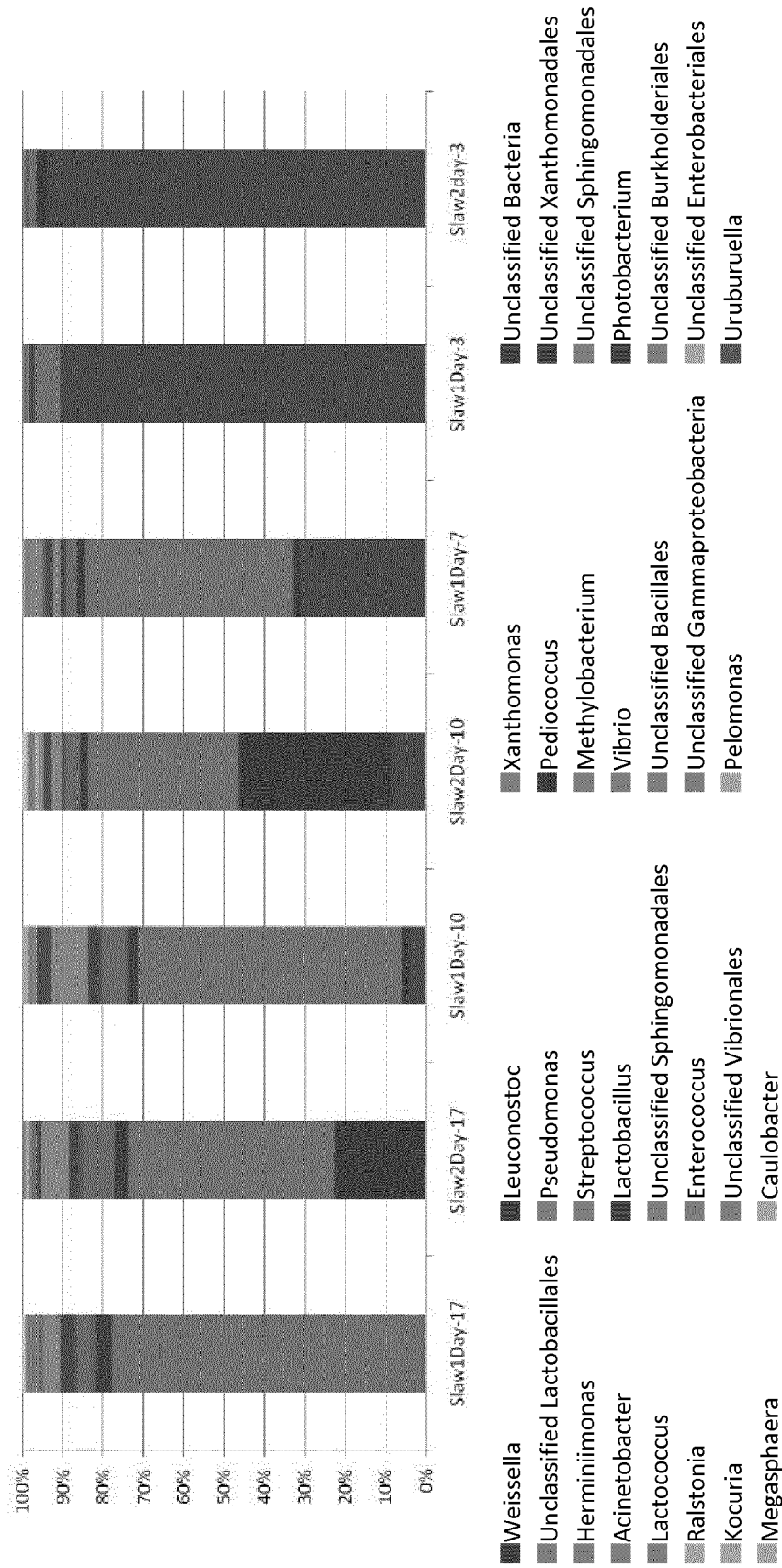


Figure 7

	DCS	Putative ID	Nisaplin	Rosemary Extract 150	Green Tea extract 150	Mustard essential oil 500	Micro GARD 100	Micro GARD 200	Micro GARD 300	Micro GARD 400	Micro GARD 430 CS 1-50	Micro GARD 730	Micro GARD 740	Nova GARD CB 1	Nova GARD NR 100	Nova GARD 285	Nova GARD LM 100	BioVia
CKS2 - B2-C12	DCS	<i>Bacillus subtilis group</i>	99.9	99	26.6	-20.8			23.7		100	71.4	99.9	99.9	99.9			58.8
CKS2 - B2-D04	DCS	<i>Leuconostoc camosum</i>	97.6	78	-32.7				98		97.6	97.3	97.3	97.7	98.4		97.3	-66.8
CKS2 - B2-E03	DCS	<i>Leuconostoc camosum</i>	98.8	59.8	98.9				98.9	Kev	98.8	98.9	98.7	98.8	98.9		99.4	51.9
CKS2 - B2-F08	DCS	<i>Leuconostoc mesen</i>	99.3	52.2	34.6				99.4		99.3	99.3	99.4	99.4	99.5		99.2	51.9
CKS2 - B3-F03	DCS	<i>Pseudomonas decepti</i>		4.8	28.8	19.5	99.7	99.7		99.8	99.7	99.8	99.8	99.8		99.8		100
CKS2 - B3-E03	DCS	<i>Pseudomonas</i>		-23.2	-4.3	53.4	99.9	99.9		99	98.4	100	100	100		100		99.8
CKS2 - B3-G04	DCS	<i>Pseudomonas</i>		54.5	0.5	-19.1	99.8	99.7		83.8	99.8	99.9	99.9	99.7		100		73.9
CKS2 - B2-A11	DCS	<i>Weissella</i>	99.6	0.8	-2.9				99.6		99.6	99.6	99.6	99.6	99.6		99.8	0.4
CKS2 - B2-E12	DCS	<i>Weissella</i>	99.5	-0.9	-18.9				99.5		99.5	99.4	99.5	99.5	99.6		98.7	-15.8

*%inhibition = 100 - ((area under growth curve of treated strain (n=3)/(area under growth curve untreated strain (n=12))*100)

Figure 8



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/067205

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68 C12Q1/04
ADD.

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)
C12Q

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)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	US 2010/129813 A1 (LEVIN ROBERT E [US]) 27 May 2010 (2010-05-27)	1,9,10, 12,13
Y	paragraphs [0008], [0017] - [0020] paragraphs [0052] - [0066]; claims 20-25	2-8,11, 14,15
Y	US 2006/115824 A1 (SAMADPOUR MANSOUR [US]) 1 June 2006 (2006-06-01) the whole document	1-15
Y	WO 2006/134349 A1 (MATFORSK [NO]; GARDNER REBECCA [GB]; RUDI KNUT [NO]) 21 December 2006 (2006-12-21) claims 1-33 the whole document	1-15
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Further documents are listed in the continuation of Box C.



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Date of the actual completion of the international search

25 November 2014

Date of mailing of the international search report

02/12/2014

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Schmitt, Anja

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/067205

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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