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Bacteria bioluminescence, a novel technique in food quality assessment

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Abstract
Rapid detection of microorganisms has becomes more important to many companies in food and beverage areas and contributes to a better control of raw materials as well as finished products. Adopting rapid technologies would allow companies for cost saving and would speed up products release. Despite clear advantages, traditional methods are still frequently used. Products are incubated in a liquid or solid culture media routinely for 2 to 7 days before getting results of contamination. This required lengthy incubation time is chiefly as a result of stressed microorganisms present in complex materials needs several days to grow to visible colonies to be detected. Moreover, In specific applications, this incubation period can be increased up to 14 days. Although simplicity is evident in these techniques, the use of economical materials and their acceptability to the regulatory authorities, the major disadvantage is the amount of time required to produce microbiological results. Thus, need for the increase in demand for quick methods of detection; many alternative technologies have been developed. In the area of rapid detection of microorganisms, bacteria bioluminescence centered on luciferine/luciferase reaction has shown great interest. The chemical reaction can take place either within or outside of the cell. In bacteria, the expression of genes related to bioluminescence is controlled by an operon called the Lux operon. Indeed, this is an excellent marker for viability and cellular contamination. Detection through bacteria luminescence technology is therefore an accepted method to replace traditional method and significantly reduce detection time without losing reliability.

Keywords: assays, bioluminescence, bacteriophage

Introduction
Bacterial Bioluminescence is the production and emission of light by a living organism as the result of chemical reaction during which chemical energy is converted to light energy (Science daily). Recent trends in nutrition and food technology impose increasing demands on food microbiologists to guarantee a safe food supply (Griffiths, *et al.,* 1993). The last 25 years have been dedicated to the development of new methods for Quality Assurance, Total Quality Management and the Hazard Analysis and Critical Control Point (HACCP) systems (Ugarova *et al.,* 1993). The HACCP system was planned as a procedure to guarantee the safety and quality of food products. It was developed by the U.S. Food and Drug Administration (FDA) for regulating and inspecting food plants (Bauman *et al.,* 1974). The basic premise is to identify possible hazards on time and set up a system of procedures, inspections, and records to minimize the possibilities of the hazards causing unsafe or of-quality end products. The points at which hazards might been countered and controlled in the course of preparation and packing of the product are indicated as Critical Control Points (CCP). The general categories of hazards include: toxic products produced by Microorganisms; Chemicals such as toxins, heavy metals and pesticide residues, and Foreign Matter (Curtis & Huskey 1974) [10], Although the most effective methods of monitoring CCP are often physical or chemical, many of the hazards associated with the food production are of microbiological nature. Microorganisms of concern to food producers can be divided into two groups: (i) the pathogens and (ii) those that can be used as indicator organisms. It is clear that any direct test for microorganisms must be rapid enough to be compatible with HACCP and it is imperative, therefore, to quit the traditional use of media and plates (Kodikara *et al.,* 1991) [24]. Of the emerging technological methods for rapid microbiological analysis, bioluminescence is purported to provide result at a short time frame. Bioluminescence involves different areas which are used in the food industry, they include: ATP bioluminescence and bacterial bioluminescence (fung *et al.,* 2002) [14]

Bioluminescent Adenosine Triphosphate (ATP) Assay
Intracellular ATP which is needed for the regulation of the stored metabolic energy, for maintaining the enzyme systems, and for biosynthesis of cellular constituents during all phases of growth is found in living cells. Storage at suboptimal conditions may reduce the intracellular ATP levels. In dead cells, autolysis helps breakdown ATP within a few minutes. ATP can thus be used as a measure of microbial biomass. Indeed, linear relationships have

been found between intracellular ATP levels and total number of colony-forming units (CFU) with bacteria as well as with yeasts. A very rapid and sensitive ATP assay, based on the firefly (*Photuris pyralis*) ATP luminescent reaction, was formed as an alternative to the traditional plate count techniques in routine microbiological analysis of food and beverages. Firefly luciferase catalyses the ATP-dependent oxidative decarboxylation of luciferin (LH2) re-sulting in the production of light as shown in the reaction (where P denotes the product oxyluciferin and hv denotes the light produced):

(LH2) + ATP + 02 Mg2+ P + AMP + PPi + CO2 + hv

Three factors led to a greater utilization of bioluminescent methods for analysis in the 1980s (Leach and Webster, 1986) [25], i.e. (1) the availability of commercially prepared reagents, (2) the commercial manufacture of suitable measuring instruments, and (3) the holding of conferences and the publishing of reports and monographs that highlight the advantages of bioluminescent techniques. Commercially available manual or automated luminometers can detect less than 0.1 pg (or 10-13 g) of ATP per cuvette, corresponding to approximately 100 bacterial cells. Quantization of the steady-state ATP levels in a variety of microorganisms revealed ATP ranges from 0.1 to 4.0 fg/CFU (average ca. 1 fg or 10-15 g/CFU) in bacteria and from 10 to 100 fg/CFU in yeasts (Leach & Webster 1986, Girotti *et al.*, 1997; Cross 1992) [25, 19, 9].

Disturbing Factors

Most food products contain no microbial sources of "intrinsic" or "somatic" ATP which must be eliminated by sample pretreatment procedures. Somatic cells have to be lysed and incubated with the enzyme apyrase or somase (ATPase) to destroy the released ATP. After this treatment, the microorganisms are chemically disrupted and the released microbial ATP is determined using purified luciferin-luciferase reagent. Physically separating microorganisms from somatic cell by differential filtration is another possibility for avoiding ATP interference, combinations of exchange resins, centrifugation and filtration, or enzymatic destruction of somatic ATP followed by differential filtration procedures (Cross *et al.*, 1992) [9]. Extinguishing light which is emitted is another factor that can adversely affect microbial ATP determination. Certain compounds from the food samples can strongly reduce the amount of light measured photometrically. Internal standards must then be incorporated in the dilutions to be tested, as has been shown with skimmed milk. Some food samples may also contain inhibitory substances influencing the luciferase activity (Leach & Webster, 1986) [25].

Application of the ATP Assay in the Food Industry

Hygiene Monitoring

Probably the most widely used current application of ATP bioluminescence in the food industry is that for the estimation of surface cleanliness. Swabbing and assayed rapidly (i.e within 5 min) can be used to extract the total ATP present on a surface with no less accuracy than that obtained using traditional techniques. The result shows the overall contamination of the surface because ATP from all microbial sources will be found. The amount of contamination determined by ATP and plate count methods correlated well in about 80o of samples (Poulis *et al.*, 1993) [31]. A number of bioluminescent-based hygiene monitoring kits are commercially available (Lumac Hygiene Monitoring Kit, Lumac Water Microbial Kit) and are used routinely to monitor critical points many rood processing operations worldwide (Anonym, 1996) [1].

Milk and Milk Products

Raw milk quality from the point of view of somatic cell count and microbial count can be monitored by ATP bioluminescent method. An indication of the somatic cell concentration in milk can be obtained from the concentration of ATP in milk following the treatment with non-ionic detergent (Webster *et al.*, 1988) [45]. This can be adopted as an index for mastitis infection. Recent ATP bioluminescent procedures may be used to discover as few as 104 CFU/ml of bacteria in milk in5 to10 min. This ensures the technique is useful as a rejection test for the incoming milk tankers at milk processing plants (Griffiths & Phillips 1989; Moore *et al.*, 2001) [28].

These tests are available commercially (Anonym, 1996) [1] and marketed by Biotrace (Bridgen, Wales) and Lumac (Laandgraaf, the Netherlands - Raw Milk Microbial Kit). ATP bioluminescent methods can adopted for pasteurized milk quality and sterility testing of UHT and other dairy products. Because ATP is an integral part of the metabolism of bacterial cells, the concentration of ATP may provide a better method of the activity of lactic acid bacteria for monitoring starter culture activity then the measurement of pH changes. A strong correlation between the acid production and ATP concentration exists for a number of lactic acid bacteria, including *Lactococcus lactis* and *Lactobacillus acidophilus,* during their growth in milk. The monitoring of the change in ATP during the growth in milk rapidly indicated the presence of antibiotic residues or phage (Gritffiths, 1993).

Concentrations as low as 0.005 U/ml of penicillin can be detected in about 90 min, An interesting application of luminescence is emerging that will have implications for the dairy industry in the future, namely the development of bioluminescent assays for detecting a variety of enzymes and other substances of importance in this industry (Griffiths,1993) [20].

Meat and Meat Products

Microbial load of meat estimated by an ATP bioluminescence assay is also impeded by interfering somatic ATP. The ATP background levels in meat Samples, for example, may be equivalent to 105 to 108 CFU/g (based on an ATP content of 1 fg/CFU). A successful application of the ATP assay, therefore, relies on a proper pretreatment procedure (Basol & Gogus, 1996) [4]. In this case, the lower detection limit was 5 x 104 CFU/g. and good correlations between ATP content and colony counts were found. Good correlations also exist between ATP content and colony counts in vacuum-packed cooked cured meat products. Many cooked meat products appear to contain relatively low contents of somatic ATP. The removal of this somatic ATP is therefore not necessary, and as consequence, the assay time can be reduced to less than 5 min. The detection limit is 105-106 CFU/g, which suffices for screening these vacuum-packed cooked cured meat products (Bautista *et al.*, 1994, 1995, Siragusa and Cutter, 1995; Russel, 1995) [35, 33]. A commercial kit also exists for checking the microbial quality of raw meat in 30 min-the Lumac Meat Microbial Kit (Anonym *et al.*, 1996) [1].

Carbonated Beverages and Fruit Juices

A lot of carbonated beverages are readily filterable and contain low or undetectable levels of somatic ATP. Simple filtration procedures are sufficient for detecting low levels of yeasts (5 CFU/m) by ATP assay. Fruit juices, however, has high level of somatic ATP and many methods exist for the pretreatment. It has been concluded that the bioluminescent ATP assay offers opportunities for quality control of fruit juice: detection limits are 103 CFU/mi for yeasts and 105 CFU/ml for bacteria (Ugarova *et al.*, 1993). A commercial kit is available from Lumac which checks fruit juice sterility. After a short preincubation period, the presence of microorganisms is tested using a bioluminescent technique. The Lumac Beer Microbial Kit is specifically designed for the rapid detection of microbial contamination or its absence in finished beers (Odebrecht *et al.*, 2000) [30]. The results are gotten within 20 hours which is faster in comparison to conventional techniques (Anonym *et al.*, 1996) [1].

Bacterial Bioluminescence

Bioluminescent bacteria (bacteria who possess the ability of emitting light) are classified into four major genera: *vibrio, Photobacterium, Alteromonas* and *Xenorhabdus*. The bioluminescent reaction, catalyzed by the enzyme luciferase, involves the oxidation of a long-chain aldehyde and reduced riboflavin phosphate (FMNH2) and results it the emission of blue green light (Baker *et al.*, 1992) [3]

FMNH2 + 02 + RCOH Iuciferase FMN+ RCOOH+ H20 + light (490 nm)

The primary source of energy for the light is supplied by the conversion of the respective aldehyde to the corresponding fatty acid. Long-chain aldenydes are essential for the luminescence reaction and the aldehyde tetradecanal appears to be the natural substrate for the luminescence reaction. The reaction is highly specific for FMNH2 which is formed by the reaction:

NADP) H + FMN FMN oxidoreductase FMNH2

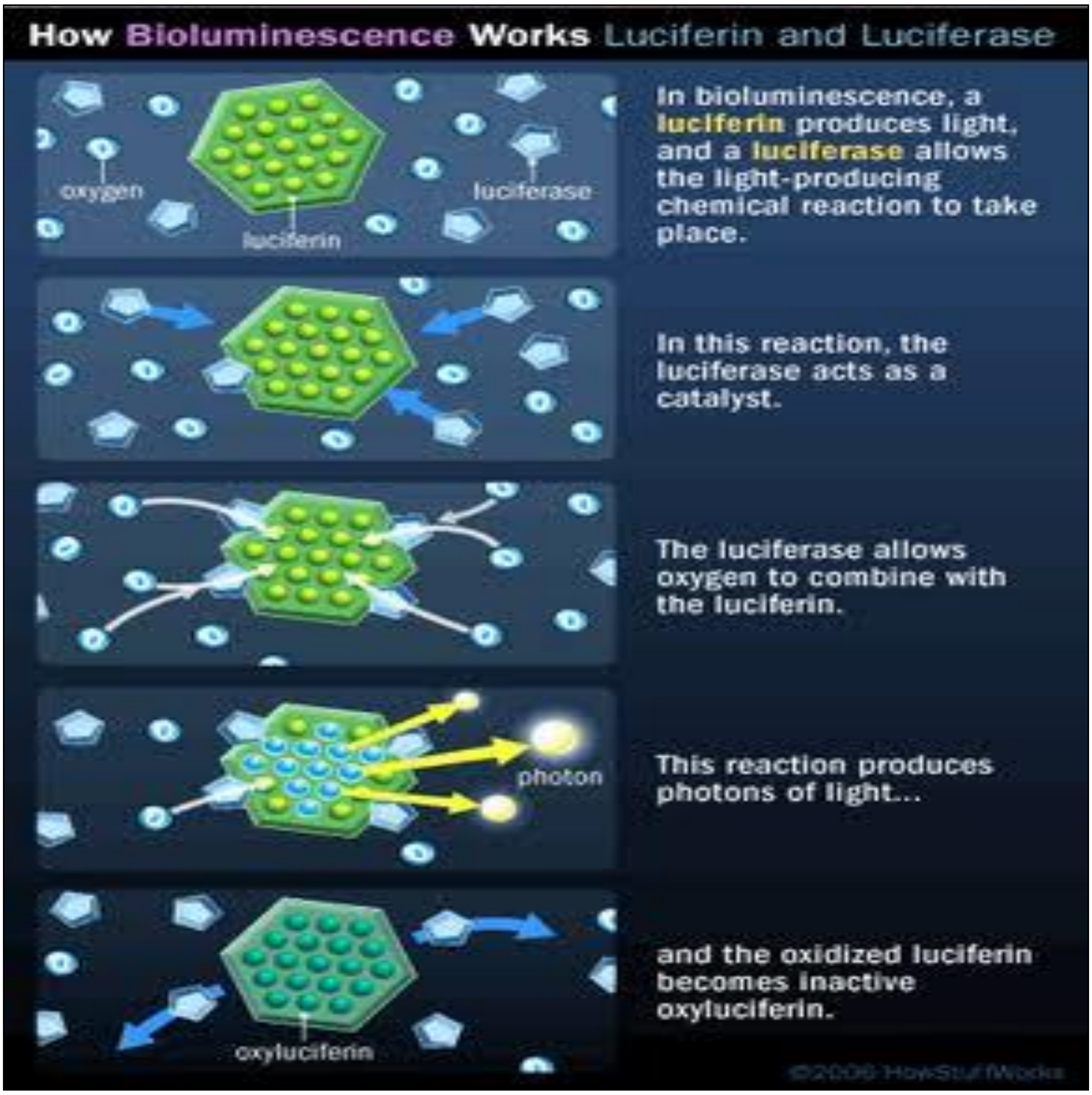
Thus, the luciferase reaction may be driven by coupling it to any system that produces FMNH2 (Girotti *et al.*, 1990, 1993a; Coulet and Blum, 1992, Girotti *et al.*, 1993b; Saul *et al.*, 1996; Micková *et al.*, 2004; Nivens *et al.*, 2004) [17, 18, 8, 34, 27] In bacteria, Lux operon is responsible for controlling of the expression of genes linked to bioluminescence.

In general, bioluminescence is a reaction that produces light with a combination of two types of reaction. One is a luciferin, or a light-producing substance. The other is a luciferase, or an enzyme that catalyzes the reaction. In some cases, the luciferin is a protein known as a photo protein, and the process of light emission needs a charged ion to activate the reaction. Neurological, mechanical, chemical or as-yet-undiscovered triggers can start the reactions that produce light.

Often, the process requires the presence of other substances, like oxygen or adenosine triphosphate (ATP). ATP is a molecule that stores and transports energy in most living organisms, including the human body. The luciferin-lucilerase reaction can also create by products like oxyluciferin and water.

Applications of Lux Gene Technology in Food Microbiology

In a review of the potentials of *in vivo* bioluminescence in microbiology, (Stewart *et al.*, 1990) [37] states that three components are necessary for novel developments in gene technology. The first component is the potential of a high level of the light output from individual cells. The second one is the rapidly expanding ability to transfer the biochemistry of the light production by cloning lux gene vectors into normally dark microorganisms. The third component involves the use of instruments, originally designed for *in vitro* ATP assays, which permit the detection of light from only a few hundred bacteria per ml. All of these are important in implementing bacterial bioluminescent assays into the realm of microbiological testing. Whereas a number of ATP luminescent techniques are presently employed in various aspects of food microbiology, the application of bacterial bioluminescence is still at the research or prototype levels. Many experiments have been and are currently made, to determine the feasibility and usefulness of the assays proposed. Some of the applications for the use in the food industry include the detection of specific bacterial pathogens and indicator microorganisms, on-line monitoring of hygiene quality, determining the effectiveness of spore destruction, monitoring starter culture integrity, biocide and Virucide challenges, and studying the recovery of sub lethally injured cells (Baker *et al.*, 1992; Hill *et al.*, 1993, Meighen *et al.*, 1993) [3, 22, 26].



How bioluminescence works, Luciferase, Luciferin

Fig 1

Detection of Specific Bacterial Pathogens and Indicator Organisms

At present, in food laboratories the determination of ATP by firefly luciferase is used to detect and determine cells, and to assess the shelf life and microbial quality of many kinds of food. The ATP assay is a rapid technique but, for the time being it lacks specificity for the identification of bacteria. This specificity can be achieved with lux genes from luminescent bacteria. Lux genes can be introduced into bacteriophages, which will then be absorbed by specific bacteria thus transferring the light-emitting genes to those bacteria (Kodikara *et al.*, 1991) [24]. By knowing what type of bacteria one wishes to detect, it is only the matter of obtaining the host specific phages for that particular organism and performing genetic manipulations. The organisms are mixed with the phages and the light emission can be quantitatively measured. The research has shown that the bioluminescent assay is rapid (usually less than 1 h), sensitive (luminometers detect as few as 500 bacteria), simple, specific, and demonstrates a good correlation between cell numbers and bioluminescence (STEWART *et al.*, 1990, Stewart and Williams, 1992; Rees *et al.*, 1995).

Dark terrestrial organisms that need to be monitored in food microbiology (pathogens, starter cultures, hygiene indicators) lack the ability to produce luciferase or fatty acid reductase, but they can supply FMNH2. Therefore, all that is needed is the transfer of luciferase genes and, in some cases, fatty acid reductase. Phage P22 is a narrow host range phage infecting only *Salmonella typhimurium*. After infection with this phage, as few as 100 cells of *S. typhimurium* could be detected in 50 min by monitoring light emission. Lux containing bacteriophages should be able to target other pathogens, example *Campylobacter spp*. and *Listeria monocytogenes*. 1here may still be a need 1or the recovery and enrichment procedures prior to phage detection, because of zero tolerance (regulatory requirements) for *L. monocytogenes*. However, due to the sensitivity (100 per ml) and specificity of the host and the phage, the enrichment time can be short providing simple same day testing for pathogenic bacteria. Indicators of poor sanitary conditions in food industries are enteric bacteria. Lux genes have been inserted into phages that infect a broad range of enteric bacteria providing a reagent for an on-line hygiene test with a detection limit of 1000 enteric per g or per cm2. After a 4-h enrichment of the sample, viable enteric counts of 10 per g or per cm can be distinguished from the background (Kodikara *et al.*, 1991; Baker *et al.*, 1992, Waddell and Poppe 2000) [24, 3, 44].

Spore Forming Organisms and Bioluminescence

Bacterial luciferase can function in gram-positive organisms, although originates from gram-negative ones. Bacillus spp. are capable of producing heat stable dormant endospores (Cook *et al.* 1993) [7]. The spores obtained from phenotyplcally bioluminescent vegetative cells are dark. When the spores geminate, the onset of electron transport and the initiation of metabolism are the early events. With the lux containing spores, germination is accompanied by the surfacing of bioluminescence. This provides a sensitive, real-time monitoring of the germination and growth processes. The spores that have been killed or injured, and are thus unable to germinate, produce no light (Baker *et al.*, 1992; Hill *et al.*, 1993) [3, 22].

Lactic Acid Bacteria and Starter Cultures

Many assays based on luminescent bacteria are used in the dairy industry. The presence of bacteriophages or antibiotics can cause starter culture failure in cheese or yoghurt manufacture. Bioluminescent lactic-acid streptococci are suitable for use as an indicator of the presence of lytic phages or antibiotics. This light-emitting component of starter cultures can be monitored for a loss of luminescence that indicates the presence of an inhibitory substance. Using a luminescent derivative of *Lactobacillus casei*, the technique allowed the detection of *Penicillin G*. to levels as low as 0.03 Hg/ml (0.05 units/ml) in 30 min, and bacteriophages at concentrations as little as 105/ml in 100 min (Ulitzur *et al.*, 1986a; Baker *et al.*, 1992; Grifiths *et al.*, 1993) [42, 3].

Advantages and Disadvantages of the Luminescent ATP Assay and Bacterial Luminescent System in Food Microbiology

The ATP bioluminescent assay is very quick but not very specific. The bacterial luminescent assays are rapid-fast enough for near on-line assays that requires immediate action, such as the detection of antimicrobials in milk. The speed detection may also, in some cases, reduce the costs. The method is simple enough to use so that plant workers could perform the test. It is sensitive very low numbers of cells can be detected with luminometers and the continued research may lower those numbers even further. In most cases it is advantageous the invivo bioluminescence assay is noninvasive. No cell disruption is required to measure light emission. It has been revealed to be an accurate method exhibiting good correlation with the cell counts- for both increasing and decreasing numbers.

When using narrow range phages to introduce the lux genes, assay's can be specific to detect a particular organism. A final advantage is that the technology or bacterial bioluminescence leads to a great deal of innovations in many areas. As with any new technique, it also has disadvantages. There could be a problem with phage or plasmid host ranges being either too specific or too wide, resulting in false negatives or false positives, respectively. Another disadvantage to the food microbiology industry is that the bacterial bioluminescent assays are still at the experimental and prototype stages, in contrast to the ATP bioluminescent assay. There are also general disadvantages with the luminescent methods. The principal disadvantage is the extinguishing of emitted light hat can severely affect the measurements. Namely some compounds from the biological samples can greatly reduce the amount of the light measured photometrically. On the other hand, there are some luminescent no microbial substances in the biological samples that can increase the intensity of the measured light.

Recent Advances in Bioluminescence Technology

Disposable Bioluminescence-Based Biosensor for Detection of Bacterial Count in Food.

A biosensor for rapid detection of bacterial count based on adenosine 5'- triphosphate (ATP) bioluminescence has been developed. The biosensor 1s composed of a key sensitive element and a photomultiplier tube used as a detector element. The disposable sensitive element is made up of a sampler, a cartridge where intracellular ATP is chemically extracted from bacteria, and a microtube where the extracted ATP reacts with the luciferin-luciferase reagent to produce bioluminescence. The bioluminescence signal is transformed into relevant electrical signal by the detector and further measured with a homemade luminometer. Parameters affecting the amount of the extracted ATP, including the types of ATP extractants, the concentrations of ATP extractant, and the relevant neutralizing reagent, were optimized. Under the optimal experimental conditions, the biosensor exhibited a linear response to standard bacteria in a concentration range from 10(3) to 10(8) colony-forming units (CFU) per milliliter with a correlation coefficient of 0.925 (n-22) within 5 min. Moreover, the bacterial count of real food samples obtained by the biosensor correlated well with those by the conventional plate count method. The proposed biosensor, with characteristics of low cost, easy operation, and fast response, provides potential application to rapid evaluation of bacterial contamination in the food industry, environment monitoring, and other fields.

Conclusion

Numerous of research in the last 30 years has been directed towards the development of the bioluminescent assays. The resurgence of interest in ATP (firefly) bioluminescence and the dramatic improvements in the reagent quality and instrument sensitivity has led to the availability of applications of direct relevance to many branches of the food industry, and many kits are now commercially available, The many advantages of assays using bacterial luciferase should increase their use, especially in food microbiology. These assays will not eliminate the classical microbiological methods, but they will enhance the importance of microbial assays in areas such as the HACCP system where rapid results are necessary. The recent advances made in genetic engineering of microorganisms aimed at making them bioluminescent, coupled with a need for rapid microbiological assays in the food industry, will make *in vivo* bioluminescence a common practice in the future. Bioluminescence, together with low level light detection systems such as photon counting and CCD imaging, will provide the food microbiologist with a very powerful tool for studying food as an ecosystem, and will offer a rare opportunity to study microbial behavior and interactions in food

Conflict of Interest

The authors declared that there is no conflict of interest regarding the publication of this manuscript.

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