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Construção de cepas de *Salmonella* bioluminescentes para utilização em microbiologia de alimentos

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Lista de abreviaturas e siglas

BARDOT - Bacteria rapid detection using optical scattering technology

BGA - Brilliant green agar

CCD - Charged couple device

FMN- Flavin mononucleotide

$FMNH_2$ - Reduced flavin mononucleotide

GFP - Green fluorescent protein

HSL - Homoserine lactone

Kan - Kanamicin

LB - Luria Bertani

MSM - Minimal Salt Medium

Na - Nalidixic acid

O_2 - Oxygen

OD - Optical density

PCR - Polymerase Chain Reaction

XLD - Xylose Lysine Deoxycholate

Sumário

RESUMO

MENDONÇA, KARLA SEQUEIRA. **Construção de cepas de *Salmonella* bioluminescentes para utilização em microbiologia de alimentos.** Tese (Doutorado) - Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Universidade Federal de Pelotas, 2013.

Os avanços na engenharia genética permitem que muitas espécies de bactérias que são normalmente não luminescentes sejam desenvolvidas, de modo que se tornem organismos bioluminescentes. Atualmente, micro-organismos como *Salmonella* spp. estão sendo geneticamente construídos a fim de produzir esse sinal mensurável. Desse modo, a utilização do cassete *luxCDABE* completo permite que a bioluminescência possa ser expressa de forma contínua, sem necessidade da adição de substratos exógenos. Assim, as bactérias biorrepórteres bioluminescentes permanecem inteiramente auto-suficientes em sua capacidade de produzir luz visível. Além disso, uma importante vantagem da bioluminescência é que as medições utilizando bactérias biorrepórteres não são invasivas e destrutivas para a célula, e ainda fornecem resultados em tempo real, o que permite a oportunidade de empregar a emissão de luz com muito sucesso em distintas aplicações em microbiologia de alimentos, como a avaliação da eficácia antimicrobiana para a inativação de agentes patogênicos. Portanto, o objetivo deste estudo foi construir cepas biorrepórteres bioluminescentes de *Salmonella* e utilizá-las para avaliar a resistência desse patógeno microbiano frente a compostos antimicrobianos naturais, bem como investigar a expressão da proteína Lux e de outras proteínas em diferentes cepas de bactérias recombinantes utilizando um sensor que capta imagens de dispersão.

Palavras-chave: alimentos. bactérias recombinantes. bioluminescência. biorrepórteres. carvacrol.

ABSTRACT

MENDONÇA, KARLA SEQUEIRA. **Construction of bioluminescent *Salmonella* strains for use in food microbiology.** Tese (Doutorado) - Programa de Pós-Graduação em Ciência e Tecnologia de Alimentos, Universidade Federal de Pelotas, 2013.

Advances in genetic engineering allow different bacterial species that are normally non-luminescent can be developed to become bioluminescent organisms. Nowadays, living microorganisms such as *Salmonella* spp. are genetically engineered to produce this measurable signal. Many studies have been using the gene *lux* to modify micro-organisms in other to produce visible light. Besides, the use of the complete *luxCDABE* cassette allows the bioluminescence can be expressed continuously, without addition of exogenous substrates. Thus, bioluminescent bioreporter bacteria remain entirely self-sufficient in its ability to produce visible light. Moreover, an important advantage is that the bioluminescence measurements using bioreporter bacteria are not invasive and destructive to the cell, and also provide real-time results, which allows the opportunity to employ light emission with great success in several applications in Food Microbiology, as evaluating the antimicrobial efficacy to inactivate pathogens. Therefore, the objective of this study was to construct bioreporter bioluminescent strains of *Salmonella* and use them to evaluate the microbial resitant against natural antimicrobial compounds, as well as to investigate Lux protein expression and other proteins in different strains of recombinant bacteria using a light-scattering sensor.

Keywords: bioluminescence. bioreporter. carvacrol. food. recombinant bacteria

1 INTRODUÇÃO

A bioluminescência bacteriana é um processo de emissão de luz que ocorre em muitas diferentes bactérias, durante o qual uma luz visível é emitida, como consequência de uma reação enzimática catalisada por uma enzima chamada luciferase (MEIGHEN, 1991). Embora as luciferases tenham sido identificadas principalmente em organismos marinhos, sabe-se que os micro-organismos bioluminescentes estão amplamente distribuídos na natureza (WILSON; HASTINGS, 1998). Sendo assim, o fenótipo bioluminescente foi encontrado principalmente em três gêneros bacterianos de diferentes ecossistemas, *Photobacterium* e *Vibrio*, do ambiente marinho e *Photorhabdus*, do ambiente terrestre (DOSTÁLEK; BRÁNYIK, 2005).

As enzimas envolvidas em todos os sistemas bacterianos bioluminescentes são basicamente codificadas em um único operon composto por cinco genes comuns *luxA*, *luxB*, *luxC*, *luxD*, *luxE* (MEIGHEN, 1994). Assim, para produzir bioluminescência, a enzima bacteriana luciferase, codificada pelos genes *luxA* e *luxB*, catalisa a oxidação da flavina mononucleótido reduzida ($FMNH_2$) e de uma cadeia longa de aldeídos graxos na presença de oxigênio molecular, com a produção simultânea de fótons, visíveis como uma luz azul-verde clara (MEIGHEN, 1993).

No entanto, em circunstâncias normais, não existem genes que codificam a luciferase no cromossoma da grande maioria das bactérias de importância em doenças transmitidas por alimentos (MEIGHEN, 1988; JIANG et al, 2006). Deste modo, com o intuito de assegurar que as bactérias não luminescentes possam adquirir o fenótipo bioluminescente, é necessário transformá-las com os genes *luxAB* ou *luxCDABE* (NUNES-HALLDORSON; DURAN, 2003). Contudo, se apenas os genes *luxAB* forem transferidos para dentro da célula bacteriana, é necessário adicionar uma longa cadeia de aldeídos graxos ao meio para produzir luz. Deste modo, utilizando-se o operon *lux* completo, as células bacterianas podem emitir luz continuamente, sem a necessidade da adição de substratos exógenos. Consequentemente, as bactérias bioluminescentes permanecem completamente auto-suficiente na sua capacidade de produzir luz visível em resposta a diferentes condições ambientais, o que facilita a compreensão dos processos de crescimento e inibição celular (RIPP et al., 2001).

No entanto, a intensidade da luz bioluminescente por célula depende do crescimento celular das bactérias bioluminescentes (MEIGHEN, 1994). Durante a fase de crescimento inicial, o cultivo celular encontra-se em baixa densidade e os genes *lux* não são expressos. Portanto, a luminescência de uma cultura de células não irá aumentar devido à limitação de substratos para a reação de bioluminescência. Contudo, durante a fase de crescimento logarítmica, a emissão de luz aumenta drasticamente devido à ativação da expressão dos genes no operon *luxCDABE*. Além disso, a taxa de indução de luciferase também depende da espécie e da composição de nutrientes dos diferentes meios de cultura (CHATTERJEE; MEIGHEN, 1995; SCHEERER et al, 2006).

Assim, o sistema *lux* bacteriano pode servir como uma excelente e eficaz ferramenta, uma vez que representa uma técnica muito interessante baseada na detecção de luz visível (GREER; SZALAY, 2002). Além disso, bactérias recombinantes bioluminescentes podem ser utilizadas como um método seguro, rápido, sensível e eficaz para uma ampla variedade de aplicações, uma vez que a maior parte dos ensaios de bioluminescência exige apenas alguns segundos (MEIGHEN, 1993; BILLARD; DUBOW, 1998; RIPP et al. , 2003; WAIDMANN et al, 2011).

Ao todo, os dados gerados neste trabalho foram divididos em três seções (capítulos), os quais foram formatados como artigos publicáveis. Na primeira seção é apresentada uma revisão sobre a aplicação de bactérias bioluminescentes como biossensores em microbiologia de alimentos. Na segunda seção, o artigo descreve a construção de cepas de *Salmonella* Enteritidis bioluminescentes e sua utilização para a avaliação da eficácia de ação de um composto antimicrobiano natural. Na última seção, apresenta-se um trabalho onde foi investigada a expressão da proteína *lux* e de outras proteínas heterólogas em diferentes cepas de *Salmonella* Enteritidis e *Escherichia coli* recombinantes, usando um sistema que capta imagens de dispersão.

2 Capítulo 1 – ARTIGO 1

A ser submetido para o periódico Food Control

Bioluminescent bacteria: *lux* genes and applications in food microbiology

Mendonça, K. S.; Mendonça, M.; Silva, W. P.; Applegate, B. M.

2.1 ABSTRACT

Bioluminescence is a process of visible light emission which occurs in living organisms as a consequence of an enzymatic reaction catalyzed by luciferases. Luciferases enzymes, as well as the corresponding *lux* genes involved in the bioluminescent *lux* systems, have been identified and isolated mainly in marine organisms and are used for several biological purposes including camouflage, repulsion, attraction, communication and illumination. Over the past decades, advances in molecular biology have allowed the characterization and manipulation of the luciferase genes, to construct bioluminescent bioreporters using some of the currently well known luciferases, by cloning and expression of the *lux* genes into bacteria that are naturally non-bioluminescent, transforming them in bioluminescent bacteria. These recent advancements have allowed the use of *lux* operon in the development of genetically engineered pathogenic bacteria including *Salmonella* spp., *L. monocytogenes* and *E. coli* O157:H7, that encode the enzyme luciferase, responsible for the photon emission as a product of its reactions. All of these bacterial microorganisms are etiological agents of foodborne diseases, which has been of great concern to the food industry. With the present review, we intend to provide an overview of bioluminescence and the utilization of bioluminescent bacteria as biosensors, which include their methodologies and applications in food microbiology, as the evaluation of antimicrobial efficacy to inactivate pathogens.

Keywords: bioluminescence, biorepoter, biosensor, food, luciferase

2.2 Introduction

The emission of light by different microorganism is of special interest in food microbiology since the advances in genetic engineering have conducted to the development of ‘‘living biosensors’’ (BILLARD; DUBOW, 1998). This specific phenomenon, known as bioluminescence, is a process of visible light emission that occurs in particular organisms as a consequence of an enzymatic reaction catalyzed by luciferases (WILSON; HASTINGS, 1998). Therefore, a large diversity of bacterial species that are naturally non-bioluminescent has been constructed in order to generate bioluminescent microorganisms by insertion of *lux* genes (LOESSNER et al., 1997; BAUTISTA et al., 1998; LOIMARANTA et al., 1998; SIRAGUSA et al., 1999; LO et al., 2006; JIANG et al., 2006; XU et al., 2010).

Since rapid detection of pathogenic and deteriorating microorganisms become a main requirement of food industries, bioluminescence-based methods has been shown as an important technique (NUTT et al., 2004; LEWIS et al, 2006; KARSI et al., 2008). The use of bacterial luciferase bioreporter allows the employment of light emission easily and successfully in a broad range of applications. Besides, using the entire bacterial *luxCDABE* operon, measurements can be performed even without adding an exogenous substrate (CHATTERJEE; MEIGHEN, 1995). In addition, bioluminescent bioreporter assays are rapid, simple, specific, sensitive, and demonstrates a trustworthy correlation between cells numbers and bioluminescence (BURLAGE; KUO, 1994; XU et al., 2010).

Therefore, the present review provides an overview of bioluminescent bacteria and rapid bioluminescent detection techniques applied in food industry. Also, it gives advantages and disadvantages of the utilization of bioluminescence bacteria, as well as the application of these bioluminescent bioreporters and the general bioluminescent mechanism are briefly described.

2.3 Bioluminescent organisms

Luminescent organisms comprise a diverse group of species, which are widely distributed and can be found in terrestrial, freshwater and marine ecosystems. Several species of annelids, cephalopods, cnidarians, crustaceans, dinoflagellates, echinoderms, insects, fungi, protozoa and bacteria have the ability to produce light

(MEIGHEN, 1991; WILSON; HASTINGS, 1998). Currently, over 700 species of bioluminescent organisms are known, with more than 80% having been isolated from marine environments (MEIGHEN, 1988; WIDDER, 2010).

Within this broad group of organisms, the bioluminescent bacteria are the most abundant and widespread luminescent microorganisms found in nature (MEIGHEN, 1993). These bioluminescent bacteria have as main habitat the marine environment, in which can be found in symbiotic, saprophytic and parasitic relationship, as well as in a free-living style (MEIGHEN, 1994; FORST et al., 1997; WOLLENBERG; RUBY, 2009). However, the bioluminescent phenotype was found predominantly in three genera of bacteria from different ecosystems, *Photobacterium* and *Vibrio*, from marine environment and *Photorhabdus*, from a terrestrial environment. Thus, enzymes from five different species have been previously isolated and often used in numerous research applications, including *Vibrio fischeri*, *Vibrio harveyi*, *Photobacterium phosphoreum*, *Photobacterium leiognathi* and *Photorhabdus luminescens* (MEIGHEN, 1993; NIVENS et al., 2004; DOSTÁLEK; BRÁNYIK, 2005; WAIDMANN et al., 2011). However, three major bacterial luciferase from *P. luminescens, V. fischeri* and *V. harveyi*, have been frequently used in several studies (Table 1).

Table 1. Main characteristics of luciferases from different microorganisms.

Luciferase	Organism (family)	Substrate	Cofactor	Composition (mass)	Localization (native)	Emission (nm)
P. luminescens	Bacteria (Enterobacteriaceae)	Long-chain aliphatic aldehyde	O_2; $FMNH_2$	Heterodimer (77kDa)	Cytoplasm	490
V. fischeri	Bacteria (Vibrionaceae)	Long-chain aliphatic aldehyde	O_2; $FMNH_2$	Heterodimer (77kDa)	Cytoplasm	490
V. harveyi	Bacteria (Vibrionaceae)	Long-chain aliphatic aldehyde	O_2; $FMNH_2$	Heterodimer (77kDa)	Cytoplasm	490

The luciferase of all bacterial bioluminescent systems is a heterodimeric protein composed of two subunits, α (encoded by *luxA*) and β (encoded by *luxB*) (MEIGHEN; DUNLAP, 1993). Light production also requires the expression of three other genes, *luxC*, *luxD* and *luxE*, and all five genes are organized in a single operon

(*luxCDABE)* (MEIGHEN, 1994). Nevertheless, it is noteworthy that even being found in different environments, the arrangement and sequence of the *lux* genes contained in *P. luminescens* is very similar to that found in *Vibrio* spp. and *Photobacterium* spp., suggesting that these genes have a common evolutionary origin (SZITTNER; MEIGHEN, 1990; FORST et al., 1997).

2.3.1 *Vibrio fischeri* and *Vibrio harveyi*

V. fischeri is found in marine environments and it is a symbiont in fish and squids, being responsible for light generation in those organisms, which use it as a defense mechanism to avoid predators (DUNLAP et al., 2008). The bioluminescent mechanism is a product of the *lux* operon, an operon that is used extensively in molecular biology as a marker. The establishment of this symbiotic relationship is quite complex and involves communication between the bacterium and the host before the colonization by bacterium (WAIDMANN et al., 2011).

V. harveyi is a free-living microorganism commonly found in tropical marine environments. This bacterium is known to form associations with eukaryotes, either as commensals in the intestinal flora of marine animals, as opportunistic pathogens or as primary pathogens of many commercially cultured invertebrate species (WILSON; HASTINGS, 1998; FARRIS et al., 2008).

These two bacteria were the first microorganisms for which the quorum sensing was described. In this mechanism, the bacterial cell to cell communications via secreted signaling molecules are used to synchronize community behavior by regulating gene expression (BRIGHT et al, 2004). The quorum sensing is known to regulate a number of genes in *Vibrio* spp. including those of the *lux* operon, whose products are responsible for the luminescent characteristic of these species (CAO et al., 2012).

Both the microorganisms, *V. fischeri* and *V. harveyi* are naturally luminescent Gram-negative bacteria, in which expression of the *luxCDABE* genes are responsible for their bioluminescent phenotype. The bacterial luciferase of these microorganisms produces light in the presence of combinations of substrates (long-chain aliphatic aldehyde) and cofactors (O_2 and $FMNH_2$), with a maximum intensity at about 490 nm (MEIGHEN, 1993).

Bioluminescence is a metabolic process very costly to the cell, corresponding for 20% (*V. fischeri*) and 12% (*V. harveyi*) of the total energy requirement of these microorganisms. However strains of the same species may differ in the level of luminescence. Moreover, it is known that the *luxAB* genes isolated from *V. fischeri* and *V. harveyi* show thermal stability at 30°C and 37°C, respectively (MEIGHEN, 1991). Thus, for the construction and establishment of bioluminescent bioreporter with an optimum temperature above 30°C, the use of *V. fischeri* luciferase it is not recommended because of lack of thermo stability (STEWART; WILLIAM, 1992; MEIGHEN, 1994).

2.3.2 *Photorhabdus luminescens*

Photorhabdus luminescens is an unusual organism, once it is symbiotic in an insect (nematode *Heterorhabditis megidis*), and pathogenic in another (insect larvae *Galleria mellonella*), the only organism that is known to exhibit this dual phenotype. This bacterium lives in the gut of an entomopathogenic nematode that attacks the insect larvae of another species. In addition, *P. luminescens*, exhibits an autoinduction pattern but appears not to involve an autoinducer, and is also capable of produce light, since this *lux* system has been used extensively as a molecular marker in many experiments. Unfortunately, the exact biological function of this bioluminescent microorganism is not known, although it is believed to have a role in protection against the other species (DUCHAUD et al., 2003).

The *luxCDABE* operon originated from the Gram-negative bacterium *P. luminescens* has been frequently used for the construction of bioreporters. An important advantage of using the *lux* operon from this microorganism as a reporter system is due to the fact that the LuxAB proteins of this species is the most thermo stable of all known bacterial luciferases, and produces a visible peak wavelength of light emission at 490 nm (CHATTERJEE; MEIGHEN, 1995; VOISEY; MARINCS, 1998).

P. luminescens lux proteins show thermal stability at 42°C and as a result, this recombinant bacterium has been proposed as a more applicable alternative for the conventional *V. fischeri* in different applications (MEIGHEN, 1991; MARQUES et al., 2005; FOUCAULT et al., 2010). However, one factor that may limit the use of the *P. luminescens luxCDABE* gene cassette in genetic manipulations is the presence of

multiple internal restriction enzyme sites, once in this *lux* operon, there are restriction sites for the enzymes *Hind*III, *Sph*I and *Xba*I, which are common in the multiple cloning site (MCS) of many plasmid vectors. Also, *P. luminescens lux* operon is A + T rich (> 69%), which can possibly have an effect when inserted into bacterial genomes with high G + C content, and that is why the *lux* genes may not be expressed efficiently in this type of microorganism (VOISEY et al., 1998; CRANEY et al., 2007).

2.4 The *lux* genes and *lux* operon

The *lux* system consists of five genes (*lux A*, *B*, *C*, *D*, *E*) contained in a single operon and required for visible light production. In all know bacterial luminescence systems, the *luxAB* genes are flanked by the *luxCDE* genes, with transcription in the order *luxCDABE* (ENGEBRECHT; SILVERMAN, 1984; MEIGHEN, 1991; FORST et al., 1997). The genetic organization of *lux* operons from *P. luminescens*, *V. fischeri* and *V. harveyi* are show in Figure 1.

The bacterial luciferase is an enzyme consisting of two non-identical subunits, α (~40 kDa) and β (~35 kDa), whose molecular weight can vary for each subunit according to the species, encoded by genes *lux*A and *lux*B respectively, which are both essential for bioluminescence (MEIGHEN; DUNLAP, 1993; NUNES-HALLDORSON; DURAN, 2003). The luciferase enzyme catalyzes the oxidation of reduced flavin mononucleotides ($FMNH_2$) and long-chain fatty aldehydes in the presence of molecular oxygen to FMN and the corresponding long-chain fatty acid, with the simultaneous production of photons, visible as blue-green light at 490 nm (MEIGHEN, 1991; O'KANE; PRASHER, 1992; MEIGHEN, 1994). Interestingly, these two subunits that compose the heterodimeric luciferase enzyme, α (40 kDa) and β (35 kDa), contain approximately 32% of genetic identity, suggesting that the β-subunit can be the result of gene duplication (MEIGHEN, 1993; WILSON; HASTINGS, 1998; WAIDMANN et al., 2011). In addition, the *lux* system also contains the *luxCDE* genes, which encode the multienzyme complex that consists of three different proteins: a reductase (encoded by *luxC*), a transferase (encoded by *luxD*) and a synthetase (encoded by *luxE*), which convert and recycle the fatty acids in aldehyde substrate for the light-emission reaction (MEIGHEN, 1991; MEIGHEN, 1993).

In addition, other *lux* genes (*luxG*, *luxH*, *luxI* and *luxR*) have been identified in specific luminescent strains. Following *luxE*, marine luminous bacteria, such as *V. fischeri* and *V. harveyi*, have an additional gene, *luxG* encoding a flavin reductase that provides the $FMNH_2$ substrate for the bacterial luminescence reaction (MEIGHEN, 1991; NIJVIPAKUL et al., 2008). However, in *V. harveyi* another gene was identified as part of the *lux* operon, *luxH* and its gene product might be involved in riboflavin biosynthesis, thus providing FMN (WAIDMANN et al., 2011). In *V. fisheri*, two regulatory genes *luxI* and *luxR* are located upstream of the *luxCDABE*. The *luxI* gene is located immediately in front of *luxC* and is part of the same operon (MEIGHEN, 1994; WAIDMANN et al., 2011). Furthermore, the *luxI* is required for the synthesis of an autoinducer that controls expression of luminescence system, while the *luxR* gene product act as a receptor for the autoinducer and activates the expression of the *lux* operon (BOSE et al., 2011).

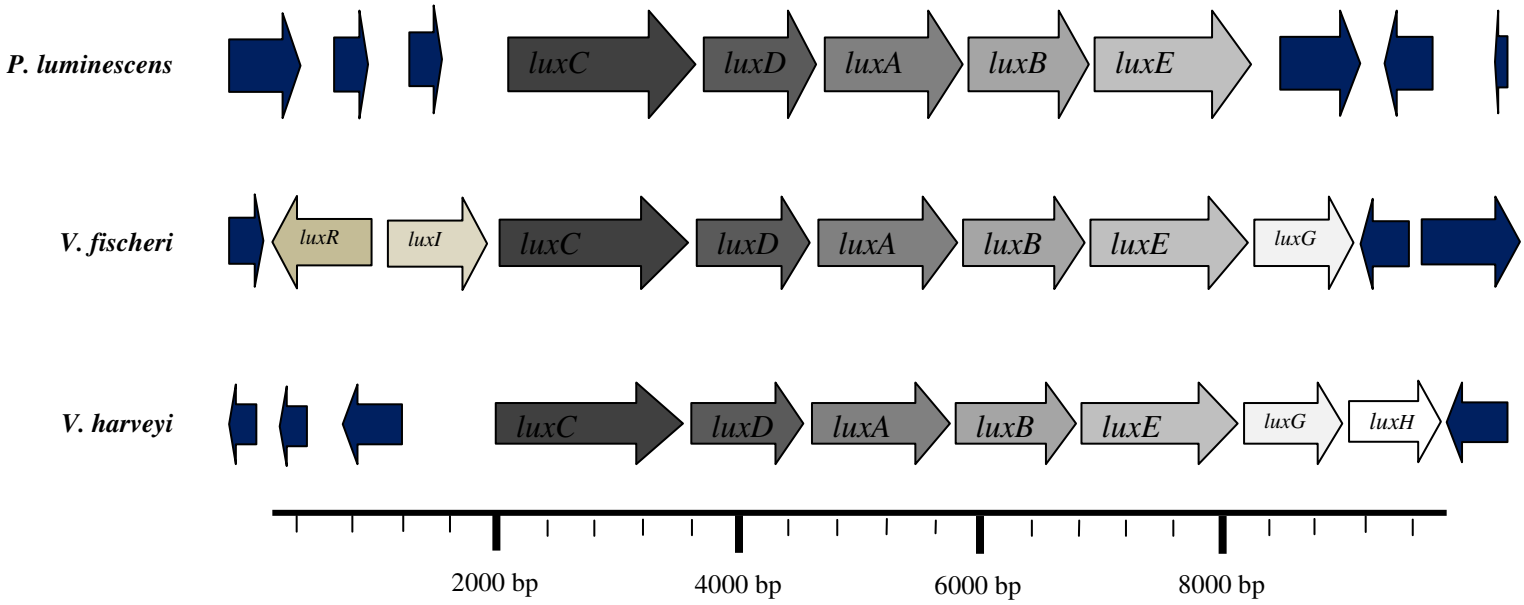

Figure 1. Genetic organization of *lux* operons of bioluminescent bacteria from *P. luminescens*, *V. fischeri* and *V. harveyi*. Genes of the core *luxCDABE* operon and accessory *lux* genes are shown in different shades of grey.

However, it is important to emphasize that with the use of the complete *luxCDABE* cassette enables that the bioluminescence can be expressed continuously, with no necessity for exogenous addition of chemicals or co-factors.

Thus, the bioluminescent bioreporter remains entirely self-sufficient in its capacity to produce visible light (CHATTERJEE; MEIGHEN, 1995).

2.5 Bioluminescent mechanism

Bioluminescence is a process of light emission that occurs among molecules formed in physiological conditions, producing visible light which occurs in living organisms, mediated through an enzymatic reaction (WILSON; HASTINGS, 1998). The enzymes responsible for catalyzing the reaction of bioluminescence are called luciferases and molecules of substrates are referred to as luciferins (GREER; SZALAY, 2002). The light emission by microorganisms is a process that requires large amounts of energy and the genetic system necessary for the expression of luminescence is very complicated (MEIGHEN, 1993).

Light is generated when genes *luxA* and *luxB* encode the enzyme luciferase, which binds to $FMNH_2$, O_2, aliphatic aldehyde and then converts these substrates to FMN, water, and an aliphatic acid, with the simultaneous production of light (Figure 2). The three other genes *luxC*, *luxD* and *luxE* are needed to encode the enzymes that convert and recycle metabolites in high-energy substrates, which regenerate aldehyde and $FMNH_2$ substrates (BILLARD; DUBOW, 1998). The bioluminescent reaction is as follows:

$$FMNH_2 + O_2 + R\text{-}CHO \xrightarrow[(\sim 490\ nm)]{\text{Luciferase}} FMN + H_2O + R\text{-}COOH + Light$$

Figure 2. Schematic plot of the light generation of enzyme luciferase

Bioluminescence is a very intensive energy demand for the bacterial cell. Some researchers estimate that the light emission process represents an expense of around 6 ATP molecules for each photon (NUNES-HALLDORSON; DURAN, 2003). Actually, this is very interesting and explains why energy is conserved in bioluminescent organisms, therefore only being expressed when physiologically required (GREER; SZALAY, 2002; NIVENS et al., 2004).

Furthermore, bioluminescence requires oxygen as an electron acceptor and under conditions in which $FMNH_2$ is continuously supplied, such as in the bacterial

cell with the complete *luxCDABE* operon, luminescence stays at a constant level (STEWART; WILLIAM, 1992; MEIGHEN, 1993). Although the processes of light emission and oxygen-dependence are common characteristics of all the bioluminescent organisms, luciferase systems have developed independently and the light reactions are relatively different for each microorganism (HASTINGS, 1983; MEIGHEN, 1991). In addition, bioluminescence reactions in many organisms require different substrates and cofactors, as well as the light emission occurs at different wavelengths (O'KANE AND PRASHER, 1992; WILSON; HASTINGS, 1998). However, as previously shown in Table 1, for the expression of luciferase from the *lux* system encoded by the three major bacteria used in the recombination processes (*P. luminescens*, *V. fischeri* and *V. harveyi*), substrates and cofactors, as well as the wavelengths for light emission, are the same. Therefore, the mechanisms of bioluminescence reaction in different bacteria have been investigated and the detail of the general biochemical reaction is shown in Figure 3. Since luciferase utilizes oxygen as a substrate, anaerobic cultures are not luminescent. However, as with all bioluminescence measurements, samples removed from anaerobic bottles, need to be stirred for about 10 seconds in order to saturate the luciferase with oxygen, prior to measuring luminescence (SEPTER et al., 2010).

Although the production of luminescence occurs frequently among different microorganisms, currently this reaction is not well understood. There are many explanations for the variable expression of *luxCDABE* (luminescence) among bacterial strains from distinct environments. Among some of the mechanisms for variability investigated are the expressions of the different *Lux* proteins, regulatory sequences associated with the *lux* promoters, other physiological elements of the strains, or even a combination of these different factors (BOSE et al., 2011).

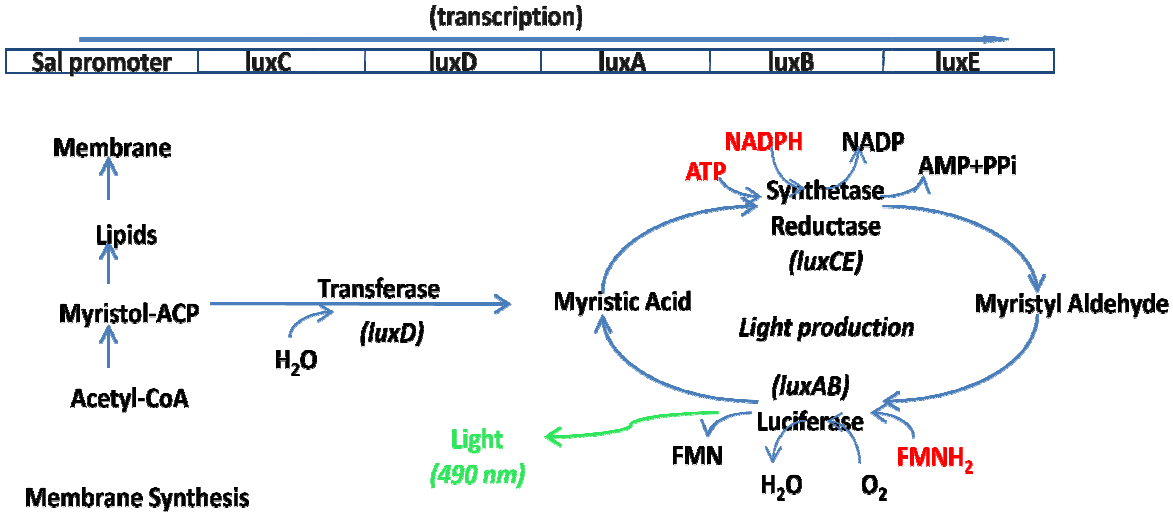

Figure 3. Bioluminescence biochemical pathway encoded by the *luxCDABE* operon (adapted from APPLEGATE et al., 1998)

2.6 Regulation of bioluminescence

Bioluminescent bacteria have the capability to limit the emission of light when at low cell densities, in order to prevent the use of energy or specific metabolites. Thus, under normal circumstances, single bacterial cells never express high levels of bioluminescence (MEIGHEN, 1988). Also, bioluminescence is always dependent on the levels of luciferase and substrate, therefore it is well known that many bacteria do not emit or emit low levels of light when diluted in culture medium (MEIGHEN, 1993). In culture, during the initial growth phase at a low cell density, the bacteria will grow but the *lux* genes will not be expressed or will be poorly expressed. Consequently, bioluminescence will dramatically decrease, probably due to low availability of substrates initially available for the reaction (MEIGHEN, 1993; WILSON; HASTINGS 1998). Thereafter, during mid to late logarithmic growth phase, light emission will increase significantly, depending on the bacterial species and the nutrient composition of the culture medium, reaching a peak in stationary phase (BILLARD; DUBOW, 1998).

Definitely, bioluminescence regulation in the most part of bacteria is cell density-dependent and the development of light emission with cellular growth has been reported as autoinduction (MEIGHEN, 1993). The autoinduction, also known as quorum-sensing, is a mechanism for communication between cells, which allow the

genes of the *lux* system to be expressed synchronically, contributing to the increase of luminescence with the increase of bacterial cell density (NUNES-HALLDORSON; DURAN, 2003; BRACKMAN et al., 2009). The main element of the autoinduction process is an autoregulator, referred to as *lux* autoinducer. The autoinducers implicated in this regulation have been identified as homoserine lactone (HSL), which are initially synthesized during the early growth phase and are then excreted into the culture medium (BILLARD; DUBOW, 1998). At low cell density, the autoinducer slowly accumulates in the medium until it reaches an ideal concentration. The high levels of bioluminescence are only observed in the final stages of cell growth (stationary phase), which reflects the dependence relationship between cell density and expression of the *lux* system (MEIGHEN, 1988; WILLIAM; STEWART, 1992; MEIGHEN, 1993; CAO et al., 2012).

The bacterial cell communication through autoinducers, leads the bacteria to interact as a group and have been demonstrated to regulate various processes which include conjugation, pathogenesis, symbiosis, biofilm formation and bioluminescence (WILSON; HASTINGS, 1998; SIMPSON et al., 2001). However, the characteristic of cell density and autoinducer dependence in different bioluminescent bacteria usually may not be reconstituted and light levels in a recombinant bacteria are still below the levels of light emission in the native strain (MEIGHEN, 1994; BOSE et al., 2011). Beyond autoinduction, numerous other mechanisms may contribute to regulate light production in bioluminescent bacteria, which includes oxygen, iron and salt concentrations, as well as the Sigma-32 protein under some stress conditions (BILLARD; DUBOW, 1998).

2.7 Advantages and disadvantages of bioluminescence

Bioluminescence represents an extremely efficient bioreporter system, through the light emission is characterized as a signal that can be measured with high sensitivity and rapidly, without any disturbance to the cell (MEIGHEN, 1988; WILSON; HASTINGS, 1998). Since rapid detection of microorganisms become a major requirement of food industries, bioluminescence-based methods has been identified as the most promising technique for the detection of bacteria, especially for applications in food processing, where the risks of contamination must be constantly

monitored (SIRAGUSA et al., 1999; NUTT et al., 2004; LEWIS et al., 2006; KARSI et al., 2008; MOZA et al., 2009).

The acknowledgment of the distinct advantages and disadvantages of bioluminescence has increased the number and diversity of applications in many different fields, enabling bacterial luciferase reporters to become valuable tools in modern molecular biology (WAIDMANN et al., 2011). Many current bioluminescent strains, which were constructed to express the *lux* genes, emit enough light to be seen with the naked eye in a dark room. However, different methods have been developed to allow light emission to be measured quantitatively (BILLARD; DUBOW, 1998; MASSEY et al., 2011).

In order to detect bacteria, bioluminescence-based assays offer numerous advantages compared to traditional methods (BILLARD; DUBOW, 1998; KARSI et al., 2008; WAIDMANN et al., 2011; ANDREU et al., 2012). This technique allows the emitted signal (luminescence) to be measured with great sensitivity as well as very quickly (RODA et al., 2004). Therefore, compared with traditional methods of detecting bacteria that require several days, a minimum time is required for the synthesis of luciferase and light emission, hence the majority of bioluminescent assays require only a few seconds (MEIGHEN, 1993; LOIMARANTA et al., 1998; FARRIS et al., 2008; HOWE et al., 2010). Moreover, another important advantage of bioluminescent detection methods is the ability to detect those bacteria that are in “viable but non-cultivable state” (DUNCAN et al., 1994; BILLARD; DUBOW, 1998).

However, the technique can also present some problems, since the bioluminescence reaction is strictly dependent on oxygen and cellular energy reserves (STEWART; WILLIAM, 1992; WILSON; HASTINGS, 1998; THORN et al., 2007). Bioluminescent strains have few limitations, which include firstly the dependence of the concentration of substrate available. This means that the requirement for $FMNH_2$ in the bacterial luminescence system is one of the major disadvantages of bioluminescence-based reporter systems (NIVENS et al., 2004; Brock et al., 2012). In addition, depending on the physiological state of bacteria, the luciferase enzyme stability and the presence of stimulating or inhibiting factors in the sample, also represent significant disadvantages of the bioluminescence system (NUNES-HALLDORSON; DURAN, 2003). Nevertheless, it is important to keep in mind that the loss of bioluminescence can be rapidly monitored compared with the time required to enumerate the number of viable cells (MEIGHEN, 1993; BILLARD;

DUBOW, 1998). Thus, with the availability of tools necessary to perform the analyses, bioluminescent bioreporter are faster and less laborious and expensive when compared with traditional tests.

2.8 Bioluminescent bioreporter bacteria

Bioluminescent bioreporters are living microorganisms that have been genetically engineered to produce a measurable signal from the detection of changes that occur in its environment (HAY et al., 2000; HARMS et al., 2006). Traditionally, the genetic construct consists of an inducible promoter fused to a reporter gene. These elements work together to detect environmental changes and control the expression of genes or operons in order to improve the physiological status of the cell (ABD-EL-HALEEM et al., 2002; NIVENS et al., 2004). Reporters genes may be native, although they are usually acquired by genetic construction. Commonly used reporter genes include: *lacZ*, *gfp*, *luc*, *luxAB* or *luxCDABE* (BILLARD; DUBOW, 1998; HAY et al., 2000). The *luxCDABE* reporter gene cassette has been used extensively in bioreporter constructs, because such a reporter produces an easily measured signal (bioluminescence) that does not require the addition of an exogenous substrate for signal production (RIPP et al., 2001; NIVENS et al., 2004; ANDREU et al., 2012).

Bacteria have become excellent candidates for bioreporters, since it is known that they can be found in almost all types of environments. Furthermore, microorganisms are easy to manipulate genetically and have a diverse group of gene regulation (SIMPSON et al., 2001). However, for the successful development of a bioluminescent reporter strain some important conditions should be carefully considered. First, a microorganism should be selected that can easily persist in the environment to be studied. Secondly, the selected microorganism must have the appropriate genetic regulatory elements to be utilized and be amenable to receive the reporter construct through recombinant DNA manipulations. Finally, the *lux* genes should be integrated inside the genetic system of the microorganism with no disruption of critical cellular functions (STEWART; WILLIAMS, 1992; SIMPSON et al., 1998). In addition, a bioluminescent reporter system should also express other positive characteristics, such as high *luxCDABE* expression and strong luminescent signal intensity, without affecting the physiology of the cell (BROCK et al., 2012).

Nevertheless, it is important to consider that bioluminescent bioreporters may also have limitations, which primarily include living bioreporters need nutrients to survive and require minutes to produce a detectable signal (NIVENS et al., 2004).

The bioluminescent bioreporters bacteria are cells which respond to specific agents, through its ability of producing visible light. Usually, the *lux* reporter genes are placed under the regulatory control of a promoter previously determined, maintained in native plasmids, broad-host-range vectors (shuttle plasmids) or chromosomally incorporated within the host, which will be induced in the presence of specific chemical and physical substances (HEITZER et al, 1998; RIPP et al, 2000). In these genetic systems, the target analyte or its degradation products act as the inducer of the bioluminescence reaction and the activation of the promoter will result in the transcription of the *lux* genes and consequently light production, the measurable signal (BRIGHT et al, 2004; IVASK et al., 2009). Therefore, the construction of bioluminescent bioreporters strains can be accomplished by insertion of just the *luxAB* complex or the complete *luxCDABE* cassette. Therefore, it is important to remember that if only the *luxAB* genes have been added to bacterial DNA, the cells should be supplemented with an aldehyde substrate, before start to produce a luminescent response. However, using the *luxCDABE* cassette, bioluminescence will be expressed continuously, and as a consequence, the bioluminescent bioreporter strain stays entirely self-sufficient in their capacity to produce visible light in response to different environmental conditions (CHATTERJEE; MEIGHEN. 1995; RIPP et al., 2001).

Bioreporters using the *luxCDABE* cassette characteristically can occur naturally in environmental isolates or be genetically constructed with constitutive promoters expressed continuously. In addition, bioreporters can be constructed with promoter-*lux* fusions for specific stress responses and constructed with gene regulatory systems that are specific for some analytes. It may also be performed multiple constructs with different promoters which can be used in a wide variety of genomes (NIVENS et al., 2004; BROCK et al., 2012).

Considerable importance must be given to the method of delivery of the *lux* reporter genes to the strain. In general, the integration of the reporter in the host cell chromosome has a better conservation than when inserted into a plasmid (BILLARD; DUBOW, 1998). Furthermore, another important characteristic is that the expression of gene *lux* should not affect the normal survival of the host. However, it is important

to note that expressions of the luciferase and *lux* proteins, as well as the process of light emission have a significant onus to the bacterial metabolism. Therefore, the effect of high level expression of *lux* system might result in disturbances of growth characteristics of the microorganism (WAIDMANN et al., 2011).

An additional advantage of bioluminescence is that measurements using bioreporter strains are not invasive and destructive to the cell (STEWART; WILLIAMS, 1992; MARQUES et al., 2005; ANDREU et al., 2012). Moreover, bioreporter assays provide results in real time, a condition which is not a normal characteristic of most conventional tests, in which sampling, processing and development of signs require a considerable time in order to obtain the results (SIMPSON et al., 1998; LO et al., 2006; FOUCAULT et al., 2010).

2.9 Applications of bioluminescent bioreporter bacteria

Bioluminescence has emerged as a powerful tool, since it represents a very interesting technique based on the detection of visible light (GREER; SZALAY, 2002). With the advancements in genetic engineering, a wide diversity of bacterial species that are normally non-bioluminescent has been genetically modified to make them bioluminescent microorganisms. This technology has been applied in microbiology, especially for some foodborne pathogens such as *Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes* (LOESSNER et al., 1997; BAUTISTA et al., 1998; SIRAGUSA et al., 1999; LO et al., 2006).

Bioluminescent based-assays are rapid, simple, specific and sensitive, demonstrating a good correlation between the number of cells and the expression of light (MEIGHEN, 1993; BURLAGE; KUO, 1994). In view of the fact that this is an extremely sensitive technique, a low number of cells are required to detect bioluminescence using only a luminometer device that can detect as few as 500 bacteria per mL (DOSTÁLEK; BRÁNYIK, 2005). Furthermore, it is important to emphasize that these tests are not invasive, so no cell disruption is required to measure the light emission. In addition, the method is cost-effective, which can easily be adjusted to a large number of samples (MEIGHEN, 1988; NIVENS et al., 2004).

The use of recombinant bacteria containing *lux* genes enables the opportunity to employ the light emission very successfully in a broad range of applications in food microbiology (DOSTÁLEK; BRÁNYIK, 2005; FARRIS et al., 2008). Some of these

applications of bacterial *lux* gene for the use in the food industry include detecting specific bacterial pathogens and indicator microorganisms in environmental samples or food matrices, evaluating and monitoring of bacterial gene expression, online monitoring of bacterial cell injury and viability, and in vivo monitoring of bacteria-host interactions (STEWART; WILLIAM, 1992; BILLARD; DUBOW, 1998; NIVENS et al., 2004; WAIDMANN et al., 2011). Obviously, these are just some examples of possible use of the bacterial *lux* genes and a brief summary of these bacterial reporter systems applications is presented in the table 2.

Bioluminescent bioreporters have three major advantages compared to classical microbiological methods which employ GFP, such as short half-life for the luciferase, the wavelength of light emission and the measurement of bioluminescence online during the experiment. Usually, the luciferase undergoes only a single catalytic cycle in most assays, since the level of chemical oxidation of $FMNH_2$ is greater than the level of turnover in the bioluminescent reaction. Consequently, the half-life of luciferase binding to its substrate is only several seconds, thus allowing the monitoring of light emission to be performed in real time (MEIGHEN, 1991; XU et al., 2010). Furthermore, since the wavelength of emitted light from the bioluminescent system is longer and thus the energy is higher, it enables a better tissue penetration while luciferase is expressed in living animals (RICE et al., 2001; DOYLE et al., 2004; FOUCAULT et al., 2010; BROCK et al., 2012). Finally, using the entire bacterial *luxCDABE* operon as a bioluminescent reporter system, the light emitted can be measured as a signal in real time during all the different experiments, even without addition of an exogenous substrate (MARQUES et al., 2005; LEWIS et al., 2006; KARSI et al., 2008). Therefore, these bioreporter are faster and less laborious and expensive compared to tests with traditional promoter test assays.

To evaluate the expression of bacterial genes, several studies have been performed and all three major bacterial luciferases (from *V. fischeri*, *V. harveyi* and *P. luminescens*) have been used as a reporter to measure the activities of bacterial promoters (APPLEGATE; KEHRMEYER; SAYLER, 1998; ABD-EL-HALEEM et al., 2002; NIVENS et al., 2004; LO et al., 2006). *P. luminescens lux* operon system was used to quantify activities of various promoters of *L. monocytogenes* during growth in culture, infection of epithelial cells *in vitro* and *in vivo* (BRON et al., 2006; RIEDEL et al., 2007). Another specific application for bioluminescent bioreporters in the

investigation of bacterial gene expression is to use the reporter as a trap to identify fragments of DNA with promoter activity in clone libraries (BJARNASON et al., 2003).

The monitoring of bacterial contamination in food or environmental samples is possible through the use of phages specific to each microorganism. In order to make this possible, it is necessary to create target-specific phages harboring all genes required for bioluminescence reaction. Whether the target bacterium is present in the sample, the phage will infect it and as a result the bacteria will transcribe the phage-encoded genes leading to bioluminescence (LOESSNER et al., 1997; RIPP et al., 2006). A similar application can be performed through the tests of efficacy, which aim to prevent contamination and growth of microorganisms in food products (LIU et al., 2008).

More recently, bioluminescence imaging is emerging as an effective tool thus becoming an innovative and noninvasive, for real-time monitoring which allows the quantification and monitoring of live bacteria in hosts. The main use of bioluminescent reporter systems is to investigate the interaction between bacteria and host. This is especially true when studied infections of bacterial pathogens in animals are realized by bioluminescence imaging, which demonstrate as an important advantage the fact that the animals used can be visualized several times during the experiment, allowing a significant reduction in the number of animals required for experiments (FOUCAULT et al., 2010; MASSEY et al., 2011; BROCK et al., 2012). Furthermore, it is noteworthy that since the light emitting reaction requires oxygen, it is believed that bioluminescence imaging *in vivo* should be possible in most of all tissues of living animals, and even in the lumen of the gastrointestinal tract, where the level of available oxygen is very low (WILES; DOUGAN; FRANKEL, 2004; RIEDEL et al., 2007. In addition, frequently, it has been verified for most microorganisms investigated by bioluminescence imaging, that luminescence correlates very well with the number of bacteria during growth *in vitro.* Nevertheless, among other organisms a significant decrease in bioluminescence has been observed. This occurs especially during the culture period immediately after entry of microorganism into the stationary phase, possibly due to a change in the availability of cofactor, substrate or even both (RIEDEL et al., 2007).

The implementation of the bioluminescence imaging for online monitoring of bacterial promoters *in vivo* in infected animals may also be performed. However, the control of bacterial promoters during *in vivo* infections is limited and just promoters of

highly expressed genes can be monitored. An important advantage of the bioluminescence imaging *in vivo* is that many of the sites of infection by pathogens may not be detected by conventional techniques (SHEEL et al., 2010; WAIDMANN et al., 2011; ANDREU et al., 2012).

Applications of bioluminescent bacteria as reporter systems can involve the use of luciferase enzymes alone (*luxAB*) or the use of the entire *lux* operon (*luxCDABE*). The *luxAB* genes have been transferred into various bacterial species resulting in a luminous phenotype on addition of an aldehyde substrate to the cells culture. However, using the complete *lux* operon cells can continuously emit light and facilitate the understanding of the processes of cell growth and inhibition, without the requirement for the addition of exogenous aldehyde (VOISEY; MARINCS, 1998; RIPP et al., 2001; MASSEY et al, 2011). The half-life of luciferase binding to its substrate is only a few seconds, so the capture of light reflects the processes that are occurring in real time, which are not artifacts of accumulated signals (MEIGHEN, 1991; XU et al., 2010). Furthermore, it is known that the luciferase activity is dependent on the metabolic integrity of the cell, whereas the number of emitted photons correlates with the biomass of live bacteria. Once the bioluminescence is related to the bacterial burden, this property may be used to measure quantitatively the load of microorganism (HUTCHENS; LUKER, 2007; XU et al., 2010).

Bioluminescent bacteria combined with methods for the detection of emitted light have been considered for the study of food microbiology, since it is an extremely sensitive method with low detection limits along with the need for rapid microbiological assays in the food industry. Moreover, this system will provide to the microbiologist an interesting technique allowing the food to be understood as an ecosystem, thus providing an important opportunity to study microbial behavior, and to investigate the interactions that occur between bacteria and host, as well as the interactions which occur in food products (BILLARD; DUBOW, 1998; SIMPSON et al., 2001; WAIDMANN et al., 2011).

In summary, bioluminescent bioreporter bacteria may cover a wide range of applications as well as being used as an effective method for monitoring cell physiology in real time by measuring cell light production.

Table 2: Applications of bioluminescent bacteria in food products

Purpose	Product	Target	Application	Reference
Pathogen tracer	Milk	*Streptococcus thermophilus*	Detection of dairy-relevant antibiotics in milk using a highly bioluminescent strain	Jacobs et al., 1995
	Salad	*Listeria*	Screening of foods and environmental samples for *Listeria* cells using luciferase reporter bacteriophage A511::luxAB	Loessner et al., 1996
	Contaminated foods (Ricotta Cheese, Chocolate pudding, Cabbage and Minced meat)	*Listeria monocytogenes*	Detection of *L. monocytogenes* in contaminated foods	Loessner et al., 1997
	Yogurt and cheese	*E. coli* O157:H7	Test growth and survival of *E. coli* cells in fermented dairy products under different storage conditions	Hudson et al., 1997
	Lactic acid-treated poultry carcasses	*Salmonella* Hadar	Monitor the effects of acid and temperature treatments on cell survival and viability	Bautista et al., 1998
	Soft cheeses	*L. monocytogenes* *E. coli* O157:H7	Test the survival of *L. monocytogenes* and *E. coli* O157:H7 in Camembert and Feta cheeses	Ramsaran et al., 1998
	Beef carcass surface tissues	*E. coli* O157:H7	Real-time monitoring of *E. coli* O157:H7 adherence to beef carcass surface tissues	Siragusa et al., 1999
	Camembert cheese	*Yersinia enterocolitica*	Direct monitoring of *Y. enterocolitica* cells on cheeses stored at 10°C	Maoz et al., 2002
	Spinach	*E. coli*	Assess the interaction of a bioluminescence-labeled *E. coli* strain with growing spinach plants	Warriner et al., 2003
	Beef	*Salmonella enterica*	Use of bioluminescence to evaluate the effects of rapid cooling on recovery of *Salmonella enterica* serovar Typhimurium DT104 after heat treatment	Lewis et al., 2006
	Hot dogs and Camembert	*Listeria monocytogenes*	Improved Luciferase Tagging System for *Listeria monocytogenes* Allows Real-Time Monitoring In Vivo and In Vitro	Riedel et al., 2007
	Chicken skin	*Salmonella*	Development of bioluminescent *Salmonella* strains for use in food safety	Karsi et al., 2008
	Chicken breast fillets	*Salmonella enteric*	Use of bioluminescent *Salmonella enterica* serovar Enteriditis to determine penetration in tumbled and hand-tumbled marinated chicken breast fillets	Moza et al., 2009
	Chicken skin	*Salmonella enteric*	Development of stable reporter system cloning *luxCDABE* genes into chromosome of *Salmonella enterica* serotypes using Tn7 transposon	Howe et al., 2010

2.10 Conclusion

The development of novel technologies for the effective control of biological contaminants is in constant evolution, and has been directed in the last years particularly for the improvement of bioluminescence assays. Different luciferase

systems were established for use in several studies, and the *lux* operon seems to be very well applicable in order to study various bacteria of importance to the food industry.

Applications of the recombinant bioluminescent bioreporter provide a sensitive approach for bacterial detection, as well as its sensitivity to different antimicrobial compounds. In addition, the use of *lux* genes can allow a real-time measurement of these genes expression, and therefore determine cell viability. However, it is important to remember that the bioluminescent activity is mainly dependent on the levels of luciferase and substrates, and is also dependent on the host in which it is expressed. Besides, since all bacterial luciferases are dependent on molecular oxygen, it is important to remember that any application under anaerobic conditions should be discarded. However studies using bioluminescent images show that these tests are possible, even in low oxygen availability.

It is also important to consider the mode of delivery of the bacterial *lux* system, which is intended to modify genetically. Usually, the chromosomal integration of a single copy of the *luxCDABE* operon should be preferred over plasmid based systems, since by using stable integration, the use of antibiotics during the experiments, which would be required for stable replication of the plasmid, can be excluded in order to avoid any effects on gene regulation.

Therefore, the many advantages presented by bioluminescent bioreporter bacterial will probably increase its use in food microbiology. Nevertheless, these bioluminescent assays will not eliminate the traditional microbiological methods currently used, since it will contribute to reinforce the importance of microbiological assays in areas that require rapid results.

In conclusion, the use of bioluminescent assays enables a series of developments in the area of microorganism detection. Furthermore, the flexibility of bioluminescent methods and its ease of use, combined with a considerable time saving in comparison with traditional methods, make it a powerful tool that can serve as a trigger for subsequent applications in food microbiology.

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3 Capítulo 2 – ARTIGO 2

A ser submetido para o periódico Applied and Environmental Microbiology

Construction of bioluminescent *Salmonella* strains by recombination and its use to evaluate the antimicrobial compound carvacrol

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3.1 ABSTRACT

Bioluminescent bioreporter bacteria are genetically constructed to respond to specific agents, through their ability to produce visible light. The requirement for reduced flavin mononucleotide (FMN) also makes bioluminescent strains excellent for rapid screening of antimicrobials, since inactivated cells are unable to produce bioluminescence. Therefore, the purpose of this study was to construct a bioluminescent bioreporter Salmonella to further investigate the microbial resistance to the natural antimicrobial compound carvacrol. After the procedure of genetic recombination by conjugation twenty *Salmonella* strains were selected expressing the bioluminescent phenotype. These recombinant strains can cover a wide range of applications and used as an effective method for real-time monitoring of cell physiology by measuring the bioluminescent activity. In this study, ten of these recombinant bioluminescent *Salmonella* strains were further used to investigate the microbial resistance to the natural antimicrobial compound carvacrol. Currently, the interest in nanoemulsions for the delivery of antimicrobial agents to improve the microbiological quality of minimally processed foods has been increased. Consequently, we also determined the bactericidal effect of carvacrol delivered by lecithin stabilized nanoemulsions using the bioluminescent strains of *Salmonella*. Light measurements were taken immediately after the addition of the nanoemulsions in the bacterial suspensions of bioluminescent *Salmonella*. It was observed that bioluminescence decreased rapidly when the carvacrol nanoemulsion was added to the bacterial cell culture. Therefore, using the bioluminescent *Salmonella* strains here

constructed, it was possible to verify the rapid effectiveness of the nanoemulsion containing carvacrol as antimicrobial compound.

Keywords: antimicrobial, bioluminescence, bioreporter, carvacrol, conjugation, nanoemulsion, vegetable

3.2 INTRODUCTION

Changes in dietary patterns by the increased number of health conscious consumers in the United States, have raising the per capita consumption of fresh minimally processed fruits and vegetables by nearly 20% between 1970 and 2000 (USDA, 2002). While illness outbreaks associated to fresh produce represented only 2% of all foodborne outbreaks between 1973-1987, this percentage increased to 13% in 2006 (HANNING et al., 2009; DOYLE; ERICKSON, 2008). The concerns has been raised mainly because organisms which had historically been associated to animal sources, such as *Salmonella* spp. in meat and poultry products have been more frequently found in products from vegetable origin, particularly minimally processed products. In fact, it is know that the largest disease outbreaks associated to fresh produce in the United States were caused by bagged leafy-greens (MATTHEWS, 2009). Products involved in recent outbreaks by *Salmonella* include peppers (FDA, 2007), lettuce and tomatoes (FDA, 2008), alfafa (CDC, 2010), mangoes and melons (CDC, 2012).

However, human pathogens are not naturally part of the native microflora of plants (WHIPPS et al., 2008). Contamination of products with pathogens occurs by transference from the environment. Sources from microbial contamination in the fields include contaminated irrigation water, poor manure fertilizer practices, wild animals and human handling (BEUCHAT; RYU, 1997). Examination of leaf surfaces reveal that bacterial colonization occurs in specific regions of the leaves (ARUSCAVAGE et al., 2006) such as stomata, depressions and grooves in the cuticle, along the veins, and in the junction zones of cut leaves (SAPERS et al., 2009). Several studies have demonstrated that bacteria attach rapidly and irreversibly to damaged leaf tissue (BEUCHAT, 1999; HASSAN; FRANK, 2004) making the elimination of pathogens during rinsing/washing procedures difficult to reach (BOYER et al., 2007).

Intervention strategies used in order to improve the microbiological quality in minimally processed plant foods, usually combine physical removal of organisms by washing, plus the use of sanitizers. However, many times these interventions fail to completely remove pathogens that proliferate to greater numbers when in contact with damaged tissue (ARUSCAVAGE et al., 2006; SOLOMON et al., 2006). Thus, there is a need for development of more efficient disinfection strategies that guarantee the safety of minimally processed products.

Since last decade, consumers have manifested a preference for products formulated with natural ingredients and have given a negative connotation to the use of chemical additives. Natural antimicrobial compounds extracted from vegetable sources represent an attractive alternative to conventional antimicrobials, while satisfying consumers demands for natural products. Essential oils extracted from herbs and spices are potent natural antimicrobial agents known since a long time ago. The toxicity of essential oils extracted from spices and other plant materials against several microorganisms is widely documented (ULTEE et al., 1999; LAMBERT et al., 2001; OUSSALAH et al., 2006; PAPARELLA et al., 2008; FISHER; PHILLIPS, 2009).

Currently, the Department of Food Science at Purdue University has been developed a study that represents a novel approach for the inactivation of attached and internalized pathogens in cut vegetables by design of a functionalized lipid-based antimicrobial delivery system capable of acting within the vascular system of cut vegetables, thus targeting pathogens that are not usually reached by common disinfection interventions. The proposed research will generate critical knowledge about the effectiveness of lipid-based nanoparticle using natural antimicrobial compounds for microbial inactivation. Also, this constitutes a new intervention for the inactivation of pathogens internalized or preferentially attached to cut or damaged surfaces, thus increasing the safety of minimally processed vegetables.

However, in order to successfully accomplish the development of the antimicrobial delivery system, the monitoring of effectiveness in decreasing internalized pathogens will be done *in situ* using genetically modified pathogenic strains. These strains contain the *lux* operon, which confers to these microorganisms constitutive bioluminescence, allowing for real time monitoring of bacterial attachment sites and effectiveness of applied treatments.

Therefore, the main purpose of this study was to construct a bioluminescent bioreporter *Salmonella* to further investigate the microbial resistance to natural antimicrobial compounds, since inactivated cells are unable to produce bioluminescence. Thus, initially was performed the production of recombinant bioluminescent strains of *S.* Enteritidis through the insertion of *luxCDABE* genes cassette into the chromosomal DNA of these bacteria, and further were evaluated the effectiveness of essential oils as natural antimicrobials agents for the inactivation of recombinant bioluminescent strains of *Salmonella*.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial strains and culture conditions

Initially, a total of 40 strains of *Salmonella* of different sources, from culture collection of Dr. B. M. Applegate Laboratory (Department of Food Science, Purdue University, IN - USA), were selected for this study. These *Salmonella* strains were transferred from the - 80^{o}C stock culture by loop inoculation to Luria Bertani (LB, Acumedia, Lansing, MI) agar plate without any antibiotics for 24 h growth. In addition to these, the *E. coli* SV17 strain from de same culture collection, containing the suicide vector pFSP125 harboring the Kan-lux*CDABE* cassette (DE LORENZO et al., 1993), was also transferred from the - 80^{o}C stock culture and spread on LB-Kan agar for 24 h. Afterwards, all the *Salmonella* strains were spontaneously mutated with nalidixic acid (Na) in order to obtain Na-resistant mutants.

3.3.2 *Salmonella* spp. nalidixic acid resistant

The forty nalidixic acid (Na) resistant strains were developed as described in and Russell (2001), with slightly modifications. Initially, these different native *Salmonella* spp. strains from a glycerol stock at - 80^{o}C were streaked on LB agar plates without antibiotics. After overnight culture, a single colony of each *Salmonella* strain were inoculated into 100 mL of LB broth and incubated at 37^{o}C for 16 h. Aliquots of 1 mL of *Salmonella* cultures were centrifuged at 13,000 rpm at room temperature for 3 min in eppendorf tubes. Then, the supernatant of each tube were

discarded and each pellet was resuspended in 100 µL Minimal Salt Medium (MSM) broth. The suspended *Salmonella* cultures were subsequently spread onto LB agar plates with 15 µg/mL Na and incubated at 37°C for 16 h. Colonies from LB-Na plates were streaked onto fresh LB-Na plates for 24h incubation at 37°C. Therefore, colonies that grew on these last plates were *Salmonella* which had been converted into Na resistant strains. Further, these *Salmonella* strains were subjected to conjugation process.

3.3.3 Insertion of the Kan-*luxCDABE* cassette into the chromosome of *Salmonella* by conjugation

All forty strains of *Salmonella* spp. Na resistant and *E. coli* SV17 containing the Kan-*luxCDABE* suicide vector pFSP125 were subjected to conjugation, resulting in the insertion of the Kan-*luxCDABE* cassette into the chromosome of *Salmonella*. Firstly, the recipient *Salmonella* spp. strains and the donor *E. coli* SV17 were all inoculated into LB-Na broth and LB-Kan broth, respectively, and incubated for 16 h at 37°C. Then, the cultures were centrifuged at 13,000 rpm at 4°C, the supernatants were discarded and the pellets were resuspended separately in 25 mL of LB medium. Washes were performed three times to remove the antibiotics and finally the cells were resuspended in 50 mL of fresh LB broth, without the presence of antibiotics. Recipient (*Salmonella* spp.) and donor (*E. coli* SV17) were mixed together in a ratio of 1:10 (50 µL of *Salmonella* spp. and 450 µL of *E. coli* SV17). Each mating mixture was centrifuged at 13,000 rpm for 5 min at room temperature and the supernatant was discarded. Following incubation at room temperature for 16 h, the cell pellets were serially diluted in MSM broth and dilutions of 25 µL were spread on LB-Kan-Na agar plates and incubated for 16 h at 37°C. The plates were then examined in the dark and individual bioluminescent colonies were selected and spread onto a Brilliant Green Agar (BGA, Difco, Detroit, MI, USA) and Xylose Lysine Deoxycholate agar (XLD, Difco, Detroit, MI, USA) for further 24 h of incubation at 37°C. Characteristics colonies of *Salmonella* in the selective agars, which were presented as bioluminescent colonies, were individually streaked in a fresh LB-Kan-Na agar plates for overnight incubation at 37°C. Incubated plates were stored at 4°C for short-time usage whereas freezer stocks were also prepared in 10% glycerol LB-Kan-Na media and stored at - 80°C for further using. Recombinant bioluminescent

strains were further screened by PCR to confirm the genus *Salmonella* with chromosomal integration of the Kan-*luxCDABE* cassette.

3.3.4 DNA extraction and amplification of *inv*A gene by PCR

The chromosomal DNA of three selected colonies for each of the twenty strains expressing the bioluminescent phenotype, were selected and isolated by the method adapted from Sambrook and Russel (2001). After, PCR was performed in order to confirm the genus *Salmonella* of these bioluminescent strains, with the integration of the Kan-*luxCDABE* in the chromosomal, using oligonucleotide sequences corresponding to the *inv*A gene of the *Salmonella* (invA–F5'CCCATATTATCGCTATCGCCATTT3' and invA–R 5'CCATGCTGACCATTGGTGATG 3'), that amplify a fragment of 109 bp. PuReTaq Ready-To-Go PCR beads (GE Healthcare) were used for PCR amplifications. Basically, 1,0 µL of forward primer, reverse primer (0.2 µM each) and chromosomal DNA, plus 22 µL of deionized autoclaved water was added to a PCR bead in a PCR tube for a final volume of 25 µL. The PCR conditions as follows: initial denaturation for 5 min at 95°C followed by 38 cycles at 95°C for 30 s; 50°C at 30 s and 30 s at 72°C for extension, and then a final extension at 72°C for another 10 min. The PCR was carried out using a DNA Engine Thermal Cycler (MJ Research Inc. San Francisco, CA) and reactions visualized in a UV transilluminator using Kodak EDAS 290 (Eastman Kodak Co., Rochester, NY). All twenty *Salmonella* bioluminescent strains confirmed by PCR were cultured onto LB-Kan-Na plates 16h at 37°C and after selected, bioluminescent colonies were cultured in LB-Kan-Na broth and stored in 10% glycerol at - 80°C for long-term usage.

3.3.5 Identification of *Salmonella* strains by sequencing of the 16S rRNA

Sequencing of 16S rRNA gene was prepared from the bacteria DNA as follows. To extract the DNA from all bioluminescent *Salmonella* strains, a simple boiling method was performed. Briefly, a single colony was suspended in 1 mL of sterile water in an eppendorf tube and boiled in water bath for 10 minutes. After, the cells were centrifuged at 5000 rpm for 5 minutes to separate lysate, with the supernatant containing the DNA template. PCR of the 16S rRNA gene were carried

out for all strains with a DNA Engine Thermal Cycler (MJ Research Inc. San Francisco, CA). Amplification of 16S rRNA was carried out using primers 16SUF (5'-AGAGTTTGATCCTGGCTCAG-3') and 16SUR (5'-TACGGCTACCTTGTTACGACTT-3'), and amplified DNA fragments with 1.3 kb. The template DNA was initially denatured at 94℃ for 5 min and a total of 30 cycles were performed under the following conditions: annealing at 94℃ for 55 s, DNA extension at 46℃ for 55 s and after extesion of 72℃ for 4 min and then PCR amplified DNAs were detected by electrophoresis in 1% agarose gel. The purified PCR fragments were sent to DNA sequencing at Purdue University Genomic Core Facility. At final, the target 16S rRNA gene nucleotide sequences were aligned with the Clustal W program and tree robustness was assessed by bootstrap resampling (1,000 replicates each).

3.3.6 Growth curve of bioluminescent *Salmonella* Enteritidis cultures

The twenty bioluminescent *S.* Enteritidis cultures were transferred from a -80^{o}C stock to a fresh LB-Kan-Na plate for 24 h growth. On the following day, LB-Kan-Na plates were observed in the dark room to identify the brightest single colony, which were transferred into LB-Kan-Na broth. Two additional 24 h subcultures were made consecutively into fresh LB-Kan-Na broth. After the final, subcultures were adjusted to 0.1 at optical density of 600 nm (OD_{600}) with a fresh LB medium and three 100 µL aliquots of each bioluminescent *S.* Enteritidis cultures were transferred into 10 mL LB-Kan-Na broth in a 50 mL Falcon tubes. The tubes were placed on an orbital shaking incubator at 37^{o} C with 100 rpm rotation. Bacterial growth was monitored by measuring the OD_{600} from 1 mL culture at 0, 1, 4, 8 and 24 h during incubation.

3.3.7 Artificial contamination in green leafy vegetables by bioluminescent *Salmonella* Enteritidis strains

A preliminary assay using recombinant bioluminescent strains was conducted in order to verify the presence of these bacteria in the vegetable tissue. Two bioluminescent *S.* Enteritidis (strains 21030 and 21058) were used to visualize the attachment sites of this pathogen in the vegetable romaine lettuce. A 16 h culture of

LB-Kan-Na of these bioluminescent strains of *Salmonella* was used to inoculate the lettuce. The green leaves were allowed to immerse into a thin layer of 5 mm of each *S.* Enteritidis culture for 5 min and 10 minutes. Prior to the test, the Coomassie blue dye was added to this broth medium, in order to facilitate the visualization by the naked eye, of the absorption of contaminated culture broth by the leaves of lettuce. Samples were then removed from the inoculation broth and excess liquid removed. An image of bioluminescent intensity was recorded using an Andor CCD photon counting camera.

3.3.8 Encapsulation of natural antimicrobial into lipid matrices

The antimicrobial emulsions used in this study, was developed in the laboratory of Dr. F. San Martin-Gonzalez (Department of Food Science, Purdue University, IN - USA). High pressure homogenization was used to create liquid lipid dispersion (emulsion) that contains the hydrophobic antimicrobial compound carvacrol, a component representative of essential oils with proven antimicrobial activity. Also, this liquid dispersion was prepared using coconut oil (a vegetable oil) as lipid phase, and lecithin was used as emulsifier. Therefore, two types of nanoemulsions, which were previously referred to as Control and Sample, were used. The emulsions were formulated as follows: the Control emulsion contained 10% (w/w) coconut oil, 1% (w/w) lecithin (Ultralec), and 89% distilled water, while the emulsion Sample was prepared with 5% (w/w) coconut oil (Sigma-Aldrich), 5% (w/w) carvacrol (98% Purity, Sigma-Aldrich), 1% (w/w) lecithin (Ultralec), and 89% distilled water. Further, nanoemulsions were made in two steps: first, a coarse emulsion was prepared by mixing the distilled water and the emulsifier with the oil phase using a Polytron PT 2100 (Kinematica). In a second step, the coarse emulsion was passed 5 times through a high pressure homogenizer NanoDeBee (Bee International) at 30 kpsi. Sample emulsion containing 5 % (w/w) of carvacrol were diluted with sterile water to 5000 ppm of carvacrol for subsequent tests.

3.3.9 Antimicrobial activity of nanoemulsions against the bioluminescent pathogen *Salmonella* Enteritidis

To evaluate the efficacy of action of both nanoemulsions (Control and Sample), ten recombinant strains of bioluminescent *S.* Enteritidis were used: strains 21052, 22150, 21061, 21101, 25078, 21030, 21046, 26017, 21060, 21058. Initially, each bacterial culture was grown in LB broth for 16 h at 37°C with the Kan-Na antibiotics. After that, each culture were adjusted to OD_{600} 0.1, being used to start a new culture, also using proper antibiotics, and incubated at 37°C. When the new cultures have reached the OD_{600} of 0.1 and 0.5, respectively, were used for the experimental assays. At this point, the bioluminescence values of each culture, was verified by the use of a luminometer (Zylux Corporation, Oak Ridge Tennessee, USA). Later, the assays were performed in triplicate using a black microtiter plate, and the bioluminescence values were verified by using the VICTOR 3 Light Luminescence Plate Reader (PerkinElmer). Thus, 100 µL of bacterial cell culture was added to each well of the microtiter plate. Firstly, in this volume of cell culture, when the culture reached OD_{600} 0,1, a 5 µL of both nanoemulsions (Control and Sample) was added, and after when the bioluminescent cell culture reached the OD_{600} 0,5, were added 10 µL of each nanoemulsion. Immediately after, the bioluminescence was measured (time zero) and, in the other three additional times: time 1 (5 minutes), time 2 (15 minutes) and time 3 (30 minutes). After the measurement of bioluminescence, some selected wells of each microtiter plate previously tested were subjected to serial dilution using MSM broth and then the dilutions were spread onto LB-Kan-Na agar plate and incubated for 16h at 37°C, in order to check the recovery rate of each bacterial cell.

3.3.10 Measurement of bioluminescence

Cell bioluminescence was measured from 1 mL culture aliquots in a FB14 luminometer (Zylux Corporation, Oak Ridge Tennessee, USA) and reported as Relative Light Units (RLUs). During the experiments using the antimicrobial nanoemulsions, bioluminescence was also measured by VICTOR 3 Light Luminescence Plate Reader (PerkinElmer).

3.3.11 Charged Couple Device (CCD) Camera

The imaging system was located in a dark room. An iXon imaging system (AndorTM Technology, South Windsor, CT) consisting of a cooled CCD camera mounted on a light-tight chamber, a camera controller, a camera cooling system and a computer system for acquisition and analysis was utilized to monitor bioluminescence activity from the agar plates. Each plate was placed in the chamber mounted with the CCD camera cooled to -75^{o}C, with a field of view set at 25 cm height above the plate. The photo emission from plates inoculated with bioluminescent *S.* Enteritidis was monitored per 20 s.

3.4 RESULTS AND DISCUSSION

3.4.1 Construction of bioluminescent bioreporter *Salmonella*

Bioluminescent *Salmonella* have received particular attention during the last years and frequently used as bioreporter, once these reporters systems are based on quantified light emission as a result of *lux* genes expression (KARSI et al., 2008; MOZA et al., 2009; HOWE et al., 2010). Here, we described the development of bioluminescent *Salmonella* which could be used in a large numbers of applications in food microbiology, which include reporter gene assays, promoter identification by insertional mutagenesis, analysis of gene expression and *in vivo* imaging (CONTAG et al., 1995; BAUTISTA et al., 1998; GREER; SZALAY, 2002; LEWIS et al, 2006; HOWE et al., 2010). The bioluminescent phenotype of these *Salmonella* strains were achieved via conjugation with *E. coli* SV17 containing a suicide vector pFSP125 (DE LORENZO et al., 1993), which carries the *luxCDABE* operon from *P. luminescens*.

Since this *lux* operon consists of genes encoding a thermo stable luciferase, the expression of the *luxCDABE* provides a system that generates bioluminescence in living cells at 37^{o}C (CHATTERJEE; MEIGHEN, 1995). Therefore, to obtain the bioluminescent phenotype, the *Salmonella* strains have to synthesize components of the *lux* pathway and provide an energy source in the form of reduced flavine mononucleotide ($FMNH_2$) (BILLARD; DUBOW, 1998). Furthermore, it is important to emphasize that while bioluminescent *Salmonella* cells are capable of producing

sufficient $FMNH_2$ and other components of *luxCDABE* pathway to sustain the bioluminescent phenotype, even small bioluminescent colonies can be easily visualized using a CCD camera (AUBRECHT et al., 2007).

Moreover, bioluminescent *Salmonella* strains expressing this bacterial luciferase have important advantages as bioreporters, which include the absence of toxic or phenotypic effects from the accumulation of signal, real-time detection and no need for addition of an exogenous substrate (BILLARD; DUBOW, 1998; KARSI et al., 2008). In this study were obtained a total of twenty bioluminescent *Salmonella* spp. strains that can be used for real-time monitoring, in order to be able to evaluate the effectiveness of essential oils as natural antimicrobials agents for the inactivation of this microbial pathogen.

3.4.2 Bacterial conjugation

Conjugation, a special type of replication, is the process of genetic transfer during which chromosomal material is transferred from the donor to the recipient cell (LLOSA et al., 2002). In this type of genetic recombination, one organism receives genetic information from a donor, while the recipient will be changed by that information (PHORNPHISUTTHIMAS et al., 2007). However, in bacterial conjugation, the transfer of genetic material is not reciprocal. Therefore, whenever one cell act as the donor, the other cell will act as the recipient (FROST; KORAIMANN, 2010).

Initially, it was believed that the conjugation was essentially a Gram-negative behavior, but nowadays the phenomenon is known to be widespread among Gram-positive bacteria (DE LA CRUZ et al., 2009). The whole process requires a lot of proteins, usually encoded by plasmid genes, including those required to form a mating pair between donor and recipient. Moreover, conjugation always requires cell-to-cell contact (LLOSA; DE LA CRUZ, 2005). Thus, previously the bacterial conjugation, the pilus is encoded and is expressed from the donor cell and binds to the recipient cell. Later, during conjugation, the pilus contracts and approximates the two cells, forming an essentially pore, which will allow the transfer of DNA. Consequently, for the DNA molecule can be transferred, bacteria require close physical contact between cells throughout the process. In addition, the DNA replication prior to conjunction ensures a complete chromosome for the donor cell after mating. As the transferred genetic material is also replicated in the recipient cell,

the genes of the donor cell can then be incorporated into the recipient chromosome through crossovers, creating a recombinant cell (GRIFFITHS et al., 1999; LLOSA et al., 2002; PHORNPHISUTTHIMAS et al., 2007; FROST; KORAIMANN, 2010).

Thus, the bacterial conjugation is a significant and important mechanism that allows bacteria to exchange genetic material (FERNANDEZ-LOPEZ et al., 2005). Furthermore, it is considered the major mechanism for horizontal DNA transfer among pathogenic bacteria, with potential for universal DNA delivery, which makes it a potential tool for genomic engineering (LIN et al., 2011). Besides, the horizontal transfer of genes provides selective advantages, which allow microorganisms can quickly respond to environmental changes (LLOSA; DE LA CRUZ, 2005).

In our study, figures 1-2 show the emission of light from recombinant bioluminescent strains of *Salmonella* which were monitored using a CCD camera. Overall, the CCD camera shows to be a very efficient tool for analyzing the activity of recombinant *Salmonella* bioluminescent strains in both non-selective and selective agar, even using a low exposure time. Furthermore, it is important to emphasize that the bioluminescent phenotype was observed in a total of 20 recombinant strains of *Salmonella*, which were identified as: bioluminescent strains numbers 21025; 21030, 21046, 21052, 21054, 21056, 21058, 21060, 21061, 21063, 21101, 22150, 23004, 25076, 25078, 25079, 26017, 27031, 27035, 28015. However, here we only shown the results for two of these strains, bioluminescent strains 21030 and 21058, since similar results were observed for all the other 18 strains of bioluminescent *Salmonella* (Figures 1 and 2).

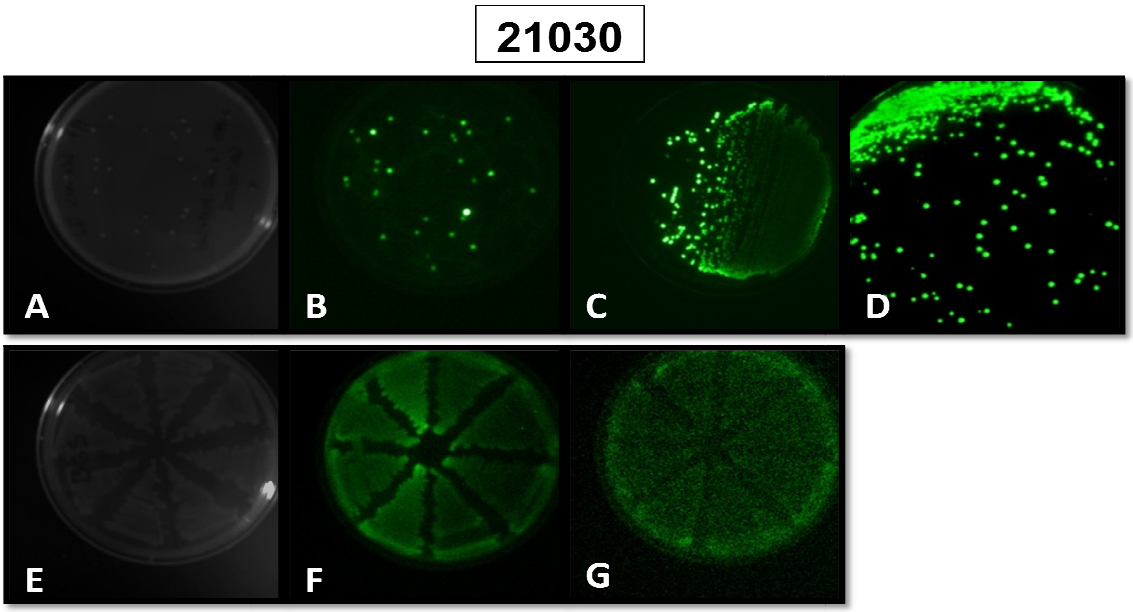

Figure 1. Sequence of plates of recombinant bioluminescent *Salmonella* 21030 expressing luciferase. **A** – LB-Kan-Na photo with natural light; **B** to **D** – LB-Kan-Na photo in the dark; **E** – BGA photo with natural light; **F** – BGA photo in the dark; **G** – XLD photo in the dark.

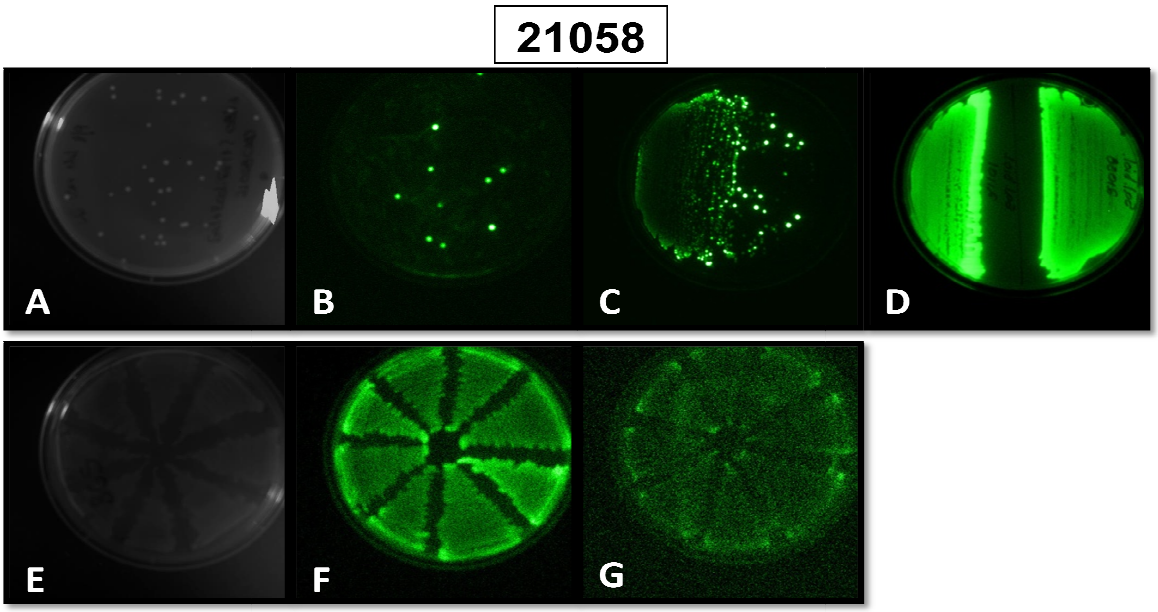

Figure 2. Sequence of plates of recombinant bioluminescent *Salmonella* 21058 expressing luciferase. **A** – LB-Kan-Na photo with natural light; **B** to **D** – LB-Kan-Na photo in the dark; **E** – BGA photo with natural light; **F** – BGA photo in the dark; **G** – XLD photo in the dark.

After, characteristic colonies expressing bioluminescence in both selective agars were transferred into 10 mL LB-Kan-Na broth, which was incubated 16 h at 37°C. Then, cell bioluminescence was measured from 1 mL culture in a FB14 luminometer. Aliquots of each bioluminescent *Salmonella* were also prepared in 10% glycerol with LB-Kan-Na media and stored at - 80°C.

Therefore, in the present study, it was found that after the conjugation process and identification of characteristics colonies in the selective agars, along with the monitoring for the presence of bioluminescent colonies, twenty *Salmonella* strains were selected expressing bioluminescence.

3.4.3 Confirmation of *Salmonella* spp. by amplification of *inv*A gene by PCR

The majority of *Salmonella* virulence genes are clustered in chromosomal regions named *Salmonella* Pathogenicity Islands (SPIs) (AMMARI et al., 2009). Also, the virulence of *Salmonella* is related to a combination of chromosomal and plasmid factors (ARAQUE, 2009; SÁNCHEZ-JIMÉNEZ et al., 2010). The chromosomally located invasion gene *inv*A is responsible for expression of the protein InvA, which is a component of set of several inner membrane proteins that form the Type III Secretion System (T3SS), which is crucial for to mediate the transfer of virulence factors from the bacterial cytoplasm into host cells in pathogenic bacteria (WORRALL et al., 2010). With this system T3SS is possible to perform the bacterial protein translocation across the membrane, which is subsequently deposited directly into the cytoplasm of the host organism (LILIC et al., 2010). Moreover, the protein InvA, involved in cellular invasion, is conserved among the different *Salmonella* and is a useful marker for molecular detection of this pathogen by PCR (AHMED et al., 2008). Furthermore, the fact that this gene is unique to *Salmonella* ensures high specificity and sensitivity in this detection assay (HADJINICOLAOU et al., 2009).

Therefore, individual colonies expressing the bioluminescent phenotype, which demonstrate themselves with typical characteristics in both selective agars XLD and BGA, were screened by PCR using *inv*A primers to confirm the bacterial genus *Salmonella*, however now with the chromosomal integration of the Kan-*luxCDABE* cassette. Therefore, the analysis using PCR for the occurrence of *inv*A gene in this study, confirmed its presence in 20 recombinant strains, confirmed as bioluminescent bioreporter *Salmonella* (Figure 3). Other studies reported similar results, which were

expected since the *inv*A is an invasion gene conserved among *Salmonella* serovars. In addition, it is important to emphasize that this result was consistent with reports that recognized the presence of *inv*A gene in all *Salmonella* independent of serovar or source (SWAMY et al., 1996; AHMED et al., 2008; GARCIA et al., 2010).

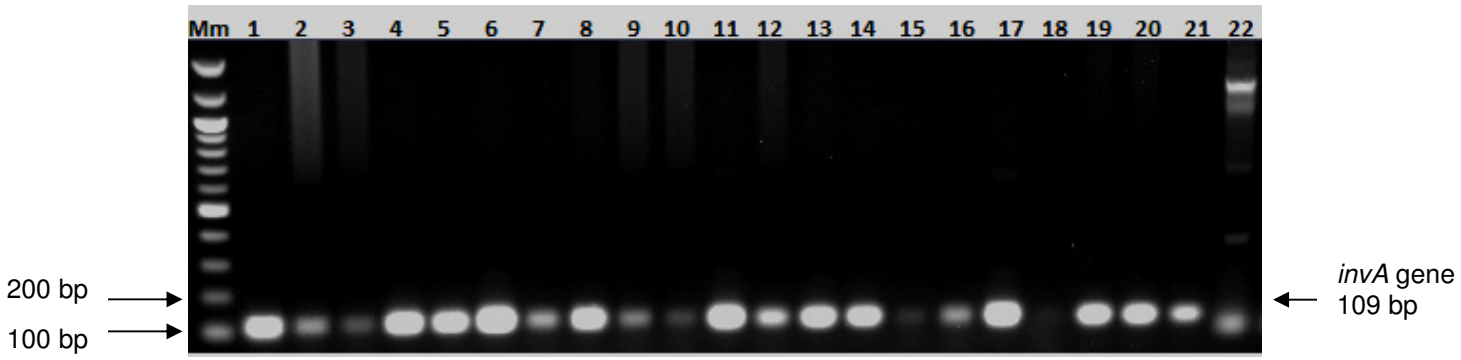

Figure 3. PCR of *inv*A gene to confirm the bacterial genus *Salmonella* spp. with the chromosomal integration of the Kan-*luxCDABE* cassette. Mm: Molecular marker 1Kb; Lines 1 to 20 – Bioluminescent *Salmonella*; Line 21: *S. poona* (Positive Control); Line 22: *E. coli* SV17 (Negative Control).

3.4.4. Identification of bioluminescent *Salmonella* strains by 16S rRNA gene sequencing

Over the last few years, automated DNA sequencing has enhanced the application of 16S rRNA gene sequence analysis to bacterial identification (PATEL, 2001). Therefore, 16S rRNA gene sequencing has been considered a well established method for identification of several different bacterial species, including *Salmonella* spp. (CLARRIDGE, 2004; WOO et al., 2008). The sequencing method uses universal primers to amplify and sequence a partial region of the 16S rRNA gene, or even if necessary, the full gene. Furthermore, the 16S rRNA gene is a multi-copy housekeeping gene that is normally conserved, but also contains many variable regions, which enable the differentiation of most microorganisms at species and subspecies level (CHAKRAVORTY et al., 2007). Usually, most of bacteria contain between 1 and 15 copies of the 16S rRNA gene, whereas *S. enterica* has been reported to have 7 copies of the 16S rRNA gene, which also allow for discrimination beyond the specie level (ACINAS et al., 2004). Current developments has also

improved the application of gene sequence analysis to bacterial detection, since recently was reported that slight sequence differences in the 16S rRNA gene could be used for *S. enterica* identification (HELLBERG et al., 2012).

Advances in PCR and sequencing technology have greatly reduced the time required associated with these methods, which along with a substantial reduction in the cost of applying these techniques, have contributed to the identification of bacterial species in a small period of time (PATEL, 2001). At the moment, a 16S rRNA gene sequence from bacterial DNA can be obtained in few hours, which is significantly shorter than the minimum of 5 days usually required for biochemical assays used to identify *Salmonella* spp. (ODUMERU; LEÓN-VELARDE, 2012). Our study analyzed the 16S rRNA gene sequence among twenty bioluminescent *Salmonella* spp. strains, in order to confirm the genus, specie and serovar of these recombinant strains (Figure 4). Besides, confirming that the DNA bacterial belongs to genus *Salmonella*, we also confirmed that all strains are belonging to subspecies *enterica* serovar Enteritidis. In addition, the results indicated that the 16S rRNA gene sequencing of *Salmonella* spp. can be used to classify this specie quickly than biochemical tests normally used.

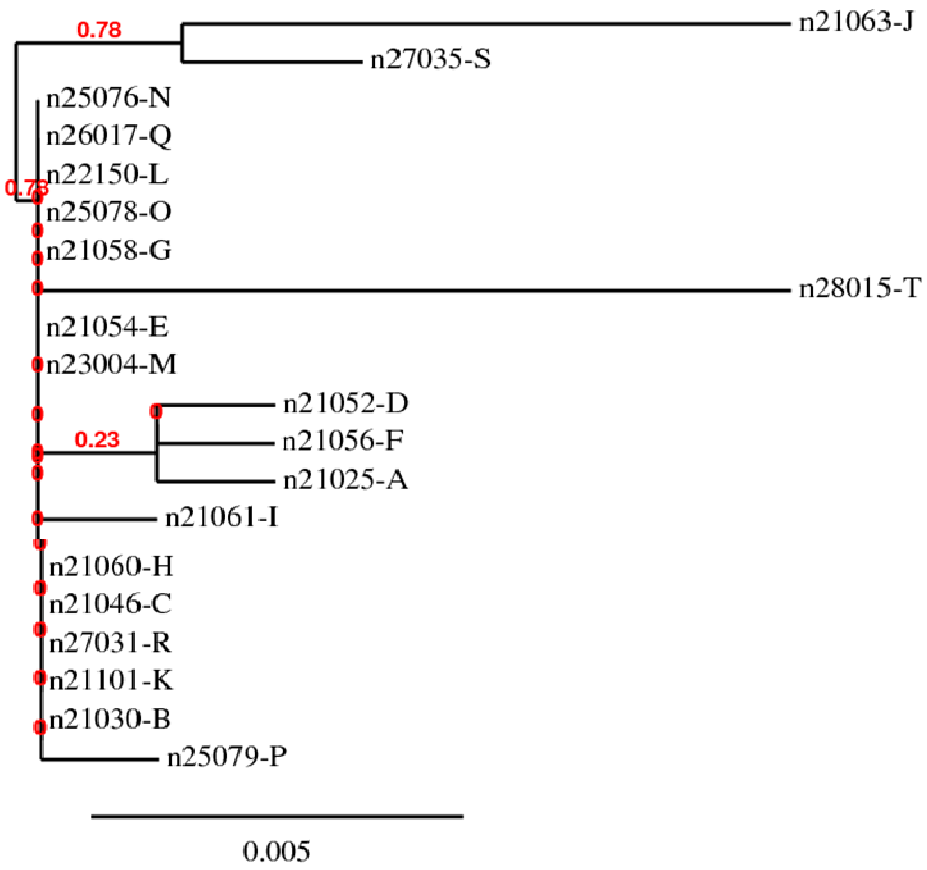

Figure 4: Phylogenetic analysis of 16S rRNA of *S.* Enteritidis strains. The multiple 16S rRNA sequences were aligned using Clustal W.

3.4.5 Visualization of bioluminescent intensity in lettuce leaves

Recombinant strains of bioluminescent *S.* Enteritidis were employed to identify attachment sites in romaine lettuce. The use of these bioluminescent *Salmonella* strains allows to visualize how and where the bacteria can attach or internalize into vascular systems of vegetable tissue. Furthermore, it shows that bacteria bind less efficiently to intact and undamaged surface tissue, than to cut surfaces, and that considerable reduction in cell numbers is achieved by simple rinse (data not show). When the tissue becomes damaged, as in cutting or slicing for example, surfaces become hydrophilic and bacteria attach very effectively as indicated by sites with higher bioluminescent intensity (Figure 5). Thus, greater photon production corresponds to sites with greater bacteria accumulation.

Both bioluminescent *S.* Enteritidis strains 21030 and 21058, attaches preferentially to cut edges as shown by photon production (Figures 5 and 6). Also, it is possible that the intense light production occurs in or on the main vascular system. However, the images generated in our assay, do not allow us to assess whether bacteria were exactly internalized into the vascular system. In these images, it was possible to view that the bottom part of the all leaves were cut. Then, the leaves were vertically immersed into a thin layer of bioluminescent *Salmonella* strains cultures for 5 minutes and 10 minutes, respectively. Immediately after, images of the bioluminescence distribution were taken, and it was possible to note that bacteria strains were adhered in the lettuce leaves, as evident by light moving up to the vegetable tissue.

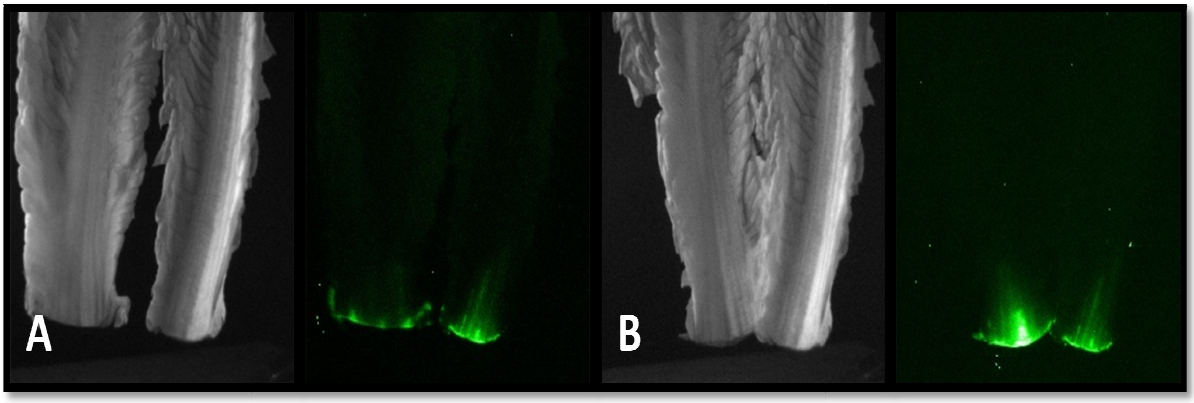

Figure 5. Lettuce artificially contaminated demonstrating the recombinant bioluminescent *Salmonella* 21030 inside the leaf. Photos took with natural light and in the dark. **A** - Exposure time of 5 minutes of leaves in the culture broth contaminated; **B** - Exposure time of 10 minutes of leaves in the culture broth contaminated.

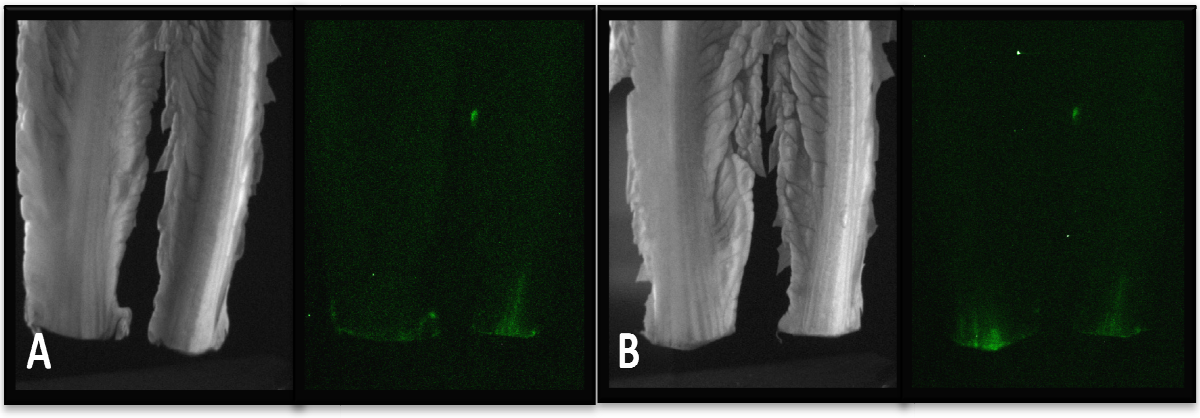

Figure 6. Lettuce artificially contaminated demonstrating the recombinant bioluminescent *Salmonella* 21058 inside the leaf. Photos took with natural light and in the dark. **A** - Exposure time of 5 minutes of leaves in the culture broth contaminated; **B** - Exposure time of 10 minutes of leaves in the culture broth contaminated.

3.4.6 Growth condition and bioluminescence activity of bioluminescent *Salmonella* Enteritidis

Bioluminescent bioreporter *Salmonella* are bacteria genetically constructed to express the *lux* genes, which have been employed to monitor growth *in vitro* and *in vivo*, using the light production as a reporter of viable and metabolically active cells. However, for the bioluminescent *S.* Enteritidis strains to constitute an appropriate tool for studying colonization, it is necessary to verify whether the insertion of *lux* is responsible for affecting its growth. Therefore, to compare the *in vitro* growth of bioluminescent *Salmonella* to its parent strain, bacterial growth was monitored by measuring the optical density, and to evaluate the *lux* expression among different bioluminescent *Salmonella* strains, the light intensity was detected using a luminometer.

The growth curves represented in figure 7 indicate that the both strains of *S.* Enteritidis, parent and bioluminescent, demonstrated the same growth pattern, at all time points into the exponential growth phase (0-24 h). Thus, it is important to emphasize that the insertion of the *lux* operon in the chromosome of *S.* Enteritidis strains, did not affect the development of essential functions involved in bacterial growth.

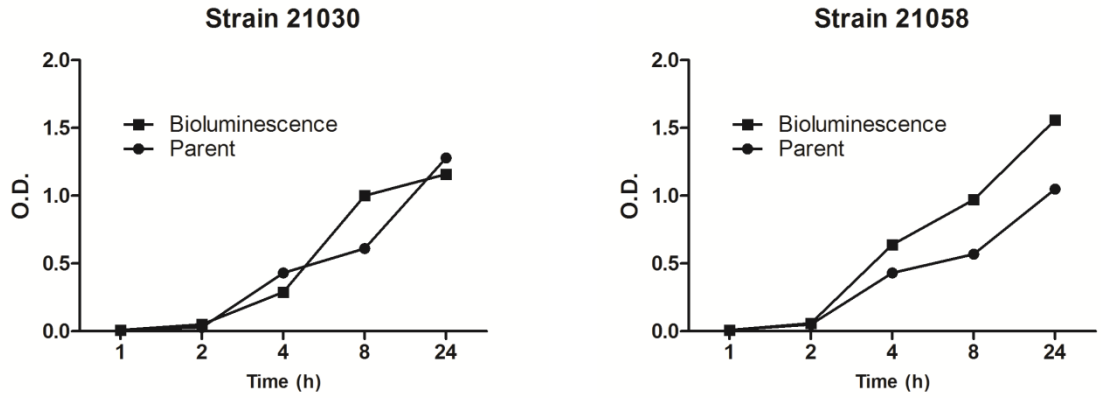

Figure 7. *G*rowth curve of *S.* Enteritidis (Parent) and *S.* Enteritidis with *luxCDABE* genes (Bioluminescent) in the LB medium and LB medium with 50 µg/mL Kanamycin and 15 µg/mL Nalidixic acid, respectively. Data are the average of 2 independent assays performed in triplicate.

Additionally, it is possible to observe through the graphs represented in figure 8, which during the initial growth phase both bioluminescent bacteria are growing but the *lux* genes are being poorly expressed. Usually, this occurs because through the initial growth phase, the cell culture is found in very low density and thus the *lux* genes are not expressed. Consequently, the light emission in a cell culture will not increase due to substrates limitation for the bioluminescent reaction. On the other hand, during mid to late logarithmic growth phase, the bioluminescence increases considerably due to the activation of genes expression in the *luxCDABE* operon, having the luciferase and fatty acid reductase activities, powerfully increased with the growth. Furthermore, luciferase induction is also dependent on the species and nutrient composition of the several growth medium (MEIGHEN, 1988; CHATTERJEE; MEIGHEN, 1995; SCHEERER et al., 2006).

As a result, in our study it was possible to verify that the bioluminescence increased significantly when both bioluminescent *Salmonella* grew in logarithmic phase and started to decrease when bacterial growth has reached the stationary phase through a rapid and strong decline in the bioluminescence emission concentration, suggesting that the highest peak of OD coincides with the decrease in the expression of *lux* genes. In both cases, during exponential growth, the bioluminescence increased concomitantly with the OD, whereas during the stationary phase light emission dramatically fell. This phenomenon is probably correlated with a

decrease in the concentration of the luciferase co-substrate, reduced flavin mononucleotide ($FMNH_2$), required for the luminescent reaction (SIMON et al., 2001). However, it is unclear whether the light was reduced, due to lack of oxygen used by the luminescent reaction, or was due to consumption by the increasing number of cells.

Finally, it is also important to note that both the bioluminescent bioreporter *Salmonella*, demonstrated a good correlation between the bacterial numbers and the luminescence expression, which allows that the bioluminescence can be used to compare the amount of bacterial, for both increasing and decreasing cell numbers. Therefore, we can conclude that the bioluminescence closely correlates with the OD of the culture, and also with the cell number. In addition, it is important to remind that no cell disruption is required to measure the light emission, and that bioluminescent based-assays are rapid, simple, specific and extremely sensitive (MEIGHEN, 1993; STEWART & WILLIAMS 1992; BURLAGE; KUO, 1994).

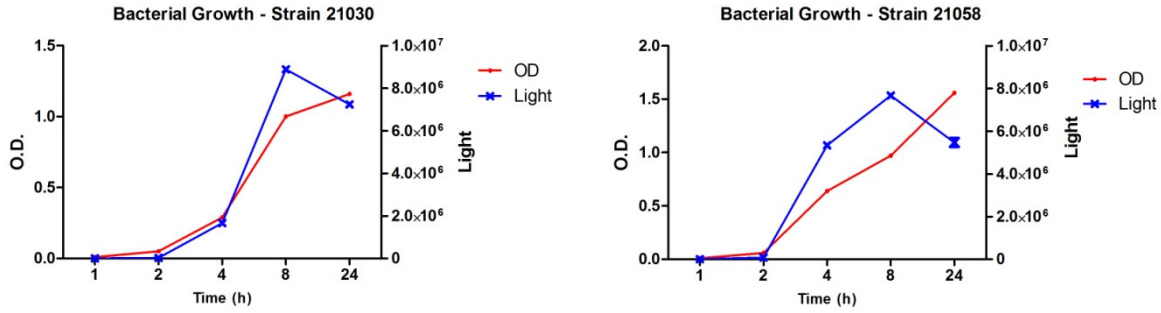

Figure 8. Growth curve and bioluminescence activity of bioluminescent *S.* Enteritidis strains 21030 and 21058 in LB medium with 50 µg/mL Kanamycin and 15 µg/mL Nalidixic acid. Bioluminescence and optical density were measured for 24 h at 37°C. Data are the average ± SD of 2 independent assays performed in triplicate.

3.4.7 Natural antimicrobial compound carvacrol

Carvacrol is an essential oil component of numerous aromatic plants, such as oregano, thyme, marjoram and wild bergamot. Usually recognized as a safe food additive, being used as a flavoring agent in several products (KISKÓ; ROLLER, 2005; OBAIDAT et al., 2010). Recently has attracted attention by its biological properties, such as a wide spectrum of antimicrobial activity, being considered

effective against bacteria, yeasts and fungi (LAMBERT et al., 2001; KNOWLES et al., 2005; NOSTRO et al., 2009; RAO et al., 2010). The biocidal mode of action of carvacrol on bacteria is similar to that of other phenolic compound (OBAIDAT et al., 2010). Carvacrol acts on the cytoplasmic membrane, resulting in bacterial membrane perturbations that lead to an increased permeability to protons and potassium ions. Furthermore, it also leads to the depletion of the intracellular ATP pool, which causes the loss of membrane integrity. Finally, these dysfunctions together, will result in cell death (ULTEE; BENNIK; MOEZELAAR, 2002; NOSTRO et al., 2009).

Despite its broad antimicrobial spectrum, the application of carvacrol in food preservation has been limited by its potent aromatic properties (KNOWLES et al., 2005), since some studies have reported that when this component is used in high concentrations can lead to the development of undesirable color, odor and flavor changes in food products (KISKÓ; ROLLER, 2005). Consequently, it is important that preventive measures are taken in the preparation of nanoemulsions, which will prevent the appearance of undesirable characteristics that may mask any flavor and aroma leading an alteration in the sensory properties of the product where this delivery system based on lipid nanoparticles are applied.

3.4.8 Effectiveness of essential oil encapsulated into lipid nanodispersion for the inactivation of the bioluminescent microbial pathogen *Salmonella* Enteritidis

The evaluation of efficacy of the antimicrobial delivery system, containing the essential oil of carvacrol encapsulated into liquid lipid nanodispersions, was performed using the genetically modified human pathogen of concern in fresh produce *S.* Enteritidis, previously constructed in this study. These strains contain the *lux* operon from *P. luminescence*, which confers to this microorganism constitutive bioluminescence, allowing for real time the monitoring of bacterial conditions front the effectiveness of applied treatments. Since bioluminescence intensity varies for each bacterial strain, correlations between emitted photons and cell concentration was generated for ten different *S.* Enteritidis bioluminescent strains.

However, besides the bactericidal activity, it is extremely important to emphasize that among the desirable characteristics of an efficient delivery system for natural antimicrobial compound, should be included: absence of toxicity to the

consumer, deliver the amount of compound required to carry antimicrobial activity, be successfully internalized into vascular systems of cut vegetables, mask any flavor or aroma that might change the organoleptic properties of the product.

Initially, experiments were conducted in order to study the effect of nanoemulsions (Sample and Control) in microbial inactivation, which was measured by decrease in the bioluminescence intensity. Although the experiment has been carried out in a total time of 30 minutes, it was observed that independent of cell concentration used (OD_{600} 0.1 and OD_{600} 0.5), immediately after the addition of the nanoemulsion Sample, which contained the antimicrobial compound carvacrol, there was a rapid decrease in the bioluminescence value emitted by these recombinant *Salmonella* strains. This demonstrates the high bactericidal potency of the antimicrobial compound carvacrol, when it is administered as an emulsion in a liquid medium. Furthermore, after the measurement of bioluminescence, cell cultures treated with nanoemulsions were subjected to serial dilutions and plated. For both strains 21030 and 21058, regardless of cell concentration (OD_{600} 0.1 and OD_{600} 0.5) it was observed no growth after adding the nanoemulsion Sample (Figure 9). Besides, the same result was observed for all other bioluminescent *Salmonella* strains also tested. However, when treated with the nanoemulsion Control, which does not contain the antimicrobial compound carvacrol, was observed increased levels of bioluminescence for *S.* Enteritidis strains 21030 and 21058, over the course of the test (Figure 9). These results were further confirmed by plate count. For both strains, for OD_{600} 0.1, the cell growth ranged from 1.0 $x10^7$ to 3.1 $x10^7$, while for OD_{600} 0.5, this bacterial cell growth ranged from 3.0 $x10^7$ to 5.1 $x10^7$. Additional results related to the tests with nanoemulsions for other bioluminescent *Salmonella* strains evaluated, can be visualized in the supplementary figures (Appendices B, C and D).

Furthermore, our results with the use of antimicrobial nanoemulsions suggest that the presence of carvacrol contributes to a rapid bioluminescent assay, in order to evaluate the inactivation of *Salmonella* in liquid media. It is important to emphasize that this assay provides real-time information on the antimicrobial activity, since only metabolically active cells are able to emit light. Therefore, it is important to note that the carvacrol exhibited greater antimicrobial activity in the inactivation of these strains of *S.* Enteritidis bioluminescent when delivered as an emulsion prepared by high pressure, and has potential to significantly impact the commercial disinfection

operations of leafy greens. However, in the future additional tests need to focus more on the effect of the concentration versus the contact time, once the inactivation, based on light emission observations, seems to occur extremely fast.

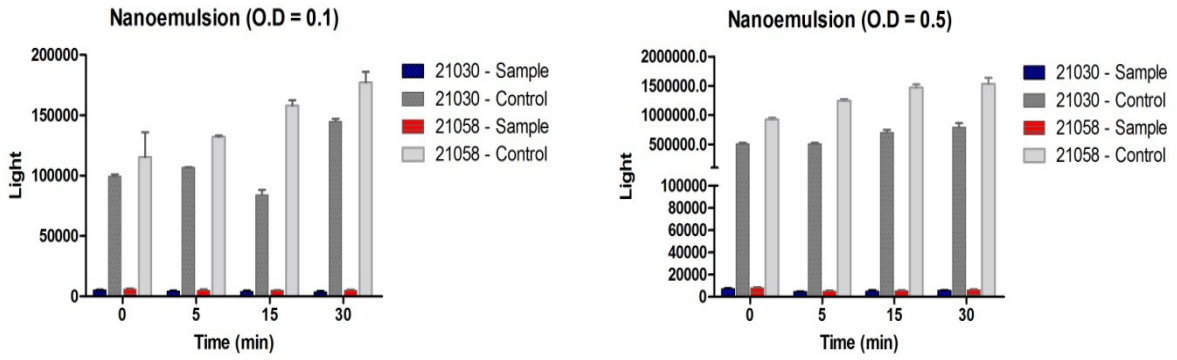

Figure 9: Bioluminescent intensity of *S.* Enteritidis strains 21030 and 21058, in the presence of nanoemulsions with and without the antimicrobial carvacrol. Assays with both nanoemulsions (Sample and Control) were performed using LB medium supplemented with 50 mg/mL Kanamycin and 15 mg/mL Nalidixic acid, with two different optical densities: OD 0.1 and OD 0.5, respectively. **Sample:** nanoemulsion with carvacrol; **Control:** nanoemulsion without carvacrol.

3.4.9 Conclusion

Through the process of bacterial conjugation, was possible to construct twenty recombinant strains of *S.* Enteritidis, which through the chromosomal integration of the Kan-l*uxCDABE* cassette are now able to express the bioluminescent phenotype. These recombinant strains can be used as bioluminescent bioreporter bacteria in a wide variety of applications, since this is an efficient method for real-time monitoring of cell physiology by measuring the bioluminescent activity. Our preliminary tests using the bioluminescent *S.* Enteritidis strains allows to visualize in real time, where bacteria preferentially attach in vegetable tissue. Furthermore, through the use of these strains, it was possible to develop a rapid bioluminescent method for evaluation of bactericidal properties. Therefore, the high bactericidal potency of the antimicrobial compound carvacrol, it was observed immediately after the addition of the nanoemulsion Sample to the bacterial culture broth, with a fast

decrease in the bioluminescence emitted by these recombinant *Salmonella* strains.

Future works intend to develop protocols of inoculation and disinfection to understand bacterial attachment at various inoculums levels. In addition, we also intend to investigate the inactivation of this pathogen using different concentrations of different antimicrobial agents, as well as, through the application of different methods, such as spraying and soaking. Therefore, incision cuts will be made on vegetable surfaces to identify potential attachment sites. The cut vegetables will be inoculated by immersion into bacterial cultures at different times. After inoculation, cut vegetables will be rinsed using different solutions, which will be done either by pouring or by immersion to simulate real rinsing conditions. Also, at each step of the process, bioluminescence images will be recorded to monitor changes in bioluminescent intensity.

Thus, the long term goal of our studies intend to reduce the incidence of outbreaks caused by minimally processed vegetables, through the design and construction of several lipid-base antimicrobial delivery nanoparticulate systems, for elimination of different foodborne pathogens, that usually become internalized into vascular tissue or even that attach to cut surfaces, as a consequence of unsatisfactory minimal processing operations in leafy-greens and other cut vegetables.

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4 Capítulo 3 – ARTIGO 3

A ser submetido para o periódico Journal of Microbiology Methods

Detection of protein expression using light-scattering sensor in *Salmonella Enteritidis* and *Escherichia coli*

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4.1 ABSTRACT

Light-scattering images from bacterial colonies have emerged as an attractive technology for rapid detection of foodborne pathogens. This technology consists of a laser when applied directly to bacterial colonies, generates scatter images and has been used for the detection of *Salmonella, Escherichia coli* O157:H7*, Listeria, Staphylococcus* and *Vibrio* isolates. However, the use of light scattering to identify differences in the same strain under different metabolic conditions, as protein expression, has not been assessed. In this study, *lux* proteins and specific proteins from different foodborne pathogens were used to evaluate the effect of specific protein expression on light-scattering images from different bacterial strains. Light-scattering images were acquired from plates with colonies using a light scattering sensor, BARDOT (**BA**cteria **R**apid **D**etection using **O**ptical scattering **T**echnology). Images of *S.* Enteritidis expressing *luxCDABE* genes were compared to their parent strains to determine visual differences associated with *lux* expression. *E. coli* expressing *lux* genes from *Photorhabdus luminescens* (*E. coli* PL lux) and *E. coli* expressing *lux* genes from *Vibrio fischeri* (*E. coli* VF lux) were compared by light-scattering images to determine visual differences within the same strain constructed with two different *lux* cassettes. Light-scattering images for the bioluminescent *Salmonella* strains showed a visual distinctive pattern as compared to the parent strains. In addition, BARDOT images resulted in two unique patterns for each bioluminescent *E. coli* expressing different *lux* genes. Also, *E. coli* Top 10 strains transformed with distinct plasmids coding for heterologous proteins, were analyzed using the light-scattering and images compared with the control, demonstrate that each one of these individual *E. coli* strains expressing different proteins, had unique

scatter patterns. Our results suggest that the light scatterometer BARDOT can be used with a high degree of accuracy for the selection and discrimination of recombinant colonies expressing different and specific proteins.

Keywords: BARDOT, bacteria, bioluminescent, optical biosensor, recombinant protein

4.2 INTRODUCTION

The evaluation of bacterial contamination in foods is conventionally monitored through a standard laboratory practice of isolation, identification and characterization of specific colonies on a selective agar medium. The methods commonly used for identification and characterization of bacteria involve traditional culture techniques, combined with the differential metabolic substrate for each microorganism, which include pre-enrichment and plating onto selective solid media (GRACIAS; MCKILLIP, 2004). After the microorganisms being identified with selective culture procedures, other methods are used to determine phenotypic characteristics of the microorganism. Besides traditional morphological, biochemical and serological techniques, many molecular tools such as ribotyping, pulsed-field gel electrophoresis, PCR and sequencing, also has been utilized for the final confirmation of microorganisms and are often used to identify different bacterial genera and species (NARAVANENI; JAMIL, 2005). Nevertheless, these traditional bacterial methods, as well as molecular methods are known to be labor intensive, time consuming, involves multiple steps, are relatively expensive and for most of bacteria, lasting more than five to seven days to conclude the tests (AKOVA et al., 2010; HUFF et al., 2012). Moreover, the cells generally are damaged by some of these types of tests and therefore, are not available for evaluation of additional confirmation (BHUNIA, 2008).

Among the emerging technologies for microbiological analysis, the light scatter-based methods provide an interesting alternative for rapid detection and classification of foodborne pathogens (BHUNIA, 2011). The light scatterometer BARDOT, was developed relying on the idea of recognizing microorganisms based on the phenotype of the colonies (BAYRAKTAR et al., 2006; BANADA et al., 2007). A bacteria colony is defined as a visible cluster of bacteria growing on the surface of

a solid medium, presumably cultured from a single cell. Furthermore, a colony is not only the bacteria itself, but also consists of extracellular materials, which are secreted while the colonies are growing (WIMPENNY, 1979). This structure can then be analyzed in order to provide distinguishable phenotypically characteristics, when using the appropriate methods. Therefore, when the laser emitted by the BARDOT system passes through a colony, these structures encode their characteristic signatures, which result in unique forward scattering patterns (BAE et al., 2007; AKOVA et al., 2010). Thus, this method is based on the recognition of image patterns to evaluate the colonies, and through this information is capable of recognizing microorganisms belonging to specific classes (BANADA et al., 2009).

The optical sensor BARDOT is capable of real-time detection and identification of colonies of several bacterial pathogens, providing highly repeatable and distinguishable forward scattering patterns of bacteria, which has been used for distinguishing bacterial cultures at the genus, species and strain level for *Escherichia* spp., *Listeria* spp., *Salmonella* spp., *Staphylococcus* spp. and *Vibrio* spp., with a high degree of accuracy and specificity (BANADA et al., 2009). This system employs a laser (635 nm) to illuminate single colonies on an agar plate and the resulting scattered light images are collected using a digital camera (BAI et al., 2009). These images are unique patterns from individual bacterial colonies used to detect and classify pathogens, creating a scatter signature that are genus and species specific, and have been shown to distinguish different strains or serotypes (BAYRAKTAR et al., 2006; BANADA et al., 2007; BAE et al., 2008; BANADA et al., 2009; BAE et al., 2011).

An important advantage of the forward-scatter BARDOT to colony phenotyping is that the characteristics from scatter patterns are easily quantified. Nevertheless, the greatest benefit of this technology is that it is non-invasive and non-destructive to the cell. Also, this technique does not require any probes or labelling reagents, and the identification results are obtained from bacterial colonies in only few seconds - approximately 3 seconds per colony (BANADA et al., 2009; HUFF et al., 2012). Furthermore, only basic microbiological practices are required for preparation of samples used in BARDOT (BHUNIA, 2008). Consequently, the costs are relatively low when compared with other traditional and molecular methods of detection and identification (BETTASSO et al., 2012).

Usually, the screening of clonal libraries from recombinant bacteria expressing unique proteins is a difficult task and predominantly relies on a detectable visual phenotypic change and molecular characterization. Previous studies using the light scattering technique to investigate the scattering patterns of different bacteria species showed significant differentiability (BANADA et al., 2009; ROBINSON et al., 2011). However, the use of light scattering to detect differences within the same strain under different metabolic conditions, such as protein expression, has not been previously evaluated. Thus, in the present study, *lux* proteins and other proteins from different foodborne pathogens, were used to evaluate the effect of these specific protein expressions on light-scattering patterns, as well as, we evaluated the application of the of this system for initial screening of clonal libraries.

Recombinant bacteria are microorganisms that have been submitted to genetic engineering. This means that these bacteria have its original contents of DNA modified by the introduction of new exogenous DNA. Currently, these three methods of genetic recombination (conjugation, transformation and transduction) have been extensively used for the construction of recombinant strains, which can be used in various applications in the food industry (WARRINER et al., 2003; RIEDEL et al., 2007; HOWE et al., 2010).

Recombinant bioluminescent reporter bacteria have been used as an effective method for real-time monitoring by measuring cell light production (SIMPSON et al., 1998). The *lux* genetic system consists of a luciferase composed of two different subunits, encoded by the genes *luxA* and *luxB* and also contains a multienzyme fatty-acid reductase consisting of three proteins (a reductase encoded by *luxC*, a transferase encoded by *luxD* and a synthetase encoded by *luxE*), which initially converts and then recycles the fatty acid to the aldehyde substrate (MEIGHEN, 1991). All genes are contained on a single operon, denoted *luxCDABE*, which are both required for the light production. Consequently, the use of the complete *lux* cassette enables that the bioluminescence can be expressed continuously, with no necessity for exogenous substrates (CHATTERJEE; MEIGHEN, 1995). Therefore, the purpose of this study was to evaluate the application of the BARDOT system to investigate the protein expression on light-scattering images from different recombinant bacterial strains.

4.3 MATERIALS AND METHODS

4.3.1 Light-scattering instrumentation

The BARDOT device (Advanced Bioimaging Systems) was used in this study for acquiring the light scattering patterns of recombinant bacterial colonies. The system included a CCD camera to acquire the images of the colony, a red laser (635 nm) to generate the scatter pattern of each bacterial colony, and a CCD chip to acquire forward-scatter patterns. The machine automatically moves the Petri-dish in order to align the colony center with the laser. The system uses this laser to illuminate individual colonies and create a forward-scattering pattern as the signature "fingerprint" of the colonies, which are collected and subsequently analyzed. Therefore, were excluded from this study, the images of all bacterial colonies that grew on the edge of the Petri dish, since these colonies did not grow on a flat area of the agar, which distorts considerably the light-scatter patterns.

4.3.2 Acquisition of scatter patterns

Agar plates containing about 20 and 30 colonies with the appropriate size of 1.3 ± 0.2 mm diameter, were analyzed with the BARDOT instrument. An average of 60 to 80 different images were randomly selected for the strains analyzed, which were subsequently processed to obtain a numerical representation of 50 images of the light-scatter patterns for all the strain evaluated. Thus, a pattern library was created consisting of scatter fingerprints for each one of the different strains.

4.3.3 Bacterial strains

A total of 55 bacterial strains from culture collection of Dr. B. M. Applegate Laboratory (Department of Food Science, Purdue University, IN – USA) were analyzed. Among these, it were used 21 parent bacterial strains (twenty *S.* Enteritidis and one *E. coli* Top 10), 22 bioluminescent bacterial strains (twenty *S.* Enteritidis and two *E. coli* Top 10) expressing de *lux* proteins and 12 recombinant bacterial strains (*E. coli* Top 10) expressing various proteins of three different bacterial genera. Bioluminescent strains of *S.* Enteritidis acquired the bioluminescent phenotype

through the process of conjugation with *E. coli* SV17, which contains the *luxCDABE* operon from *P. luminescens* (MENDONÇA et al. 2013 - unpublished data). Bioluminescent *E. coli* strains were transformed with plasmids encoding for the expression of *Lux* protein genes from two different microorganisms, *P. luminescens* (E. coli PL lux) and *V. fischeri* (E. coli VF lux). Recombinant *E. coli* Top 10 strains were transformed with plasmids coding for specific proteins from different foodborne pathogens, with five strains of *E. coli* Top 10 expressing genes of *E. coli* O157:H7 (genes *rfbE*, *stxI*, *stxII*, *eaeA*, and *hlyC*), five strains of *E. coli* Top 10 expressing different genes of *S. enterica* (genes *invA*, *sipB*, *spvC* and *fimY*) and three strains *E. coli* Top 10 expressing genes of *L. monocytogenes* (genes *iap*, *hlyA* and *inlA*) (DOMINGUEZ, 2004).

4.3.4 Bacterial cultures and growth condition

Each bacterial culture, stored at - 80°C in Luria Bertani (LB) broth-glycerol stock was initially cultivated in a fresh LB broth with the appropriate antibiotics. *Salmonella* strains, parent and recombinant bioluminescent strains, were cultured respectively in LB broth and LB broth containing Kanamycin (Kan) + Nalidix acid (Na) for 16 - 18h at 37°C. The *E. coli* recombinant bioluminescent strains were cultivated in LB broth containing Kan and *E. coli* Top 10 was cultured in LB broth without antibiotic, for 16 - 18h at 25°C for *E. coli* VF lux and 37°C for the other *E. coli* (*E. coli* VP lux and *E. coli* Top 10) strains. Cultures were then decimally diluted in MSM broth and dilutions were spread on the surface of LB agar plates, with the proper antibiotics. The plates were incubated at 25°C or 37°C until reached the desired colony size, and then were analyzed with the BARDOT instrument. All recombinant *E. coli* Top 10 strains were cultivated in LB broth with Kan at 37°C, overnight. Untransformed *E. coli* Top 10 was used as a control. Cultures were decimally diluted in MSM broth and dilutions were spread on LB agar plates containing Kan individually, in pairs, and in groups of three e four strains together. Plates were incubated at 37°C until the appropriate colony size (1.3 ± 0.2 mm diameter) was reached. Colonies were then analyzed using light-scattering and images compared with the control.

4.3.5 Scatter images

Light-scattering images were acquired from plates with colonies using a light scattering sensor BARDOT. Images of *S.* Enteritidis. expressing *luxCDABE* genes were compared to their parent strains to determine visual differences associated with *lux* expression. *E. coli* Top 10, *E. coli* expressing *lux* genes from *P. luminescens* (*E. coli* PL lux) and *E. coli* expressing *lux* genes from *V. fischeri* (*E. coli* VF lux) were compared by light-scattering images to determine visual differences within the same strain constructed with two different *lux* cassettes. Images of recombinant *E. coli* Top 10 strains, coding for specific exogenous proteins, were compared among themselves and with the control *E. coli* Top 10 untransformed, in order that could be detected visual differences occurring in the same strain, on different metabolic conditions.

4.4 RESULTS AND DISCUSSION

In this study, we evaluated the performance of the BARDOT system in a set of distinct strains, consisting of recombinant strains of *S.* Enteritidis and *E. coli* expressing the *lux* proteins, and recombinant strains of *E. coli* expressing particular proteins from different bacteria pathogens, in order to investigate the influence of protein expression on light-scattering images.

It is known that the bacterial contamination in food and other products usually are monitored through a standard laboratory practice of isolation, identification and characterization of the microorganisms. The gold standard method to detect and isolate bacteria species is to grow the sample in a Petri dish, which will form colonies on solid media. Typically, a bacteria colony is defined as a group of individual bacteria clustered together. However, a colony consists of not only bacteria, but also of extracellular materials, which are secreted during colony growth. Nevertheless, if used properly, these combined structures can be analyzed, since provide phenotypic characteristics distinguishable. Also, there are a variety of methods for identifying bacteria, as morphological, serological, proteomic and genomic tests, which are extremely laborious and lengthy (GRACIAS; MCKILLIP, 2004; NARAVANENI; JAMIL, 2005). In addition, samples are usually destroyed by these tests and hence are unavailable for additional confirmatory evaluation assays (BANADA et al., 2009).

Therefore, it is essential to develop techniques that allow rapid identification in real time of most epidemiologically important bacterial pathogen, in order to ensure the supply of safe foods (BHUNIA, 2011). Thus, if used the phenotypic characteristics expressed by bacterial colonies together with the appropriate equipment and tests, these can become an important tool for rapid detection, identification and investigation of metabolic conditions related to microbial growth.

A novel method of applying a forward scattering technique was developed and has been applied to detect and identify colonies of different pathogens in real time (BHUNIA, 2008). The light scatterometer BARDOT is a label-free forward light-scattering sensor (i.e., without use of biochemical reagents or genetic probes), which has been demonstrated as great promise for distinguishing bacteria cultures with highly accurate classification and broad applicability (GUO, 2004; BAYRAKTAR et al., 2006; BANADA et al., 2007). The BARDOT system uses a laser to scan the bacteria colonies and produces individual images (forward-scatter signature). These scatter images are then collected and consequently analyzed after matching with the scatter image library (BAE et al., 2008; BAI et al., 2009). Some previous studies have established scattering properties for *Listeria* species, *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Escherichia coli*, *Enterobacter aerogenes*, *Enterococcus faecalis*, *Bacillus cereus*, *Lactococcus lactis*, *Aeromonas hydrophila*, *Vibrio parahaemolyticus* and *Staphylococcus aureus* (BAYRAKTAR et al., 2006; BANADA et al., 2007; BAE et al., 2008; BAI et al., 2009; BANADA et al., 2009; HUFF et al., 2012). Interestingly, these images are both genus and specie-specific and have been shown to differentiate between strains or serotypes as well (BANADA et al., 2009; BAE et al., 2011; BETTASSO et al., 2012).

Furthermore, Rajwa et al. (2010) demonstrated that this phenotypic method of laser scattering can not only be used to classify biological samples into previously known categories, but may also contribute as an independent detection system, which can be used as an inexpensive tool that allows the screening of a large numbers of strains. However, according Banada et al. (2007), as the polysaccharides constitute the major structural component of bacterial cells, it is believed that these constituents represent an important contributing factor influencing scattering signatures. Thus, as the amounts of exopolysaccharide produced by bacteria lead to cellular changes during different stages of bacterial growth, probably result in strikingly different scatter signatures (BAE et al., 2007; BANADA et al., 2007).

Also, it is important to emphasize those changes in scatter signature of bacterial colonies are usually affected by the accumulation of metabolic by-products and distribution of cells within a colony (BANADA et al., 2009). Beyond the medium composition, oxygen, water activity and humidity are well known factors that can affect the bacterial growth rate and the colony phenotype, which supports the observation of medium-dependent difference in colony scatter patterns, verified by Bae et al. (2010), Mialon et al. (2012) and Bettasso et al. (2012). Also, it was demonstrated that genetic mutations have the capacity to modify colony scatter images if the mutation is really significant to growth and cell function (BETTASSO, 2010).

Nevertheless, the use of light scattering to detect differences within the same strain under distinct metabolic conditions has not been evaluated. Therefore, in this study, BARDOT was employed to differentiate various recombinant strains of *S.* Enteritidis and *E. coli*, particularly to detect protein expression. Light-scattering images for the bioluminescent *S.* Enteritidis showed a visual distinctive pattern as compared to the parent strains (Figure 1). In addition, BARDOT imaging resulted in two unique patterns for each *E. coli* expressing different *lux* genes, as previously expected. Moreover, these two strains of recombinant bioluminescent *E. coli* when compared to the images presented by the strain control *E. coli* Top 10, also exhibited a distinct scatter pattern (Figure 2). Besides, in the same study, it was possible to verify that the light scatterometer BARDOT can be used as well, to select and discriminated recombinant colonies of *E. coli* Top 10, expressing different proteins from distinct bacteria, even when these bacteria were spread together in the same agar plate (Figure 3).

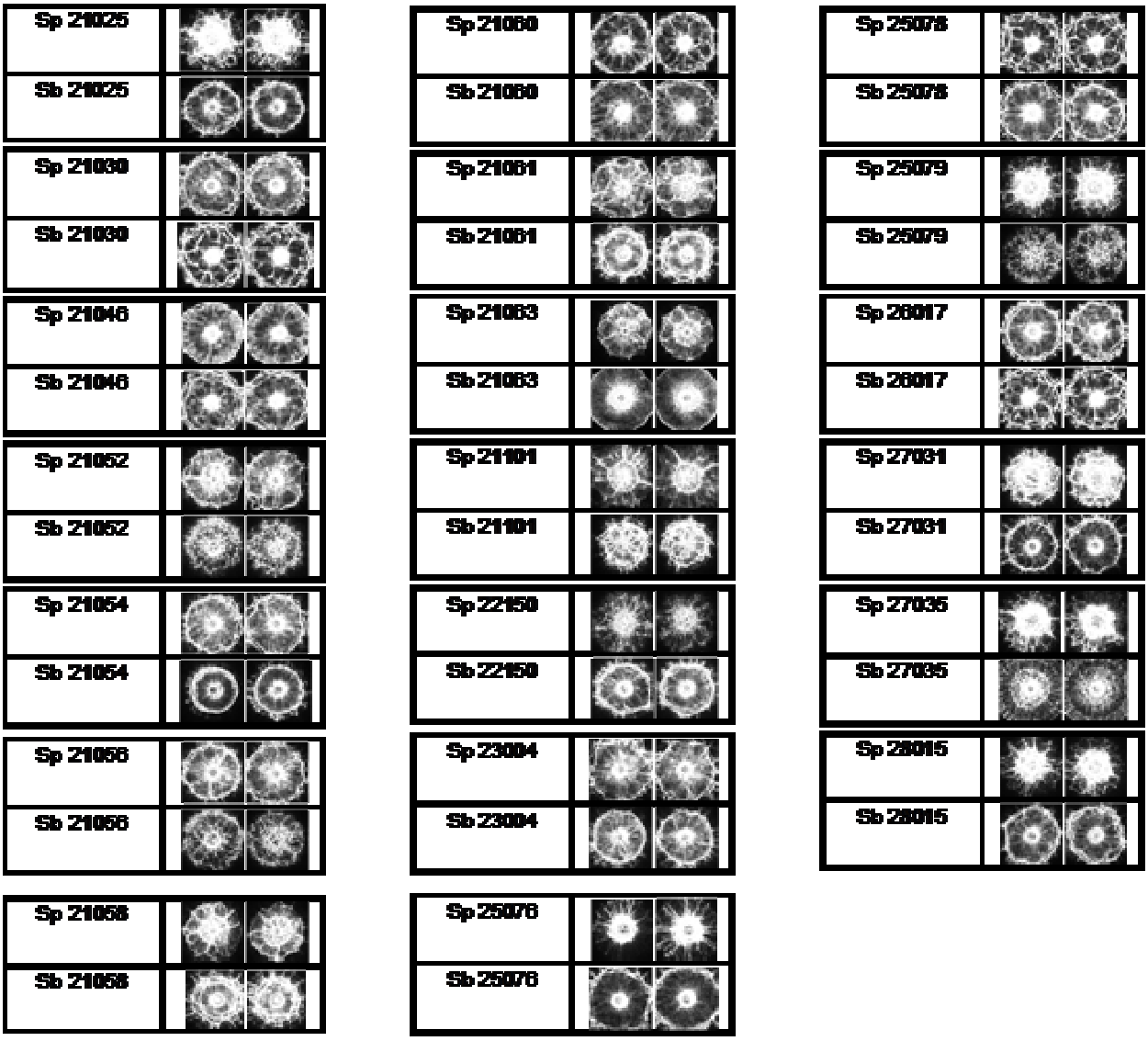

Figure 1. Comparison of scatter patterns between *S.* Enteritidis parent and *S.* Enteritidis bioluminescent expressing *luxCDABE* genes. Sp: *Salmonella* parent; Sb: *Salmonella* bioluminescent.

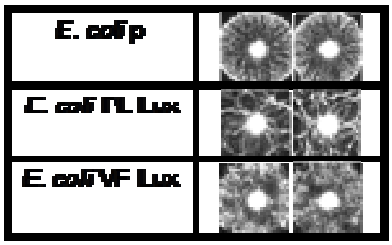

Figure 2. Comparison of scatter patterns among *E. coli* Top 10 parent and bioluminescent *E. coli* Top 10 expressing different *luxCDABE* genes. *E. coli* p: *E. coli* Top 10 parent; *E. coli* PL lux: bioluminescent *E. coli* Top 10 expressing the *Lux* proteins from *P. luminescens*; *E. coli* VL lux: bioluminescent *E. coli* Top 10, expressing the Lux proteins from *V. fischeri*.

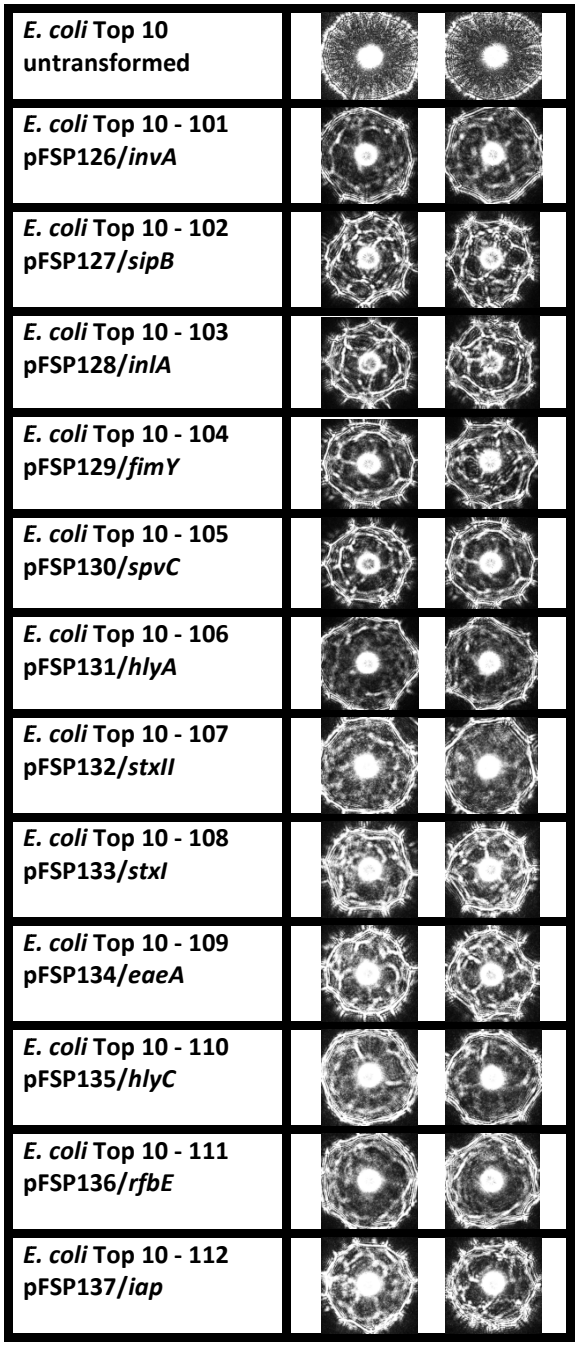

Figure 3. Comparison of scatter patterns among *E. coli* Top 10 untransformed and *E. coli* Top 10 strains transformed with distinct plasmids encoding for specific proteins from three different foodborne microorganisms. Recombinant *E. coli* Top 10 strains were transformed with plasmids coding for specific proteins from different foodborne pathogens, with five strains of *E. coli* Top 10 expressing genes of *E. coli* O157:H7 (strains 107, 108, 109, 110 and 111), five strains of *E. coli* Top 10 expressing different genes of *S. enterica* (strains 101, 102, 104 and 105) and three strains *E. coli* Top 10 expressing genes of *L. monocytogenes* (strains 103, 106 and 112).

The collected patterns for *E. coli* recombinant strains appear to be visually distinct from each other, when compared among them and with the control strain *E. coli* Top 10. Initially, each one of the recombinant *E. coli* strains expressing different proteins were spread individually in LB agar plates in order to starting the library of scatter images. Later, these strains were plated in pairs as well as in groups of 3 to 4 strains in the same LB agar plate and even being plated as a mixture, the BARDOT sensor was able to differentiate all these strains. Therefore, in order to prove the efficiency of the BARDOT system for the differentiation of recombinant *E. coli*, strains expressing distinct proteins from different types of microorganisms, first all the twelve recombinant strains were plated in pairs at the same LB-Kan agar plate. Thus, were grown together the strains 101 and 102, 103 and 104, 105 and 106, 107 and 108, 109 and 110, 111 and 112. Detailed analysis using imaging the software DotBar showed a difference of 90,0% to 93,0% for strains 101 and 102, while for the strains 103 and 104, the difference was 60,8% to 84,6%. Already, the recombinant strains of *E. coli* 105 and 106, demonstrated a difference of 83,2% to 86,5%, whereas strains 107 and 108 exhibited a difference of 83,4% to 86,6%. In addition to these, when analyzed the recombinants *E. coli* strains, numbers 109 and 110 demonstrated a difference of 55,3% to 93,4%, as well as the strains numbers 111 and 112, exhibited a difference of 81,5% to 88,1%, respectively (Figure 4).

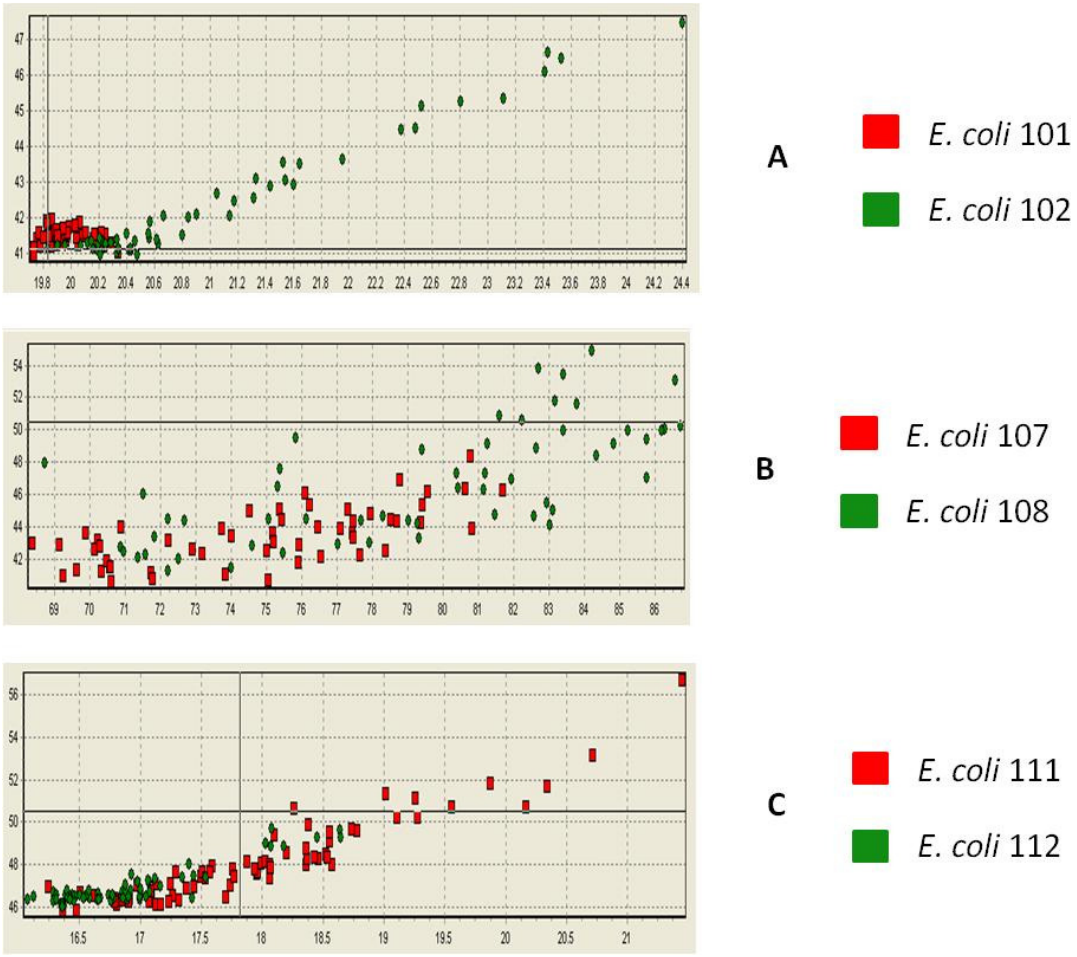

Figure 4: DotBar analysis of recombinant *E. coli* strains scatter patterns. Detailed analysis using imaging software showed a significant difference between the patterns obtained from both recombinant *E. coli* strains.

Also, other interesting results were observed when strains of recombinants *E. coli* plated on pairs were subjected to analysis comparing the scatter patterns obtained from these plates with patterns of each individual strains, stored in the scatter pattern library. Analysis using the software DotBar showed a similarity of 90,0% and 96,1% for strains of *E. coli* 101 and 102, respectively, when compared with previously stored scatter patterns. For the recombinant strains 103 and 104 were observed similarity of only 58,6% for the 103 strain, whereas for strain 104, this similarity was 89,1%. A similarity of 97,9% and 80,2% was found for strains of *E. coli* 105 and 106, respectively, when its patterns were compared to the scatter patterns library. The strains 107 and 108 exhibited a similarity of 85,3% and 86,2% with the patterns of the library, whereas for the strains 109 and 110, this similarity was 55.3%

and 93.4%, respectively. And for the recombinant *E. coli*, strains 111 and 112, the similarity observed with the scatter patterns found in the library, were respectively of 84,2% and 86,5%. Some graphics with the results obtained from the analysis with the software DotBar are shown in Figure 5.

Moreover, the *E. coli* strains were further cultured in groups of three and four strains on the same plate. However, for the great majority of these recombinant strains plated in groups, the similarity results obtained were usually less than 70% when compared to the pattern stored in the scatter library. Our results for recombinants *E. coli* cultured in a group of three strains was observed for strains 101, 102 and 103, which have shown a similarity of 88.3%, 74.4% and 70.4%, respectively, with those scatter previously found (Figure 5). In addition, another good result was observed when the strains were grown as a group of four different recombinants *E. coli*, and strains numbers 105, 106, 107 and 108, respectively exhibited a similarity percentage of 78.8%, 71.6%, 62.3% and 73.1%, with the patterns of scatter library (Figure 5).

Consequently, we found that measurements using the BARDOT system were highly reproducible with the grayscale images being used directly as a quick and easy way to distinguish the recombinant strains. However, these similarity values are very relevant, since it is important to emphasize that our most significant result was found when comparing the patterns shown by the recombinant *E. coli* strains with the pattern exhibited by the strain of *E. coli* Top 10 untransformed.

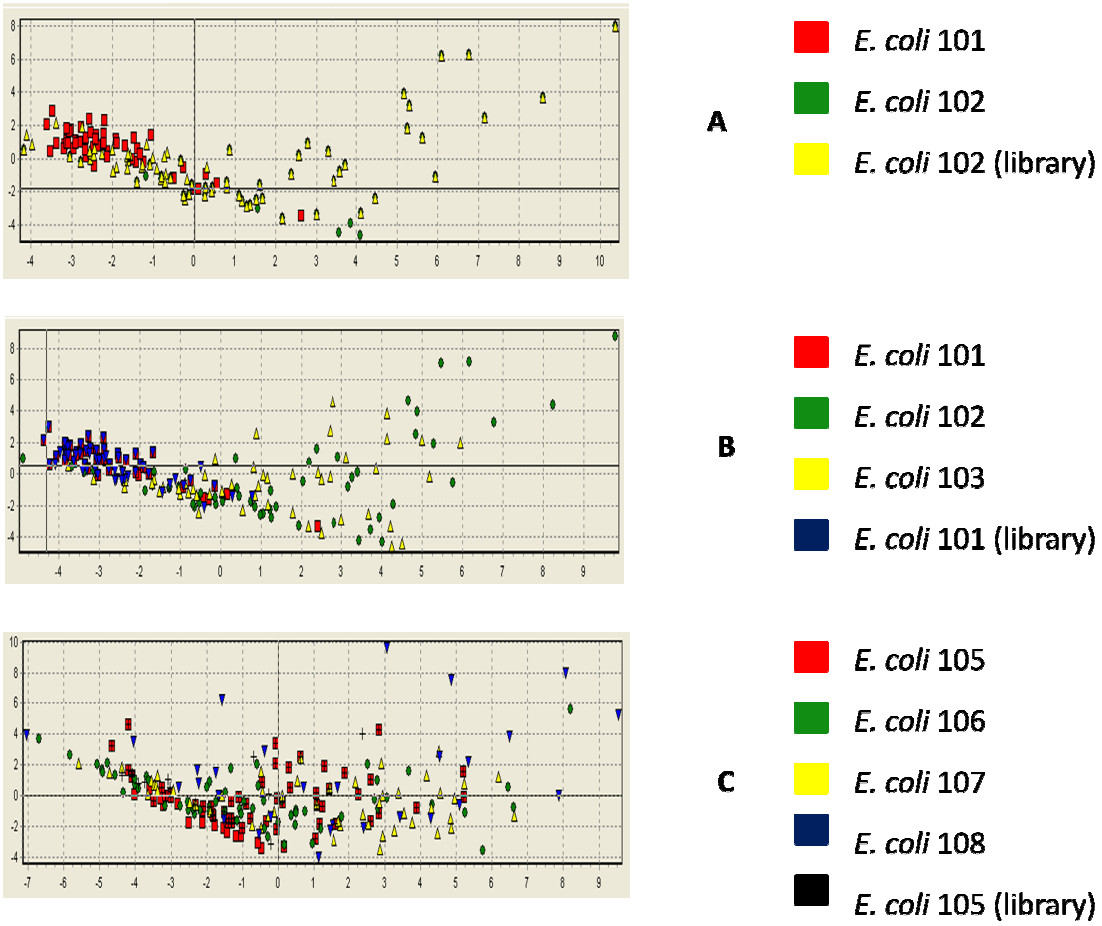

Figure 5: DotBar analysis of recombinant *E. coli* strains scatter patterns. Detailed analysis using imaging software showed a significant similarity between the patterns obtained from both recombinant *E. coli* strains.

Furthermore, it is important to explain that in this forward-scattering measurement, we excluded all bacterial colonies growing on the border of the Petri dish, since these colonies do not grow in a flat agar area, which will probably distorts the forward-scattering pattern considerably (BAE et al., 2009). As a result, our study showed that the BARDOT system was able to detect recombinant colonies on agar plates without the need for costly and time-consuming microbiological tests, thus reducing the time to identify recombinant pathogenic bacteria. In addition, the BARDOT system has proved to be a powerful tool allowing for non-invasive and non-destructive differentiation of various bacteria strains. Moreover, it is important to emphasize that these initial analysis were performed without the use of biochemical

or molecular techniques, ensuring that the colonies are available for further analysis. Also, BARDOT does not require any probes or labeling reagents and the sample preparation is quite simple, since the laser can be emitted directly on bacterial colonies, displaying the images that are captured to define the predominant scatter pattern.

In summary, we have demonstrated a method for the identification of recombinant strains of *S.* Enteritidis and *E. coli*, using a forward light-scattering sensor BARDOT. This method offers a fast and reliable way to detect recombinant strains on agar plates. However, since this is the first study investigating the scatter pattern of recombinant strains, additional studies with different bacteria are necessary to improve the BARDOT library, in order to make the BARDOT system more functional. In addition, it is important to keep in mind that the construction of such a library could be extremely difficult due to the high variety of sequences that may be introduced into the microorganisms by genetic recombination.

4.5 CONCLUSION

Our results suggest that the light scatterometer BARDOT can be used for the selection and discrimination of recombinant colonies, since this system was able to detect the expression of *luxCDABE* genes in *S.* Enteritidis and differentiated proteins expressed from *lux* genes in *E. coli* PL *lux* and *E. coli* VF *lux*, once these recombinant bioluminescent strains expressing the bioluminescent phenotype showed distinct visual pattern when compared to the parent strains. Also, the BARDOT system, proved able to select and discriminate recombinant colonies of *E. coli* expressing different proteins from other microorganisms, as these strains had unique scatter patterns.

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5 Conclusão

A bioluminescência é um processo de emissão de luz que ocorre em organismos vivos específicos, como consequência de uma reação enzimática. Nesse processo, a energia química produzida por estes organismos é convertida em luz visível, a qual fornece inúmeras vantagens e tem sido utilizada para diversos fins biológicos, os quais incluem camuflagem, repulsão, atração, comunicação e iluminação. Atualmente, com os constantes avanços na engenharia genética, muitas espécies de bactérias normalmente não-luminescentes tem sido geneticamente modificadas a fim de tornar estes micro-organismos bioluminescentes. Assim, através da utilização do cassete *luxCDABE* completo é possível que a bioluminescência possa ser expressa de forma contínua, sem necessidade de substratos exógenos. Portanto, as bactérias biorrepórteres bioluminescentes permanecem inteiramente auto-suficiente em sua capacidade de produzir luz em resposta a diferentes condições ambientais. Além disso, os ensaios bioluminescentes fornecem resultados em tempo real, uma condição que não é uma característica normal da maioria dos testes convencionais.

Consequentemente, as bactérias biorrepórteres bioluminescentes podem ser utilizadas como um método eficaz para controlar a fisiologia celular bacteriana em tempo real. Assim, em nosso estudo, através da construção de cepas biorrepórteres bioluminescentes de *S.* Enteritidis foi possível avaliar a resistência desse patógeno microbiano frente ao composto antimicrobiano natural carvacrol, bem como investigar a expressão da proteína Lux e de outras proteínas em diferentes cepas recombinantes de *S.* Enteritidis e *E. coli*, utilizando o sistema BARDOT, um sensor que capta imagens de dispersão de colônias bacterianas isoladas em placas de meio de cultivo.

Portanto, nós podemos concluir que o uso de ensaios de bioluminescência vem permitindo uma série de desenvolvimentos na área de microbiologia de alimentos, os quais devido a sua facilidade de uso, em combinação com um ganho considerável de tempo, tornam este método uma ferramenta poderosa e que pode servir para diversas aplicações como a avaliação de eficácia antimicrobiana para inativação de patógenos e o monitoramento de viabilidade e lesão celular.

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Apêndices

APÊNDICE A – Tabela 1

Table 1. *Salmonell*a enterica strains serotype Enteritidis and isolation sources

***Salmonella* serovar/ Strain Number**	**Source**
S. Enteritidis/21025	Mouse spleen
S. Enteritidis/21030	Mouse spleen
S. Enteritidis/21046	Egg contamination
S. Enteritidis/21052	Egg yolk
S. Enteritidis/21054	Egg yolk
S. Enteritidis/21056	Egg yolk
S. Enteritidis/21058	Egg albumen
S. Enteritidis/21060	Egg albumen
S. Enteritidis/21061	Egg albumen
S. Enteritidis/21063	Chicken spleen
S. Enteritidis/21101	Chicken feces
S. Enteritidis/22150	Egg contamination
S. Enteritidis/23004	Homogenized egg
S. Enteritidis/25076	Outbreak NC2005-001080 pattern D
S. Enteritidis/25078	Outbreak NC2005-000836 pattern D
S. Enteritidis/25079	Outbreak NC2005-000875 pattern B
S. Enteritidis/26017	Chicken Gilmour PT13 Quebec
S. Enteritidis/27031	Chicken Liver
S. Enteritidis/27035	Chicken lower oviduct
S. Enteritidis/28015	MocR22079 KanR

APÊNDICE B – Tabela 2

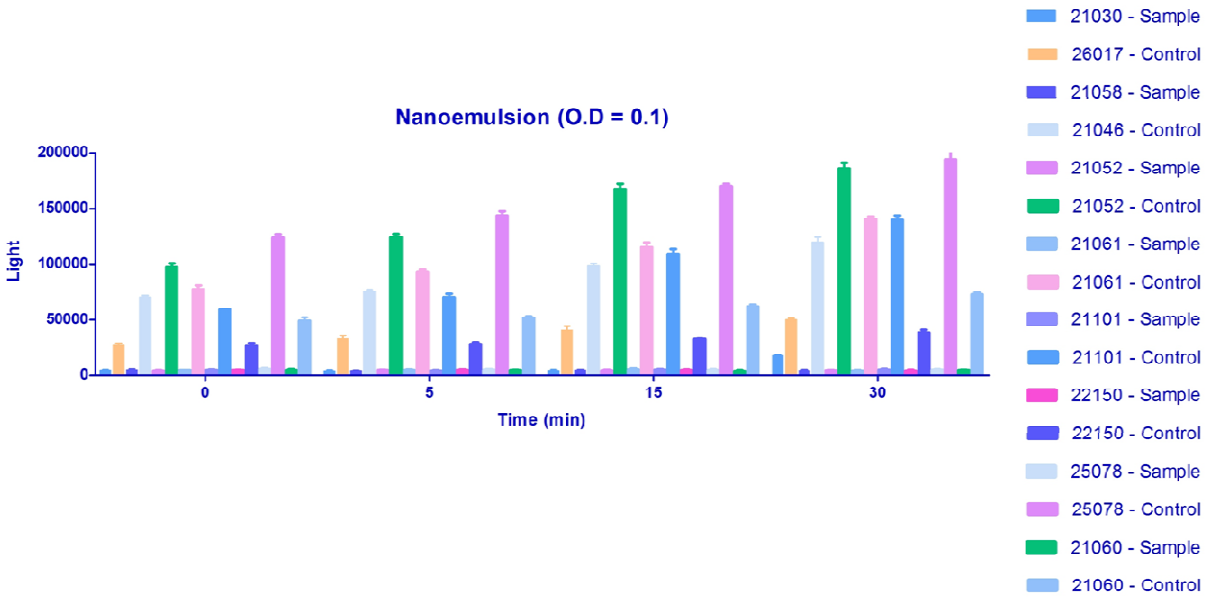
Table 2. Plate count after application of nanoemulsions (Sample and Control)

Strains Number	Sample	Control
Optical density (OD) 0.5		
21030	*WG	3,1 X 10^7
21058	*WG	5,0 X 10^7
21052	*WG	3,5 X 10^7
22150	*WG	1,6 X 10^7
21101	*WG	3,0 X 10^7
25078	*WG	1,8 X 10^7
21052	*WG	3,5 X 10^7
22150	*WG	1,6 X 10^7
21061	*WG	5,9 X 10^7
21046	*WG	1,9 X 10^7
26017	*WG	1,5 X 10^7
21046	*WG	6,0 X 10^6
21060	*WG	1,9 X 10^7
Optical density (OD) 0.1		
21030	*WG	1,0X 10^7
21058	*WG	3,1 X 10^7
21052	*WG	2,0 X 10^6
22150	*WG	1,0 X 10^6
21101	*WG	3,0 X1 0^6
25078	*WG	1,4 X 10^7
21052	*WG	2,0 X 10^6
22150	*WG	1,0 X 10^6
21061	*WG	3,0 X 10^6
21046	*WG	1,0 X 10^6
26017	*WG	1,5 X 10^7
21046	*WG	1,0 X 10^6
21060	*WG	3,0 X 10^6

*** WG:** Without Growth

APÊNDICE C – Figura 1

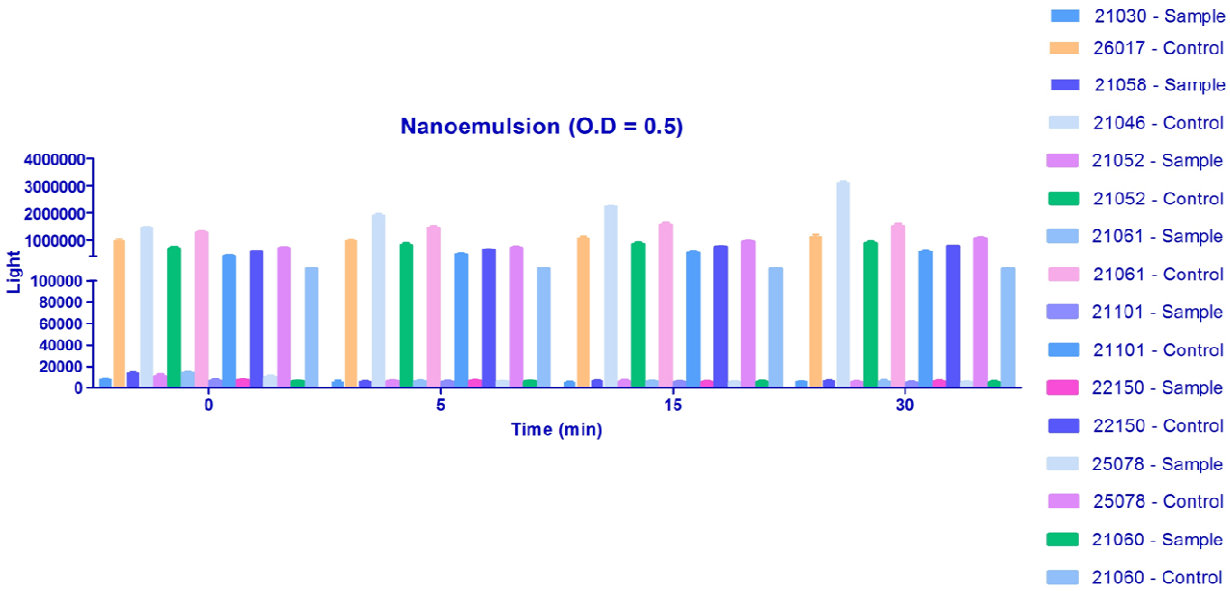
Figure 1. Effect of nanoemulsions (Sample and Control) for inactivation of bioluminescent *Salmonella enterica* strains



Sample: nanoemulsion with carvacrol; **Control:** nanoemulsion without carvacrol.

APÊNDICE D – Figura 2

Figure 2. Effect of nanoemulsions (Sample and Control) for inactivation of bioluminescent *Salmonella enterica* strains



Sample: nanoemulsion with carvacrol; **Control:** nanoemulsion without carvacrol.

Anexos

ANEXO A – Tabela 1

Table 1. Genes selected for detection of *E. coli* O157:H7.

Genes	References	Description
rfbE	Bilge *et al.* (1996) Stroeher *et al.* (1992)	This gene is a homolog of *rfbE* found in *Vibrio cholerae*. The gene encodes the perosamine synthetase O1, found in association with the lipopolysacharides in the cell wall. The expression of this gene is necessary for the expression of the O157 antigen of *E. coli* O157:H7.
stxII	Strockbine *et al.* (1986)	These genes are homologous to the Shiga toxins produced by *Shigella dysenteriae*. These toxins, named Shiga-like toxin I and Shiga-like toxin II, cause paralysis and death in mice, and accumulate in rabbit ileal segments. *E. coli* produces two genetically related but antigenically distinct cytotoxins with similar biologic activities. Strains of *E. coli* that produce elevated levels of one of these toxins or both have been associated with the clinical syndromes of diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome.
eaeA	Louie *et al.* (1993) Yu and Kaper (1992) Jerse *et al.* (1990)	The *eae* operon is responsible for the attaching and effacing histopathology characteristics of *E. coli* EHEC and *E. coli* EPEC. The *eaeA* gene, from this operon, produces a 94-kDa outer membrane protein, intimin, which is necessary, although not sufficient, to produce the attaching and effacing lesion.
hlyC	Schmidt *et al.* (1995)	The EHEC-*hlyC* produces a 19.9 kDa peptide constituent of the EHEC hemolysin protein. The EHEC hemolysin was responsible for the enterohemolytic phenotype as it occurs in all O157 strains tested.

Source: DOMINGUEZ, W. Multiplex PCR for the detection of foodborne pathogens: *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes*. **Master of Science**. Purdue University, West Lafayette, IN, 2004.

ANEXO B – Tabela 2

Table 2. Genes selected for detection of *S. enterica*

Genes	References	Description
invA	Scholz *et al.* (2001) Galan *et al.* (1992)	**The *invA* gene is a necessary gene of the *inv* operon for intestinal epithelium cell invasion. PCR-based identification has been successful with *S. typhimurium*.**
sipB	Wood *et al.* (1996) Hermant *et al.* (1995)	The *sopE* gene, which codes for *Salmonella* outer protein E, is an invasion-associated secretory protein of *S.* Dublin. This protein is dependent on the *sip* (*Salmonella* invasion proteins) operon. The *sipB* is essential for the ability of the pathogen to invade epithelial cells. The five adjacent genes, *sipEBCDA*, were also identified in *S.* Typhi and were designated as being essential for cell invasion.
spvC	Chu *et al.* (1999) Krause *et al.* (1992) Barrow and Lovell (1989) Jones *et al.* (1982)	The cluster of five plasmid genes, designated *spvR*, *spvA*, *spvB*, *spvC*, and *spvD*, is sufficient to express the plasmid-related virulent phenotype in *S.* Dublin. These genes are located in a large plasmid, responsible for adhesive, invasive, and virulent phenotypes. This plasmid is also present in *S.* Typhimurium, *S.* Choleraesuis, *S.* Enteritidis, *S.* Gallinarum, and *S.* Pullorum.
fimY	Tinker and Clegg (2000) Yanisch-Perron *et al.* (1985)	Type 1 fimbriae are bacterial adhesins characterized by their ability to mediate mannose-sensitive binding to eukaryotic cells in vitro, thus being necessary for pathogenicity. In *Salmonella enterica*, as in other microorganisms, this external organelle is coded by the *fim* operon. The expression of type 1 fimbriae was significantly reduced when mutants of *fimY* and *fimZ* were studied, suggesting that these two genes upregulate the expression of this binding mediator.

Source: DOMINGUEZ, W. Multiplex PCR for the detection of foodborne pathogens: *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes*. **Master of Science**. Purdue University, West Lafayette, IN, 2004.

ANEXO C – Tabela 3

Table 3. Genes selected for detection of *L. monocytogenes*.

Genes	References	Description
iap	Kuhn and Goebel (1989)	The invasion-associated protein p60, coded by the *iap* operon, is responsible for *L. monocytogenes* invasion characteristics. Mutants impaired in the synthesis of this protein were unable to invade nonprofessional phagocytic 3T6 mouse fibroblast cells.
hlyA	Mengaud *et al.* (1988)	Listeriolysin 0, coded by the *hlyA* gene, is responsible for the hemolytic, lysis of red blood cells, characteristics of *L. monocytogenes*. This gene has been detected in all *L. monocytogenes* strains, even in the nonhemolytic strains, using DNA-DNA hybridization, but has not been detected in other *Listeria* species.
inlA	Pizarro-Cerda *et al.* (2004) Rousseaux *et al.* (2004) Cai *et al.* (2002) Jonquieres *et al.* (1998)	Internalin, coded by the *inlA* gene, is a surface protein that mediates entry of *L. monocytogenes* into epithelial cells in humans and poultry. This gene has been successfully employed previously for identification purposes.

Source: DOMINGUEZ, W. Multiplex PCR for the detection of foodborne pathogens: *Escherichia coli* O157:H7, *Salmonella enterica*, and *Listeria monocytogenes*. **Master of Science**. Purdue University, West Lafayette, IN, 2004.