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Detection of *Listeria monocytogenes*, *Escherichia coli* and *Salmonella species* in Ready-to-eat Salad by Rapid Microbiological Detection Methods (Redox potential measurement + Real-time PCR)

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1. List of Abbreviations

BBL	Brilliant green bile broth
CDC	Centers for Disease Control and Prevention
Cfu	Colony forming unit
DNA	Deoxyribonucleic acid
E. coli	Escherichia coli
ELISA	Enzyme Linked Immunosorbent Assays
HUS	Hemolytic uremic syndrome
ISO	International Organization for Standardization
LEB	Listeria enrichment broth
MSZ	Magyar Szabványügyi Testület
NaCl	Sodium chloride
No.	Number
PCR	Polymerase chain reaction
RVS	Rappaport-Vassiliadis Soya Peptone Broth
Spp.	Subspecies
TTD	Time to Detection
UN	United Nations

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2. Introduction

When it comes to food and well-being, consumers are more health conscious than they were a few decades ago. There is a growing need for people to eat better and lower their calorie intake, and in many parts of the world, the star of healthy eating is the salad. But we are also living in an era where time is a precious commodity, a lifestyle where people opt for the fastest and cheapest way to fulfil these basic life necessities. Previously, cooking had been a daily chore, taking up long hours of the day as meticulous details and thought were put into providing a delicious meal for the family. Now, it has become a tedious activity for many. Rather than buying, cleaning, cutting, and preparing their daily serving of vegetables from scratch, people are opting for readymade salads from supermarket shelves instead.

The advances in technology in this modern age has changed how people live. With vehicles, machines, and screens decreasing people's mobility, the result of a lack of physical exercise are obesity and unhealthy snacks. Eating a diet of vegetables has been linked with a decrease of a numerous amount of fatal diseases ranging from gastrointestinal to cardiovascular problems. (Dias, 2012).

Vitamins specifically A and C, minerals, antioxidants and phytochemicals are one of the most important health benefits of eating vegetables in a human diet in addition to providing a big source of dietary fibre. (Joanne, 2012). However, time is still an issue, and grabbing your on-the-shelf-ready to toss salad bag is a big-time saver.

Since these food products are eaten raw and are minimally processed there is an increased risk for pathogenic contamination. Moreover, they are usually eaten as well without further washing and decontamination. Recently these products have been recognized as potential vehicles of food contamination with harmful pathogens.

The aim of this study is to examine the possibility of application of the redox potential based measurement method for the detection of *Listeria monocytogenes, salmonella, and Escherichia coli* in Ready-to-eat salad. In case of detection of *Listeria monocytogenes and Salmonella* further confirmation will be done by real-time PCR. In the next step, samples obtained from supermarkets were examined to detect *Listeria monocytogenes, Salmonella and E. coli.*

3. Literature review

3.1. Microbiological risk of Salad consumption

However, one of the issues regarding the safety consumption of these salads is the possibility of contamination during the farming phase and the final packaging of the product. Microbiological risk might occur at any of the production phases, since they are minimally processed and are generally consumed raw. (Tyrrel et al., 2006). Contaminated water possibly joint with animal pasture could be responsible for input of pathogens into the irrigation system of these vegetables, especially if the water source itself is contaminated. (Berger et al. 2010). Manure used as fertilizer for the soil also possesses risk for contamination, not to mention the possibility of insects and fomites coming in contact with the vegetables during the preharvest process. (Park et al., 2012). The equipment used during harvest packaging of the product could also contribute heavily to the contamination of the greens with a high microbial count. (Splittstoesser, 1970). Even postharvest, washing of the leafy vegetables could pose a risk with unhygienic water during the cooling and hydrating of the vegetables. (Gombas et al., 2017).

The integrity of this readymade product relies on good hygiene methods from farm to fork. Since vegetable crops will always be affected by microbial agents, expecting otherwise would be unrealistic. Therefore, the importance of washing and decontamination, as well as storage and distribution control of appropriate temperatures, is highly important to control the presence of pathogens. (Sagoo et al., 2003). Decontamination of the ready-to-eat salad is done while washing the leafy greens with 50 to 200 ppm chlorine water, and then sent for cutting and packaging, but recontamination of the product with pathogens could occur during the cutting operation. (Jay et al., 2005).

The safety of consumers could be compromised due to the occurrence of pathogens in these salads (Gurler et al.,2014), especially seeing that they eat the vegetables without further cooking post purchase, posing a major food-safety risk associated with ready-to-eat (RTE) products. (Ricke et al.,2013). In a Turkish study performed on 261 samples of RTE salads, the packages were found to contain 8% of *salmonella sp*, and 10% *of listeria sp* and 4% *E. coli.* (Gurler et al.,2014). Another study in Sao Paolo, Brazil showed the prevalence of *listeria monocytogenes* in 3.1% of the samples from a total of 512 packages, with 5 samples having counts between 1.0×10^1 and 2.6×10^2 CFU/g. (Anderson et al.,2011). In 2001, another study on RTE salads showed that 0.5% of the samples were of unsatisfactory microbiological value,

posing a health risk to the public due to the presence of *salmonella sp* in 5 samples and a *listeria monocytogenes* count of 660 CFU/g. (Sagoo et al., 2003).

Due to the increasing request of improving food safety and health for the public, the demand for testing of pathogens has been improved. (Balachandran et al., 2012). Modern methods have become less time consuming and more efficient compared to the previous culture based methods. (Gouws et al., 1998). It takes 3 to 4 days to obtain the results of the conventional culturing method, which puts food producers and lab technicians at a disadvantage. Meanwhile, screening big quantities of samples nowadays takes less than a day. (Fang et al., 2003).

3.1.1. *Salmonella spp.*

The most important gram-negative bacteria causing foodborne gastroenteritis is the genus *Salmonella.* (Jay et al., 2005). This bacterium is found abundantly in nature and its primary reservoir is both humans and animals. The primary cause of food poisoning is due to the ingestion of a big number of toxic strains of *Salmonella*. (Jay et al., 2005). It is estimated to cause 1.4 million illness and 600 deaths per year in the united states alone. (Chittick et al., 2006). The *Salmonellae* are indistinguishable from E. coli under the microscope due to their similar morphology both being small, gram negative, non-spore forming rods. (Jay et al., 2005). The genus *Salmonellae* are a member of the enterobacteria family. This is due to them being oxidase negative, and their ability to ferment glucose and reduce nitrate to nitrites. (Ricke et al., 2013). They can grow at a wide range of temperatures between 2-47°C; the optimum being 25-43°C. The optimal PH is between 6.5 to 7.5 with an NaCl concentration of ≥ 3%. (D'Aoust, 2001).

There are around 2600 known serotypes of *Salmonella* species that can be differentiated by the agglutinating properties of the somatic O, flagellar H, and capsular Vi antigens. (Guibourdenche et al. 2010). Diseases caused by *Salmonella* species depend on the serotype and its host specificity. (Uzzau et al. 2000). Host specific diseases for humans include severe systemic illnesses like septicaemic typhoid syndrome caused by *Salmonella Typhi*. Other forms of diseases caused by non-typhoidal, food Bourne *Salmonella* species could potentially be the source of multiple gastroenteritis infections. (Acheson and Hohmann, 2001). Humans typically contract these Salmonellosis infections through contaminated water or food. (Alcaine et al., 2007).

With antimicrobial resistance increasing through the years due to an increase of antibiotic use in animal production, Salmonellosis infection has become a big issue in human health. Once in the blood stream, drugs of choice are not being as effective, but instead are hindering the treatment process of the patient. In immunocompromised individuals especially, being infected by Salmonellosis can be an extremely invasive and detrimental disease. (Tauxe, 1991).

Contamination of crops with *Salmonella* species can occur by using wastewater during irrigation (Melloul et al., 2001), or by using bovine manure as fertilizer for vegetables. (Islam et al., 2004). Moreover, human handling, and passing wildlife animals and insects could all be a potential source of the bacteria. (Buck et al., 2003).

While the majority of outbreaks regarding Salmonella are linked to products of animal origin, many outbreaks can be traced back to plant based produce. (Berger et al., 2010). According to the Public Health Laboratory Service, in the year 2000, infections of two strains linked to the consumption of ready-to-eat lettuce: *Salmonella Typhimurium* DT 104 and *Salmonella Typhimurium* DT 204b caused two outbreaks in England and Wales, resulting in one death and affecting 174 people. There was a similar outbreak in Iceland, affecting 183 people. It was also caused by the consumption of imported lettuce contaminated with *Salmonella Typhimurium* DT 204b. Not to mention several affected individuals in Scotland and Germany who were also identified to be affected by the same serotype, *Salmonella Typhimurium* DT 204b. Another outbreak in Finland in 2008, caused by pre-chopped iceberg lettuce contaminated with *Salmonella*, affected 100 people resulting in the death of two women aged 85 and 102. (Lienemann et al., 2011).

A study was done to detect pathogen in ready-to-eat salads which showed that out of 3,845 samples 0.1% of the samples were contaminated with *Salmonella* species. In 3 of the samples, *Salmonella Umbilo, and Salmonella Newport and Durban* were each found in a different sample. (sagoo et al., 2002).

3.1.2 Escherichia coli

Escherichia coli is a gram-negative, facultative anaerobic, non-spore forming bacteria, that resides in the lower intestine of humans as a harmless inhabitant in the gut flora. (Singleton, 1999). According to the CDC, the majority of the *E. coli* strains don't pose any harm on human health, but some serotypes can cause serious damage in their host. There are 6 types of

pathogenic *E. coli*. The most significant strain being the Shiga toxin-producing *E. coli* by means of *E. coli* O157:H7, a major culprit of foodborne outbreaks. If one is lucky, it can manifest as an uncomplicated diarrhoea but it can also be the culprit of a severe haemorrhagic colitis or sometimes reach a fatal outcome due to haemolytic uremic syndrome. (Noris and Remuzzi, 2005). But even other non O157:H7 serotypes were also identified as having a role in severe diseases caused by *E. coli*, having a higher prevalence in sporadic caused HC and HUS than the strain O157:H7.

E. coli O157:H7 can be found in the majority of wild and domesticated species, but the chief host of that serotype resides in asymptomatic colonized ruminants. (Dipineto et al., 2006). People can become infected with this strain from food or water directly, or indirectly through faeces of animals or humans. (CDC, 2017). The possibility of *E. coli* O157:H7 entering the edible part of a lettuce through migration from contaminated irrigation water is possible. And once contaminated, the removal of the pathogen through a process of disinfection is not very efficient. (Solomon et al. 2002). Kudva et al and Chalmers et al indicated in their studies that *E. coli* can survive for a long time in soil and water.

While harvesting leafy vegetables, there is a possibility of damage and handling from farmers, which poses a risk of contamination by opportunistic bacteria. Unfortunately, there isn't sufficient data backing up the degree of contamination of *E. coli* during that process. (Delaquis et al., 2007). Throughout the processing period, the leafy vegetables go through numerous steps including trimming, cutting and washing, all of which can drastically affect the integrity of the leaves and pose a threat for the inoculation of *E. coli*. (Bolin et al., 1977). After packaging, the temperature control during storage, transportation and on supermarket shelves of the ready to eat salads is of extreme importance. Delaquis et al, 2007 proved that the survival and growth of *E. coli* O157:H7 can predominate at temperature of greater or equal to 8 degrees.

During the 1980's, the main source of *E. coli* illness in humans was thought to be solely from the ingestion of animal products contaminated with the pathogenic strain (Riley et al., 1983). But in the 1990's, due to many outbreaks caused by the consumption of agricultural produce, it was evident that plants can be indirectly contaminated with *E. coli*, and the ingestion of leafy vegetables could cause diseases with the strain O157:H7. (Harris et al., 2003). Between 1982 and 2002, 21% of outbreaks where caused by fresh produce, with lettuce being one of the major causes. (Rangel et al., 2005). Lettuce was another source of an *E. coli* O157:H7 outbreak in 1995 in western Montana, Maine, Ontario, and Alberta. (Lammerding, 1996). The outbreak of

40 confirmed laboratory O157:H7 *E. coli* cases in Montana traced back to purchased lettuce being the origin of the foodborne illness. (Ackers et al., 1998).

The infective dose of O157:H7 is less than 1000 cells, therefore even low amounts in leafy vegetables can pose a health risk to humans. (Ackers et al., 1998).

3.1.3 Listeria monocytogenes

Listeria monocytogenes is a gram positive intracellular bacterium responsible for a number of outbreaks. (Peterkin, 1991). *Listeria* species are catalase-positive, oxidase-negative, methyl red positive, Voges-Proskauer positive and negative for citrate utilization, indole production and urea hydrolysis. (Benson, 2002). *L. monocytogenes* can grow under both aerobic and anaerobic conditions, although it grows better in an anaerobic environment (Sutherland et al. 2003). It is found naturally in decaying plant material, soil, animal faeces, sewage, water, and animal feeds. (Schuchat et al., 1991)

Listeria monocytogenes can cause a serious disease called Listeriosis, especially in high risk patients, where it often becomes fatal. (Doganay, 2003). Even if *listeria monocytogenes* is the most harmful of the *listeria* species, the presence of any species of *listeria* in food indicates poor hygiene practice. (Sagoo et al., 2003)

L. monocytogenes is across the board in nature and can taint an extensive variety of foods. It is most ordinarily connected with chilled ready-to-eat food, for example, cooked cut meats, smoked fish, cooked shellfish, delicate form aged cheeses, pate and pre-arranged sandwiches and salads that don't require additionally cooking or warming. Such foods have a high processing level which in turn stretches their shelf life, thus providing *Listeria monocytogenes* adequate time to spread. (McLauchlin, 1993)

L. monocytogenes can be found in a lot of raw products especially leafy vegetables (Beachat, 1998). Due to the prevalence of *listeria* causing disease in people and the potential of it being fatal, there have been a lot of studies focusing on the detection of the bacteria in vegetables. (Beachat, 1998). The first ever documentation about a listeria outbreak was in Canada in 1980, due to the consumption of contaminated coleslaw (Schlech et al., 1983). In Malaysia, a study found 22% of their samples to contain the pathogen in leafy vegetables. (Arumugaswamy et

al., 1994). Another study in Australia that includes 120 packages of bagged lettuce, found *Listeria monocytogenes* in 2.5% of the samples. (Szabo et al., 2000).

Since the pathogen is predominantly found in the soil, and the roots of leafy vegetables have a high contact potential, then they are more likely to have an increase in contamination risk. (Heisick et al., 1989). It can also survive for 56 days in the soil (Everis, 2004). Although everyone agrees that there should be minimal contamination of this bacteria in food, countries differ in the contamination level, for example in the United States there is a zero-tolerance policy where there should not be any presence of the bacteria in 2 samples of 25g of food. On the contrary, some levels in Europe demand <100cells of the bacteria in 1 gram of food when being consumed. (Nørrung, 2000).

Listeria monocytogenes can survive and grow over an extensive variety of natural conditions, like refrigeration temperatures, low pH, and high salt concentration. This enables the pathogen to defeat food preservation and safety limits, and represents a potential hazard to human wellbeing. (Gandhi and Chikindas, 2007)

South Africa is currently in the middle of the biggest *listeria* outbreak ever seen, according to the UN World Health Organization (WHO 2018). The outbreak has killed 180 people since January 2017, with nearly 1,000 cases reported.

3.2 Detection Methods

3.2.1 Traditional culture methods

Standard cultural method has been the primary method relied on for bacterial detection dating back to the 17th century. It is a very important diagnostic tool for both medicine and food hygiene practices. (Hunter-Cevera and Belt, 1996). The big advantage of this method is that it is cost effective and very sensitive. But on the other hand, it is labour-intensive as well as very time consuming, needing at least 5 days to obtain results. In a world where it is extremely demanding that routine food samples that have shelf -lives of 5 to 7 days must be examined for microbiological criteria, standard cultural methods are disappointing to the manufacturers. (Fabiani et al., 2017). 48 hours are needed just for the cultivation of the bacteria, with the rest of the time frame used to detect and identify the microorganisms involved. (Deisingh and Thompson, 2002).

Traditional culture methods consist of 5 steps, starting with pre- enrichment phase with allows bacteria to multiply and grow if they are found in low numbers or injured. This procedure alone is time consuming and takes around 24-72 hours to complete. (Busch, 2010). It is followed by a selective enrichment phase, where both a solid or liquid media can be used. When using a selective media specific chemical are added to prevent the growth of other bacteria and aid the proliferation of the Bacteria specific to the media chosen. (Ray and Bhunia, 2014). The other processes involve selective plating, biochemical screening and serological confirmation. (Vunrcrzant and Pllustoesser, 1987). With the two final steps as their name entails are used to confirm that they are the targeted species. (Benson, 2002)

3.2.2 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) also known as an enzyme immunoassay (EIA), is an immunological method used to detect an antibody or antigen of a specific pathogen in a sample. (Joseph and Carlos, 2012). Perlmann and Engvall were the first to discover this method in 1971, its advantage is that it can detect pathogen in a much shorter time than traditional

culture methods. It can detect them in less than 24 hours compared to the average 5 days with the culturing technique. (Jasson et al., 2010).

A sample with an unknown amount of antigen is restrained on a solid plate, then a specific antibody is added to the surface so it can bind to the antigen. This antibody is linked to an enzyme and in the final step the enzymes substrate reacts to it producing a detectable reaction usually a colour change of the substrate. Between each step the plate is washed with a mild solution removing any unbound antibodies and proteins. (Joseph and Carlos, 2012).

Unfortunately, this method is not without its advantages, it has a high limit of sensitivity of > 10^5 cfu/mL (Cox, 1988), also cross reactivity is possible (Westerman et al. 1997), and acetylation causing changes to the antibodies (Kim and Slauch, 1999). Moreover, when this method is used to detect pathogens in food there is a detection limit of 10^3 to 10^5 cfu/ml, therefore the direct detection is not possible for food-borne pathogens hence a 16-24hour enrichment phase is required. (Mandal et al., 2011)

3.2.3 Biochemical methods

Biochemical tests rely on the biochemical actions of pathogens. This method works by the ability of the bacteria to use different substrates challenged by different circumstances. The pathogens are cultivated in microtiter plates using different substrates of carbon sources causing a visible reaction in colour change. (Preston-Mafham et al., 2002).

3.2.4 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) is a technique based on the amplification of certain DNA segments. (Mullis et al. 1986) It has been developed by Kary Mullis in 1983. (Bartlett and sterling, 2003). This method entails of 3 important steps, starting with the denaturation process consisting of a heating process of 94 – 98 degrees Celsius for 20-30 seconds causing DNA melting of the double stranded template by breaking hydrogen bonds, creating two single-stranded DNA molecules. Annealing would be the next step where the temperature is lowered to 50–65 degrees Celsius for 20 to 40 seconds, allowing the primers to interact with each of the single stranded molecules. Followed by the last step which is the extension process where the temperature used depends on the type of DNA polymerase used. (Chien et al. 1976).

The final process then forms new strands complementary to the primary strand. (Smith, 2009). The newly formed strands can be wither analysed or stored for future use at – 20 degrees Celsius. (Mahon, 2000)

Although this type of traditional PCR is specific under enhanced circumstances, it has some disadvantages compared to some newer methods, it can be time consuming due to the post amplification steps. (McKillip and Drake 2004). Moreover, the PCR method cannot differentiate between living and dead cells which can lead to a false negative. (Biswas et al. 2008).

3.2.5 Real-Time PCR

Real-Time PCR was discovered by Higuchi in 1990. It is also known as Kinetic PCR or homogenous PCR. The advantage of this method is that with the help of fluorescence staining it can detect the accumulation of PCR products in real time unlike in conventional PCR, hence eliminating the post amplification steps resulting in a more time saving method. (Willhelm and Pingoud, 2003). This method consists of 4 phases, the linear ground phase, early exponential phase, log-linear phase and finally the plateau phase. (Tichopad et al.,2003)

3.2.6 Biosensors

Biosensors are particularly advantageous when it comes to the detection of pathogens since they are rapid, cost effective, and have both high specificity and selectivity. (Liu et al., 2016). Biosensors function by producing signals when they detect a target molecule by using biological materials like antibodies and antimicrobial compounds. The device is made up of four biological components. (Saha et al., 2012) The biological recognition element is the most important component which works by having a very similar to the target pathogen being analysed. (Sekretaryova et al., 2016)

3.2.7 MicroTester- Redox Potential Method

A novel method used for the detection of pathogen contamination is the MicroTester which is based on redox potential measurement. It was created by the Department of Food Hygiene at the University of Veterinary Medicine in Budapest in partnership with the Department of Physics and Automation, Faculty of Food science at the Corvinus University of Budapest. It detects a reduction in the environment when microbial growth occurs due to the consumption of oxygen. This method is very advantageous regarding acquiring both a quantitative and qualitative aspect of a given sample.

4. Materials and methods

The experiments were carried out in the Food Hygiene Laboratory (Accredited Microbiological Laboratory), Department of Food-Hygiene at the University of Veterinary Medicine, Budapest.

4.1. Methods

4.1.1. Standard methods:

Detection of *Listeria monocytogenes*: ISO 11290-1:1998

Detection of *Salmonella*: ISO 6579:2006

Detection of *E. coli*: MSZ ISO 16649-2:2005

4.1.2. Bacterial strains:

Listeria monocytogenes (ATCC 19111)

Escherichia coli (ATCC 10536)

Salmonella typhimurium (ATCC 13311)

4.1.3. Media used with Redox potential measurement:

Listeria monocytogenes: *Listeria* Enrichment Broth (LEB) Base according to FDA/IDF-FIL (Merck) with Oxford *Listeria* Selective Supplement (Merck) at the incubation temperature of 37 °C was used.

Escherichia coli: Selective medium: BBL broth (Merck). Incubation temperature was 44 °C.

Salmonella spp.: Rappaport-Vassiliadis *Salmonella* selective broth (RVS broth) MERCK 107666 was used as culture medium at the incubation temperature of 42 °C.

The living cell concentrations of the microbial suspensions were determined by plate counting.

4.1.4. Redox potential measurement based method

The experiments were carried out by a 32-channel redox potential measuring instrument, the MicroTester. The parts of the MicroTester system are a water bath thermostat (with an accuracy of ± 0.2 °C) test cells, which in this case were plastic test tubes of 20 ml volume equipped by Schott BlueLine 31 RX redox-electrodes, PC drive data collection and evaluating unit, and a

monitor. The data collection is continuous as all channels are monitored. The measured data are saved when it is required as per channel settings.

Prior to measurement, the electrodes were disinfected with 3% H2O2, rinsed in 70% ethanol and finally were immersed in the aseptically inoculated test tubes. After putting the test tubes into the water bath, the electrodes were connected to the data collector, the software was initialized and the measurement started. The TTD values were determined from the redox curves automatically by the program. Upon completing the measurement, the electrodes were disinfected in Na-hypochlorite (1:10 diluted commercial Hypo solution) for 30 min, then rinsed in water and stored in 3M KCl solution.

The changes in the test tubes are continuously measured and the data is saved. At a certain point, which is easily detected by the instrument, the change in redox potential exceeds the threshold value. This means that the change is significantly bigger than the random changes. This value is the detection criterion. The time it takes to reach this point is called TTD (*time to detection*). There is a close linear correlation between the TTD and the logarithm of the initial viable count. This relationship is represented by the calibration curve that makes it possible to calculate the initial viable microbe concentration as a function of the TTD. Beyond the selectivity of the medium, owing to the characteristic pattern of the redox curves, the system usually makes it possible to identify the growing microflora. (Reichart et al., 2007)

4.2. Calibration curve

The equation of the calibration curve is calculated by linear regression from the logarithm of the initial viable cell numbers of the measuring cells (log N determined by plate counting) and the TTD values, determined instrumentally. The equation is fed into the computer. The measuring system constructs the redox curve, determines the detection time calculates the initial viable count of the sample by using of the calibration curve.
The equation of calibration curve:

$$TTD\ (h) = a \log N\ (cfu/cell) + b$$

$$\text{if } \log N = 0, \text{ then } N = 1$$

$$\text{in that case } TTD\ (h) = b$$

Time of detection of single cell is equal with the intercept of the calibration curve (b).

4.3. Real-Time PCR for confirmation of *Salmonella* and *Listeria monocytogenes*

The redox potential measurement could screen the samples which did not contain *L. monocytogenes* or *Salmonella* as an enrichment process. Only if a presumably positive sample was detected would it be investigated further by real-time PCR to identify the given bacterium. Genomic DNA was isolated from 1 ml of enriched food sample using the Mericon DNA Bacteria and Bacteria Plus Kit (Qiagen) per the manufacturer's instructions. Due to the loss during the process, 100 µl of the DNA isolate contains 25% of the total DNA originating from a 1-ml sample of the enrichment culture. DNA (isolate) = 0.25·DNA (sample). Real-time PCR amplification was performed in SLAN® Real-Time PCR System (Hongshi) using the Mericon *L. monocytogenes* Kit (Qiagen) and Mericon *Salmonella* spp. Kit (Qiagen) designed for the qualitative detection of the certain microbes in food and animal feed after enrichment. PCR was done in a final volume of 20 µl including 9.2 µl DNA isolate and 10.8 µl Multiplex PCR Master Mix containing target-specific primers and probes, as well as the internal control. DNA (PCR) = 0.092·DNA (isolate). Detection limit: the assay can detect down to 10 copies of *L. monocytogenes* DNA in a reaction, which – by calculation – provides 435 DNS copies/ml sample. Detection of pathogens using real-time PCR was based on the amplification of a specific region of the relevant pathogen genome. The amplified product was detected by using target-specific fluorescent probes. As the PCR product accumulated, there was an increased fluorescent signal from the bound probes. Monitoring the fluorescence intensities during the PCR run (i.e. in real time) allowed the detection of the accumulating PCR product without the need to reopen the reaction tubes afterward. A positive result is visible as a final point of the fluorescence curve that lies clearly above the threshold. The number of cycles belonging to this point is the quantification cycle value. As a guideline, the uninhibited internal control should give a quantification cycle value ranging between 28 and 32. A quantification cycle value over 33 indicates inhibition. If there is no visible point till the 38th cycle (CT ≥ 38), the result is negative. The isolated pure bacterial DNA was assayed using a streamlined real-time PCR protocol.

4.4 Samples

We examined ready-to-eat salad packages obtained from the local market. 5 types of mixed salads were obtained from supermarkets. Mixed salad 1 contained Romana, Frisee, Lollo rosso, Baby spinach, Tatsoi, Red chard leaves. The mixed salad 2 contained Endive, Frisee and Red beet. Mixed salad 3 contained White cabbage, Sweet corn, Carrot. The 4th salad contained Iceberg, Cicorinno rosso and carrots. And finally, salad 5 contained only Iceberg leaves. We examined the whole salads.

5. Result and Discussion

The redox potential measurements of *Listeria monocytogenes* were performed in LEB with Oxford supplement containing the 10 g salad sample. For the determination of calibration curve 1 slant agar of the Listeria strain was washed down and a tenfold dilution series was prepared with peptone water to the 6th dilution level. From each dilution, 1 ml was pipetted into a redox measuring cell containing 90 ml LEB with Oxford supplement.

The redox potential measurements of *Salmonella typhimurium* were performed in RVS broth containing the 10 g salad sample. For the determination of calibration curve 1 slant agar of the *Salmonella* strain was washed down and a tenfold dilution series was prepared with peptone water to the 6th dilution level. From each dilution 1 ml was pipetted into a redox measuring cell containing 90 ml RVS broth.

The redox potential measurements of *Escherichia coli* were performed in BBL broth containing the 10 g salad sample. For the determination of calibration curve 1 slant agar of the *Escherichia coli* strain was washed down and a tenfold dilution series was prepared with peptone water to the 6th dilution level. From each dilution 1 ml was pipetted into a redox measuring cell containing 90 ml BBL broth.

The equipment automatically determined the detection times belonging to the different dilution levels. After inputting the viable count of the undiluted inoculum (determined by plate counting), the software computed the calibration curve. The equations of the calibration curves

were calculated by linear regression from the logarithm of the initial viable cell numbers in the measuring cells (log N) and the TTD values.

5.1. Calibration curve of *Listeria monocytogenes*

The calibration curve of *Listeria monocytogenes* is shown in **Fig. 1**. The calibration curve was constructed in LEB broth with Oxford supplement. The temperature of the culturing was 37 °C.

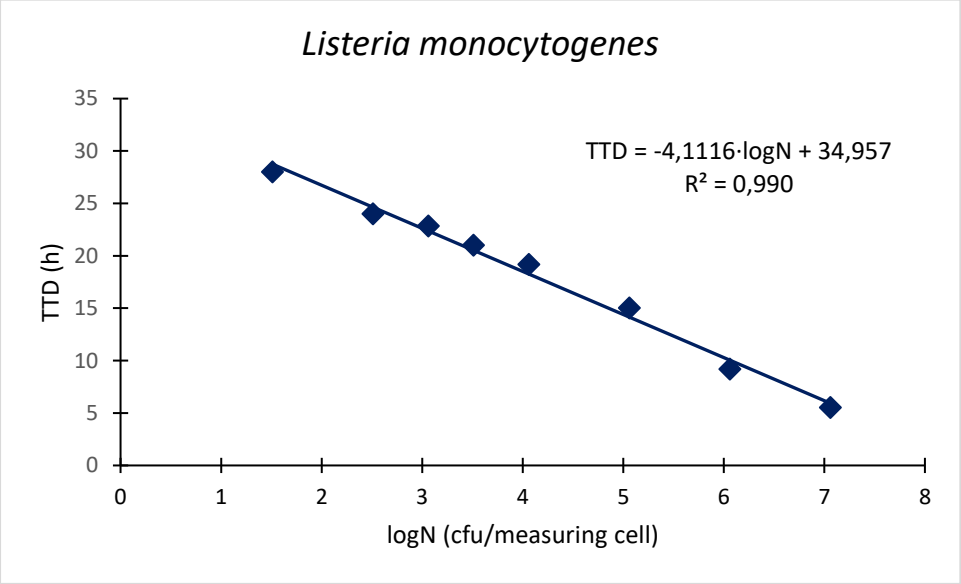

Fig. 1: Calibration curve of *L. monocytogenes* in LEB with oxford supplement T=37 °C

Table 1: Regression analysis of *Listeria monocytogenes*

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	408.9810	408.9810	632.51	2.60E-07
Residual	6	3.8796	0.6466		
Total	7	412.8606			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	34.9568	0.7287	47.9743	5.50E-09	33.1739	**36.7398**
X Variable 1	-4.1116	0.1635	-25.1497	2.60E-07	-4.5117	-3.712

The theoretical time requirement of detection of one target microorganism in the measuring cell (log N=0) can be calculated from the intercept TTD (0) of the calibration curve. **Table 1** shows that the upper limit of the intercept is 36.7398. Thus, the time requirement of screening the *L. monocytogenes*-negative samples is 37 h. So, if we do not obtain TTD within 37 h, the inoculum of the measuring cell is free of *L. monocytogenes* with high probability. In case of detectable contamination this time significantly decreases. Getting a TTD value means a presumably positive sample. To confirm the presence of *L. monocytogenes* real-time PCR technique was used. To obtain TTD value indicated that the microbe concentration in the test cell reached about 10^6cfu/ml, so the same suspension is ready for direct DNA isolation and real-time PCR assay. The identification from the enriched suspension by real-time PCR required another 3 hours.

5.2. Calibration curve of *Salmonella typhimurium*

The calibration curve of *Salmonella Typhimurium* is shown in **Fig. 2**. The calibration curve was constructed in RVS broth. The temperature of the culturing was 42 °C.

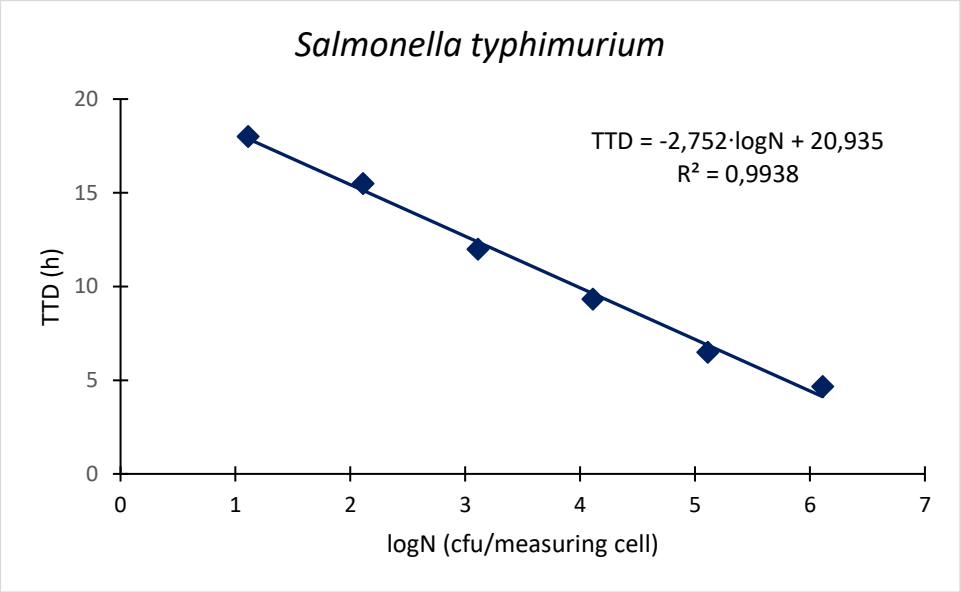

Fig.2: Calibration curve of *Salmonella Typhimurium* in RVS broth T=42 °C

Table 2: Variance analysis of Salmonella *Typhimurium*

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	132.5363	132.5363	645.35	1.43E-05
Residual	4	0.8215	0.2054		
Total	5	133.3578			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	20.9347	0.4326	48.3898	1.09E-06	19.7336	**22.1359**
X Variable 1	-2.7520	0.1083	-25.4038	1.43E-05	-3.0528	-2.4512

Evaluating the result of variance analysis (**Table 2**) it can be said that the time requirement of screening the *Salmonella*-negative samples is 22 h. In case of *Salmonella* contamination this time decreases. Instead of the biochemical confirmation real-time PCR was used.

5.3. Calibration curve of *Escherichia coli*

The calibration curve of *Escherichia coli* is shown in **Fig. 3**. The calibration curve was constructed in BBL broth. The temperature of the culturing was 44 °C.

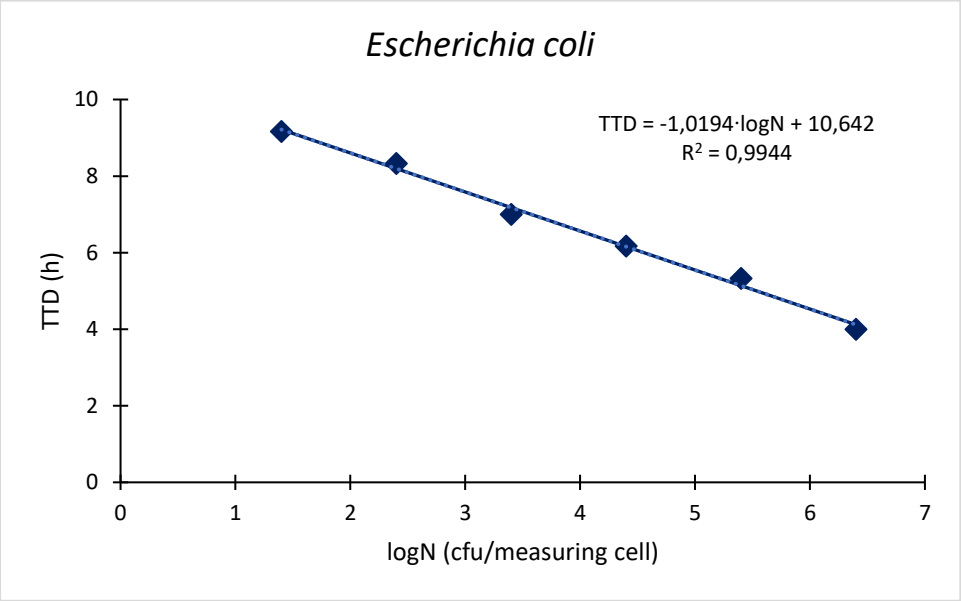

Fig.3: Calibration curve of *Escherichia coli* in BBL broth T=44 °C

Table 3: Variance analysis of *Escherichia coli*

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	18.1866	18.1866	710.92	1.18E-05
Residual	4	0.1023	0.0256		
Total	5	18.2889			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	10.6424	0.1628	65.3785	3.28E-07	10.1905	**11.0944**
X Variable 1	-1.0194	0.0382	-26.6630	1.18E-05	-1.1256	-0.9133

Table 3. shows that the maximum measuring time in case of *Escherichia coli* is 11 h. In case of *E. coli* contamination this time decreases.

Using the calibration curve, we determined the microbial count of the bacteria. The equation of the calibration curve was loaded into the equipment and the measurement was then started. The equipment determines the TTD (hour) and based on the calibration curve calculates the count of microbes of the sample.

5.4 Control Examination

We compared the Redox potential method and the traditional culture method to check if there is a difference in the detection of the quantity of the specific pathogen. We contaminated the mixed salad with 3 different levels and we checked for the results.

Table 4. Shows the results obtained by redox potential method and standard method (log N ± SD) for Listeria Monocytogenes using the 3 different contamination levels.

Salad 1		MicroTester	Standard
	TTD	logN	logN
	15.5	4.73	4.52
	15.33	4.77	4.81
	15.33	4.77	4.67
	20.67	3.48	3.51
	21	3.40	3.49
	20.67	3.48	3.52
	27.17	1.92	2
	27.33	1.88	1.95
	27.5	1.84	2.1

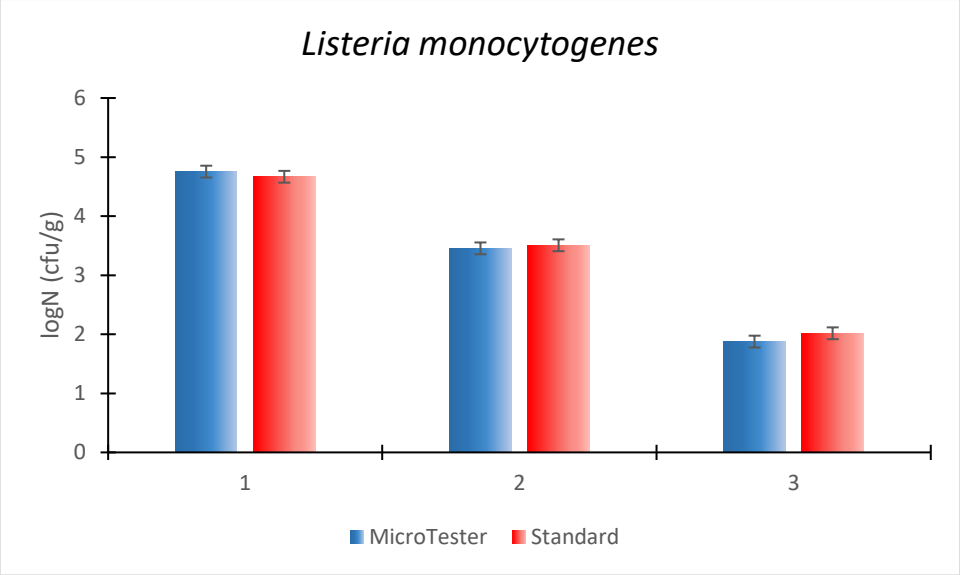

Fig. 4: Comparison of the results obtained by redox potential method and standard method (log N ± SD) for *Listeria monocytogenes*

Table 5. Shows the results obtained by redox potential method and standard method (log N ± SD) for Salmonella typhimurium using the 3 different contamination levels.

Salad 2		MicroTester	Standard
	TTD	logN	logN
	6.33	5.30	5.68
	6	5.42	5.46
	6.17	5.35	5.5
	10.5	3.79	3.63
	11.17	3.55	3.71
	10.83	3.67	3.65
	16.33	1.69	1.62
	16.5	1.62	1.62
	16	1.80	1.71

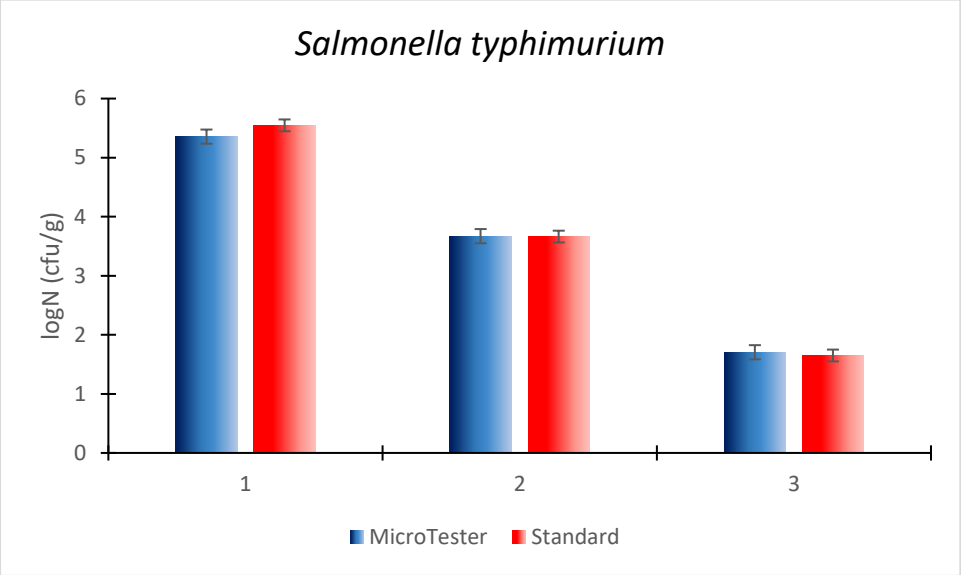

Fig. 5: Comparison of the results obtained by redox potential method and standard method (log N ± SD) for *Salmonella typhimurium*

Table 6. Shows the results obtained by redox potential method and standard method (log N ± SD) for *Escherichia coli* using the 3 different contamination levels.

Salad 3		MicroTester	Standard
	TTD	logN	logN
	4.33	6.18	6.23
	4.33	6.18	6.26
	4.67	5.85	6.26
	6.83	3.74	3.78
	6.67	3.90	3.71
	6.83	3.74	3.76
	9.5	1.14	1.24
	9.33	1.30	1.31
	9.67	0.97	1.26

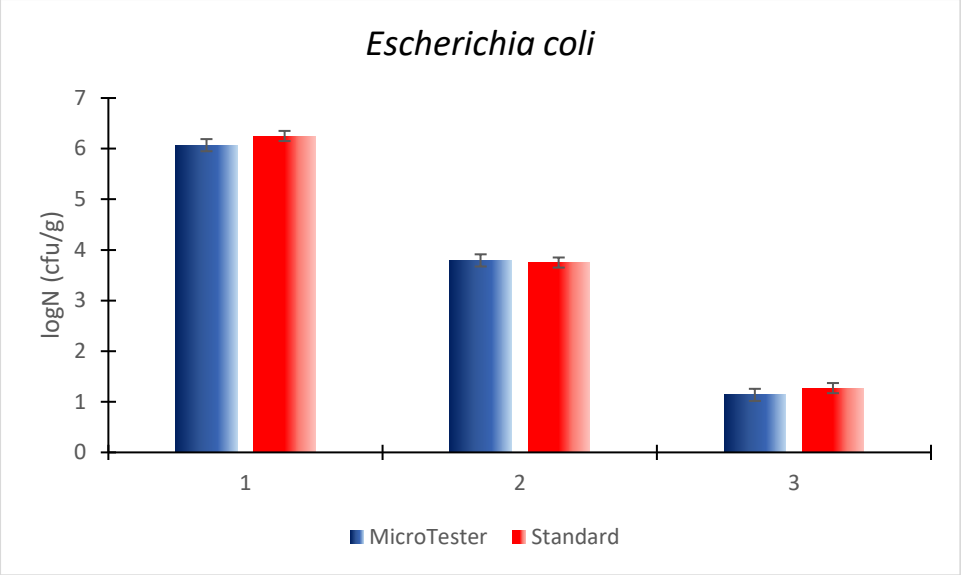

Fig. 6: Comparison of the results obtained by redox potential method and standard method (log N ± SD) for *Escherichia coli*

The results of the mixed salad samples measured by redox potential measurement and conventional methods are shown in **Fig. 4, Fig. 5 and Fig. 6.** There is no significant difference between the results obtained by the two separate methods.

5.5. Examination of samples obtained from the market

We examined 5 types of mixed salads obtained from the local market. In our studies mixed salad 1 contained Romana, Frisee, Lollo rosso, Baby spinach, Tatsoi, Red chard leaves. The mixed salad 2 contained Endive, Frisee and Red beet. Mixed salad 3 contained White cabbage, Sweet corn, Carrot. The 4th salad contained Iceberg, Cicorinno rosso and carrots. And finally, salad 5 contained only Iceberg leaves We examined the whole salads.

In case of a positive result (when we got a TTD), we examined the samples with real-time PCR for confirmation (see also Materials and Methods).

Table 7 shows the time requirement of the detection of *Listeria monocytogenes* using the combination of the redox potential measurement and PCR in contrast to the time requirement of the standard methods.

Table 8 and **9** show the time requirement of the redox potential measurement in contrast to the time requirement of the standard methods for detecting *E. coli* and *Salmonella*.

Table 7: Results of the detection of *Listeria monocytogenes*

Samples	**Redox potential measurement**		**real-time PCR**	**Redox +PCR**	**Standard method**
	result	time requirement (h)	result	time requirement requirement (h)	time requirement (h)
Salad1 n=3	-	37	-	37	120
	-	37	-	37	120
	-	37	-	37	120
Salad 2 n=3	-	37	-	37	120
	-	37	-	37	120
	-	37	-	37	120
Salad 3 n=3		37	-	37	120
	-	37	-	37	120
	-	37	-	37	120
Salad 4 n=3	-	37	-	37	120
	-	37	-	37	120
	-	37	-	37	120
Salad 5 n=3	-	37	-	37	120
	-	37	-	37	120
	-	37	-	37	120

Table 8: Results of the detection of *Salmonella*

Samples	**Redox potential measurement**		**real-time PCR**	**Redox +PCR**	**Standard method**
	result	time requirement (h)	result	time requirement requirement (h)	time requirement (h)
Salad 1 n=3	-	22	-	22	96
	-	22	-	22	96
	-	22	-	22	96
Salad 2 n=3	-	22	-	22	96
	-	22	-	22	96
	-	22	-	22	96
Salad 3 n=3		22	-	22	96
	-	22	-	22	96
	-	22	-	22	96
Salad 4 n=3	-	22	-	22	96
	-	22	-	22	96
	-	22	-	22	96
Salad 5 n=3	-	22	-	22	96
	-	22	-	22	96
	-	22	-	22	96

Table 9: Results of the detection of *Escherichia coli*

Samples	**Redox potential measurement**		**Standard method**
	result	time requirement (h)	time requirement (h)
Salad 1 n=3	-	11	24
	-	11	24
	-	11	24
Salad 2 n=3	-	11	24
	-	11	24
	-	11	24
Salad 3 n=3		11	24
	-	11	24
	-	11	24
Salad 4 n=3	-	11	24
	-	11	24
	-	11	24
Salad 5 n=3	-	11	24
	-	11	24
	-	11	24

It can be said, that all samples of the mixed salads from the local market were free from *E. coli*, *Listeria monocytogenes* and *Salmonella typhimurium.*

6. Conclusion

In this study, the possibility of the detection of *Salmonella typhimurium, Listeria monocytogenes* and *Escherichia coli* in Ready-to-Eat Salad was established by the redox potential measurement and by the further combination of the real-time PCR method.

During the redox potential measurement, the presumably *Salmonella typhimurium, Listeria monocytogenes* and *Escherichia coli* positive samples can be screened by the redox potential measurement technique. Moreover, with the case of *salmonella* and *Listeria monocytogenes* the positive samples were sent for further identification processes by implementing the real-time PCR technique. The identification of the positive samples by real-time PCR requires an additional 3 hours.

From the calibration curves of the redox potential measurement it could be deducted that in case of *Listeria monocytogenes* presence the result is given in maximal 37 hours. In case of *Salmonella* presence, the maximal measuring time is 22 hours. Furthermore, when *Escherichia coli* is present in the sample, the time requirement is maximal 11 hours. If we get no TTD in this time the samples are probably free of the examined bacteria.

The results obtained by the redox potential measurement method or in case of Salmonella and Listeria monocytogenes by the combination of the two instrumental methods (redox potential + PCR) were totally identical with those of the classical nutrient methods. However, the time constraints of the investigations were significantly different.

The detection of *Listeria* absence in Ready-to-Eat Salads obtained from the local supermarket requires 120 hours or even 168 hours when using conventional culture methods. On the other hand, with redox potential measurement and PCR the time requirement is to be a maximum of 37 hours which is notably shorter than the conventional method.

With conventional culture methods, the detection of *Salmonella* absence requires 96 hours, but with the redox potential measurement and PCR the time requirement was substantially less requiring only a maximal of 22 hours. Which is only a fraction of the total essential time compared with traditional culture method.

Furthermore, the detection of *E. coli* absence with traditional culture methods requires 24 hours. Compared with the redox potential measurement and PCR the time requirement utilized was only 11 hours, almost less than half the time requirement with traditional methods.

In conclusion, the previous data shows us that the redox potential measurement with the combination of redox potential method and PCR can provide a time saving, efficient, minimal cost and reliable tool to detect pathogens in Ready-to-Eat salad.

7. Summary

With the increase in awareness of people consuming a healthy nutritious diet, vegetables are a great way to add vitamins, minerals and dietary fibre to our everyday food regimen. Which has led to an increase in the demand of these Ready-to-Eat salads. Since these food products are eaten raw and are minimally processed there is an increased risk for pathogenic contamination.

To provide a safe product for consumers and minimize the risk of people getting contaminated with pathogens from eating these products. The detection of bacteria before sending edible products to the shelves in markets are a must. Conventional culture methods have been the choice for pathogen detection for a long time but unfortunately, they are time consuming and labour intensive, taking almost an average of 5 days to get results, which puts food producers at a disadvantage when their food products have a short shelf life.

Therefore, in this study we have used redox potential based measurement in combination with real-time PCR for the detection of three different potentially harmful pathogens to human health. We used *Salmonella typhimurium, Listeria monocytogenes* and *Escherichia coli* as the pathogens in Ready-to-Eat salad obtained from local supermarkets. All the samples were also examined by the conventional culture methods as a comparison.

The MicroTester was developed by the researchers of the department of Food Hygiene at the university of Veterinary Medicine in Budapest and the department of Physics and Automation, Faculty of Food Science at the Corvinus University in Budapest, which detects redox potential changes caused by microbial growth of pathogens.

The results obtained by the redox potential measurement method and PCR were totally identical to the results of the conventional culture method. But there was a significant difference in the time requirement between the two methods. On average the time requirement of pathogen detection using redox potential measurement took 20% of the time required to detect pathogens using the standard culture method. If we get no TTD in this time the samples are probably free of the examined bacteria.

To sum up the redox potential method used in combination with Real-time PCR was a more time saving and a better alternative to detect pathogens in Ready-to-Eat Salad than conventional culture methods.

8. References

Acheson, D. & Hohmann, E.L., 2001. Nontyphoidal Salmonellosis. Clinical Infectious Diseases, 32(2), pp.263–269.

Ackers, M. et al., 1998. An Outbreak of Escherichia coli O157:H7 Infections Associated with Leaf Lettuce Consumption, The Journal of Infectious Diseases, Volume 177, Issue 6, 1 June 1998, Pages 1588–1593

Alcaine, S.D., Warnick, L.D. & Wiedmann, M., 2007. Antimicrobial Resistance in Nontyphoidal Salmonella. Journal of Food Protection, 70(3), pp.780–790.

Althaus, D. et al., 2012. Bacteriological Survey of Ready-to-Eat Lettuce, Fresh-Cut Fruit, and Sprouts Collected from the Swiss Market. Journal of Food Protection, 75(7), pp.1338–1341.

Arumugaswamy, R., Gulam Rusul Rahamat Ali and Siti Nadzriah Bte Abd. Hamid (1994). Prevalence of Listeria monoctogenes in foods in Malaysia. International Journal of Food Microbiology, 23(1), pp.117-121.

Balachandran, P. et al., 2012. Rapid Detection of Salmonella in Pet Food: Design and Evaluation of Integrated Methods Based on Real-Time PCR Detection. Journal of Food Protection, 75(2), pp.347–352.

Bartlett, J.M.S. & Stirling, D., 2003. A Short History of the Polymerase Chain Reaction. PCR Protocols, pp.3–6.

Benson, H.J. & Benson, H.J., 2002. Instructors handbook to accompany Microbiological applications: laboratory manual in general microbiology, complete and short versions, eighth edition, Boston: McGraw-Hill.

Berger, C. N., Sodha, S. V., Shaw, R. K., Griffin, P. M., Pink, D., Hand, P. and Frankel, G. (2010), Fresh fruit and vegetables as vehicles for the transmission of human pathogens. Environmental Microbiology, 12: 2385–2397.

Beuchat, L.R. (1998). Surface Decontamination of Fruits andVegetables Eaten Raw: A Review. Geneva, Switzerland:World Health Organisation, Food Safety Unit.WHO／FSF／FOS／98.2.

Biswas, A. et al., 2008. Microbial profiles of frozen trimmings and silver sides prepared at Indian buffalo meat packing plants. Meat Science, 80(2), pp.418–422.

Blackburn, C.de W., 2009. Foodborne pathogens: hazards, risk analysis and control, Cambridge: Woodhead Publishing Ltd.

Bolin, H.R. et al., 1977. Factors Affecting The Storage Stability Of Shredded Lettuce. Journal of Food Science, 42(5), pp.1319–1321.

Buck, J.W., Walcott, R.R. & Beuchat, L.R., 2003. Recent Trends in Microbiological Safety ofFruits and Vegetables. Plant Health Progress.

Busch, Ulrich. Molekularbiologische Methoden in der Lebensmittelanalytik: Grundlegende Methoden und Anwendungen. Heidelberg, Germany: Springer-Verlag, 2010

CDC National Center for Emerging and Zoonotic Infectious Diseases. "Escherichia coli". Retrieved 2 October2012.

Chien, A., Edgar, D.B., Trela, J.M. 1976, "Deoxyribonucleic acid polymerase from the extreme thermophile Thermusaquaticus", Journal of Bacteriology, vol. 127, no. 3, pp. 1550-1557.

Chittick, P. et al., 2006. A Summary of National Reports of Foodborne Outbreaks of Salmonella Heidelberg Infections in the United States: Clues for Disease Prevention. Journal of Food Protection, 69(5), pp.1150–1153.

Cox N.A. 1988. Salmonella methodology update. Poultry Science 67:921-927.

D'Aoust, J.-Y. & Maurer, J., Salmonella Species. Food Microbiology: Fundamentals and Frontiers, Third Edition, pp.187–236.

Deisingh, A.K. & Thompson, M., 2002. Detection of infectious and toxigenic bacteria. The Analyst, 127(5), pp.567–581.

Delaquis, P., Bach, S. & Dinu, L.-D., 2007. Behavior of Escherichia coli O157:H7 in Leafy Vegetables. Journal of Food Protection, 70(8), pp.1966–1974.

Dias, J.S., 2012. Nutritional Quality and Health Benefits of Vegetables: A Review. Food and Nutrition Sciences, 03(10), pp.1354–1374.

Dipineto, L. et al., 2006. Presence of Shiga toxin-producing Escherichia coli O157:H7 in living layer hens. Letters in Applied Microbiology, 43(3), pp.293–295.

Doganay, M., 2003. Listeriosis: clinical presentation. FEMS Immunology & Medical Microbiology, 35(3), pp.173–175.

Everis, L. (2004) Risks of Pathogens in Ready-to-eat Fruits, Vegetables, and Salads Through the Production Process. Chip-ping Campden, UK: Review no. 44, Campden and Chorleywood Food Research Association Group.

Fabiani, L. et al., 2017. ELIME assay vs Real-Time PCR and conventional culture method for an effective detection of Salmonella in fresh leafy green vegetables. Talanta, 166, pp.321–327.

Farber, K.M. P.I. Peterkin. Listeria monocytogenes, a food-borne pathogen. Microbiol. Rev 55, 1991: 476-611.

Fang, Q. et al., 2003. Improved Detection of Salmonella spp. in Foods by Fluorescent In Situ Hybridization with 23S rRNA Probes: A Comparison with Conventional Culture Methods. Journal of Food Protection, 66(5), pp.723–731.

Gandhi, M. & Chikindas, M.L., 2007. Listeria: A foodborne pathogen that knows how to survive. International Journal of Food Microbiology, 113(1), pp.1–15.

Gombas, D. et al., 2017. Guidelines to Validate Control of Cross-Contamination during Washing of Fresh-Cut Leafy Vegetables. Journal of Food Protection, 80(2), pp.312–330

Gouws, P.A., Visser, M. & Brözel, V.S., 1998. A Polymerase Chain Reaction Procedure for the Detection of Salmonella spp. within 24 Hours. Journal of Food Protection, 61(8), pp.1039–1042.

Guibourdenche, M. et al., 2010. Supplement 2003–2007 (No. 47) to the White-Kauffmann-Le Minor scheme. Research in Microbiology, 161(1), pp.26–29.

Gurler, Z. et al., 2015. The microbiological quality of ready-to-eat salads in Turkey: A focus on Salmonella spp. and Listeria monocytogenes. International Journal of Food Microbiology, 196, pp.79–83.

Harris, L. et al., 2003. Outbreaks Associated with Fresh Produce: Incidence, Growth, and Survival of Pathogens in Fresh and Fresh-Cut Produce. Comprehensive Reviews in Food Science and Food Safety, 2(s1), pp.78–141.

Heisick, J.E., Wagner, D.E., Nierman, M.L. and Peeler, J.T.(1989) Listeria spp. found on fresh market produce. Applied and Environmental Microbiology 55, 1925–1927.

Hunter-Cevera, J.C. & Belt, A., 1996. Preface. Maintaining Cultures for Biotechnology and Industry, pp.xiii-xiv.

Islam, M. et al., 2004. Persistence of Salmonella enterica Serovar Typhimurium on Lettuce and Parsley and in Soils on Which They Were Grown in Fields Treated with Contaminated Manure Composts or Irrigation Water. Foodborne Pathogens and Disease, 1(1), pp.27–35.

Jasson, V. et al., 2010. Alternative microbial methods: An overview and selection criteria. Food Microbiology, 27(6), pp.710–730.

Jay, J. M., Loessner, M. J., & Golden, D. A. (2005). Modern food microbiology. New York: Springer. (p.511)

Joanne L. Slavin, Beate Lloyd, “Health Benefits of Fruits and Vegetables,” Advances in Nutrition, Volume 3, Issue 4, 1 July 2012, Pages 506–516.

Johnson, R.P. et al., 1996. Growing Concerns and Recent Outbreaks Involving Non-O157:H7 Serotypes of Verotoxigenic Escherichia coli. Journal of Food Protection, 59(10), pp.1112–1122.

Joseph, A., & Carlos, G., (2012). Salmonella Detection Methods for Food and Food Ingredients. Salmonella - A Dangerous Foodborne Pathogen.

Kim, M., 1999. Effect of acetylation (O-factor 5) on the polyclonal antibody response to Salmonella Typhimurium O-antigen. FEMS Immunology and Medical Microbiology, 26(1), pp.83–92.

Kudva, I. T., K. Blanch, and C. J. Hovde. 1998. Analysis of Escherichia coli O157:H7 survival in ovine or bovine manure and manure slurry. Applied and Environmental Microbiology. 64:3166–3174.

Lammerding, A. M. (ed.). 1996. Food Safety Issues Update, March, 1996, no. 14. Food Safety Risk Assessment Unit, Health of Animals Laboratory, Agriculture and Agri-Food Canada, Guelph, Ontario.

Lienemann, T. et al., 2011. Iceberg Lettuce as Suggested Source of a Nationwide Outbreak Caused by Two Salmonella Serotypes, Newport and Reading, in Finland in 2008. Journal of Food Protection, 74(6), pp.1035–1040.

Liu, X. et al., 2016. Biosensors based on modularly designed synthetic peptides for recognition, detection and live/dead differentiation of pathogenic bacteria. Biosensors and Bioelectronics, 80, pp.9–16.

Mahon, C. R. and G. Manuselis. Textbook of diagnostic microbiology. WB Saunders company, 2000.

Mandal, P. et al., 2011. Methods for Rapid Detection of Foodborne Pathogens: An Overview. American Journal of Food Technology, 6(2), pp.87–102.

Mckillip, J.L. & Drake, M., 2004. Real-Time Nucleic Acid–Based Detection Methods for Pathogenic Bacteria in Food. Journal of Food Protection, 67(4), pp.823–832.

Mclauchlin, J., 1987. Listeria monocytogenes, recent advances in the taxonomy and epidemiology of listeriosis in humans. Journal of Applied Bacteriology, 63(1), pp.1–11.

Melloul, A., Hassani, L. and Rafouk, L. (2001). World Journal of Microbiology and Biotechnology, 17(2), pp.207-209.

Noordhout, C.M.D. et al., 2014. The global burden of listeriosis: a systematic review and meta-analysis. The Lancet Infectious Diseases, 14(11), pp.1073–1082.

Noris, M., Galbusera, M. & Remuzzi, G., 2009. Hemolytic Uremic Syndrome/Thrombotic Thrombocytopenic Purpura. Management of Acute Kidney Problems, pp.349–364.

Nørrung, B., 2000. Microbiological criteria for Listeria monocytogenes in foods under special consideration of risk assessment approaches. International Journal of Food Microbiology, 62(3), pp.217–221.

Park, S. et al., 2012. Risk Factors for Microbial Contamination in Fruits and Vegetables at the Preharvest Level: A Systematic Review. Journal of Food Protection, 75(11), pp.2055–2081.

Preston-Mafham, J., 2002. Analysis of microbial community functional diversity using sole-carbon-source utilisation profiles – a critique. FEMS Microbiology Ecology, 42(1), pp.1–14

Public Health Laboratory Service. 2000. Outbreaks of Salmonella typhimurium DT 204b infection in England and Wales and elsewhere in Europe. Communicable Dis. Rep. Wkly. 10:349

Rangel et al., 2005. Epidemiology of Escherichia coli O157:H7 outbreaks, United States, 1982-2002. Emerging Infectious Diseases.

Ray, B., & Bhunia, A. K. (2014). Fundamental food microbiology. Boca Raton: CRC Press.

Riley, L.W., 1983. Hemorrhagic Colitis Associated With A Rare Escherichia Coli Serotype. The Pediatric Infectious Disease Journal, 2(4), pp.345–346

Sagoo, S.K. et al., 2003. Microbiological Study of Ready-to-Eat Salad Vegetables from Retail Establishments Uncovers a National Outbreak of Salmonellosis. Journal of Food Protection, 66(3), pp.403–409.

Saha, K. et al., 2012. Gold Nanoparticles in Chemical and Biological Sensing. Chemical Reviews, 112(5), pp.2739–2779.

Santana, A.S. et al., 2012. Prevalence, populations and pheno- and genotypic characteristics of Listeria monocytogenes isolated from ready-to-eat vegetables marketed in São Paulo, Brazil. International Journal of Food Microbiology, 155(1-2), pp.1–9.

Schlech, W.F., 1996. Overview of listeriosis. Food Control, 7(4-5), pp.183–186

Schuchat, A., Swaminathan, B. & Broome, C.V., 1991. Epidemiology of human listeriosis. Clinical Microbiology Reviews, 4(2), pp.169–183.

Sekretaryova, A.N., Eriksson, M. & Turner, A.P., 2016. Bioelectrocatalytic systems for health applications. Biotechnology Advances, 34(3), pp.177–197.

Singleton P (1999). Bacteria in Biology, Biotechnology and Medicine (5th ed.). Wiley. pp. 444–454.

Smith, John E. Biotechnology 5th edition. Cambridge, 2009.

Solomon, E.B., Yaron, S. & Matthews, K.R., 2002. Transmission of Escherichia coli O157:H7 from Contaminated Manure and Irrigation Water to Lettuce Plant Tissue and Its Subsequent Internalization. Applied and Environmental Microbiology, 68(1), pp.397–400.

Splittstoesser, D.F., 1970. Predominant Microorganisms on Raw Plant Foods. Journal of Milk and Food Technology, 33(11), pp.500–505.

Sutherland PS, Miles DW, Laboyrie DA (2003) Listeria monocytogenes. Ch 13 In: Hocking AD (ed) Foodborne microorganisms of public health significance. 6th ed, Australian Institute of Food Science and Technology (NSW Branch), Sydney, p. 381–443

Szabo, E., Scurrah, K. & Burrows, J., 2000. Survey for psychrotrophic bacterial pathogens in minimally processed lettuce. Letters in Applied Microbiology, 30(6), pp.456–460.

Tauxe, R.V., 1991. Salmonella: A Postmodern Pathogen. Journal of Food Protection, 54(7), pp.563–568.

Tichopad, A., 2003. Standardized determination of real-time PCR efficiency from a single reaction set-up. Nucleic Acids Research, 31(20).

Tyrrel, S.F., Knox, J.W. & Weatherhead, E.K., 2006. Microbiological Water Quality Requirements for Salad Irrigation in the United Kingdom. Journal of Food Protection, 69(8), pp.2029–2035.

Uzzau, S. et al., 2000. Host adapted serotypes of Salmonella enterica. Epidemiology and Infection, 125(2), pp.229–255.

Vunrcrzant, C. and D.F. pllustoesser, 1987. Compeodium of Methods for Microbiological Examination of Food. 3rd Edn., American Public Health Association, New York..

Westerman, R., He, Y., Keen, J., Littledike, E. and Kwang, J. (2018). Production and characterization of monoclonal antibodies specific for the lipopolysaccharide of Escherichia coli O157

WHO 2018. http://www.afro.who.int/news/listeriosis-outbreak-south-africa

Wilhelm J. & Pingoud A. 2003: Real Time Polymerase Chain Reaction. ChemBiochem; 4, 2003: 1120-1128

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