ROMA TRE
UNIVERSITÀ DEGLI STUDI

Scuola dottorale in Biologia
In Scienze e Tecnologie Biomediche

XXX Ciclo

“New applications of the Micro Biological Survey method (MBS)”

Ottavia Stalio

Docente guida: Prof. Giovanni Antonini

Coordinatore: Prof. Paolo Visca

INDEX

ABSTRACT – Nuove applicazioni del metodo Micro Biological Survey (MBS) 4

ABSTRACT – New applications of the Micro Biological Survey method (MBS) 8

1. **INTRODUCTION - Importance of microbiology in the quality evaluation of water and food** 12

1.1. Microbiological safety and quality of water 13

1.1.1. Traditional methods for drinking water analysis and related problems 16

1.2. Microbiological safety and quality of foods: pathogen detection 24

1.2.1. Traditional methods for *Salmonella spp.* and *Listeria monocytogenes* detection 26

1.3. The Micro Biological Survey method (MBS) 33

1.3.1. Micro Biological Survey method biochemical principles 34

1.3.2. Micro Biological Survey method: analytical procedure 37

1.3.3. Micro Biological Survey method Multireader 38

2. **AIMS** 40

3. **MATERIALS AND METHODS** 41

3.1. Application of the Micro Biological Survey method in the evaluation of purifying activity of *Moringa oleifera* seeds in water samples 41

3.1.1. Application of the analytical protocol on field in the evaluation of *Moringa oleifera*'s purifying activity in Latium, Italy 44

3.2. Application of the Micro Biological Survey method for *Salmonella* spp. detection in food samples 46

3.2.1. Development of the experimental enrichment broth 49

3.2.2. Application of the experimental enrichment broth to the Micro Biological Survey method 53

3.3. Application of the Micro Biological Survey method for the selective detection of *Listeria monocytogenes* in food samples 55

3.3.1. Optimization of MBS reagent for *Listeria* spp. detection 55

3.3.2. Development of the experimental enrichment broth 59

3.3.3. Application of the experimental enrichment broth to the Micro Biological Survey method 65

4. RESULTS AND DISCUSSION 67

4.1. Application of the Micro Biological Survey method in the evaluation of purifying activity of *Moringa oleifera* seeds in water samples 67

4.1.1. Application of the analytical protocol on field in the evaluation of *Moringa oleifera*'s purifying activity in Latium, Italy 76

4.2. Application of the Micro Biological Survey method for *Salmonella* spp. detection in food samples 80

4.2.1. Development of the experimental enrichment broth 82

4.2.2. Application of the experimental enrichment broth to the Micro Biological Survey method 99

4.3. Application of the Micro Biological Survey method for the selective detection of *Listeria monocytogenes* in food samples 105

4.3.1. Optimization of MBS reagent for *Listeria* spp. detection 105

4.3.2. Development of the experimental enrichment broth 116

4.3.3. Application of the experimental enrichment broth to the Micro Biological Survey method 122

5. CONCLUDING REMARKS 124

6. REFENCES 127

ABSTRACT – Nuove applicazioni del metodo Micro Biological Survey (MBS)

La sicurezza alimentare e dell'acqua è oggi una questione centrale per quanto riguarda la salute pubblica. A questo proposito, gli organismi di ispezione europei e globali hanno adottato numerosi regolamenti e linee guida, allo scopo di individuare contaminanti e patogeni eventualmente presenti nelle matrici alimentari e nell'acqua per il consumo umano e fissare limiti di accettazione per diversi microrganismi e matrici in fase di analisi. I metodi microbiologici tradizionali sono ancora i più popolari nonché ampiamente usati, vista la loro affidabilità, la relativa semplicità e il consenso internazionale da parte delle agenzie di regolamentazione. Essi sono infatti considerati come "gold standard" nella diagnostica alimentare e idrica e quindi ben accettati. Questi metodi standardizzati sono per la maggior parte metodi di coltura classica che si basano su supporti specifici per enumerare e isolare un organismo bersaglio mentre viene inibita la flora contaminante. Questi metodi sono sensibili, facilmente adattabili e possono fornire informazioni sia qualitative che quantitative sul numero e sulla natura dei microrganismi presenti nel campione, ma mostrano diversi limiti. A causa dei loro complessi protocolli operativi, essi comportano una notevole quantità di tempo e di manodopera, nonché un'alta probabilità di errore manuale; i processi sono fortemente dipendenti dall'operatore che esegue le analisi e, di conseguenza, il numero di batteri può variare ampiamente quando viene calcolato da diversi operatori e in laboratori diversi, con conseguente interpretazione soggettiva dei risultati. Inoltre, i costi associati a questi metodi possono essere elevati, non solo considerando i costi del lavoro, ma anche i costi dei materiali necessari per condurre i test. Il limite più importante è che il tempo necessario per ottenere risultati, che possono richiedere diversi giorni e la necessità di laboratori attrezzati, rendano questi metodi poco pratici in diverse situazioni. Per tali ragioni, il mio progetto di dottorato si è concentrato sul miglioramento del metodo Micro Biological Survey (MBS), un metodo colorimetrico alternativo per l'analisi microbiologica. Questo metodo misura l'attività catalitica degli enzimi redox dei principali percorsi metabolici dei batteri, consentendo una correlazione inequivocabile tra l'attività enzimatica osservata e il numero di cellule vitali presenti nei campioni. Questa correlazione è ottenuta utilizzando fiale monouso con all'interno reagenti originali per il rilevamento selettivo dei microrganismi. L'analisi può essere automatizzata

utilizzando un dispositivo denominato MultiReader (MR), che può eseguire contemporaneamente 8 analisi indipendenti a temperature diverse. L'MR è controllato da un computer con un software specifico che rileva la modifica dei colori delle fiale e calcola il numero di microrganismi presenti nel campione. Il metodo può essere applicato in molti campi e riduce significativamente il tempo necessario per ottenere risultati di test comparabili ai test tradizionali di microbiologia e la sua semplice interfaccia e funzionamento richiede un livello minimo di formazione e capacità operativa. Ho concentrato perciò la mia attenzione su diversi campi applicativi, come l'utilizzo del metodo MBS per il controllo della qualità dei prodotti alimentari, per implementare l'applicazione di un piano di autocontrollo nel sistema alimentare a livello industriale utilizzando il metodo MBS per prevenire la mancanza di qualità alimentare; la sua applicazione per il controllo della qualità dell'acqua, al fine di aumentare il monitoraggio microbiologico delle nuove fonti d'acqua già esistenti, l'ottimizzazione dei mezzi già utilizzati dal metodo MBS, per renderli sempre più veloci secondo le caratteristiche del metodo e risolvere qualsiasi problema che le matrici differenti potrebbero rappresentare verso il metodo, e le possibili e totalmente nuove e originali applicazioni del metodo in altri settori diversi da quelli già presenti, per ampliare la flessibilità e l'applicabilità del metodo. Oggi l'uso della pianta tropicale *Moringa oleifera* come uno strumento di purificazione alternativo delle acque sta diventando molto popolare nei paesi in via di sviluppo, motivo per cui il primo obiettivo della mia ricerca è stato verificare se questa pianta potesse essere efficace nella purificazione dell'acqua utilizzando il metodo MBS, sviluppando un protocollo di analisi facilmente applicabile nei paesi in via di sviluppo. Il secondo obiettivo era lo sviluppo di un brodo di arricchimento per la rilevazione degli agenti patogeni, in particolare per la rilevazione di *Salmonella* spp. e *Listeria monocytogenes*. Questi brodi, secondo i principi MBS, possono consentire l'analisi di 25 g di campione e una significativa riduzione del tempo di analisi, senza influenzare negativamente l'affidabilità dei risultati. Queste caratteristiche rendono questa nuova procedura più conforme agli standard di riferimento, ma sempre seguendo i principi del metodo MBS. Per quanto riguarda l'uso possibile di Moringa oleifera (MO) come strumento di purificazione alternativa, dopo lo studio su quale parte della pianta avesse la migliore attività antimicrobica, l'analisi di diversi ceppi batterici, al fine di valutare eventuali differenze di azione e la valutazione della sua azione al variare di parametri chimici, come il pH, ho condotto in test sul campo, per dimostrare che i miei dati di laboratorio potrebbero essere confermati su ceppi batterici ambientali. Dopo aver verificato la validità del sistema, è stata valutata la possibilità che il protocollo utilizzato in laboratorio possa essere applicato ai

batteri comunemente presenti nell'acqua. Per questo motivo sono stati analizzati diversi siti di fiumi della regione del Lazio, testando sia i parametri chimico-fisici dell'acqua che l'indice microbiologico della conta batterica totale con il metodo MBS, per misurare la qualità e la sicurezza dell'acqua. L'analisi microbiologica è stata condotta prima e dopo il trattamento con MO, per monitorare l'azione del trattamento, dimostrando l'uso del metodo MBS come strumento di monitoraggio per le analisi dell'acqua, verificando l'azione purificante della MO nei campioni di acqua, anche se il suo utilizzo non potrebbe essere adatto nei paesi in via di sviluppo a causa del fatto che MO modifica in modo significativo le caratteristiche organolettiche dell'acqua, rendendola indesiderabile al consumo. Per quanto riguarda lo studio su un brodo di arricchimento per la ricerca di *Salmonella* spp. applicabile al metodo MBS per consentire l'analisi su 25 g di campione, rendendo il metodo MBS più conforme agli standard di riferimento, i risultati ottenuti da questa ricerca hanno dimostrato che il brodo di arricchimento MBS promuove una crescita selettiva di *Salmonella* spp. L'efficacia di questo brodo è stata dimostrata anche in campioni contaminati artificialmente con contaminazioni crociate. Prima dell'introduzione del brodo di arricchimento, le analisi usando le fiale specifiche sviluppate dal metodo MBS avevano una durata di circa 72 ore. Dopo l'introduzione del brodo di arricchimento, nella procedura MBS è stata registrata una consistente riduzione del tempo massimo di analisi, da 72 ore a 48 ore circa. Questo intervallo di tempo tiene conto della fase di arricchimento e dell'analisi con le fiale MBS. In conclusione, questa nuova procedura permette l'analisi su 25 g di campione e una significativa riduzione del tempo di analisi, senza influenzare negativamente l'affidabilità dei risultati. Queste caratteristiche rendono questa nuova procedura più conforme agli standard di riferimento, ma sempre seguendo i principi del metodo MBS. Infine, lo stesso tipo di ricerca è stata condotta per la rilevazione di *Listeria* spp.. In questo caso, il reagente originariamente sviluppato dal metodo MBS per la rilevazione di *Listeria* spp. non era sufficientemente sensibile e selettivo, quindi si è resa necessaria un'ottimizzazione del reattivo . I risultati ottenuti dalla ricerca hanno dimostrato che l'MBS LY è stato ottimizzato per l'analisi quantitativa con una diminuzione dei risultati falsi positivi, e il brodo di arricchimento MBS promuove una crescita selettiva di *Listeria monocytogenes* verso altre specie batteriche concorrenti. Una riduzione del tempo massimo di analisi è stata registrata nelle analisi quantitativa MBS, approssimativamente da 72 ore a 48 ore. L'analisi del campione dopo la fase di arricchimento selettivo nelle fiale MBS LY modificate non ha mostrato risultati altrettanto incoraggianti, comportando perciò ulteriori ricerche future per modificare nuovamente il reattivo MBS LY che potrebbe essere utilizzato

per eseguire analisi qualitative secondo il metodo MBS. In conclusione, l'ottimizzazione del reagente già presente ha determinato una diminuzione del rapporto di risultati falsi positivi e una consistente riduzione del tempo di analisi e il di brodo selettivo formulato permette l'analisi su 25 g o ml di campione con un significativo arricchimento selettivo di *Listeria monocytogenes*, generalmente molto difficile da rilevare seguendo le procedure di riferimento. È comunque necessario proseguire la ricerca per modificare nuovamente il reagente MBS LY di modo da renderlo adatto all'analisi successivamente alla fase di arricchimento, secondo i principi del metodo, per renderlo in linea con gli standard qualitativi.

ABSTRACT - New applications of the Micro Biological Survey Method (MBS)

Food and water safety is nowadays an extremely central issue concerning public health. In this respect, European and global inspection bodies have enacted many regulations and guidelines with the aim of detecting contaminants and pathogens possibly found in food matrices and water for human consumption, and set limits of acceptance for different microorganisms and matrices under analysis. Traditional microbiological methods of analysis are still the most popular and widely used, due to their reliability, relative simplicity and international consensus by regulatory agencies. They are in fact considered the "gold standards" in food and water diagnostics and thus overall well accepted. These standardized methods are most of the time classical culture methods that rely on specific media to enumerate and isolate viable target organism while suppressing the indigenous background flora. These methods are sensitive, easily adaptable, and can give both qualitative and quantitative information on the number and the nature of microorganisms present in the sample, but display several limitations. Due to their complex operating protocols, they involve a significant amount of time and labor, as well as a high chance of manual error; the processes are highly operator-dependent, and as a result the bacterial count can vary widely when calculated by different operators and different labs resulting in a subjective interpretation of results. Moreover, the costs associated with these methods can be high, not only considering the labor costs, but also the cost of materials necessary to conduct the testing. Most importantly the long time required for obtaining results, which can take up to several days, and the need for equipped laboratories make these methods unpractical in several situations. For these reasons, my PhD project focused on the improvement of the Micro Biological Survey Method (MBS), an original colorimetric method for microbiological analysis. This method measures the catalytic activity of redox enzymes of the main metabolic pathways of bacteria, allowing an unequivocal correlation between the observed enzymatic activity and the number of viable cells present in the samples. This correlation is obtained using ready-to-use disposable analytical vials filled with original reagents for selective counting of microorganisms. Analysis can be automated using a device, called Multi Reader (MR), which can perform simultaneously 8 independent analysis, at different temperatures. The MR is

controlled by a computer with a specific software which detects the color change of the vials and calculates the number of microorganisms present in the sample. The method can be applied in many fields and significantly reduces the time required to obtain test results comparable to traditional microbiology testing and its simple interface and operation requires a minimal level of training and operator skills. In particular, I focused my attention on different application fields, such as the use of the MBS method for the quality control of food, to implement the appliance of a self-control plan in the industrial-scale food system using the MBS method, in order to prevent the lack of food quality; its application for the quality control of water, in order to increase water microbiological monitoring of the new and already existing water sources, the optimization of media already used by the MBS method, to make them always faster according to the characteristics of the method, and solve any problem different matrixes could represent towards the method, and really new and original applications of the MBS method in other fields different from the ones already present, to enlarge the flexibility and applicability of the method. Nowadays, the use of the tropical plant *Moringa oleifera* as an alternative purification tool is becoming very popular in these countries, so the first aim of my research was to verify if this plant could be effective in water purification using the MBS method, developing a protocol of analysis easily applicable in developing countries. The second aim was the development of an enrichment broth for pathogens detection, in particular for *Salmonella* spp. and *Listeria monocytogenes*. These broths, in accordance with MBS principles, allow the analysis on 25 g of sample and a significant analysis time reduction, without negatively affecting reliability of results. These characteristics make this new procedure more in accordance with the reference standards, but always following the principles of the MBS method. Concerning the possible use of *Moringa oleifera* (MO) as an alternative purification tool, after the study on which part of the plant could have the best antimicrobial activity, the analysis on different bacterial strains, in order to evaluate any differences of its action, and the evaluation of its action varying chemical parameters, such as pH, I conducted in field tests, in order to prove that my laboratory evidences could be confirmed on environmental bacteria. After verifying the validity of the system, it was evaluated the possibility that the protocol used in laboratory could be applied to bacteria commonly present in water. For this reason, different sites of rivers in Lazium region were analyzed, testing both chemical-physical parameters of water and microbiological index of total viable count with the MBS method, in order to measure the quality and safety of water. Microbiological analysis were conducted before and after MO treatment, to monitor

the action of the treatment, demonstrating the use of the MBS method as a monitoring tool for water analyses, verifying the purifying action of MO on water samples, although its use could not be suitable for human consumption in developing countries due to the fact that MO significantly modifies the organoleptic characteristics of water, making it undesirable to drink. Concerning the study on an enrichment broth for *Salmonella* spp. detection, which could be applied to the MBS method in order to allow the analysis on 25 g of sample, making the MBS method more in accordance with reference standards, results obtained from this research demonstrated that the MBS enrichment broth promotes a selective growth of *Salmonella* spp. towards other bacterial species biochemically related to *Salmonella*. The effectiveness of this broth was also demonstrated in artificially cross contaminated samples, in order to repeat naturally contaminated food samples. Before the introduction of the enrichment broth, analyses using the specific vials developed by the MBS method lasted about 72 hours. After the enrichment broth introduction, a consistent reduction of maximum time of analysis was recorded in the MBS procedure using the MBS enrichment broth, from 72 hours to 48 hours approximatively. This maximum time frame accounts the enrichment phase and the analysis with the reaction vials. In conclusion, this new procedure allows the analysis on 25 g of sample and a significant analysis time reduction, without negatively affecting reliability of results. These characteristics make this new procedure more in accordance with the reference standards, but always following the principles of the MBS method. At last, the same kind of research was conducted on *Listeria* spp. detection. In this case, the reagent originally developed by the MBS method for *Listeria* spp. detection was not sensitive and selective, so it had to be optimized. Results obtained from the research demonstrated that the MBS LY was optimized for quantitative analysis with a decrease of false positive results, and the MBS enrichment broth promotes a selective growth of *Listeria monocytogenes* towards other competitor bacterial species. A reduction of maximum time of analysis was recorded in the MBS quantitative analyses, from 72 hours to 48 hours approximatively. The inoculum of the sample after the selective enrichment phase in the modified MBS LY vials was not so encouraging in results, prompting to further researches, in order to modify again the MBS LY reagent that could be used to perform qualitative analyses according to the method. In conclusion, the optimization of the reagent already present led to a decrease in false positive results ratio, and a consistent analysis time reduction, and the selective broth formulated allows the analysis on 25 g or ml of sample with a significant selective enrichment of *Listeria monocytogenes*, generally very difficult to detect following reference standards' procedures,

which is faster than the reference broth. It is however necessary to continue the research to modify again the MBS LY reagent that could be suitable for the analysis including an enrichment step, in accordance to the principles of the method in order to make it also in line with qualitative standards.

1. INTRODUCTION - Importance of microbiology in the quality evaluation of water and food

Food and water safety is nowadays an extremely central issue concerning public health. European and global inspection bodies have enacted many regulations and guidelines with the aim of detecting contaminants and pathogens possibly found in food matrices and water for human consumption, and set limits of acceptance for different microorganisms and matrices under analysis.

Traditional microbiological methods of analysis are still the most popular and widely used, because of their reliability, relative simplicity and international consensus by regulatory agencies. They are in fact harmonized open access methods looked at as the “gold standards” in food and water diagnostics and thus overall well accepted. These standardized methods are most of the time classical culture methods that rely on specific media to enumerate and isolate viable target organism while suppressing the indigenous background flora. These conventional test methods are sensitive, easily adaptable, and can give both qualitative and quantitative information on the number and the nature of microorganisms present in the sample.

Traditional culture-based methods however display several limitations. Due to their complex operating protocols, they involve a significant amount of time and labor, as well as a high chance of manual error; the processes are highly operator-dependent, and as a result the bacterial count can vary widely when calculated by different operators and different labs resulting in a subjective interpretation of results [1,2]. Moreover, the costs associated with these methods can be high, not only for labor costs, but also for materials costs necessary to conduct the testing. Most importantly the long time required for obtaining results, which can take up to several days, and the need for equipped laboratories, make these methods unpractical in several situations. For this reason, there is a continuous request for the development of more robust, efficient, sensitive, and cost-effective analytical methodologies to guarantee a faster analytical response in order to improve safety and quality, in compliance with legislation and consumers’ demands. In the last few years, several alternative methods have been developed for detection and identification of bacteria, focusing on different aspects,

such as sensitivity, selectivity, quickness, discrimination among vital and non-vital cells, and suitability for *in situ* analysis [3]. These alternative methods range from simple dip-stick-type tests to very complex automated systems that perform a variety of tests using a variety of techniques taken from chemistry, molecular biology, biochemistry, immunology, immunochemistry and molecular electronics.

All the alternative methods however display several limitations for their adoption in terms of variable reliability, cost, novelty and the need of highly equipped laboratories. A lot of work still needs to be done to ensure their reliability, decrease the labor necessary to prepare and handle the sample and ensure compatibility to on field testing [4]. All analytical methods need to be validated prior to their introduction into routine use [5]. Validation schemes have been established from the major global authorities to assess whether these methods perform at least as well as the corresponding reference methods [6-7]. In addition, a European and International Standard, EN ISO 16140 (2003), has been developed to provide a common reference protocol for the validation of alternative methods, as well as to determine general principles for their possible subsequent certification [8].

1.1. Microbiological safety and quality of water

The World Health Organization (WHO) stated that “Water entering the distribution system must be microbiologically safe and ideally should also be biologically stable.” In this context, the term ‘biological stability’ refers to the concept of maintaining microbial water quality from the point of drinking water production up to the point of consumption [9].
Water sources must have particular characteristics to be used for human consumption: they have to be clear, fresh, colorant and odor free, pathogens and toxic compound free, and their mineral composition has specific concentration limits. Therefore, water needs to show chemical and microbiological purity, in order to be defined as suitable for human consumption. It is important to consider sources of water provisioning and the treatments to perform to obtain water suitable for human consumption. Although the huge water availability on our planet, not all of sources are suitable for human consumption due to environmental pollution and anthropic activities; therefore it is fundamental to perform drinking water treatments [10].

Moreover, the World Health Organization (WHO) estimates that almost the 80% of diseases are directly or indirectly connected with water contamination [11].
Waterborne diseases are defined as human and animal infective illnesses directly or indirectly associated with water consumption [12].
In developing countries, the absence of hygienic treatment on sewage urban water (90% is discharged directly in rivers, lakes or in the sea), and the lack of sanitary structures and water supply is one of the main causes of infections and illnesses. The majority of deceases of children and undernourished people due to water contamination is reported in African and Asian middle-East countries [13].
Infecting agents are mainly present in feaces, where flies and mosquitos may reproduce, spawn and spread infection. More than one hundred percent of pathogens were estimated in contaminated waters (between bacteria, viruses, parasites and fungi) [14].
There could be a high risk of infection due to the serotype and the MID (Minimum Infecting Dose) of these microorganisms; moreover, water persistence and drinking water treatment resistance are two other main factors of persistence of waterborne diseases.
It has already been highlighted the importance of different purity parameters that drinking waters have to respect, such as the organoleptic characteristics, chemical composition and pathogens absence [15].
Drinking water must be colorless and odorless: temperature and electrical conductibility are the main physical parameters used to control these characteristics, but also chemical parameters, such as pH, fixed residue, water hardness and the presence of toxic compounds are fundamental to ensure water colorless and odorless.
Water is usually a neutral or slightly acidic reaction. Too high or too low values of pH may have an indirect hazard significance due to solubility of pipe components that can lead to a real toxic risk.
Fixed residue expresses the total amount of water salt. Salts presence is essential for drinking use due to the need of human body of a certain amount of salts. A discrepancy in salt levels could lead to nutritional deficiencies, when their level is too low, or could alter its orgaloleptic characteristics, when their level is too high.
Hardness is due to the content of soil salts, mainly calcium and magnesium. There are two hardness categories: "temporary" hardness, so called because it disappears after prolonged boiling, mainly due to bicarbonates and, in part, phosphates and silicates, and a "permanent"

hardness due to chlorides, sulphates and nitrates. Medium or high hardness can only cause changes in the taste of water.

Water can be declared suitable for human consumption only when analyzed, as well as from a chemical-physical point of view, even from the microbiological point of view. In particular, the microbiological control aims to ensure that water is not a vehicle for the transmission of pathogenic microorganisms. In a recent global study involving 20,000 children in seven developing countries, rotaviruses, protozoa of the genus *Cryptosporidium* and *Escherichia coli* bacteria were among the most pathogenic agents associated with severe forms of diarrhea [16]. The increase in *E. coli* contamination in domestic water is associated with an increase in childhood diarrhea [17].

Since pathogenic microorganisms detection and quantification methods are often articulated, costly and requiring specialized operators and are not available to all microorganisms, the microbiological quality evaluation of water is based on the definition and the research for indicator organisms established by regulations.

An organism must meet the following requirements to be considered an indicator:

- must be present in the water every time the pathogenic microorganisms are present;
- its concentration in water must be related to that of pathogenic microorganisms and should in any case reflect the level of microbiological pollution;
- its survival in water must be similar to that of pathogenic microorganisms;
- resistance to treatment and disinfection treatment must be similar to that of pathogenic microorganisms;
- must not be pathogenic;
- its indicator role must be valid in any type of water that requires a monitoring program;
- its characteristics must not change over time;
- it must not be able to multiply or grow in water;
- must be detectable by simple, accurate, rapid and economical methods.

The use of indicator organisms does not permit direct estimation of pathogenic microorganism presence in aquatic environment, but it allows the assessment of the likelihood that it will be present.

Traditionally selected microbiological markers for fecal contamination are coliforms, total and faecal, and faecal streptococci. However, in recent years, the various studies and experimental evidence have led to a review of the health significance of traditional fecal

contamination indicators, as caused by raw and untreated sewage and diffuse impacts from anthropogenic, pasture and agricultural activities [18]. Another important parameter seldom used for a general evaluation of the hygienic state of water is the total estimate of bacterial numbers in water. This measure is called Heterotrophic plate count (HPC) or TVC.
The two major international bodies, the United States Environmental Protection Agency (USEPA), and the European Union (EU) both include *E. coli* as a mandatory microbial indicator, and the USEPA regulates for total coliforms, via the Total Coliform Rule. Most drinking water guidelines also refer to the use of total estimates of bacterial numbers in water. This measure is generally called 'total heterotrophic plate count' (HPC) or 'standard plate count bacteria', and is considered to represent the general cleanliness of drinking water [19;20]. Legislative point of view, D.lgs. 31/01, for many of the chemical, physical and microbiological contaminants, limit values or "concentration thresholds" that have to be exceeded; if one or more contaminants are present in water in excess of the limit value it no longer has the potency requirements.

1.1.1. Traditional methods for drinking water analysis and related problems

Microbiological contamination is the most common and widespread danger associated with drinking water. The quality of drinking water continues to represent a main concern to consumers, water suppliers, regulators and most importantly public health authorities. Microbiological monitoring offers the most sensitive test for the detection of recent and potentially dangerous contamination, thereby providing a hygienic assessment of water quality and ensuring safe supplies of drinking water with high sensitivity and specificity [21].

There are many microorganisms of interest when dealing with water quality control. Ideally detection of individual specific pathogens could be effective but certainly unpractical since the concentration of these bacteria is low compared to other non-pathogenic organisms and methods to detect them are costly in time and money. Therefore, tests for the detection and quantification of indicators organisms which are expected to predict the potential presence of

pathogenic microorganisms in the water, are used and accepted by regulatory and public health agencies worldwide [22].

The current reference techniques for the routine detection and enumeration of indicator bacteria are: the multiple tube fermentation (MTF), plate count techniques or membrane filtration (MF).

All methods use media that are designed to select for the growth and identification of specific organisms. Generally, each method also includes inhibitors, restrictive temperatures, and/or limited nutrients to restrict growth of unwanted species [23].

Heterotrophic plate count standard methods. Heterotrophic plate counts (HPC) or total viable counts (TVC) are enumerations of the general population of heterotrophic bacteria present in water supplies. The enumerations may give information not only about bacteria whose natural habitat is the water environment, but also about those that have originated from soil or vegetation. HPC measurements are mainly used to indicate the effectiveness of water treatment processes, thus as an indirect indication of pathogen removal, or to monitor the performance of filtration or disinfection processes. HPC testing may be used in investigating aesthetic quality, and it is used by some authorities as a marker for some of the underlying causes of some aesthetic problems [24].

Heterotrophic plate count standard methods provide a standardized means of determining the density of aerobic and facultative anaerobic heterotrophic bacteria in water. Standard methods outline three techniques for determining a standard plate count: pour plate, or standard late count, spread plate, and membrane filtration (MF) [25]. The standard plate involves pouring 1 ml of the sample into a plate and pouring an agar solution over the top. The sample is allowed to incubate after the plate is ready. This is an effective method for quantifying organisms, and also allows more space for the colonies to grow. The spread plate method differs from the pour plate method because the agar plates are poured and are solidified before inoculation with samples and the quantity of spreading sample is 0.1 ml and allowing it to incubate. This method gives more 3-dimensional colonies than the pour plate method that are much easier to identify and count. The pour plate or spread plate methods are typically used for process water and drinking water [26].

The membrane filtration technique is best suited to test large volumes of low-turbidity, low-count (less than 1 to 10 Colonies Forming Units (CFU) /ml) water. Membrane filtration involves filtering a particular volume of a sample to get a higher concentration of bacteria, then rinsing the filter with a sterile buffer before placing it on an agar plate. This is generally the only method used to test sterile water. It can also be used to test drinking water depending on the application. This method provides good colony morphology, making it a good method for identifying the bacteria present in a sample [27].

All of these standard test methods require a significant amount of time and labor required, and the chance of manual error is high; the processes are highly operator-dependent, and as a result the CFU can vary widely when calculated by different operators and different labs [85]. Moreover, the costs associated with these methods can be high, in particular for labor costs and the cost of materials necessary to conduct the testing. The time required to get results is about 48-72 hours, and if the sample is improperly diluted so the results are too high or not enough to count, the sample must be retested. Once testing is completed, the process of counting the number of bacterial colonies is complicated and time-consuming, and it is easy to make mistakes during this process.

Total coliform and *Escherichia coli* count standard methods. Coliform is a subgroup of the family *Enterobacteriaceae,* but are further defined by functional characteristics rather than systematic genus and species [28]. The World Health Organization has defined coliforms as any rod-shaped, non-spore-forming, gram-negative bacteria capable of growth in the presence of bile salts or other surface-active agents. Continuing, the definition states that coliforms are cytochrome-oxidase negative and able to ferment lactose at either 35 or 37 °C with the production of acid, gas, and aldehyde within 24 to 48 hours [29]. The total coliform group is the most inclusive indicator classification, and their presence indicates inadequate disinfection of drinking water; but it does not always correlate properly with the presence of fecal pollution. Total coliforms monitoring at the treatment plant and in the distribution and storage systems is carried out to provide information on the adequacy of drinking water treatment and on the microbial condition of the distribution system. Their presence in groundwater indicates that it is vulnerable to contamination from the surrounding environment, so their detection in water which leaves the well should trigger further actions [29;30]. *Escherichia coli* is a thermophilic coliform that produces indole from tryptophan, but also defined now as coliform able to produce β-glucuronidase (although taxonomically up to 10% of environmental *E. coli*

may not). The bacterium's only natural habitat is the large intestine of warm-blooded animals and although *E. coli,* with some exceptions, generally does not survive well outside of the intestinal tract, its presence in environmental samples, food, or water usually indicates recent fecal contamination or poor sanitation practices in food-processing facilities. Also *E. coli* monitoring provide information on the adequacy of drinking water treatment and on the microbial condition of the distribution system [31].

The US Environmental Protection Agency (EPA) and AFNOR have approved several methods for coliforms and *E. coli* detection: the multiple-tube fermentation (MTF) technique and the membrane filter (MF) technique [32]. The MTF test uses a specified number of tubes (based on the expected population in the sample) with a specific medium and sample water. After incubation, each tube is examined for growth of the target organism(s). The number of tubes that shows growth are matched to a statistically determined table of numbers to yield the most probable number (MPN) of organisms in the sample. The number of presumptive *E. coli* is determined by enzymatic testing on samples that give a positive total coliform test [33]. The lauryl tryptose broth added with MUGlu as the medium for the multi-tube fermentation (MTF) technique was first proposed for rapid detection and immediate confirmation of *E. coli* in food and water samples. The presence of methylumbelliferone due to the hydrolysis of MUGlu (positive samples) is detected by exposure to long-wave UV light and visualization of blue-white fluorescence [34] .

The use of MTF for coliform bacteria detection significantly affected by different factors, especially during the presumptive phase. High numbers of non-coliform bacteria, as well as the inhibitory nature of the media have been identified as factors that could lead to an underestimation of coliform abundance. The MTF technique lacks precision in qualitative and quantitative terms. Results are obtained in a time that is higher than with the membrane filter technique, that has replaced the MTF technique in many instances for the systematic examination of drinking water. However, this technique remains useful, especially when turbid or colored waters do not allow the use of the membrane filter technique. MTF is easy to implement and can be performed by a technician with basic microbiological training, but this method can become very tedious and labor intensive due to many dilutions that have to be processed for each water sample. However, it is also relatively inexpensive, as it requires unsophisticated laboratory equipment. Nevertheless, this method is extremely time-

consuming, requiring 48 h for presumptive results, and necessitates a subculture stage for confirmation which could take up to a further 48 h [35].

The last technique is the Membrane Filtration (MF) method. It consists of filtering a water sample on a sterile filter with a 0.45-mm pore size which retains bacteria, incubating this filter on a selective medium and enumerating typical colonies on the filter. If the sample is contaminated with microorganisms of interest, colonies will grow on the membrane filter. Colonies are examined at 10–15X magnification with a stereoscopic microscope and then identified by size, color, shape and sheen. Typical colonies are counted and the result is reported as the number of colonies per 100 ml of sample. This technique is fully accepted and approved as a procedure for monitoring drinking water microbial quality in several countries. *E. coli* is determined from total-coliform-positive samples by the means of specific confirmation media. Different commercial agar media are now available for the detection of *E. coli.* They include classical agar media modified with specific chromogenic and/or fluorogenic substrates for the detection of β-Dglucuronidase activity. Many media and incubation conditions for the MF method have been tested for optimal recovery of coliforms from water samples [36].

The main concern about MF is its inability to recover stressed or injured coliforms, because different chemical and physical factors involved in drinking water treatment, such as disinfection, can cause sub lethal injury to coliforms, resulting in a damaged cell which is unable to form a colony on a selective medium. Bacterial exposure to products like chlorine may result in injury and increased sensitivity to bile salts or to the replacement surface-active agents (sodium desoxycholate or Tergitol 7) contained in some selective media. Other authors have suggested that chlorination affects various functions of coliform bacteria activity, such as catalase enzymatic activity. Metabolically active bacteria produce hydrogen peroxide (H_2O_2), which is usually rapidly degraded by the catalase. Injured coliforms with reduced catalase activity accumulate toxic hydrogen peroxide, to which they are extremely sensitive [37]. The high number of modified media in use is a consequence of the fact that no universal medium currently exists which allows optimal enumeration of various coliform species originating from different environments and present in a wide variety of physiological states. A significant advantage of the MF technique over the MTF method is that with MF, the examination of larger volumes of water is feasible, which leads to greater sensitivity and reliability, and it offers a quantitative enumeration comparatively to the semi-quantitative

information given by the MTF method. MF is a useful technique for the majority of water quality laboratories as it is a relatively simple method to use. Many samples can be processed in a day with limited laboratory equipment by a technician with basic microbiological training. However, since this method is not sufficiently specific, a confirmation stage is always needed, but this could take a further 24 h after the first incubation period on selective media.

Comprehensively these methods have limitations, such as duration of incubation, antagonistic organisms interference, lack of specificity to the coliform group and a weak level of detection of slow-growing or stressed coliforms. Indeed, depending on the environmental system, only a small portion (0.1–15%) of the total bacterial population can be enumerated by cultivation-based methods. The proportion of non-culturable bacteria may be affected by unfavorable conditions for bacterial growth during culturing or by their entry into viable or active but non-culturable states (VBNC or ABNC) [38].

Water purification techniques

The concept of water safety traces back to the nineteenth century, but it is only in the last century that appropriate criteria have been established to define its quality.
Water must have specific organoleptic characteristics and, above all, its use must not be a health risk, as the complete absence of pathogenic agents and dangerous chemicals.
The potable processes consist of a series of physical, chemical and biological treatments that give the water such indispensable properties.
Before being distributed, the water is subjected to various treatments: primary disinfection, coagulation, flocculation, filtration and final disinfection.
Primary disinfection: It is done to deactivate viruses, parasites such as Giardia intestinalis and protozoa such as Cryptosporidium. In many cases a primary disinfectant can also be used as oxidant. The most used oxidants / disinfectants are gaseous chlorine, sodium hypochlorite and chlorine dioxide. Chlorine, a powerful disinfectant, reduces hydrogen sulphide, removes the color and controls the smell and taste of the water. It helps remove particles and decreases turbidity as well as controlling the growth of algae and biofilms in the treatment plant tanks. [39]

Coagulation: Most of the impurities in the waste water are colloidal particles, which are not easily deposited. The undesirable most difficult substances to eliminate from water are those that have small size; these ones are finely dispersed in suspension and produce turbidity and color of water, and generally have negative charge. The addition in water of a coagulant causes compression of the double layer [40] and the neutralization of the electrostatic surface area of the particles. The resulting destabilized particles tend to aggregate forming agglomerates. During the treatment of water to remove particulate matter (eg bacteria), inorganic coagulants and / or polymers positively charged are usually used. The addition of the coagulant is performed while the water is shaken to dissolve the coagulating agent rapidly. The coagulant reaction is rapidly produced in hot water (1 to 10 sec) and can be strongly slowed down in cold water (<4 ° C). Numerous chemicals are used in coagulation and flocculation processes.

Flocculation: The purpose of the flocculation process is to aggregate the particles in a larger particles or "flakes" that will adhere to the bottom in the subsequent sedimentation process. Through slow and prolonged agitation, the suspended particles collide with each other and form flakes. Mixing should be enough to cause the particles to collide, but also delicate to prevent flocculate particles from falling. It is likely however that some flakes will disperse.

Clarification: This process is to clarify what is intended to produce clarified water. There are various types of sedimentation tanks (also called clarifiers) used for the treatment of drinking water.

Filtration: The purpose of filtration is to remove small particles and pathogens not eliminated by coagulation, flocculation and clarification processes. The most used filtration is on a granular medium that can be either sand or sand and anthracite, based on the physical trapping of small particles; this process removes over 99% of the microorganisms originally present in the water.

Final disinfection: The purpose of the final disinfection is to reduce the level of microorganisms in order to minimize the health risk to the users, since water disinfection means removal, deactivation or killing of pathogenic microorganisms [41]. Disinfection differs from sterilization as the first involves selective microorganisms destruction, while the second aims the elimination of all microorganisms.

Various products are used for disinfection, each one with its advantages and disadvantages, but the most used are gaseous chlorine and hypochlorite. To reduce the consumption of chlorine, it is advisable to check the pH of the treated water by bringing it to about 7.0.

Due to the limited availability and high cost of these chemicals used for the treatment of drinking water, in developing countries, there is an urgent need to find alternative water purification solutions that guarantee a level of hygiene and purity suitable for human consumption.

To date, it is undergoing a procedure that uses as a purifying element a natural extract present in the seeds of the *Moringa oleifera* plant, capable to flocculate the particles present in waters. The hypothesis is that this property, very interesting, is due to the fact that the seeds of this tropical tree contain a high amount of proteins with a relatively low molecular weight (6-16 kDa) and an isoelectric pH value of 10 [42], acting as cationic polyelectrolyte, capable of bonding strongly to the contaminant particles surfaces, charged negatively once they are added to raw water. Seeds can act directly on microorganisms by inhibiting their growth. It is probable that this action of *Moringa oleifera* is due to the presence of particular antimicrobial peptides acting or interrupting the cell membrane or inhibiting essential enzymes [43]. Sutherland et al. (1990) reported that this antimicrobial effect of the seeds is attributed to compound 4 (α-L-ramnopyranosyloxy) benzyl isothiocyanate [44].

Literature provides a simple treatment. It starts by picking white almonds from ripe seeds, then grind finely with a mortar and mix well with a small amount of water. After the coagulant addition, it is very important to have a rapid agitation of at least 5 minutes to ensure uniform dispersion of flour in the water, in addition to increasing the possibility of particle-to-particle contact. It is important not to use metal tools as this could introduce metallic ions. One liter of water can be treated with a quantity between 50 and 150 grams of *Moringa* seed flour based on the type of impurities initially present.

During mixing, fine particles and bacteria will begin to aggregate forming flakes that tend to deposit on the bottom for precipitation. This step can take up to one hour, after which all is removed by a filtration process using a clean cloth [45]. The results of this treatment are not yet fully clear and there is no adequate scientific evidence of its use as a purifying agent.

1.2. Microbiological safety and quality of foods: pathogen detection

Food science and the technology of food production and illness caused by foodborne pathogens continues to be a major problem to health and it is of economic significance. Estimates of the incidence of foodborne illnesses vary greatly, due mainly to large differences in the sources of data and in surveillance systems. Nevertheless, there is agreement that foodborne illness is one of the principal causes of human morbidity. In industrialized countries, the percentage of people suffering from foodborne diseases each year has been estimated to be up to 30% [46;47]. The most common pathogens responsible for foodborne gastroenteritis were pathogenic *Escherichia coli*, Norovirus, *Campylobacter* and non-typhoidal *Salmonella* [48]. It can be assumed that the burden of foodborne illness is of the same order of magnitude in most industrialized countries. It should also be taken into account that only a small proportion of these cases are ever brought to the attention of health authorities. Even when the incidence rate is low, the public health impact of foodborne illnesses can be high, due to the severe consequences these diseases have for certain segments of the population (e.g., young children, elderly people, pregnant women or immuno-compromised people) and the high mortality rate of certain diseases (e.g., listeriosis or botulism). Estimates have been made of the economic consequence of foodborne illness, where costs are incurred by individuals who become ill (including sequelae), by their employers, their families, health-care agencies and the food companies and businesses involved. Beyond productivity losses, additional costs may be incurred by industry or in some cases by governments. [49]

It has been the practice for governments to develop and enact basic food laws, and to set standards for safety and quality, including composition and labelling. The organization of inspection and compliance vary widely in different countries but it is essential that government and industry work together constructively. Powers have been given to local government inspectors to sample in factories to ensure that codes of Good Manufacturing Practice (GMP) and Good Hygienic Practice (GHP) are followed and meet regulations, and at retail and in food service establishments. Food Safety/Food Hygiene Regulations commonly cover general requirements for food premises (hygienic design, construction operation and sanitation of premises and equipment, hygiene of food preparation and processing operations,

education and training of personnel). Compliance has been largely by inspection, although shortcomings have long been recognized. Laws often contain vague terms that are open to different interpretations by inspectors. Sometimes important factors to food safety were given less importance than factors that were largely aesthetic or a matter of opinion. However, the sampling plans in the past decades were unable to detect hazards/defects occurring at low frequencies and negative results did not guarantee complete absence, e.g. from pathogens. Many food processes and practices developed after a problem occurred e.g. spoilage during transportation or human illness, its microbiological cause was identified, and a method determined to control it. For example, pasteurised milk was introduced in Denmark in 1870 and in the US in 1874; frozen meats were first transported from Australia to the UK in the 1880s; drinking water was chlorinated in the UK in 1905. As the scientific basis for safe food processing was strengthened, particular hazards were identified and processes for their control designed e.g. heat processing for low acid canned foods to eliminate spores of *Clostridium botulinum*. In the 1960's an alternative approach to food control was developed, initially to ensure microbiologically safe foods for US astronauts. The Hazard Analysis Critical Control Point (HACCP) approach offered a rational and structured approach to the control of food safety by identifying the hazards, their severity and risk; determining critical control points (CCPs) that will eliminate or control those hazards; establishing procedures that check that the CCPs are under control; and identifying appropriate corrective actions should they not be [50]. The adoption of the HACCP approach, with its records of details of the raw materials and processes applied, has greatly reduced the need for microbiological testing and limited to the validation and verification purposes. In 1962 a Joint FAO/WHO Codex Alimentarius Commission (Codex) was established to protect the health of consumers and to ensure fair practices in international food trade. Members develop documents that are a consensus based on available science, taking into account the needs of the food industry and the impact on international trade. [51]

For many years authorities have set limits or criteria for microbiological contaminations in foods without strictly following the framework laid down by the International Commission on Microbiological Specifications for Foods [52] and the Codex Alimentarius [53]. Criteria have often been set based upon experience of food production and processing, research and expert opinions of what was considered achievable in relation to the application of good hygienic practices on the one hand, and what was necessary to ensure food safety on the other. The risk

analysis framework, laid down by the Codex Alimentarius during the past ten years, has made it increasingly possible to link food safety activities to public health via risk assessment. Based on ‘the formal risk analysis approach’ concepts that have evolved include Appropriate Level of Protection (ALOP), Food Safety Objective (FSO) and Performance Objective (PO). Furthermore, this new framework emphasizes that Performance Criteria (PC), Process Criteria (PrC) and Microbiological Criteria (MC) should be scientifically based. Neither the well-established criteria nor these new concepts have been used consistently. Importantly, with the new concepts, microbiological criteria must be discussed in a broader perspective than previously[51].

1.2.1. Traditional methods for *Salmonella spp.* and *Listeria monocytogenes* detection

Salmonella spp. detection

Salmonellae are organisms that conform to the definition of the Enterobacteriaceae. Most strains are motile. Apart from a few exceptions that form acid only, they produce acid and gas from glucose and mannitol, and usually also from sorbitol; they ferment sucrose or adonitol rarely, and rarely form indole. Acetylmethylcarbinol is not formed. They do not hydrolyze urea or deaminate phenylalanine, usually form H_2S on triple sugar iron agar, and use citrate as sole carbon source. They form lysine and ornithine decarboxylases. The many serotypes in the group are closely related to each other by somatic and flagellar antigens and most strains show diphasic variation of the flagellar antigens. Salmonellae are primarily intestinal parasites of vertebrates; they are pathogenic for many species of animals, giving rise to enteritis and to typhoid-like diseases [54].

Over the years, numerous techniques have been developed to highlight these pathogens in foods. However, Regulation (EC) 2073/05 [55] is shown in the analytical protocol in ISO 6579: 2008 [56], which describes a horizontal method for the search for Salmonella spp. in foodstuffs intended for human consumption and zootechnical use (Figure 1). This procedure is used for qualitative research (therefore only presence / absence) in foods. It involves four

steps (pre-enrichment, enrichment, isolation, identification) because Salmonella can be present in very small quantities and is often accompanied by considerable amounts of other microorganisms especially belonging to the *Enterobacteriaceae* family [57].

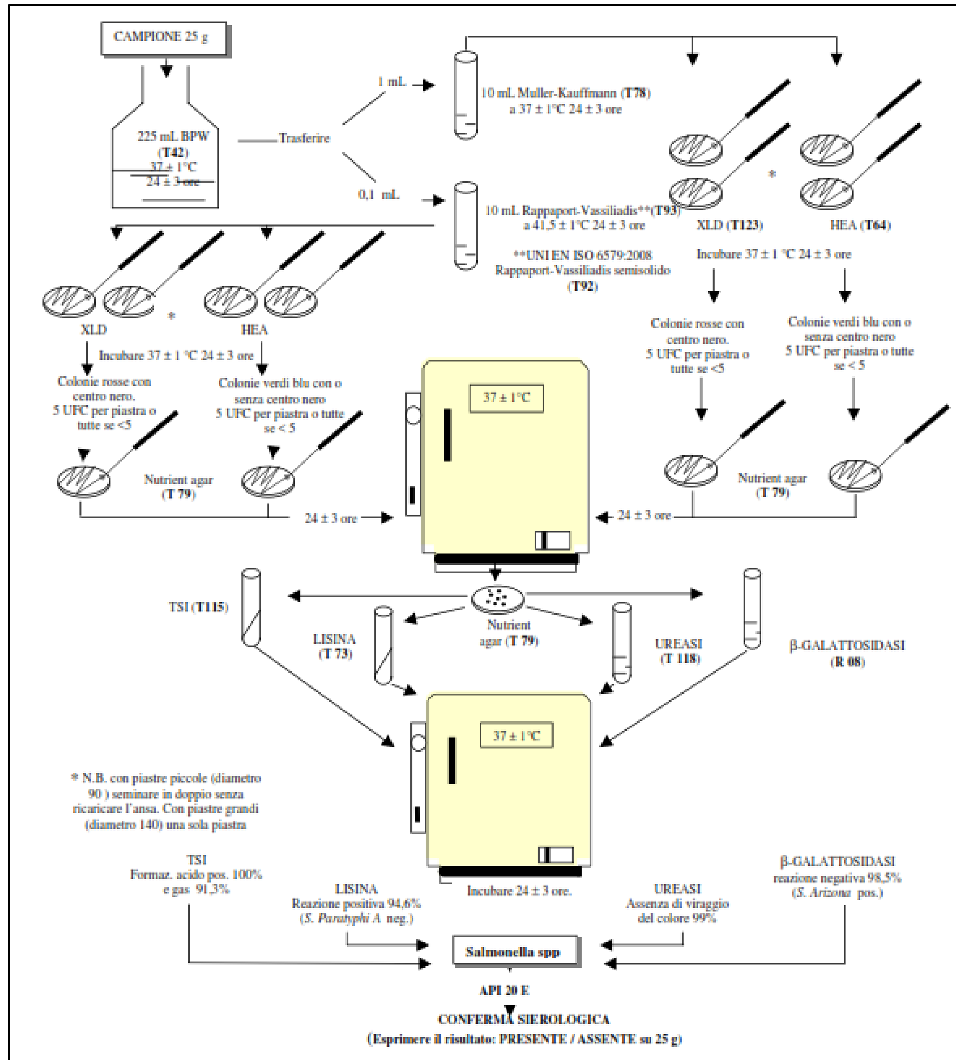


Figure 1. UNI EN ISO 6579:2008

Pre-enrichment is primarily intended to revitalize low concentrations of Salmonella spp. eventually present in the sample and is carried out by taking 25 grams (or ml) of the product to be analyzed and then inoculated into 225 ml of a pre-enrichment broth, called Buffered Peptone Water (BPW). After homogenization, the pre-enrichment broth is incubated at 37 ° C for 24 hours.

After 24 hours of enrichment in BPW, pre-enrichment broths were transplanted with a sterile loop on two Xylose Lysine Deoxycholate Agar (XLD agar) selective solid medium plates and two plates of another selectable and / or differential soil at the operator's choice, such as Brilliant Green Agar (BGA) or Hektoen Enteric Agar (HEA). In addition to inhibiting Gram-positive bacteria, these media discriminate *Salmonella* species by detecting the presence of sulphurous hydrogen with black colonies, the formation of acids by the fermentation of the sugars present, or the lysine decarboxylation.

At the same time, two aliquots of 1 ml and 0.1 ml of the solution are taken in BPW and placed in two tubes: one containing 10 ml of Muller Kauffmann enrichment broth which will be incubated at 37 ° C for 24 hours and one containing 10 ml of Enrichment broth Rappaport Vassiliadis that will be incubated at 41.5 ° C for 24 hours. After 24 hours of enrichment, both broths were sown with a sterile loop on two selectable solid medium XLD agar plates and two plates of another selective and / or differential soil of the operator's choice, as in the case of sowing after pre-enrichment in BPW.

As a result, both the plates prepared from the pre-enrichment broth and from the two enrichment broths (after further incubation), on solid media will develop growth units described as colonies or colony-forming units (Colony- Forming Unit, CFU) that are defined as suspect if they have certain morphological characteristics: red with the black center on XLD agar, green with or without black center on HEA, red colonies surrounded by red alone on BGA. After the identification of suspected colonies, 5 CFUs (or all colonies present if <5) should be transferred to species-specific Nutrient agar preparation medium, then incubated for 24 hours at 37 ° C followed by isolation of *Salmonella* spp.

This isolation phase of suspected colonies follows the biochemical identification of Salmonella species and subspecies with a series of tests including, for example, urea, lysine, decarboxylase, β-galactosidase and so on. Only cultures where biochemical confirmation of Salmonella presence will be serologically typed [54].

This method, although is the only legally approved, clearly requires quite long times, from 4 to 5 days, which does not meet the need for a pathogen detection system which is faster and at the same time reliable[58].

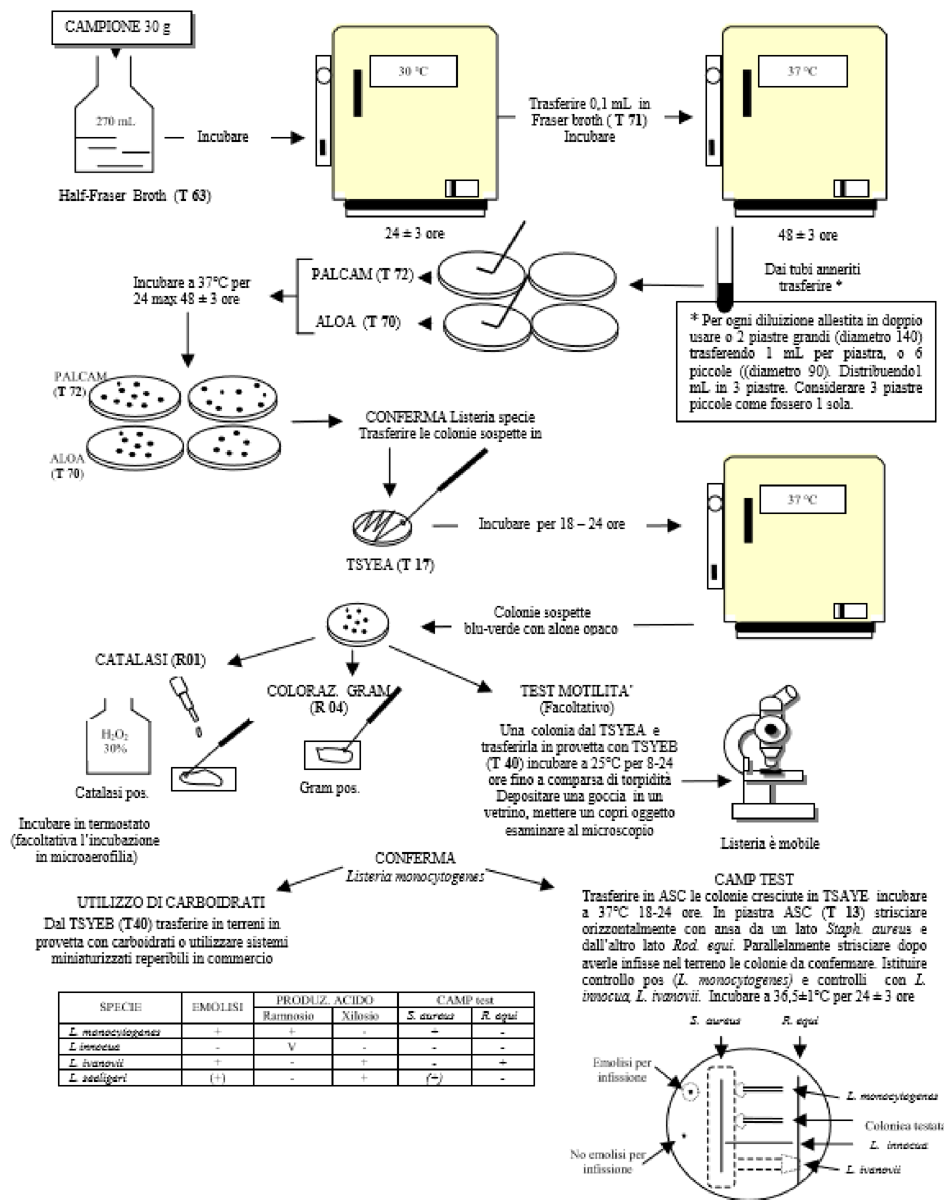
Listeria spp. detection

Listeria are coccobacillary- to bacillus-shaped gram-positive organisms. They are nonsporing and motile by peritrichate flagella. They are aerobic and microaerophilic organisms that grow between <0 and 45°C. The organisms exhibit fermentative activities on carbohydrates, producing predominantly lactate and no gas from glucose. Listeria spp. are catalase-positive and oxidasenegative. The genus Listeria consists of six species: monocytogenes, grayi, innocua, ivanovii, seeligeri, and welshimeri. L. monocytogenes is the major pathogen for a wide variety of animals, including man, causing septic lesions in various organs. The G + C content of DNA is 36–42 mol% and the type species is *Listeria monocytogenes* [59].

The high mortality rate associated with the listeriosis and the associated risk with the presence of L. monocytogenes in foods and environments make reliable and rapid methods for the detection of this bacterial species to be of paramount importance for the preservation of public health. Microbiological risk associated with *Listeria monocytogenes* is also associated with the detection of other *Listeria* species, for example *Listeria innocua*, in foods, since the presence of *Listeria* spp. is an index for the development of *L. monocytogenes*, since all the leaflets grow in very similar habitats [60]. Beyond the method provided by food safety regulations, following the long Standard Operating Procedures (POS) based on methods validated by the International Standard Organization (ISO), there are several methods for quicker detection of Listeria spp. In the EC Regulation 2073/2005 [55] on microbiological criteria for foodstuffs, the analytical protocols in UNI-EN-ISO 11290-1 [61] and UNI-EN-ISO 11290- 2 [62], specifying respectively the horizontal method for research (qualitative analysis) and the quantitative analysis of L. monocytogenes in foodstuffs intended for human or animal consumption.

The standard UNI-EN-ISO 11290-1 for qualitative analysis requires the total absence of the bacterium in 25 g of the sample analyzed. It is applied as a reference analysis method for the research of L. monocytogenes in ready-to-eat foods infants and ready-to-eat foods for special medical purposes and ready-to-eat foods that are favorable to the growth of *L. monocytogenes* other than those intended for infants and for special medical purposes. This food safety criterion is applied in the first case to products placed on the market during their shelf life and in the latter case before the foodstuffs are no longer under the direct control of the food business operator that produces them, that is, before 'food leaves the factory where it was

produced. This standard describes an analysis protocol, schematically represented in Figure 2, divided into 4 steps: pre-enrichment, enrichment, isolation and identification. The first two steps are of utmost importance because they allow the selective growth of *L. monocytogenes*, which may initially be present in foods at low concentrations and often along with many other bacterial genres. Pre-enrichment involves the inoculation of X g (or ml) of product to be analyzed in 9X ml of a low selectivity pre-enrichment broth, called Half Fraser Broth. After homogenization, the sample in pre-enrichment broth is incubated at 30 ° C for 24h. In the enrichment step, 0.1 ml of the culture obtained in the previous step is inoculated into 10 ml of increased selectivity enrichment broth called Fraser Broth and placed at 35-37 ° C for 48 hours . Then, by enrichment, 10 µl of a sterile strain are sowed on two selectable soluble media for *Listeria* spp .: Agar Listeria according to Ottaviani and Agosti (ALOA) and Oxford or PALCAM (Polymyxin-Acriflavin-Lithium Chloride-Ceftazidime -Asculin-Mannitol) agar. Thus, the plates are incubated at 35 ° -37 ° C for 24h or 48h according to the medium used. In presence of *Listeria* on plates, growth units will be considered as colonies or colony-forming units (CFUs) with certain morphological characteristics, depending on the culture media used: green-blue surrounded by mature sole on the ground solid ALOA; small, greyish with black alone and centered on solid OXFORD Agar; greyish-gray with black sole and sometimes with black center on PALCAM Agar. After this phase there is the identification of suspected colonies: they are drawn from each plate 5 CFU (or all suspected colonies if less than 5) with a sterile loop and spread on the surface of plates containing Tryptone Soya Yeast Extract Agar (TSYEA), such plates are incubated at 35 ° -37 ° for 24 hours or more, in general for the time required for satisfactory growth. Finally, the colonies of *Listeria* spp., which will appear colorless, opaque, with uniform edges and with a diameter of 1-2 mm on this medium, will be used in morphological, physiological and biochemical tests aimed at the definitive identification of L. monocytogenes or more generally of bacteria belonging to the *Listeria* genus.



SPECIE	EMOLISI	PRODUZ. ACIDO		CAMP test	
		Ramnosio	Xilosio	*S. aureus*	*R. equi*
L. monocytogenes	+	+	-	+	-
L. innocua	-	V	-	-	-
L. ivanovii	+	-	+	-	+
L. seeligeri	(+)	-	+	(+)	-

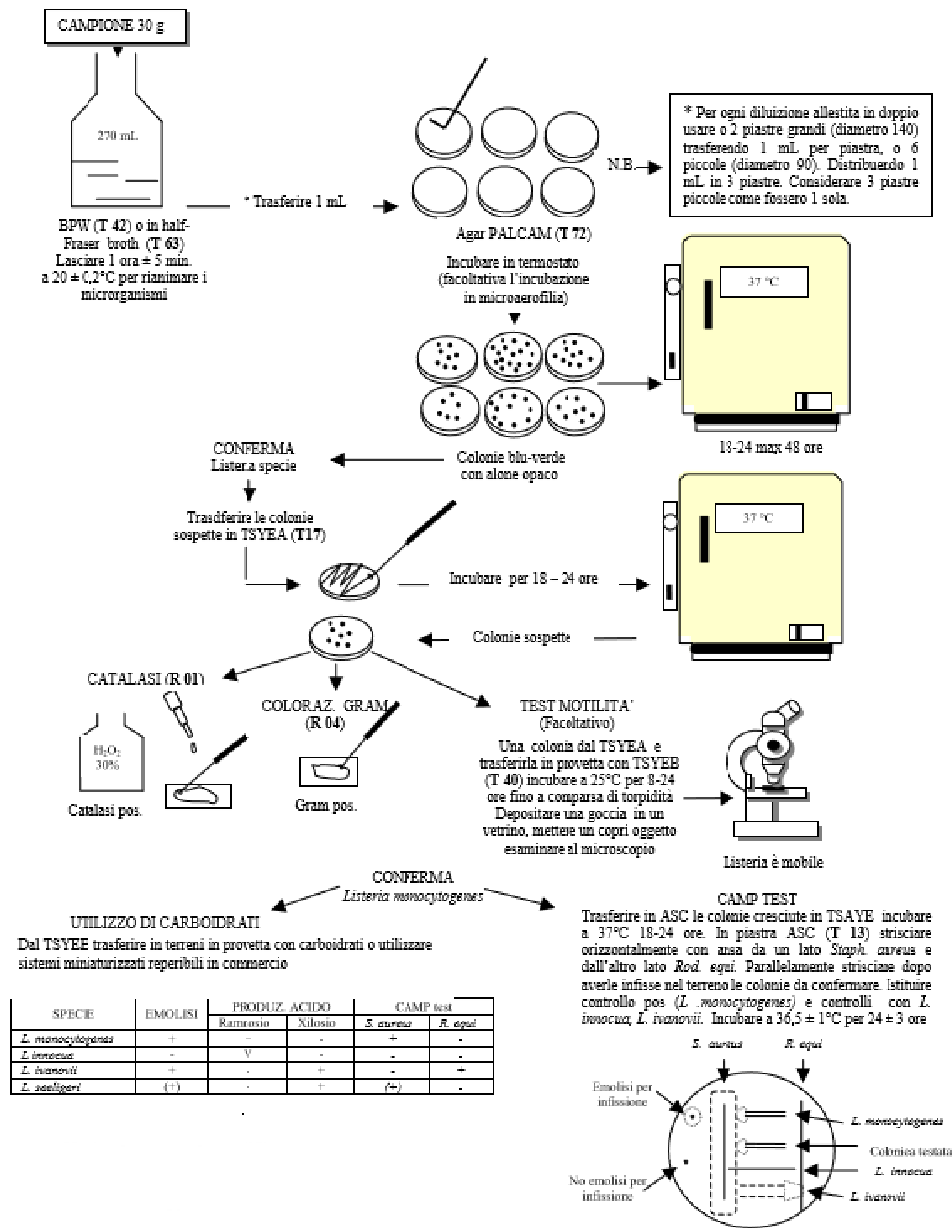
Figure 2. UNI EN ISO 11290-1

The UNI-EN-ISO 11290-2 [62] procedure for quantitative analysis for the verification of a L. monocytogenes CFU number of less than 100 colony forming units per sample 1g is carried out on foodstuffs which are favorable to the growth of *L. monocytogenes* other than those intended for infants and for special medical purposes and ready-to-eat foods that are not favorable to the growth of L. monocytogenes, other than those intended for infants and for special medical purposes, and is applied in both cases during l the entire shelf life of the product. This procedure, diagrammatically represented in Figure 3,is divided in 4 steps: initial suspension of the food sample in a non-selective liquid medium for *Listeria*, then sowing on plates and incubation, counts on the Listeria characteristic colonies and confirm the specific identity of the bacterium. The sample to analyze is suspended in a liquid such as peptonate

water or Half Fraser Broth without antibiotics and placed at 20 ° C for approximately 1h in order to revitalize the microorganisms present in the sample if the sample conditions, transport and storage of food can cause stress and metabolic inhibition in the associated bacteria, preventing their subsequent detection through selective growth passages. After this first incubation, an amount of 0.1 ml is taken from the culture, spread on a PALCAM plate and incubated at 37 ° C for 24-48h. Once the time has elapsed for the emergence of suspected colonies, the regulation establishes the counting of these colonies only in plates with a maximum of 150 CFU of the bacterium of interest. As in the case of UNI-EN-ISO 11290-1 [61], UNI-EN-ISO 11290-2 [62] also envisages the selection of 5 suspected colonies on each plate (or all suspected colonies if less than 5), their spreading on TSYEA plates, and incubation such plates at 35-37 ° C for 24h. The suspected colonies will be used in various confirmatory tests (morphological, physiological and biochemical) necessary for the identification of the genus *Listeria*, including the pathogenic *L. monocytogenes*.

The main confirmatory tests adopted by these two ISO standards for genetic and species confirmation are: Gram staining, catalase reaction, motility test, β-hemolysis in ground-based blood, excl. hydrolysis and the production of acids free from gas from glucose, maltose and salicin, but not mannitol. Biochemical confirmation can be made faster using Listeria-specific identification systems such as the Listeria API test strip, allowing simultaneous testing of various biochemical and a species-level identification.

Another test used to confirm the identity of Listeria spp. is the Christie-Atkins-Munch-Peterson (CAMP) test. It allows to distinguish the various species of Listeria spp. depending on the ability to produce a co-hemolysin called CAMP factor: this protein contributes to the destabilization of the red blood cell, making it more apparent a β-hemolysis zone. *L. monocytogenes* shows a positive CAMP reaction while. *L. innocua*, however, is negative to this test. Listeria isolates can also be confirmed and characterized by serological typing analysis. Typization is important for the identification of the responsible strain of the epidemic cases, due to the ingestion of contaminated foods and for the observation of the correlation between the strain found in food sample and the environmental one.



SPECIE	EMOLISI	PRODUZ. ACIDO		CAMP test	
		Ramnosio	Xilosio	*S. aureus*	*R. equi*
L. monocytogenes	+	-	-	+	-
L. innocua	-	V	-	-	-
L. ivanovii	+	-	+	-	+
L. seeligeri	(+)	-	+	(+)	-

Figure 3. UNI EN ISO 11290-2

1.3. The Micro Biological Survey method (MBS)

The Micro Biological Survey method (MBS) is an alternative, fast and simple colorimetric system for quantitative and qualitative microbiological analyses validated according to ISO 16140 [8;63;64]. The MBS analysis is performed in disposable ready to use reaction vials that contain the specific reagent for the analysis to be performed. The presence of bacteria is revealed by a color change of the reagent present in the vials in times that are inversely proportional to the bacterial concentration in the sample. In order to allow selective detection

and quantification of bacteria and meet nutritional requirements of different microorganisms, MBS reagents contain different classes of compounds: nutrients, selective agents, indicators, reducing agents.

Nutrient. These include sources of organic carbon, nitrogen, minerals (phosphorus and sulfur) as well as growth factors (amino acids, purines, pyrimidines) vitamins and trace elements (Mg, Fe, Mn) that stimulate bacterial growth.

Selective agents. The selectivity is accomplished in several ways. For example, organisms that can utilize a given sugar are easily screened by making that sugar the only carbon source in the medium. On the other hand, selective inhibition of some types of microorganisms can be achieved by adding organic substances, antibiotics, salts or other specific inhibitors which affect the metabolism or enzyme systems of the organisms.

Indicators. Organic substances capable of changing color as a result of variations of the chemical and physical state of the medium.

Reducing agents. Organic substances capable of releasing electrons to the respiratory chain, and that are not easily oxidized by the oxygen dissolved in solution.

Vaseline. Organic substance used to avoid an external input of oxygen into the reaction vial; as highly hydrophobic compared to reagent, it sets at the interface with the air excluding the oxygen input.

1.3.1. Micro Biological Survey method biochemical principles

In contrast with traditional methods that measure the capability of cells to grow in discrete colonies, the MBS method measures the catalytic activity of redox enzymes in the main metabolic pathways of bacteria allowing an unequivocal correlation between enzymatic activity and bacterial concentration in the samples. Detection of bacterial metabolism (enzymatic activity) is possible thanks to the presence of a redox indicator, the TMPD (N, N, N ', N'tetrametil-p-phenylene diamine hydrochloride).

In its oxidized form, TMPD displays a blue coloration, while it becomes yellow in its reduced form; its potential, of about 250 mV, is very close to the one of cytochromes belonging to the mitochondrial electron transport chain of aerobic and anaerobic bacteria, which goes from100 to 300 mV. For this reason, this indicator can be easily reduced by cytochromes and, at the

same time, can be directly oxidized by oxygen, which has a potential of about 820 mV. TMPD is an anphipathic molecule, capable of crossing the cytoplasmatic and mitochondrial membranes and rapidly reduce cytochrome c [65] (Figure 4).

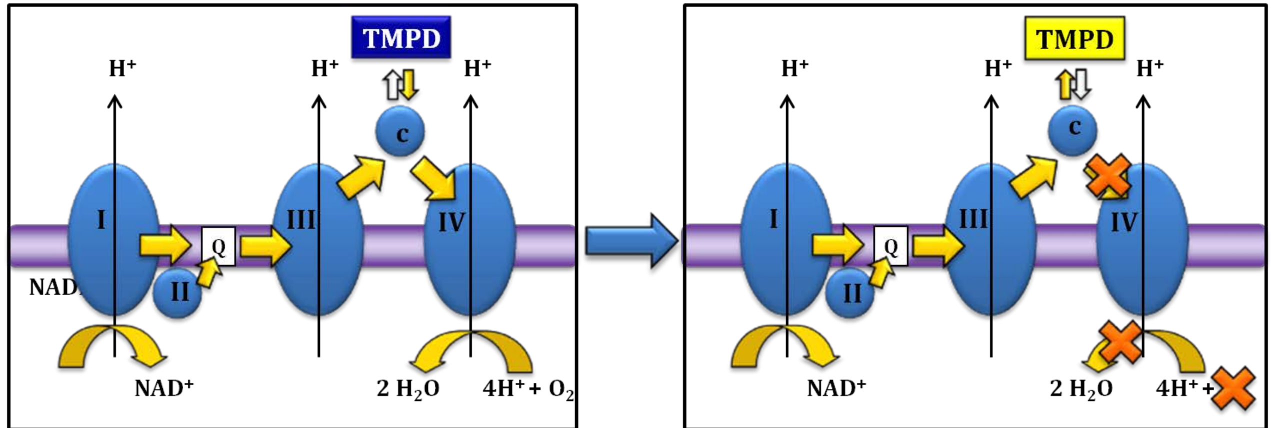


Figure 4. TMPD oxidation of TMPD in presence of oxygen (left); reduction of TMPD in absence of oxygen (right).

Starting from the inoculation of the sample, bacteria are in a lag phase and, consequently, the oxygen present in the vial oxidizes the indicator, which becomes blue. When bacterial growth reaches the log phase, being in the presence of oxygen, all electrons are transported toward the IV complex of the respiratory chain favoring reduction of oxygen, leaving TMPD in its oxidized blue form. Once all the oxygen present in the vial has been consumed by bacteria and is completely reduced to a point of saturation, electrons are transferred to the TMPD, which will be reduced, turning yellow. To prevent the passage of atmospheric oxygen in the solution, making this reaction possible, an appropriate amount of vaseline (1.5 ml) is included in the reaction vials acting as a "cap".

This kind of reaction is well visible for the MBS reagent, i.e. total viable count reagent (TVC vials), for which the starting color blue becomes yellow in the presence of bacteria thanks to the reduction of TMPD (Figure 5).

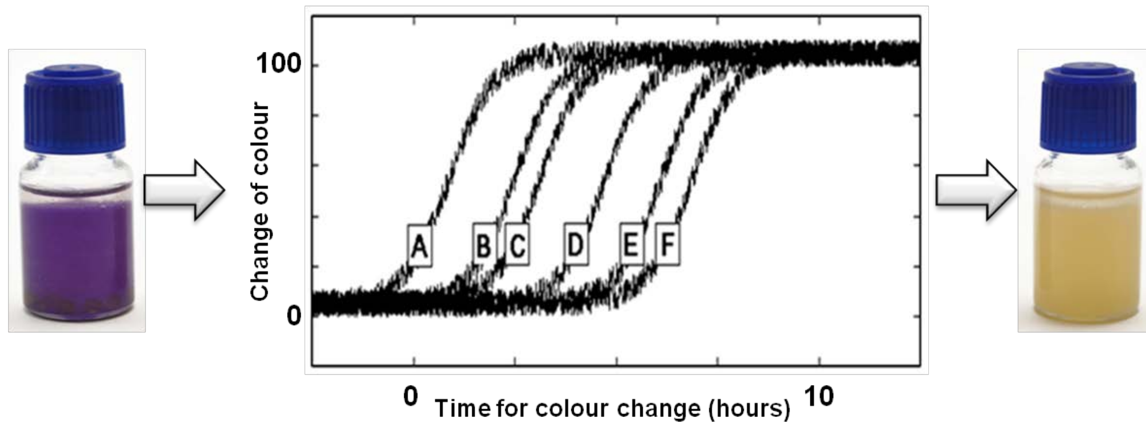


Figure 5. Color change of the MBS vials. The starting color blue (left), changes to yellow (right) in the presence of bacteria. In the absence of bacteria the color remains blue. The time required for color change is inversely related to bacterial concentration ([A]>[B]>[C]>[D]>[E]>[F])

In some MBS reagents, instead, the color change is determined by the presence of Phenol Red, an indicator of soil acidification that occurs through fermentation processes. This indicator changes color from red to yellow between the pH range that goes from 8.2 to 6.4. For these MBS reagents it is, therefore, possible to observe the color change, from red to yellow, once TMPD is completely reduced, only in the presence of bacteria able to grow in the selective medium and to ferment the source of carbohydrates of the medium, for example in the MBS reagent for detection and quantification of total coliforms and *E. coli* (COLI vials) [63].

The time required for a color change is inversely related to the logarithm of bacterial concentration in the sample: like an enzymatic reaction, the greater the number of bacteria, the faster the color change [66]. Table 1 shows an example of the correlation between the time taken by an MBS reagent to change color (expressed in hours) and the bacterial concentration of the sample analyzed (expressed as log CFU/ml or g). Each MBS reagent shows different times of reaction (meaning the time taken to change color in relation to bacterial concentration of the sample), therefore each reagent has its own specific calibration curve.

Table 1. Example of correlation between bacterial concentration (expressed as CFU/g or ml) and time taken for the vials to change color (expressed as hours).

Time for color change (hours)	Contamination (CFU/g or ml)
< 3.30	> 10^6
3.30	10^6
6.30	10^5
9.30	10^4
12.30	10^3
16.00	10^2
19.00	10
22.00	1
> 24.00	0

1.3.2. Micro Biological Survey method analytical procedure

The analytical procedure of the MBS method is extremely simple, allowing analysis also in the absence of an equipped laboratory and specialized personnel. The simple execution and interpretation of results, together with the analytical principle of the method, results in a significant reduction of time and costs while assuring the same performance, in terms of sensitivity, repeatability and reproducibility, of traditional methods of analysis [64].

For the MBS analysis, samples can be directly inoculated in the reaction vials without any preliminary treatment. Before starting the analysis vials must be opened, filled with 10 ml of sterile distilled water and shaken until the reagent is completely dissolved. The final color required to start the analysis (specific for each type of reagent) is achieved in 5-10 minutes. To start the analysis, 1 ml for liquid samples (water or a homogenate of a solid or semi-solid matrix), and approximately 1g for solid samples (or directly the swab for surfaces), can be directly introduced in the vials. The vials must be then incubated in a thermostatic device that maintains the given temperature of incubation depending on the type of microorganisms being sought (30°, 37° or 44°C). Results are obtained monitoring color variation of the vials at

different times, referring to the color scale provided with the test kit. After the analysis, the safety cap is pushed down; this opens the tank that releases the sterilizing compound into the vials; in a few minutes all microorganisms are eliminated and vials can be disposed as non-pathogenic waste.

To complete the analysis it is necessary to relate the time taken by the vial to change color to the bacterial concentration of the sample. This can be done using the reference table provided with the kit.

1.3.3. Micro Biological Survey Multireader

The MBS Multireader (MR) is a modular optical device that is made to incubate multiple samples, automatically detect the color change of the reaction vials and calculate the bacterial concentration in the sample. This device automates the analysis process, performing simultaneously 8 independent analyses and makes it unnecessary to check the color change of the vials. The device stations are independent from each other, therefore it is possible to perform at the same time analyses at different temperatures.

The MR is controlled by a computer with a specific software that is able to automatically detect the color change of the vials and calculate, according to the time taken for the vials to change color, the number of microorganisms in the sample. The analysis progression can be followed and monitored in real time. The final result is automatically recorded in a database from which it is possible to recover the results of interest and build a printable analysis report (Figure 6).

Figure 6. MBS Multireader

Each MBS reagent displays the characteristics of linearity and accuracy. Linearity, according to ISO 16140 [8], is the ability of the method when used with a given matrix to give results that are in proportion to the amount of analyte present in the sample, that is, an increase in analyte corresponds to a linear or proportional increase in results. For the MBS method the time required for a color change is inversely related to the logarithm of bacterial concentration in the sample: like an enzymatic reaction, the greater the number of bacteria, the faster the color change [66]. Accuracy, according to ISO 16140 [8], is the degree of correspondence between the response obtained by the reference method and the response obtained by the alternative method on identical samples.

2. AIMS

The incidence of waterborne and foodborne diseases is a severe problem and international food and water trade are disrupted by frequent disputes over safety and quality requirements. The foremost responsibility of controls is to enforce law(s) protecting the consumer against unsafe, impure and fraudulently presented products by prohibiting their consumption [67]. Microbiological control of water and food products is regulated, and the "gold standard" analyses are generally culture-based methods. These methods are sensitive, quite inexpensive, and simple [4]. However, these methods are time consuming, require specialized personnel and equipped laboratories, so it became important to develop and refine alternative microbiological methods of analysis, quicker and easier to perform than the corresponding reference methods. They minimize manipulation, provide results in less time, and reduce costs. They also normally involve some form of automation, and often capture data electronically. In particular alternative microbiological method technologies aim to provide more sensitive, accurate, precise, and reproducible test results when compared with conventional, growth-based methods. In this context, the alternative Micro Biological Survey (MBS) method has been developed and found successful application in water and food quality testing with the aim to propose a simple, economic and effective method to evaluate the microbiological quality of water from different sources and in diverse contexts. Previous studies demonstrated the possibility to use MBS method as a valid tool in the biomonitoring field, also in developing countries [83], where water disinfection is very difficult to achieve with traditional methods. Nowadays, the use of the tropical plant *Moringa oleifera* as an alternative purification tool is becoming very popular in these countries, so the first aim of my research was to verify if this plant could be effective in water purification using the MBS method, developing a protocol of analysis easily applicable in developing countries. The second aim was the development of an enrichment broth for pathogens detection, in particular for *Salmonella* spp. and *Listeria monocytogenes*. These broths, in accordance with MBS principles, allow the analysis on 25 g of sample and a significant analysis time reduction, without negatively affecting reliability of results. These characteristics make this new procedure more in accordance with the reference standards, but always following the principles of the MBS method.

3. MATERIALS AND METHODS

3.1. Application of the Micro Biological Survey method in the evaluation of purifying activity of *Moringa oleifera* seeds in water samples

Experiments have been carried out at the beginning in order to evaluate the effective amount of flour obtained from the *Moringa oleifera* (MO) seeds for the breakdown of the bacterial load present in the artificially contaminated water samples. The establishment of the effective amount of MO flour has been carried out to assess whether such treatment could be applicable to bacteria commonly found in polluted waters and if the purifying action of MO could vary according to the contaminating bacterial species.

Moringa oleifera seeds *Moringa oleifera* seeds used in research come from Asmara, the capital of Eritrea. They were delivered in dried form and then stored in normal envelopes at room temperature. To obtain fine flour, these seeds were crushed with a mortar. Flour, obtained by crushing the whole seed (almond and external tegument), was then stored in sterile falcon. Experiments were then conducted using increasing amounts of flour corresponding to a definite number of seeds, to achieve the effective reduction in bacterial water loading. The mean weight of the whole seeds of *Moringa oleifera* is 0.22 g and the quantity in grams of flour corresponding to the number of seeds selected for the experiments was weighed and used when needed.

Bacterial strains *Escherichia coli* (ATCC 25992); *Salmonella enterica serovar typhimurium* (ATCC14028); *Enterococcus faecalis* (ATCC 29212)

Culture media All dehydrated media were dissolved in distilled water, sterilized in an autoclave at 121°C for 15 minutes, under the pressure of 1 atmosphere.

Brain Heart Infusion (BHI) (VWR International S.r.l., Milan) is a liquid, enriched, non-selective, general-purpose medium suitable for the cultivation and maintenance of a wide

variety of microorganisms, including fast-growing growth pathogens such as molds and yeasts, using suitable temperature and incubation times. It is recommended as a universal ground for aerobic bacteriology (Table 1).

Tryptic Soy Agar (TSA) (Sigma Aldrich, St Luis, MO, USA)is used for the cultivation of mesophilic bacterial charge, allows the growth of non-demanding and / or moderately demanding microorganisms and is widely used for water and food analysis. Due to its nutritional characteristics, the absence of inhibitors and the possibility of being supplemented with the most diverse compounds, this medium is well suited for the isolation of pathogenic and growth-causing organisms.

MacConkey Agar (MC) *(Liofilchem, Roseto degli Abbruzzi, Italy)*. Selective and differential culture medium for bacteria used for the isolation and differentiation of Gram-negative enteric bacilli, and it conforms to Harmonized USP/EP/JP. Requirements is widely used for the research and isolation of Gram-negative enterobacteria because the presence of bile and crystalline-violet salts makes the selective substrate for this group of microorganisms [69;72].

Reference method. The standard plate count method for the enumeration of colony forming units (CFU/ml) on Tryptic Soy Agar medium, MacConkey agar; was used as the reference method for the assessment of bacterial concentration in the samples. 1 ml of the samples was plated using the pour plate technique and incubated in a thermostat at 37°C for 24 hours. The results were obtained counting the plates with a number of colonies between 30 and 300. All experiments were carried out in triplicate.

Preparation of artificially contaminated samples To obtain artificially contaminated samples, distilled water was collected in glass bottles, then sterilized in autoclave at 121° C at the pressure of 1 atmosphere for 15 minutes, cooled to a temperature close to 25° C and aliquoted in sterile falcon tubes of 50 ml. Serial dilutions were then set up in 1:10 sterile physiological solution starting from stationary bacterial cultures of the microorganism used for experimentation; 100 μl of dilution were used to contaminate sterile water samples. The artificially contaminated samples were then treated with MO flour.

Sample treatment with *Moringa oleifera* flour The protocol followed for the artificially contaminated water samples treatment with flour obtained from *Moringa oleifera* seeds was

that described in the literature [45]. All the steps have been taken in order to reproduce the conditions of a possible use in developing countries.

The amount of flour selected for experimentation was added in the falcon tubes containing artificially contaminated water samples. Samples were vigorously shaken with hand for 5 minutes to ensure uniform dispersion of flour and increase flocculation capacity of the flour. The samples was left upright with the conical bottom of the falcon facing down for 60 minutes in order to facilitate the flour sedimentation. The samples were then filtered through a cotton cloth previously washed in water and bleach and allowed to dry at room temperature. Subsequently, the samples were inserted into sterile falcon tubes and analyzed in order to evaluate the residual bacterial charge against a control of an artificially contaminated water sample but not treated with MO flour.

Evaluation of the effect of *Moringa oleifera* depending on pH. For all the tests distilled water has been used, which has a neutral pH. In naturally contaminated water samples however, there are many substances that could affect its value. For this reason, the effect of *Moringa oleifera* was evaluated at pH variation. These experiments were conducted both with acidified water and with alkaline water.

Various substances such as acetic acid (CH_3COOH; pKa = 4.76) and hydrochloric acid (HCl) were used to acidify water for variation in pH, while tri- (tris- (2-hydroxymethyl) - aminomethane, $C_4H_{11}NO_3$, pKa = 8.07) and sodium carbonate (Na_2CO_3) to make it alkaline. This change was performed shortly after sterilization of sterilized distilled water by adding a sterile stock of the substance used to vary the pH.

Electronic FIB Microscopy The different samples were analyzed by the focused ionic beam microscope (FIB) in order to detect MO's surface mechanics. The FIB (Focused Ion Beam) microscope is nothing more than a SEM (electronic scanning microscope) where, however, in place of the electronic beam, an ion beam is generated by a liquid gallium ion source that is focused through electromagnetic lenses and then projected onto the sample area to be examined. The FIB can also be encapsulated in a system with both ion and electron columns, allowing the same aspect to be analyzed using both the beams and the bundles. While the ion beam explores the sample, the surface of the same interacts with it, emitting electrons that are subsequently harvested and used to form the enlarged, high-resolution image visible in the fluorescent screen. The microscope used is the FEI Helios NanoLab ™ 600: this instrument

uses an ion bundle that is appropriately focused to display, cut (milling) or apply, depending on the applied current, particular materials Measurements were made with the Agilent 8453 spectrophotometer in visible light (between about 400 and 700 nm), from near ultraviolet to near infrared.

In a first spectrophotometric measurement two different conditions were analyzed; water samples treated with filtered MO, in absence of bacteria, and diluted samples 1: 5 (600 µl of H_2O treated and 2.4 ml of white). The analysis was carried out by inserting 3 ml of each sample into different cuvetes and analyzed in the visible light at a wavelength of 600 nm. In a subsequent spectrophotometric analysis the variability of the turbidity of different samples of water treated with a known amount of MO (15 seeds, equivalent to 3.3 grams of flour) and the sample not diluted and diluted to 1:5. This measurement took place at different times (T = 0 h, 6 h, 14 h, 24 h) in order to evaluate a variability of turbidity according to time.

3.1.1. Application of the *Moringa oleifera* for the purification of several surface waters of Lazio

In the next phase of the research, further experiments were carried out to evaluate the effectiveness of the flour obtained from MO seeds in the treatment of naturally contaminated water samples. In particular, the protocol described above has been applied to samples of several surface waters of Lazio.

Study and sampling area Samples of water were collected from several rivers in the Lazio region (Figure 7) in April 2015. Samples were harvested using sterile falcon tubes or sterile pirex glass bottles previously sterilized in autoclave at 121 ° C for 15 minutes, and brought to our laboratory in a refrigerated container where the sample was stored at a temperature between 2 and 8 ° C. All samples were analyzed within 8 hours after sampling.

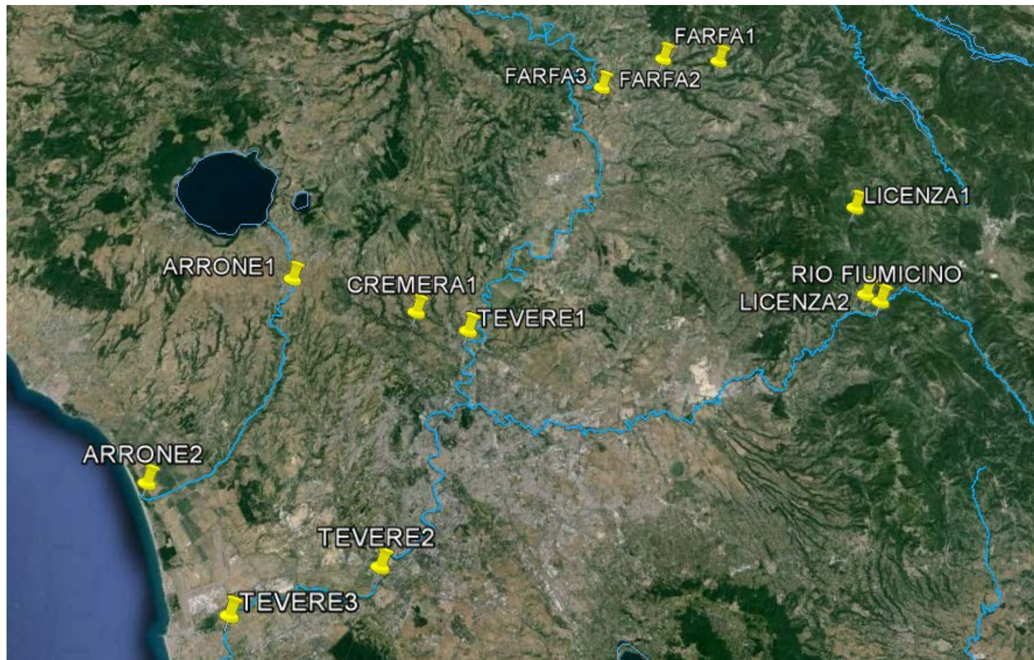


Fig. 7. Sampling sites in Latium region.

Reference method. The standard plate count method for the enumeration of colony forming units (CFU/ml) on Tryptic Soy Agar media was used as the reference method for the assessment of bacterial concentration in the samples. 1 ml of the samples was plated using the pour plate technique and incubated in a thermostat at 37°C for 24 hours. The results were obtained counting the plates with a number of colonies between 30 and 300. All experiments were carried out in triplicate.

MBS Method The MBS vials are made in PET (Polyethylene Terephthalate), provided with a screw cap for a secure closure and a safety cap that includes a tank containing a sterilizing chemical compound that must be manually injected in the vial, after testing, for proper disposal. All the components of the MBS reagents are micronized together (granulometry < 10 micron) in a mortar and then distributed in defined amounts, in each vial. Before closure, 1.5 ml of vaseline is added to each vial. After preparation, the vials are sterilized, using Gamma (γ) rays (5kGy), by a specialized company. Vials so sterilized are ready for use. MBS vials' shelf-life is generally 6 months at room temperature and 1 year at 4°C.

MBS TVC vials. The MBS TVC reagent allows the growth, detection and quantification of nonfastidious as well as moderately fastidious aerobic or microaerophilic microorganisms. The TVC reagent was validated according to ISO 16140 [8]. The presence of bacteria is detected by a color change from blue to yellow.

Sample treatment with flour obtained from *Moringa oleifera* seeds All the naturally contaminated water samples were treated with the flour obtained from MO seeds, following the protocol described for *in vitro* experiments. Water samples were then added with 3.3 grams of flour (corresponding to 15 seeds) for 50 ml of water and after rapid agitation of 5 minutes, they were left standing upright to allow flour sedimentation. After one hour, the flour was removed by filtration of water through a clean cotton cloth. For each water sample, the microbiological quality was analyzed before and after the treatment with MO flour, taking into account the Total Viable Count parameter at 20-25 ° C.

3.2. Application of the Micro Biological Survey method for *Salmonella* spp. detection in food samples

Bacterial strains Microorganisms from the ATCC (American Type Culture Collection) database, all belonging to the *Enterobacteriaceae* family, were used in this thesis work, in particular: *Salmonella* spp. (*Salmonella typhimurium* ATCC 14028, *Salmonella enteritidis* ATCC 13076); *Citrobacter freundii* (ATCC 13316); *Enterobacter cloacae* (ATCC 13047); *Escherichia coli* (ATCC 25992); *Klebsiella pneumoniae* (ATCC 33495).

Each freeze-dried ATCC strain of these microorganisms was prepared from stationary stage cultures using the BHI (Brain Heart Infusion) broth and overnight incubation at 37 ° C.

Culture media All dehydrated media were dissolved in distilled water, sterilized in an autoclave at 121°C for 15 minutes, under the pressure of 1 atmosphere.

Brain Heart Infusion Broth (BHI) (VWR International S.r.l., Milan) is a liquid, enriched, non-selective, general-purpose medium. Suitable for the cultivation and maintenance of a wide variety of microorganisms, including annoying growth pathogens, molds and yeasts, using suitable temperature and incubation times. It is recommended as a universal ground for aerobic bacteriology.

Tryptic Soy Agar (TSA) (Sigma Aldrich, St Luis, MO, USA) is used for the cultivation of mesophilic bacterial charge, allows the growth of non-demanding and / or moderately demanding microorganisms and is widely used for water and food analysis. Due to its

nutritional characteristics, the absence of inhibitors and the possibility of being supplemented with the most diverse compounds, this medium is well suited for the isolation of pathogenic and growth-causing organisms.

Brilliant Green Agar: (BGA) *(Liofilchem, Roseto degli Abbruzzi, Italy)*is a selectable soil for the isolation of Salmonella spp., With the exception of Salmonella Typhi and Salmonella Paratyphi A from fecal matter, dairy products or other samples with suspected contamination. The presence of bright green tends to inhibit the growth of enterobacteria by selectively favoring *Salmonella* spp. growth, except *S. Typhi*.

MacConkey Agar (MC) *(Liofilchem, Roseto degli Abbruzzi, Italy)*. Selective and differential culture medium for bacteria used for the isolation and differentiation of Gram-negative enteric bacilli, and it conforms to Harmonized USP/EP/JP. Requirements is widely used for the research and isolation of Gram-negative enterobacteria because the presence of bile and crystalline-violet salts makes the selective substrate for this group of microorganisms [69;72].

Violet Red Glucose Agar (VRBGA) (VWR International S.r.l., Milan) is a selective and differential soil for isolating and counting enterobacteria in foods. The medium presents as fermentable carbohydrate the glucose, the fermentable substrate as well as the coliforms, also from pathogenic Enterobacteriaceae, so that all enterobacteria grow with red colonies with or without bile salt precipitation, with a diameter equal to or greater than 0.5 mm .

Muller Kauffmann Broth (Sigma Aldrich, St Luis, MO, USA) is an enrichment broth for Salmonella spp. in water, in food and in faecal samples. There is no preparation for this ground because it is sold in 100 ml ready-to-use tubes. Triptone and soy peptone are the sources of carbon, nitrogen, vitamins and minerals. Calcium carbonate is the buffer. Sodium thiosulfate is the source of sulfur. The bile salts and brilliant green mainly inhibit the growth of Gram positive bacteria, favoring the isolation of salmonella.

MBS method The MBS vials are made in PET (Polyethylene Terephthalate), provided with a screw cap for a secure closure and a safety cap that includes a tank containing a sterilizing chemical compound that must be manually injected in the vial, after testing, for proper disposal. All the components of the MBS reagents are micronized together (granulometry < 10 micron) in a mortar and then distributed in defined amounts, in each vial. Before closure, 1.5 ml of vaseline is added to each vial. After preparation, the vials are sterilized, using Gamma

(γ) rays (5kGy), by a specialized company. Vials so sterilized are ready for use. MBS vials' shelf-life is generally 6 months at room temperature and 1 year at 4°C.

MBS SL vials The MBS SL reagent allows the growth, detection and quantification of Salmonella species, defined by WHO as a gram negative rods genus belonging to the *Enterobacteriaceae* family [70]. The SL reagent was validated according to ISO 16140 [8]. The presence of bacteria is detected by a color change from red to yellow.

The formulation of this reagent required bibliographic researches, which were carried out in order to identify the compounds capable of ensuring the selective development of the species of interest. Since the medium is patented, it is not possible to show the composition in its entirety, the names of the substances present in the MBS SL reagent are indicated by acronyms:

Table 2. Composition of MBS SL reagent

Composition	Quantity g/l
Carbon and Nitrogen source B	20
Selective agent C	10
Vitamin source Y	1
Glucide M	10
Buffered system P	20
Buffered system H	5
Selective agent Sc	5
Selective agent T	9
Color indicator PR	0.2
Selective agent Vb	0.005
Selective agent S	0.045
Selective agent Su	2
Selective agent Am	2
Aminoacid L	1

Reference method. The standard plate count method for the enumeration of colony forming units (CFU/ml) on Tryptic Soy Agar (TSA); Brilliant Green Agar (BGA); MacConkey (MC); Violet Red Bile Glucose Agar (VRBGA); Violet Red Bile Lactose Agar (VRBLA) was used as the reference method for the assessment of bacterial concentration in the samples. 1 ml of the samples was plated using the pour plate technique and incubated in a thermostat at 37°C for 24 hours. The results were obtained counting the plates with a number of colonies between 30 and 300. All experiments were carried out in triplicate.

3.2.1. Development of experimental enrichment broth

In the initial stages of the work, the possibility of using directly naturally contaminated food samples was evaluated. Given the difficulty in finding food samples naturally contaminated by Salmonella spp., And to exclude the interference of food matrices [57], the enrichment broth was tested in vitro with artificially contaminated water samples.

Preparation of artificially contaminated samples In the first phase of the experiment, the artificially contaminated samples were prepared by contaminating 25 ml sterile water with ATCC strains of *Salmonella* spp. (*Salmonella typhimurium*, ATCC 14028 and *Salmonella enteritidis* ATCC 13076) and *Enterobacter cloacae* (ATC 13047).

Starting from sterile BHI overnight culture broths from different strains of interest, serial dilutions in sterile physiologic were set up. A presumed count of the bacterial concentration levels present in the various dilutions was carried out the day before experiments by sowing two ml of the different selective digestion dilutions (BGA for Salmonella spp. And VRBGA for Enterobacter cloacae). The results were obtained by counting CFU/ml after 24 hours of incubation at 37 ° C.

On the day of the experiment, 24 ml of sterile water were aliquoted in 50 ml sterile falcon. Each sample was contaminated with a single microorganism: 1 ml of the selected dilution was inoculated into the sterile water sample.

Preparation of the different formulations of the experimental broth

The various formulations of the experimental broth were prepared by dissolving the ingredients in 225 ml of sterile water. Once a homogeneous solution was obtained to assure sterility of starting, the experimental broth was filtered using filters of 0.45 μm and then aliquoted in 400 ml sterile feed envelopes. So prepared soil was stored in a refrigerator at 4 ° C.

The table shows the various ingredients used in the first phase of the experiment and the concentrations tested. As the soil in question could be patented, it is not possible to show the composition in full, the names of the substances present in the MBS reactive are indicated by acronyms.

Table 3. Composition of MBS SL enrichment broth and the reagent's concentration tested

Composition	Quantity (g/l)
Carbon and nitrogen source B	20
Vitamin source Y	5
Glucide X	10; 12
Selective agent T	1; 2; 5; 9; 10
Salt S	5; 7
Selective agent Vb	0.0125
Selective agent S	0.25; 0.5; 0.7; 1; 2; 4
Selective agent SD	2; 3; 4.75
Selective agent Vm	0.01
Selective agent I	4.75

The sterility of the reagent was verified the day before the experiment by inoculating 3 ml of broth filtered into TSA and counting CFU / ml after 24 hours at 37 ° C.

Evaluation of the selective enrichment capacity of the different formulations of the experimental broth The artificially contaminated samples prepared as previously described were inoculated into the experimental broth and incubated for 48 hours at 37 ° C. In order to evaluate the replication capacity of the various inoculated microorganisms within the experimental broth, it was necessary to verify its initial and final concentration (after the enrichment step) using the reference method. The results were obtained by counting CFU/ml after 24 hours of incubation at 37 ° C. The exact concentration of bacteria initially inoculated in the experimental broth before enrichment was calculated according to the formula:

$$Cf = (Ci \ x \ Vi) \ / \ Vf$$

where Cf = Concentration of bacteria initially inoculated in the experimental broth; Ci = CFU / ml of the selected dilution; Vi = Inoculum volume; Vf = Final volume of the experimental broth

Growth levels of the different inoculated microorganisms in the experimental broth were evaluated after 24 and 48 hours of enrichment at 37 ° C. Serial dilutions of the contaminated enrichment broth prepared in sterile physiological solution: 1 ml of the different dilutions were seeded in duplicate on selective medium (BGA for Salmonella spp. And VRBGA for Enterobacter cloacae). The plates thus prepared were incubated for 24 hours at 37 ° C. The results were calculated as previously described.

Evaluation of the enrichment capacity of the experimental broth with respect to the enrichment broth ISO Muller Kauffmann

Preparation of artificially contaminated samples. In the second phase of the experiment the sample quantities were reduced. In order to allow the same sample to be analyzed with both enrichment broths, at this stage the artificially contaminated samples consist of 10 ml sterile water contaminated with different concentrations of ATCC strains of *Salmonella* spp. (*Salmonella typhimurium*, ATCC 14028. *Salmonella enteritidis* ATCC 13076), *Enterobacter cloacae* (ATCC 13047), *Escherichia coli* (ATCC 25992), *and Klebsiella pneumoniae* (ATCC 33495). Samples were contaminated with a single microorganism or combinations of different microorganisms. Dilutions of interest were chosen using the preselected count method as previously described.

To evaluate the susceptibility of the experimental enrichment broth to the Muller Kauffmann broth, 9 ml of sterile water was aliquoted into 15 ml sterile salmon and contaminated with 1 ml of the dilution selected to obtain starting concentrations of *Salmonella* spp. of about 10^2 CFU / ml: 5 ml of the so prepared specimens were inoculated into 45 ml of experimental broth and the remaining 5 ml in 50 ml Muller Kauffmann broth, following the ratio specified in ISO 6579: 2008 [56].

To evaluate the selectivity of the enrichment broth, 9 ml of sterile water was aliquoted into 15 ml sterile falcon tubes and contaminated with 1 ml of the dilution selected to obtain starting concentrations of *Citrobacter freundii, Enterobacter cloacae, Escherichia coli* and *Klebsiella pneumoniae* of about~ 10^6 CFU / ml: 5 ml of the so prepared specimens were inoculated into 45 ml of experimental broth and the remaining 5 ml in 50 ml of Muller Kauffmann broth.

To assess the selectivity of the enrichment broth in case of cross-contamination 8 ml of sterile water were aliquoted in 15 ml sterile falcon and contaminated with 1 ml of the two dilutions selected so as to obtain different combinations of *Salmonella* spp. and enterobacteria selected for experimentation: 5 ml of the so prepared specimens were inoculated into 45 ml of experimental broth and the remaining 5 ml in 50 ml of Muller Kauffmann broth.

Enrichment with broth Muller Kauffmann. Samples prepared as previously described were inoculated in sterility in 50 ml of Muller Kauffmann broth and incubated for 48 hours at 37° C.

Evaluation of the sensitivity and selectivity of the experimental broth and Muller Kauffmann broth. The artificially contaminated specimens, prepared as above, were inoculated in the experimental broth and in parallel in the broth Muller Kauffmann and incubated for 24-48 hours at 37 ° C.

Growth levels of the different microorganisms, inoculated in both the experimental broth and Muller Kauffmann broth, were evaluated after 24 and 48 hours of enrichment at 37 ° C. Serial dilutions of both broths were sterile physiological: 1 ml of the different dilutions were seeded in double on selective soil (BGA for *Salmonella spp*., VRBLA for *Citrobacter freundii*, and *Klebsiella pneumoniae*, VRBGA for *Enterobacter cloacae*, MacConkey for *Escherichia coli*). The plates thus prepared were incubated for 24 hours at 37 ° C and the results were calculated according to the plate count method previously described.

In cross-contamination cases, considering the final application of the enrichment broth to the MBS analysis, the concentration of strains analyzed following enrichment in both broths was evaluated only after 24 hours. In cases of single-cell bacterial contamination, the starting concentration of inoculated micro-organisms in the sample and subsequently in the experimental broth was evaluated as previously described.

In cross-contamination conditions, the starting concentration was calculated as previously described. Concerning the final concentration of the different strains (contaminants and cross contaminants) in the experimental broth and Muller Kauffmann broth, after the enrichment passage, serial dilutions of both broths were sterile physiological: 1 ml of the various dilutions were sown in double on selective BGA plates. The plates thus prepared were incubated for 24 hours at 37 ° C and the results were calculated according to the plate count method previously described. Since the BGA medium is selective and differential, differentiating the microorganisms according to the different coloring of colonies (red-purple for *Salmonella* spp. and yellow-green for *Citrobacter freundii, Klebsiella pneumoniae, Enterobacter cloacae* and *Escherichia coli*) the *Salmonella* spp. concentration was calculated counting the number of colonies morphologically related to *Salmonella* spp. (red-purple colonies surrounded by a red color of medium), and enterobacterial concentration by counting the number of colonies morphologically characterized by yellow to yellow-green colonies and surrounded by yellow-green areas.

Statistical analysis. All the results shown in the thesis, mean values and standard deviations, refer to an average of three independent experiments in which each sample was analyzed in triplicate. To evaluate the significance of these results, the data were analyzed with the t-test, using GraphPad Prism6 software. The differences with a p-value ≤ 0.05 and those with a p-value ≤ 0.01 were statistically significant.

3.2.2. Application of the experimental enrichment broth to the Micro Biological Survey method

The last phase of the experiment aims to evaluate the benefits of introducing into the analytical procedure of the MBS method of enrichment step. The enrichment was carried out in the experimental broth and Muller Kauffmann broth.

Preparation of artificially contaminated samples The artificially contaminated specimens were prepared as previously described.

Analysis with the Micro Biological Survey method To perform the analysis in the MBS SL vial, the standard procedure provided by the method was followed. The contents of the SL vials were rehydrated with 10 ml of sterile water and the vials were agitated until complete dissolution of the vial. Subsequently, 1 ml of the sample was inoculated directly into the vial and the vials were incubated for 72 h at 37 ° C in MBS Multireader as required by the original protocol.

Evaluation of sensitivity and selectivity of SL reagent before enrichment. To evaluate the sensitivity and selectivity of the SL vials before the enrichment step, 1 ml of sample was inoculated in triplicate in the MBS SL vial and the vials were incubated in MR for 72 hours at 37° C to verify the presence / absence of color changing. The results were provided directly by MR in terms of hours of output.

To verify that the output was due to the actual presence of *Salmonella* spp. and to ensure that lack of color changing was not due to the absence of enterobacteria, , 1 ml of the same samples (and any dilutions in case of artificially contaminated samples with high

enterobacteria concentrations) was plated in duplicate on selective media (BGA for *Salmonella* spp., VRBLA for *Citrobacter freundii*, *Klebsiella pneumoniae*, VRBGA for *Enterobacter cloacae*, MacConkey for *Escherichia coli*) in parallel to the MBS analysis. The plates were then incubated for 24 hours at 37° C and the results were calculated according to the plate count method previously described.

Evaluation of sensitivity and selectivity of the SL reagent after enrichment. 5 ml of artificially contaminated samples prepared as previously described were inoculated in 45 ml of experimental broth and incubated for 24 hours at 37° C; 5 ml of the same samples were inoculated into 50 ml of the Muller Kauffmann broth in parallel. After the enrichment step, 1 ml of contaminated broths were inoculated in triplicate in the SL vials. The MBS SL vials were incubated in MR for 72 hours at 37 ° C to verify the presence / absence of toner. The results were provided directly by MR in terms of hours of output.

To verify that the output was due to the actual presence of Salmonella spp. and to ensure that the lack of toning was not due to the absence of enterobacteria, in parallel to the MBS analysis, serial dilutions of both enrichment broths were made sterile physiologically: 1 ml of the different dilutions were plated in duplicate on selective media (BGA for *Salmonella* spp., VRBLA for *Citrobacter freundii,* and *Klebsiella pneumoniae*, VRBGA for *Enterobacter cloacae*, MacConkey for *Escherichia coli*). The plates thus prepared were incubated for 24 hours at 37 ° C and the results were calculated according to the plate count method previously described.

In cross-contamination conditions, the same analytical procedure was performed only with the experimental broth. In this case, to verify that the output was due to the actual presence of Salmonella spp. and to ensure that cross-contaminant enterobacteria were present, in parallel to MBS analysis, serial dilutions on sterile physiological solution of the experimental broth were made: 1 ml of the various dilutions were seeded in double on selective BGA and the results were calculated as previously described.

3.3. Application of the Micro Biological Survey method for the selective detection of *Listeria monocytogenes* in food samples

3.3.1. Optimization of MBS reagent for *Listeria* spp. detection

Bacterial strains Microorganisms from the American Type Culture Collection (ATCC) database, chosen on their frequent detection in food or because they are considered important indicators of fecal contamination: *Listeria* spp. (*Listeria monocytogenes* ATCC 7644, *Listeria innocua* ATCC 33090); *Staphilococcus aureus* (ATCC 29212); *Enterococcus faecalis* (ATCC 29212); *Escherichia coli* (ATCC 25922).

Culture media. All dehydrated media were dissolved in distilled water, sterilized in an autoclave at 121°C for 15 minutes, under the pressure of 1 atmosphere.

Brain Heart Infusion Broth (BHI) (VWR International S.r.l., Milan) is a liquid, non-selective, and general-purpose medium. It is suitable for the cultivation and maintenance of a wide variety of microorganisms, including fastidious pathogens, molds and yeasts, using the characteristic temperature and the incubation time of the microorganism that is to be cultivated. It is recommended as a universal ground for aerobic bacteriology and is used for the preparation of aerobic bacterial cultures since it allows a lively multiplication of suspended cells.

PALCAM Listeria agar (VWR International S.r.l., Milan) is an indicator and selective medium for *Listeria* spp. Isolation and detection. from food and clinical specimens. The medium selectivity is due to the presence of sodium chloride, polymyxin B sulfate, acriflavine, lithium chloride and ceftazidime, which inhibit the growth of most of the non-*Listeria* species. Differentiation on PALCAM medium is based on the hydrolysis of the exculine and fermentation of mannitol. [61;62].

Baird-Parker Agar (Liofilchem S.r.l., Roseto degli Abruzzi, TE) is a moderately selective differential soil for Staphylococcus aureus isolation and counting in foods. It is a soil that utilizes the ability of staphylococci to reduce tellurium in tellurium and reveal the presence of lecithinase from egg lecithin [71].

Bile Esculin Azide agar (Liofilchem S.r.l., Roseto degli Abruzzi, TE) is a selective and differential soil for the isolation and identification of enterococci.

MacConkey Agar (MC) *(Liofilchem, Roseto degli Abbruzzi, Italy)*. Selective and differential culture medium for bacteria used for the isolation and differentiation of Gram-negative enteric bacilli, and it conforms to Harmonized USP/EP/JP. Requirements is widely used for the research and isolation of Gram-negative enterobacteria because the presence of bile and crystalline-violet salts makes the selective substrate for this group of microorganisms [69;72].

Buffered Peptone Water (Liofilchem Srl, Roseto degli Abruzzi, TE) or peptonate water is a non-selective liquid medium for the pre-enrichment step in *Salmonella* isolation procedures in foodstuffs [56] for the pre-enrichment step for the detection and counting of L. monocytogenes in foods [62] and as a microorganisms diluent [73]. This medium is prepared with a universal pepton, particularly rich in nutrients, which allows optimal recovery of Salmonella even when present in the low-charged sample.

Listeria Fraser Broth (VWR International Srl, Milan) is the enrichment broth recommended by regulations [61;62] for the research and enumeration of *L. monocytogenes* in food products.

Reference method. The standard plate count method for the enumeration of colony forming units (CFU/ml) on PALCAM agar; Baird-Parker agar (BPA); Bile Esculine Azide agar (BEA); MacConkey (MC); Tryptic Soy Yeast Exceptional Agar (TSYEA); was used as the reference method for the assessment of bacterial concentration in the samples. 1 ml of the samples was plated using the pour plate technique and incubated in a thermostat at 37°C for 24 hours. The results were obtained counting the plates with a number of colonies between 30 and 300. All experiments were carried out in triplicate.

MBS method. The MBS vials are made in PET (Polyethylene Terephthalate), provided with a screw cap for a secure closure and a safety cap that includes a tank containing a sterilizing chemical compound that must be manually injected in the vial, after testing, for proper disposal. All the components of the MBS reagents are micronized together (granulometry < 10 micron) in a mortar and then distributed in defined amounts, in each vial. Before closure, 1.5 ml of vaseline is added to each vial. After preparation, the vials are sterilized, using

Gamma (γ) rays (5kGy), by a specialized company. Vials so sterilized are ready for use. MBS vials' shelf-life is generally 6 months at room temperature and 1 year at 4°C.

MBS LY vials. The MBS LY reagent allows the growth, detection and quantification of *Listeria* species, The LY reagent was validated according to ISO 16140 [8]. The presence of bacteria is detected by a color change from blue to yellow.

The original formulation of this reagent showed several problem in terms of sensitivity toward *Listeria* spp. so it was able to detect low concentrations of interest strains in very long times, and low selectivity towards other bacterial strain different from *Listeria* spp.
For this reason, several bibliographic researches were carried out in order to identify the compounds capable of ensuring the selective development of the species of interest. Since the medium is patented, it is not possible to show the composition in its entirety, the names of the substances present in the MBS SL reagent are indicated by acronyms (Table 4).
According to the MBS method, the presence of Listeria spp. it is verified whether the dye from yellow to yellow to vials occurs within 72 hours. The absence of toning in this time interval indicates the absence of the bacterium in the sample under consideration.

Table 4. Original formulation of MBS LY reagent.

Composition	Quantity (g/l)
Carbon and nitrogen source B	10
Carbon and nitrogen source T	10
Vitamin source Y	5
Buffered system P	9
Buffered system H	1
Selective agent SC	20
Selective agent LC	13
Energy source P	2
Selective agent B	0,01
Selective agent N	0,02
Selective agent C	0,02
Redox indicator T	0,5
Redox indicator M	0,004

pH 6,8. Color: dark blue.

Modified MBS LY reagent analyses The MBS method for analysis of ATCC collection strains provided for the preparation of MBS reactive for *Listeria* spp. in liquid and sterilized by filtration on cellulose acetate membrane (pore diameter: 0.45 µm). Each sterile glass vial is aliquoted in succession with 10 ml sterile MBS sterile vials, 1 to 2 ml Vaseline, sterilized by autoclave, and 1 ml of bacterial dilution obtained by serial dilutions, starting from overnight cultures. The vials are subsequently sealed with rubber caps decontaminated through heat treatment. The vials are then incubated in a 37 ° C incubator for 48h-72h or placed in the Multireader, set to perform the assay.

Determination of MIC (Minimum Inhibitory Concentration) of substances with antibiotic activity. The antibiotic concentrations present in the new composition were determined based on the results obtained by analyzes for the determination of their Minimum Inhibitory Concentration (MIC). The results are expressed in terms of growth / non-growth of

the analyzed microorganisms. For these analyzes 96-well sterile microtiter plates were used. The protocol envisages the preparation of serial dilutions based on 2 of the substance under consideration in the plate wells, to which the microorganisms analyzed will be added. The minimum inhibitory concentration value is defined as the concentration value of the examined antibiotic corresponding to the first well in which no observed microorganism growth is observed. Initially, a non-selective growth medium (BHI Broth) was used; Subsequently, the analyzes were repeated using the MBS reagent, in order to observe the characteristics of the substance considered within both the compounds analyzed.

3.3.2. Development of experimental enrichment broth

In the first phase of the research, the possibility of using directly naturally contaminated food samples was evaluated. Given the difficulty in finding food samples naturally contaminated by *Listeria* spp., And to exclude the interference of food matrices [57], the enrichment broth was tested in vitro with artificially contaminated water samples.

Bacterial strains. Microorganisms from the American Type Culture Collection (ATCC) database, chosen on their frequent detection in food or because they are considered important indicators of fecal contamination: *Listeria* spp. (*Listeria monocytogenes* ATCC 7644, *Listeria innocua* ATCC 33090); *Staphilococcus aureus* (ATCC 29212); *Enterococcus faecalis* (ATCC 29212); *Escherichia coli* (ATCC 25922); *Salmonella enterica subsp. enterica serovar Enteritidis* (ATCC 13076); *L. delbrueckii ssp. lactis* (ATCC 12315); *L. rhamnosus* (ATCC 11974); *L. casei ssp casei* (ATCC 393); *Bacillus cereus* (ATCC 11778); *Bacillus subtilis* (ATCC 6633)

Starting from sterile BHI overnight culture broths from different strains of interest, serial dilutions in sterile physiologic were set up. A presumed count of the bacterial concentration levels present in the various dilutions was carried out the day before experiments by plating two ml on different selective media (PALCAM agar for *Listeria* spp.; Baird-Parker agar (BPA) for *S. aureus*; Bile Esculine Azide agar (BEA) for *E. faecalis*; MacConkey (MC) for *E. coli* and *S. enteritidis*; MRSA for *Lactc Acid Bacteria*; Tryptic Soy Agar (TSA) for *B. cereus*

and *B subtilis*). The results were obtained by counting CFU/ml after 24 hours of incubation at 37 ° C.

On the day of the experiment, 24 ml of sterile water were aliquoted in 50 ml sterile falcon. Each sample was contaminated with a single microorganism: 1 ml of the selected dilution was inoculated into the sterile water sample.

Culture media. All dehydrated media were dissolved in distilled water, sterilized in an autoclave at 121°C for 15 minutes, under the pressure of 1 atmosphere.

Brain Heart Infusion Broth (BHI) (VWR International S.r.l., Milan) is a liquid, non-selective, and general-purpose medium. It is suitable for the cultivation and maintenance of a wide variety of microorganisms, including fastidious pathogens, molds and yeasts, using the characteristic temperature and the incubation time of the microorganism that is to be cultivated. It is recommended as a universal ground for aerobic bacteriology and is used for the preparation of aerobic bacterial cultures since it allows a lively multiplication of suspended cells.

PALCAM Listeria agar (VWR International S.r.l., Milan) is an indicator and selective medium for *Listeria* spp. Isolation and detection. from food and clinical specimens. The medium selectivity is due to the presence of sodium chloride, polymyxin B sulfate, acriflavine, lithium chloride and ceftazidime, which inhibit the growth of most of the non-*Listeria* species. Differentiation on PALCAM medium is based on the hydrolysis of the exculine and fermentation of mannitol. [61;62].

Baird-Parker Agar (Liofilchem S.r.l., Roseto degli Abruzzi, TE) is a moderately selective differential soil for Staphylococcus aureus isolation and counting in foods. It is a soil that utilizes the ability of staphylococci to reduce tellurium in tellurium and reveal the presence of lecithinase from egg lecithin [71].

Bile Esculin Azide agar (Liofilchem S.r.l., Roseto degli Abruzzi, TE) is a selective and differential soil for the isolation and identification of enterococci.

MacConkey Agar (MC) *(Liofilchem, Roseto degli Abbruzzi, Italy).* Selective and differential culture medium for bacteria used for the isolation and differentiation of Gram-negative enteric bacilli, and it conforms to Harmonized USP/EP/JP. Requirements is widely used for the

research and isolation of Gram-negative enterobacteria because the presence of bile and crystalline-violet salts makes the selective substrate for this group of microorganisms [69;72].

Buffered Peptone Water (Liofilchem Srl, Roseto degli Abruzzi, TE) or peptonate water is a non-selective liquid medium for the pre-enrichment step in *Salmonella* isolation procedures in foodstuffs [56] for the pre-enrichment step for the detection and counting of L. monocytogenes in foods [62] and as a microorganisms diluent [73]. This medium is prepared with a universal pepton, particularly rich in nutrients, which allows optimal recovery of Salmonella even when present in the low-charged sample.

Listeria Fraser Broth (VWR International Srl, Milan) is the enrichment broth recommended by regulations [61;62] for the research and enumeration of *L. monocytogenes* in food products.

de Man-Rogosa-Sharp Broth (MRS) and de Man-Rogosa-Sharp agar(MRSA): The MRS formula was developed by de Man, Rogosa and Sharpe (1960) to provide a and that could favor a lush growth of all of lactobacilli species. MRS medium is recommended for preparing lactobacilli cultures of food, oral and fecal origin.

Tryptic Soy Agar (TSA) (VWR International S.r.l., Milan) is used for the cultivation of mesophilic bacterial charge, allows the growth of non-demanding and / or moderately demanding microorganisms and is widely used for water and food analysis. Due to its nutritional characteristics, the absence of inhibitors and the possibility of being supplemented with the most diverse compounds, this medium is well suited for the isolation of pathogenic and growth-causing organisms.

Reference method. The standard plate count method for the enumeration of colony forming units (CFU/ml) on PALCAM agar; Baird-Parker agar (BPA); Bile Esculine Azide agar (BEA); MacConkey (MC); Tryptic Soy Yeast Exceptional Agar (TSYEA); de Man-Rogosa-Sharp Agar (MRSA); was used as the reference method for the assessment of bacterial concentration in the samples. 1 ml of the samples was plated using the pour plate technique and incubated in a thermostat at 37°C for 24 hours. The results were obtained counting the plates with a number of colonies between 30 and 300. All experiments were carried out in triplicate.

Preparation of the various formulations of the experimental broth The various formulations of the experimental broth were prepared by dissolving the ingredients in 225 ml of sterile water. Once a homogeneous solution was obtained to assure sterility of starting, the experimental broth was filtered using filters of 0.45 µm and then aliquoted in 400 ml sterile feed envelopes. So prepared soil was stored in a refrigerator at 4 ° C.

The sterility of the reagent was verified the day before the experiment by inoculating 3 ml of broth filtered into TSA and counting CFU / ml after 24 hours at 37 ° C.

Table 5. Composition of MBS LY enrichment broth and the reagent's concentration tested**.**

Ingredients	g/l
Carbon and nitrogen source B	5; 10
Carbon and nitrogen source T	5; 7,5; 10
Carbon and nitrogen source P	5; 7,5
Vitamine source Y	5; 10
Salt N	5; 10
Buffer system K	9
Buffer system H	1
Selective agent L	5; 7
Selective agent A	0.025
Selective agent Cp	0,001; 0,0015

Evaluation of the selective enrichment capacity of the different formulations of the experimental broth The artificially contaminated samples prepared as above indicated were inoculated into the experimental broth and incubated for 48 hours at 37 ° C. In order to evaluate the replication capacity of the various inoculated microorganisms within the experimental broth, it was necessary to verify its initial and final concentration (after the enrichment step) using the reference method. The exact concentration of bacteria initially inoculated in the experimental broth before enrichment was calculated according to the formula:

$$Cf = (Ci \ x \ Vi) \ / \ Vf$$

Where: Cf = Concentration of bacteria initially inoculated in the experimental broth; Ci = CFU/ml of the selected dilution; Vi = Inoculum volume; Vf = Final volume of the experimental broth

Growth levels of the different inoculated microorganisms in the experimental broth were evaluated after 24 and 48 hours of enrichment at 37 ° C. Serial dilutions of the contaminated enrichment broth were made sterile physiologically: 1 ml of the different dilutions were plated in duplicate on selective medium. The plates thus prepared were incubated for 24 hours at 37° C. The results were calculated as previously described.

Evaluation of the enrichment capacity of the experimental broth with respect to the enrichment broth ISO Fraser Broth

Preparation of artificially contaminated samples

In the second phase of the experiment the sample quantities were reduced. In order to allow the same sample to be analyzed with both enrichment broths, at this stage the artificially contaminated samples consist of 10 ml sterile water contaminated with different concentrations of ATCC strains of *Listeria* spp. (*Listeria monocytogenes* ATCC 7644, *Listeria innocua* ATCC 33090); *Staphilococcus aureus* (ATCC 29212); *Enterococcus faecalis* (ATCC 29212); *Escherichia coli* (ATCC 25922); *Salmonella enterica subsp. enterica serovar Enteritidis* (ATCC 13076); *L. delbrueckii ssp. lactis* (ATCC 12315); *L. rhamnosus* (ATCC 11974); *L. casei ssp casei* (ATCC 393); *Bacillus cereus* (ATCC 11778); *Bacillus subtilis* (ATCC 6633). Samples were contaminated with a single microorganism or combinations of different microorganisms. Dilutions of interest were chosen using the preselected count method as previously described.

To evaluate the susceptibility of the experimental enrichment broth to the Fraser Broth broth, 9 ml of sterile water was aliquoted into 15 ml sterile salmon and contaminated with 1 ml of the dilution selected to obtain starting concentrations of *Listeria* spp. of about 10^2 CFU / ml: 5 ml of the so prepared specimens were inoculated into 45 ml of experimental broth and the remaining 5 ml in 50 ml Fraser broth, following the ratio specified in [61].

To evaluate the selectivity of the enrichment broth, 9 ml of sterile water was aliquoted into 15 ml sterile falcon and contaminated with 1 ml of the dilution selected to obtain starting concentrations of *Staphilococcus aureus* (ATCC 29212); *Enterococcus faecalis* (ATCC 29212); *Escherichia coli* (ATCC 25922); *Salmonella enterica subsp. enterica serovar Enteritidis* (ATCC 13076); *L. delbrueckii ssp. lactis* (ATCC 12315); *L. rhamnosus* (ATCC 11974); *L. casei ssp casei* (ATCC 393); *Bacillus cereus* (ATCC 11778); *Bacillus subtilis* (ATCC 6633) of about ~ 10^6 CFU / ml: 5 ml of the so prepared specimens were inoculated into 45 ml of experimental broth and the remaining 5 ml in 50 ml of Fraser broth.

To assess the selectivity of the enrichment broth in case of cross-contamination 8 ml of sterile water were aliquoted in 15 ml sterile falcon and contaminated with 1 ml of the two dilutions selected so as to obtain different combinations of *Listeria* spp. and other species selected for experimentation: 5 ml of the so prepared specimens were inoculated into 45 ml of experimental broth and the remaining 5 ml in 50 ml of Fraser broth.

Enrichment with Fraser Broth Samples prepared as described above were inoculated in sterility in 50 ml of Muller Kauffmann broth and incubated for 48 hours at 37 ° C.

Evaluation of the sensitivity and selectivity of the experimental broth and Fraser Broth

The artificially contaminated samples, prepared as previously described, were inoculated in the experimental broth and in parallel in the Fraser Broth and incubated for 24-48 hours at 37° C.

Growth levels of the different microorganisms, inoculated in both the experimental broth and Muller Kauffmann broth, were evaluated after 24 and 48 hours of enrichment at 37 ° C. Serial dilutions of both broths were prepared in sterile physiological solution: 1 ml of the different dilutions were plated in double on selective media . Thus thee plates were incubated for 24 hours at 37° C and the results were calculated according to the plate count method previously described. In cross-contamination conditions, considering the final application of the enrichment broth to the MBS analysis, the concentration of strains analyzed following enrichment in both broths was evaluated only after 24 hours. In cases of single-cell bacterial contamination, the starting concentration of inoculated micro-organisms in the sample and subsequently in the experimental broth was evaluated as previously described.

In cross-contamination conditions, the starting concentration was calculated as previously described. Concerning the final concentration of the different strains (contaminants and cross contaminants) in the experimental broth and Fraser broth, after the enrichment phase, serial dilutions of both broths were prepared in sterile physiological solution: 1 ml of the various dilutions were then plated in duplicate on selective PALCAM agar, BPA agar and BEA agar. Thus the plates were incubated for 24 hours at 37 ° C and the results were calculated according to the plate count method previously described.

3.3.3. Application of the experimental enrichment broth to the Micro Biological Survey method

The last phase of the research aims to evaluate the benefits of introducing into the analytical procedure of the MBS method of enrichment phase. The enrichment was carried out in the experimental broth and Fraser broth.

Preparation of artificially contaminated specimens The artificially contaminated specimens were prepared as previously described. Quantities have been doubled to allow analysis with both enrichment broths.

Evaluation of the sensitivity and selectivity of the LY reagent before enrichment. The MBS LY reagent has been modified in order to be more sensitive towards *Listeria* spp., and contemporarily more selective against other *Listeria* competitor bacterial species, as previously described.

Evaluation of the sensitivity and selectivity of the LY reagent after enrichment

5 ml of artificially contaminated samples prepared as above indicated were inoculated in 45 ml of experimental broth and incubated for 24 hours at 37 ° C; At the same time 5 ml of the same samples were inoculated into 50 ml of the Fraser broth. After the enrichment step, 1 ml of contaminated broths was inoculated in triplicate in the LY vial. The MBS LY vials were incubated in MR for 72 hours at 37 ° C to verify the presence/absence of color changing. The results were provided directly by MR in terms of hours of output.

To verify that the output was due to the actual presence of *Listeria* spp. and to ensure that the lack of color changing was not due to the absence of other bacterial species, serial dilutions of both enrichment broths in sterile physiological solution were prepared in parallel to the MBS analysis: 1 ml of the different dilutions were plated in duplicate on selective media (PALCAM agar for *Listeria* spp.; Baird-Parker agar (BPA) for *S. aureus*; Bile Esculine Azide agar (BEA) for *E. faecalis*). The plates were incubated for 24 hours at 37 ° C and the results were calculated according to the plate count method previously described.

In cross-contamination conditions, the same analytical procedure was performed only with the experimental broth. To verify that the output was due to the actual presence of *Listeria* spp. and to ensure that the lack of color changing was not due to the absence of other bacterial species, serial dilutions of both enrichment broths in sterile physiological solution were prepared in parallel to the MBS analysis.

4. RESULTS AND DISCUSSION

4.1. Application of the Micro Biological Survey method in the evaluation of purifying activity of *Moringa oleifera* seeds in water samples

The protocol described in literature for the water treatment with *Moringa oleifera* involves the use of only internal almond [45]. Since the almond separation procedure from the tegument is laborious and takes a long time, the possibility of using the whole seed was evaluated at the initial stage of the research, and it was also verified how much the effect could vary according to the part of seed used and which part of the seed was more likely to behave as a natural flocculant.

For a generic evaluation of MO purifying capabilities, the first experiments were conducted on artificially contaminated water samples with a standard *Escherichia coli* (*E. coli*) concentration, a representative organism of water faecal contamination. These samples were treated with three different types of flour obtained from whole seed, from the internal almond and from the external tegument. Once the average weight of each seed of the plant was established, the amounts in equivalent grams of the different flours were used for the treatment. The flour was added to the samples of artificially contaminated water, after that the samples were vigorously agitated for 5 minutes to favor MO activity and filtered after 1 hour. The total bacterial charge was evaluated by the reference plate count related to an untreated control.

The averages of the results obtained are shown in Table 6 below

Table 6. Bacterial load after the MO treatment with flour obtained from different part of the seed

***Moringa oleifera* flour n° of seeds/50 ml**	**CFU/ml on Tryptic soy agar**		
	Inner almond	**External integument**	**Whole seed**
0	>300	>300	>300
4	>300	>300	30
5	>300	>300	25

In samples treated with flour obtained from whole seed, it was possible to observe a reduction in total bacterial load, whereas in samples treated with flour obtained from only almond and tegument, this abatement was not found. Since the use of only internal almond in the literature is strongly recommended in the literature, the experiment was repeated following the same protocol by treating artificially contaminated *E. coli* samples with increasing amounts of flour obtained from whole seed or sole almonds. In particular to confirm the previously obtained result and to evaluate more precisely the bacterial charge within the treated samples serial samples dilutions were made after treatment and untreated control.

The averages of the results obtained are shown in the following tables (Table 7 a, b). The results were reported for the undiluted sample indicating the total bacterial count obtained by plating 1 ml of the undiluted sample and for the CFU/ml averages obtained from the various serial dilutions of the undiluted one.

a) Samples treated with Moringa oleifera flour from whole seed

Farina Moringa oleifera/50 ml

Gr	n of seeds	cfu/ml Tq TSA	average cfu/ml TSA
/	0	> 300	1,4*105
0,66	3	> 300	2,7*103
0,88	4	8	1,7*103
1,1	5	5	1,2*103
1,32	6	3	680
1,54	7	3	550
1,76	8	2	350
1,98	9	0	55
2,2	10	0	25

b) Samples treated with Moringa oleifera flour from the inner almond

Farina Moringa oleifera/50 ml

Gr	n of seeds	cfu/ml Tq TSA	average cfu/ml TSA
Gr	equivalente in semi	cfu/ml Tq TSA	media cfu/ml TSA
/	0	> 300	1,4*105
0,45	3	> 300	9,5*103
0,60	4	> 300	3,5*103
0,75	5	240	2,5*103
0,90	6	232	1,3*103
1,05	7	170	950
1,2	8	74	830
1,35	9	26	675
1,5	10	3	440

From the results of the table 7 it is evident that the count obtained from the 1 ml of the undiluted sample is not representative as it substantially differs from that obtained using serial dilutions, probably due to a bacteriostatic MO effect which is eliminated in subsequent dilutions .

However, the greater effect of flour obtained from whole seed was confirmed in this experiment and for this reason it was decided to continue experimentation using this kind of flour.

Determination of the protocol for the purification of water through the use of flour obtained from the *Moringa oleifera* seeds. In developing countries, microbiological control analyzes, as well as water purification treatments, are difficult due to limited availability and high cost. This condition results in high consumption of polluted water, which is one of the most important causes of waterborne diseases. For this reason, the research has been carried out in order to evaluate the possibility of applying *Moringa oleifera* seeds as a domestic scale treatment for water purification. The first experiments were conducted on artificially contaminated water samples with a standard concentration of *Escherichia coli* (*E. coli*), a representative organism of water faecal contamination, in order to determine the amount of seeds needed to breakdown the bacterial load, to lead water suitable for human consumption. These samples were treated with MO flour obtained from the dried whole seed. Once the average seed weight of the plant was established, amounts in equivalent grams of flour were used for the treatment: flour was added to the artificially contaminated water samples, then the samples were vigorously shaken for 5 minutes to facilitate MO action and then filtered with a clean cotton cloth after 1 hour. In order to verify the efficacy of such treatment in the reduction of bacterial charge, the traditional plate counting method on both the treated and untreated samples was performed.

The mean results obtained from multiple replicates of the same experiment are given in Table 6. In particular, for each sample treated with a different amount of flour/seeds, the results are compared in terms of total bacterial load (expressed as UFC/ml) obtained from the direct analysis of 1 ml of the undiluted sample and as the average UFC/ml obtained analyzing the various serial dilutions of the same sample.

Table 8. Verification of the Effectiveness of Different Quantities of Moringa oleifera in the abatement of total bacterial load. For each sample the results are expressed in terms of total bacterial load (expressed as UFC/ml) obtained from the direct 1ml of undiluted sample and as the mean of the serial dilution analysis of the same sample.

***Moringa oleifera flour*/50 ml**		**Total Viable Count (CFU/ml)**	
Gr	**n° of seeds**	**Tq**	**Average**
/	0	> 300	$1,4*10^5$
0,66	3	> 300	$2,7*10^3$
0,88	4	8	$1,7*10^3$
1,1	5	5	$1,2*10^3$
1,32	6	3	680
1,54	7	3	550
1,76	8	2	350
1,98	9	0	55
2,2	10	0	25
3,3	15	0	0

The results show that the minimum effective seed count is 15 seeds in 50 ml, equivalent to 3.3 grams/50 ml (300 seeds per liter). In addition, it is evident that the count obtained by plating 1 ml of the undiluted treated sample is not representative as it really differs from that obtained using serial dilutions; this is probably the consequence of the bacteriostatic effect of MO which is eliminated in subsequent dilutions.

In order to understand the relationship between the bacterial load expressed as UFC/ml log and the number of MO seeds used, the results shown in Table 7 were charted (Figure 8). The bacterial load of each sample, shown in the graph, corresponds to the UFC/ml average obtained from serial dilutions.

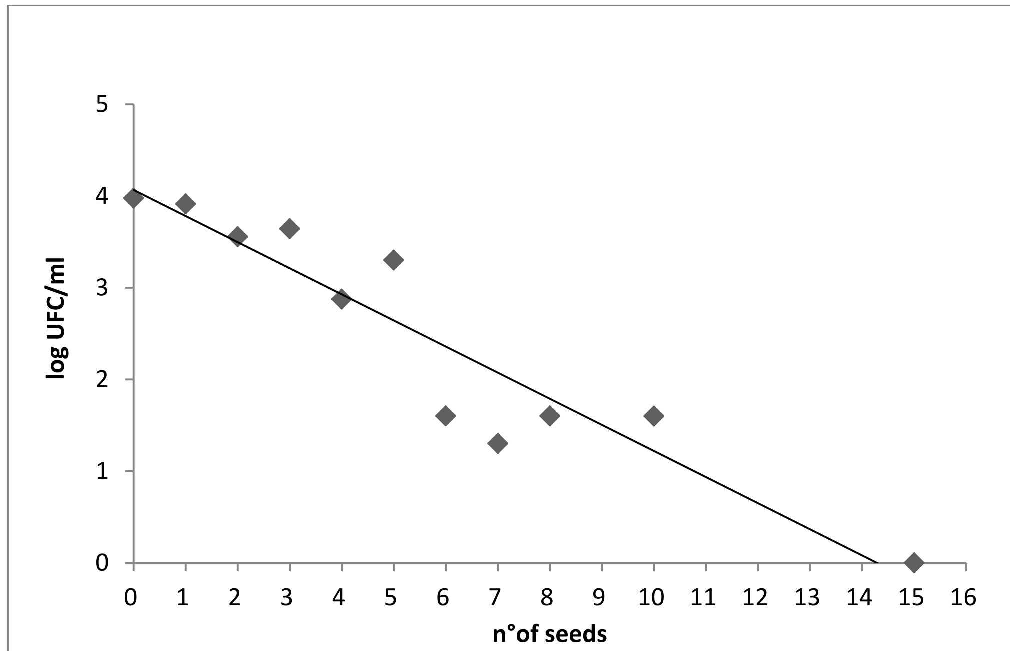


Figure 8. Relationship between the reduction of bacterial load and the number of seeds of *Moringa oleifera*

***Moringa oleifera* effect on different bacterial strains**. Since the preliminary phase of the research was focused on the treatment of artificially contaminated *E. coli* samples, in the second phase of the experimentation, MO activity was investigated on other bacterial species responsible for water contamination and in particular on *Enterococcus faecalis* and *Salmonella enterica subsp typhimurium*. Analyses were carried out according to the protocol prepared, artificially contamining samples with known *E. coli*, *E. faecalis* and *S. Typhimurium* concentrations with increasing amounts of flour obtained from whole seed using a quantity of flour equivalent to a number of seeds equal to 5, 8 and 10, so as not only to evaluate MO activity on different bacterial species, but also to evaluate whether it has different effects depending on contaminating bacteria. To evaluate more accurately the bacterial charge within the treated samples serial samples dilutions were made after treatment and untreated controls.

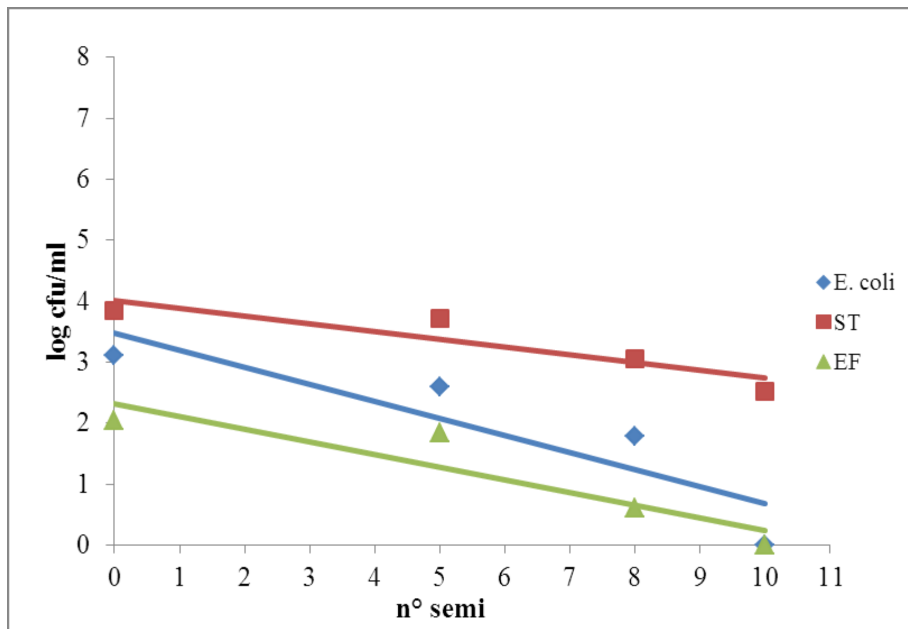
The mean results obtained are shown in the following table (Tables 9).

Table 9. Total bacterial count in water samples artificially contaminated with E. coli, E. faecalis and S. Typhimurium treated with different amounts of *Moringa oleifera* flour obtained from whole seed.

Moringa oleifera flour/50 ml		*E. coli*		*E. faecalis*		*S. typhimurium*	
Gr	**N° of seeds**	**cfu/ml Tq TSA**	**average cfu/ml TSA**	**cfu/ml Tq TSA**	**average cfu/ml TSA**	**cfu/ml Tq TSA**	**average cfu/ml TSA**
/	0	> 300	$1,3*10^3$	96	403,3	> 300	$1,5*10^5$
1,1	5	2	250	48	53,5	35	4,2*102
1,76	8	1	6	16	40	10	870
2,2	10	0	0	11	25	3	26

The results show that the purifying effect of MO flour is not equivalent to the different contaminating bacteria.

In the graph below (Figure 9), bacterial concentration of samples after treatment was expressed as log cfu/ml, depending on the number of MO seeds used, to graphically show the relationship between the abatement of the microbial load and the number of MO seeds used for the treatment. It is also possible to observe the differences in MO activity respect to the various contaminating bacteria. The concentration of the sample was calculated on the basis of the average of cfu/ml calculated by the dilutions



.

Figure 9. Relationship between the reduction of microbial charge and the number of seeds of *Moringa oleifera*.

Overall, from the observation of results, it is possible to observe that the reduction of the total bacterial load is linear in relation to the amount of MO used, but is more apparent in the samples artificially contaminated with *E. coli* and *E. faecalis*, whereas it is less significant in the samples artificially contaminated with *S. typhimurium*.

It has therefore been suggested that this result may be determined by a different interaction of the flour particles with the different bacteria and that to breakdown the bacterial load of artificially contaminated *S. Typhimurium* samples it was necessary to increase the amount of flour.

Further experiments were then conducted by increasing the amount of MO flour used for treating the samples. The experiments were carried out according to the protocol previously prepared, treating artificially contaminated samples with known *E. coli, E. faecalis* and *S. typhimurium* concentrations with increasing amounts of flour obtained from whole seed using a quantity of flour equivalent to a number of seeds equal to 10, 15 and 20.

To precisely evaluate the bacterial load within the treated samples, serial samples dilutions after treatment and untreated controls were set up.

The mean results obtained are shown in the following table (Tables 9).

Table 10. Total bacterial count in water samples artificially contaminated with *E. coli*, *E. faecalis* and *S. Typhimurium* treated with different amounts of Moringa oleifera flour obtained from whole seed

Moringa oleifera flour/50 ml		***E. coli***		***E. faecalis***		***S. typhimurium***	
Gr	**N° of seeds**	**cfu/ml Tq TSA**	**average cfu/ml TSA**	**cfu/ml Tq TSA**	**average cfu/ml TSA**	**cfu/ml Tq TSA**	**average cfu/ml TSA**
/	0	> 300	$8,3*10^4$	> 300	~103	> 300	$8,7*10^4$
2,2	10	6	12	98	26	280	240
3,3	15	0	1	46	7	146	83
4,4	20	0	1	6	1	78	53

Study on pH variation. Several experiments have been conducted to determine whether a pH alteration could affect the action of *Moringa oleifera*.

Two different acids, acetic acid (CH_3COOH) and hydrochloric acid (HCl), and two bases, tris ($C_4H_{11}NO_3$) and sodium carbonate (Na_2CO_3) were used to alter the pH.

These experiments were conducted using the protocol described above, but varying the pH of the contaminated water. The results are shown in the table below (Table 10 a, b, c).

Table 11 a, b, c. Total bacterial count of treated samples at different pH values: a) ph/; b) pH 4; c) pH 9

a) MO flour n° seeds/50 ml	Artificially contaminated water samples	
	pH 7	
	CFU/ml	Average CFU/ml
0	>300	~10^3
10	4	12
15	1	0
20	0	0

b) MO flour n° seeds/50 ml	Artificially contaminated water samples			
	pH 4 (CH_3COOH)		pH 4 (HCl)	
	CFU/ml	Average CFU/ml	CFU/ml	Average CFU/ml
0	> 300	$8,9*10^3$	> 300	$1,3*10^4$
10	4	4	192	$1,6*10^3$
15	1	3	162	630
20	0	0	114	92

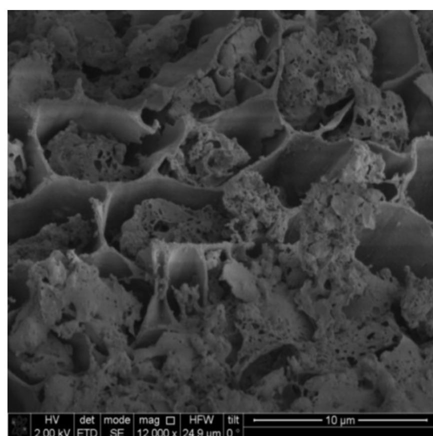
c) MO flour n° seeds/50 ml	Artificially contaminated water samples			
	pH 9 ($C_4H_{11}NO_3$)		pH 9 (Na_2CO_3)	
	CFU/ml	Average CFU/ml	CFU/ml	Average CFU/ml
0	> 300	$6,1*10^3$	> 300	$2,5*10^3$
10	3	4	119	670
15	2	1	3	1
20	0	0	0	0

These results indicate that acetic acid is able to support the flocculating action of *Moringa*,, amplifying its effect, while for the same protocol with hydrochloric acid it has been observed that, on the contrary, it acts on *Moringa* decreasing its action.

As evidenced by the results, the alkaline pH does not seem to significantly affect the flocculant action of the *Moringa oleifera* seeds.

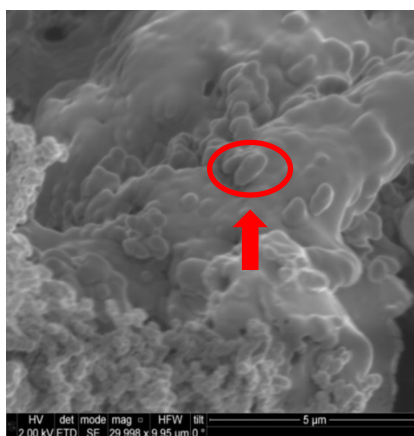
FIB analysis. To obtain information on morphology of *Moringa oleifera* flour surface after treatment and, consequently, to understand the dynamics of flocculant action, electronic microscopy has been used, a technique that allows sample observation with magnification and resolution 1000 times superior to ordinary optical microscopy. The scanning electron microscope allows you to get three-dimensional black and white images with a depth of field up to a few micrometers. The depth of field represents the distance between two planes, above and below the sample to be observed, simultaneously focused on the lens. This allows you to observe the minutest details in the specimen morphology by varying the beam energy, in fact, it is possible to see details of the actual surface of the sample producing high resolution images. In order to determine the mechanism through which the *Moringa oleifera* can perform its characteristic flocculant effect of the bacteria present in water, the different flour samples obtained by filtration on the cloth after treatment have been observed at the focused ionic beam microscope (FIB).

Moringa oleifera



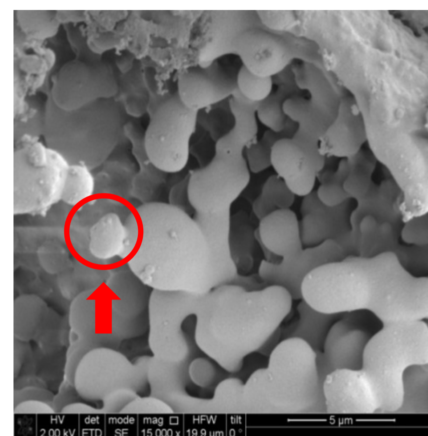
10µm

Moringa oleifera* with *E. coli



5µm

Moringa oleifera* with *E. faecalis



5µm

Figure 10. iIages of samples at FIB

4.1.1. Application of the analytical protocol on field in the evaluation of *Moringa oleifera*'s purifying activity in Latium, Italy

The results described above have highlighted the different efficacy of the purifying action of the flour obtained from MO seeds against different microbial strains derived from ATCC cultures. Therefore, in order to verify the effectiveness of this flour on bacteria naturally present in the waters destined for such treatment, the experimental protocol has been applied to naturally contaminated water samples. In particular, the surface waters of several rivers in the Lazio region have been used (Table 8).

Table 12. Sampling stations of the Lazium region for the analysis of microbiological quality before and after treatment with flour obtained from the *Moringa oleifera* seeds.

Sito	Località	Geographic Positioning System (GPS)
S1	ARRONE 1	**42° 2'0.63"N / 12°18'30.90"E**
S2	ARRONE 2	**41°52'10.19"N / 12°10'57.43"E**
S3	CREMERA	**42° 0'20.87"N / 12°26'26.72"E**
S4	FARFA 1	**42°13'52.89"N / 12°46'6.47"E**
S5	FARFA 2	**42°14'3.79"N / 12°42'21.24"E**
S6	FARFA 3	**42°12'28.81"N / 12°38'10.92"E**
S7	LICENZA 1	**42° 5'36.03"N / 12°54'23.72"E**
S8	LICENZA 2	**42° 1'7.79"N / 12°54'35.47"E**
S9	RIO FIUMICINO	**42° 0'46.81"N / 12°55'28.87"E**
S10	TEVERE 1	**41°59'25.05"N / 12°29'43.41"E**
S11	TEVERE 2	**41°48'28.72"N / 12°25'6.57"E**
S12	TEVERE 3	**41°46'25.00"N / 12°16'41.01"E**

For each water sample, microbiological quality was checked before and after treatment with flour obtained from MO seeds, taking into account the parameter Total Bacterial Charge at 20-25 ° C. The evaluation of total bacterial load allows to determine the general degree of water pollution due to the presence of microorganisms. Incubation temperature below 22 ± 1 ° C, promotes the growth of psycrophilic bacteria that can be derived from environmental sources and naturally develop in water at room temperature (20-25 ° C). The mean results obtained expressed as UFC/ml log are shown in the following Figure 11.

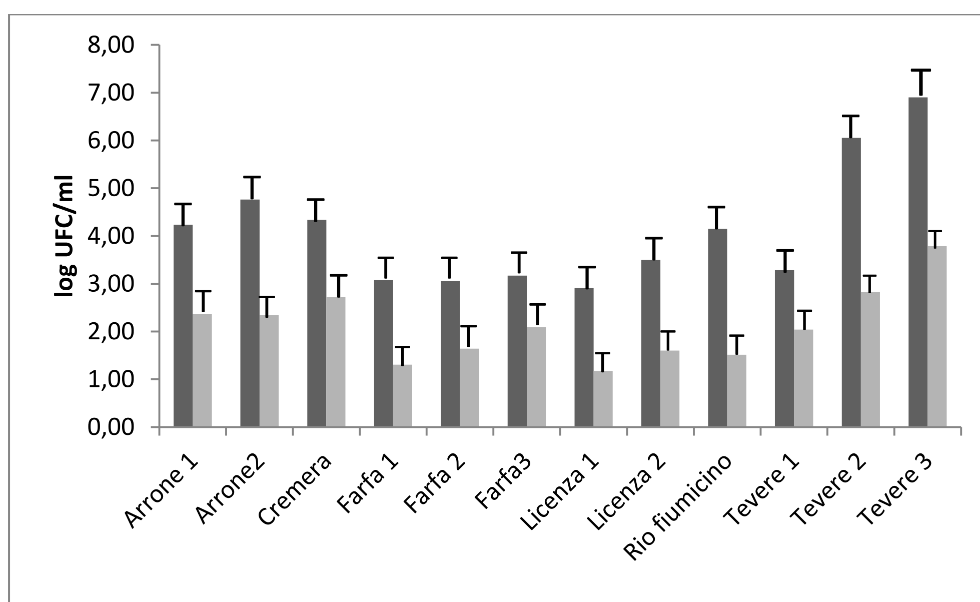


Figure 11.. Evaluation of Total Viable Count at 20-25 ° C in untreated naturally contaminated water samples and after treatment with Moringa oleifera flour obtained from whole seed (15 seeds / 50 ml) and filtration (t = 1 h) . For each untreated sample (black bars) and treated (grey bars), the results are expressed in terms of total bacterial load (expressed as UFC/ml log) obtained as mean ± DS of the serial dilution analysis of the same sample.

The results shown in the graph show that *Moringa oleifera* flour's purifying action can be observed in all naturally contaminated water samples. In particular, a general reduction of total bacterial loading of about 2 log is observed for most of the treated samples. This abatement is superior to about 3 logs for samples from the Rio Fiumicino (S9) and Tier 2 (S11) and Tevere 3 (S12) streams

.

Application of the MBS method for the evaluation of the purifying activity of Moringa oleifera. The final aim of the research is the ability to use, in developing countries, a protocol for the treatment of contaminated water, simple and easy to implement. Therefore, after verifying the effectiveness of the action of MO flour on several artificially and naturally contaminated water samples using the plate counting method, further experiments were carried out using the MBS colorimetric method in parallel to the traditional method. In non-industrialized countries where MO is processed, well-equipped microbiological laboratories and specialized personnel are virtually absent, so the simplicity, convenience and speed of the MBS method make it a valid tool for field testing of the *Moringa oleifera* activity. These experiments were carried out in order to evaluate possible interference due to MO's flour in the MBS method, using the CBT (CBT) detection reagent. To this end, experiments were carried out on artificially contaminated water samples with a standard *E. coli* concentration and treated with MO flour (3.3g/50ml). As mentioned above, the total bacterial load of the treated and untreated samples was evaluated using the traditional method of plate counting in parallel with the MBS method. Given the previously obtained data showing the MO's bacteriostatic effect in the undiluted sample, the MBS analysis was performed by analyzing 1 ml of the undiluted sample, as by standard MBS procedure, which allows sample inoculation without preliminary treatments, and 100 µl of the sample. The vials were incubated at 37 ° C in the Multilayer which automatically provided the shrinkage times.

The averages of the results obtained are shown in the following tables (Table 12). Figure 12 show vials color change according to the bacterial concentration; this concentration refers only to dilution 1:10, as the results obtained with the undiluted sample have not been considered reliable because the same problems as described above have been observed.

Table 13. Evaluation of the effectiveness of different concentrations of *Moringa oleifera* flour obtained from whole seed in the reduction of Total Viable Count using the traditional method of plate counting in parallel with the MBS method. For each artificially contaminated sample, the results are expressed in terms of total bacterial load (expressed as UFC/ml) and hours of color changing.

***Moringa oleifera flour*/50 ml**		**Total Viable Count**	
Gr	**n° of seeds**	**Trafitional method (CFU/ml)**	**MBS method hrs for color change**
/	0	$9,5 * 10^3$	9,1
0,66	3	$8,2*10^3$	11,59
0,88	4	$3,6*10^3$	13,02
1,1	5	$4,4*10^3$	12,97
1,32	6	750	14,54
1,54	7	1000	13,76
1,76	8	40	16,03
1,98	9	20	15,57
2,2	10	20	16,61

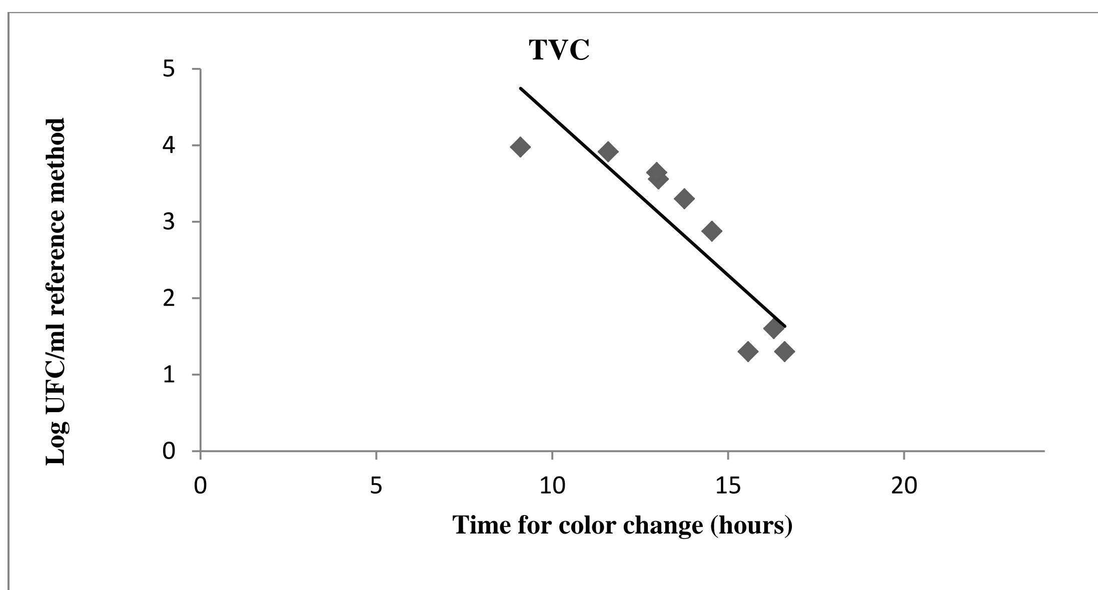


Figure 12. Variation of color change depending on bacterial concentration.

From the graphs it is shown that, as expected, the times for color changing obtained analyzing 100μl of sample are inversely proportional to the bacterial charge present in the samples. In addition, the lines show a similar pattern to the standard lines used for the evaluation of total bacterial load with the MBS TVC reagent.

4.2. Application of the Micro Biological Survey method for *Salmonella* spp. detection in food samples

The selective research for *Salmonella* spp. in a sample according to ISO 6579: 2008 [56] provides a number of steps to determine the presence/absence of this pathogen in 25 grams of food sample, a laborious process that takes 4 to 5 days. This procedure also presents limitations on the possibility of obtaining false positive results in case of high contamination by different kinds of enterobacteria with which *Salmonella* spp. is metabolically correlated [57].

The MBS method involves the inoculum of 1 g or 1 ml of sample (or homogenate) directly into sterile disposable vials containing the specific reagent for the assay to be conducted. Once inoculated, the vials can be incubated at the required temperature for analysis (30, 37 or 44° C) in the MBS Multireader (MR). The presence of microorganisms of interest, in a time inversely proportional to the present bacterial charge, induces a color change that can be detected automatically by the MR. The MBS Reagent for selective search of *Salmonella* spp. (MBS SL), already developed, detects the presence of *Salmonella* spp. thanks to a color change of the reagent from red to yellow within 72 hours. This change within 72 hours indicates the presence of the pathogen. There is no pre-enrichment step in the original experimental protocol. In addition, the possibility of obtaining false positive results is not negligible, because of the presence of cross contaminants that often interfere with the color change of the vial, decreasing the significance of the results. The limit of selectivity for microorganisms belonging to the *Enterobacteriaceae* family other than *Salmonella* spp. is in fact equal to ~ 10^6 CFU/ml. This means that in the presence of a concentration of enterobacteria other than *Salmonella* spp. ≥ 10^6 CFU/ml, SL vials may change color even in the absence of *Salmonella* spp.

The aim of the research was the development of a selective enrichment broth, to be used preliminarily for MBS analysis, which could meet the characteristics of sensitivity, selectivity and rapidity of this method, with the ultimate aim of optimizing the method for *Salmonella* spp. in food samples of different origin. In the initial stages of the work, the possibility of using directly naturally contaminated food samples was evaluated. However, due to the difficulty in obtaining such samples, the enrichment broth was tested in vitro with artificially contaminated water samples.

In the first phase of the research, based on information from the literature [75], an enrichment broth was formulated with the aim of selectively favoring the growth of *Salmonella* spp., towards other enterobacteria. Various tests have been conducted in order to seek nutritional and at the same time selective agents to ensure the best condition for the detection and growth of *Salmonella* spp. and slow down or inhibit the growth of metabolically related bacteria.

In the second phase, once the first satisfactory formulation of the broth was established, the efficiency of the experimental broth in the enrichment of *Salmonella* spp. was compared to that of the ISO broth, Muller Kauffmann. The ability to slow down or inhibit the growth of metabolically related bacteria has also been verified by evaluating the growth of several bacterial strains belonging to the *Enterobacteriaceae* family, alone or in combination with strains of *Salmonella* spp. to simulate conditions in a naturally contaminated sample and estimate the presence of any interference [57]. At the end of this phase, the optimum formulation of the experimental broth was found to be the best even in cross contaminated conditions.

In the third and final phase of this research, the new experimental broth was used as a selective enrichment broth before the MBS method analysis. The introduction into the protocol of an enrichment passage through a medium that can simultaneously carry out both the functions of the non-selective enrichment broth and the selective enrichment may, in accordance with the general principles of the MBS method, allow analysis of 25 g sample, as expected by ISO, facilitating the detection of *Salmonella* spp. even in presence of high concentrations of metabolically related enterobacteria.

4.2.1. Development of the experimental enrichment broth

The "horizontal method for the research of *Salmonella* spp." ISO 6579: 2008 provides for the detection of these bacterial strains in 25 grams of a food sample (Regulation (EC) No 2073/2005). The standard regulation proposes a series of steps: a non-selective pre-enrichment, enrichment, selective isolation, and incubation.

Non-selective pre-enrichment is performed in buffered peptonate water (BPW) which, if present, increases the number of salmonellae so that they are detectable and compete with the other microbiological species present in the sample. The second stage of enrichment in the broth Muller Kauffmann, selectively promotes the growth of *Salmonella* spp. and inhibits gram positive bacteria. Isolation of bacteria on selective media visually indicates the presence of colonies of *Salmonella* spp. Each suspected colony is plated on a Petri dish and incubated for 15 hours to identify the isolated species. After that the obtained colonies are then identified by molecular, biochemical, or other methods. This process therefore requires 4 or 5 days to confirm or deny the presence of *Salmonella* spp. in the analyzed sample.

In the first phase of the research a selective enrichment broth that could selectively favor the growth of *Salmonella* spp. was developed.

Evaluation of the selective enrichment capacity of the different formulations of the experimental broth. In the first phase of the research the artificially contaminated samples were prepared inoculating in 25 ml of sterile water different concentrations of ATCC strains of *Salmonella* spp. (*Salmonella typhimurium*, ATCC 14028, *Salmonella enteritidis* ATCC 13076) and *Enterobacter cloacae* (ATCC 13047), chosen as a representative microorganism of the *Enterobacteriaceae* family.

First formulation of the experimental enrichment broth Taking into account data reported in the literature on the different types of enrichment media for *Salmonella* spp., common components have been highlighted and accordingly a first formulation of the experimental broth was established (Table 13). As the broth could be patented, it is not possible to show the full composition, so the names of the compounds in the MBS broth are indicated by acronyms.

Tabella 14. First composition of the experimental enrichment broth**.**

Ingredients	**g/l**
Carbon and nitrogen source B	20
Vitamin source Y	5
Glucide X	10
Selective agent T	10
Salt S	7
Selective agent Vb	0.0125
Selective agent S	4
Selective agent SD	4.75

The sterility of the experimental broth was assured by filtering and checked for each experiment.

Water samples were contaminated with single strains through serial dilutions obtained from overnight culture to verify the replication capacity of each strain analyzed in the experimental broth without taking into account cross-contamination situations. A preliminary presumption of contamination strains was performed to determine the correct inoculum to be used to prepare the samples. The artificially contaminated samples were prepared to obtain a concentration of about 10^2 CFU/ml for *Salmonella* spp. and about 10^6 CFU / ml for *E. cloacae*, in order to simulate the conditions most likely to be observed in natural samples, The samples were inoculated into 225 ml of experimental broth and incubated at 37 ° C for 48 hours.

The starting concentrations of the inoculated strains in the experimental broth were verified using the traditional counting method (Brilliant Green Agar for *Salmonella* spp. and Violet Red Bile Glucose Agar for *E. cloacae*). The enrichment capacity of the experimental broth was evaluated after 24 and 48 hours. The results obtained are shown in the table (Table 14) and refer to an average of three independent experiments in which each sample was analyzed in triplicate.

Table 15. Concentration of contaminated samples before and after enrichment of the first formulation of the enrichment broth. The results refer to an average of three independent experiments in which each sample was analyzed in triplicate.

Contaminants strains	CFU/ml selective medium		
	Before enrichment	**After 24 hours**	**After 48 hours**
S. typhimurium	1	2,9 $E10^3$	1,7 $E10^1$
S. enteritidis	2	3,7 $E10^2$	2,3 $E10^1$
E. cloacae	2,3 $E10^5$	1,1 $E10^8$	6,5 $E10^7$

The results show that the enrichment broth thus formulated does not meet our needs. After 24 hours, though a modest growth of *Salmonella* spp. strains was observed, about 3 orders of magnitude for *Salmonella typhimurium* (*S. typhimurium*) and about 2 orders of magnitude for *Salmonella enteritidis* (*S. enteritidis*), of *E. cloacae*'s growth was instead of three orders of magnitude. The result is even more significant after 48 hours when a concentration decrease is observed for all strains analyzed but more evident for *Salmonella* spp. strains.

Second formulation of the experimental enrichment broth. The formulation of the experimental broth was modified by eliminating the selective agent Vb and varying the concentration of glucide X, salt salt and selective SD agent (Table 15).

Table 16. Composition of experimental broth: second formulation.

Ingredients	g/l
Carbon and nitrogen source B	20
Vitamin source Y	5
Glucide X	12
Selective agent T	10
Salt S	5
Selective agent S	4
Selective agent SD	2

The experimental conditions did not change and no significant variation in the results obtained (data not shown) was observed.

Third formulation of the experimental enrichment broth. Based on the data reported in the literature and the previously results, we decided to vary the combination of the selective T agent and the selective agent S, two reagents that were found to be important for the selective growth of *Salmonella* spp. Experiments were carried out with 25 different conditions, as shown in the table (Table 17).

Selective agent S (g/l)	**Selective agent T(g/l)**				
	0	**1**	**2**	**5**	**10**
0	A	B	C	D	E
0,5	F	G	H	I	J
1	K	L	M	N	O
2	P	Q	R	S	T
4	U	V	W	X	Y

The results in the table, which refer to an average of three independent experiments in which each sample was analyzed in triplicate, show that combinations "C" and "H" have proved to be the best (Table 18). In other cases, no significant improvement was observed with respect to the previous results (data not shown).

Contaminant strains		**Before enrichment**	**MBS broth conditions CFU/ml selective media**	
			After 24 hours	**After 48 hours**
S. typhimurium	**C**	1	3,6 E10^7	6,8 E10^7
	H		6,7 E10^5	3,2 E10^4
S. enteritidis	**C**	1	1,8 E10^6	5,2 E10^6
	H		3,7 E10^5	4,3 E10^3
E. cloacae	**C**	1,6 E10^5	2,7 E10^8	4,1 E10^8
	H		3,2 E10^6	9,2 E10^5

Fourth formulation of the experimental enrichment broth. Results until now obtained suggested that we should test intermediate concentrations of selective agent S by continuing to vary the concentrations of selective T agent, as shown in Table 19.

Selective agent S (g/l)	Selective agent T (g/l)		
	0	**2**	**5**
0	A	B	C
0,25	D	E	F
0,5	G	H	I

The best results obtained from the "B" and "E" combinations are shown in Table (Table 20) and refer to an average of three independent experiments in which each sample was analyzed in triplicate.

Contaminant strains		**Before enrichment**	**MBS broth conditions CFU/ml selective media**	
			After 24 hours	**After 48 hours**
S. typhimurium	**B**	1	3,4 $E10^{7}$	5,8 $E10^{7}$
	E		7,9 $E10^{6}$	8,8 $E10^{6}$
S. enteritidis	**B**	3	4,6 $E10^{6}$	6,7 $E10^{6}$
	E		1,9 $E10^{6}$	5,8 $E10^{6}$
E. cloacae	**B**	2,1 $E10^{5}$	3,4 $E10^{8}$	4,5 $E10^{8}$
	E		6,1 $E10^{7}$	4,7 $E10^{6}$

The results obtained have temporarily determined the concentration of the selective T agent at 2 g/l to continue to vary the concentrations of the selective agent S. Moreover, since *E.cloacae's* growth was also significant , we tried to introduce two selective agents into the broth: Vm and I. The results obtained, however, led to the exclusion of both the selective agents as they did not improve the selectivity of the experimental broth (data not shown).

Based on results obtained from the first phase of the research, a first formulation of the broth was chosen which most satisfies our requirements, as shown in the table (Table 21).

Ingredients	g/l
Carbon and nitrogen source B	20
Vitamine source Y	5
Glucide X	12
Selective agent T	2
Salt S	5
Selective agent S	0,25
Selective agent SD	2

Evaluation of selective enrichment capacity of the experimental broth respect to the enrichment broth ISO Muller Kauffmann. In this second phase of the research, the efficiency of the experimental broth in the selective enrichment of *Salmonella* spp. was compared to that of the ISO broth, Muller Kauffmann. The ability to promote of *Salmonella* spp. growth and slowing down or inhibiting the growth of metabolically related enterobacteria has been verified by evaluating the replicating capacity of interest strains in the experimental broth and in the ISO broth. Since the first formulation of the experimental broth still allowed *E. cloacae* growth and had not been tested under cross-contamination conditions, further variations in the experimental broth were tested in the various experiments.

Using artificially contaminated water samples instead of food samples allows the elimination of the interference of the food matrix itself [57] and to keep the homogeneity of the sample intact constant. These characteristics, added to the large number of variables and modifications to be tested, have led to a decrease in sample quantities to 10 ml (5 ml of artificially contaminated water sample in 45 ml of experimental broth and 5 ml of sample in 50 ml of broth Muller Kauffmann), maintaining the ratio of the ISO 6579:2008 [56] (25 grams in 225 ml) to the experimental broth unchanged.

Growth level of interest strains after enrichment in the experimental broth and broth Muller Kauffmann were evaluated after 24 and 48 hours of enrichment. Although the speed is one of the characteristics of primary interest in view of engagement of the enrichment step in the analytical protocol the MBS method, and the results obtained after 24 hours are of greatest

interest with respect to the final objective, we decided to continue to monitor the growth trend even at 48 hours.

Evaluation of the efficiency of the experimental broth compared to the Muller Kauffmann broth. Artificially contaminated samples were prepared to obtain a concentration of about 10^2 CFU/ml for *Salmonella* spp. and 10^6 CFU/ml for *E. cloacae*; 5 ml of the so prepared specimens were inoculated separately in 45 ml of experimental broth and 5 ml in the ISO Muller Kauffmann broth. Both broths were incubated at 37° C for 48 hours.

Although the formulation of the experimental broth has now been defined, we have decided to continue to vary the concentrations of the selective agent S. The following conditions have been tested: (A) selective agent S 0 g/l; (B) Selective agent S 0.25 g / l; (C) Selective agent S 0.5 g/l. Concentration of contaminating strains following enrichment in the experimental broth and ISO Muller Kauffmann broth was evaluated after 24 and 48 hours. The results are shown in Table 20 and refer to an average of three independent experiments in which each sample was analyzed in triplicate.

Table 22. Single contamination concentrations before and after enrichment step monitored for 24 and 48 hours. The results refer to an average of three independent experiments in which each sample was analyzed in triplicate.

Contaminants strains	**MBS broth conditions**		**MBS enrichment broth CFU/ml selective media**		**Muller Kauffmann broth CFU/ml selective media**	
		Before enrichment	**After 24 h**	**After 48 h**	**After 24 h**	**After 48 h**
S. typhimurium	A	2	5,6 E10^7	2,3 E10^8	7,5 E10^6	9,1 E10^6
	B		9,6 E10^6	7,1 E10^7		
	C		4,3 E10^5	6 E10^6		
S. enteritidis	A	1	6,4 E10^6	5,6 E10^7	3,2 E10^6	4,5 E10^6
	B		4,7 E10^6	5,6 E10^7		
	C		8,7 E10^5	5,6 E10^7		
E. cloacae	A	3,1 E10^5	3,3 E10^8	7,2 E10^7	1,2 E10^8	7,3 E10^7
	B		4,3 E10^7	8,1 E10^6		
	C		1,8 E10^6	5,2 E10^5		

It is evident from results reported in the table above that the best condition of the experimental broth, both for the promotion of *Salmonella* spp. growth which for the partial inhibition of growth of *E. cloacae*, is the condition "B" (selective agent S, 0.25 g / l). The concentration of *Salmonella* spp. in the experimental broth with this formulation, after 24 hours, is in fact about six orders of magnitude, while that of *E. cloacae* is about 2 orders of magnitude. The result is even more evident after 48 hours, when the concentration of *Salmonella* spp. continues to increase as *E. cloacae* decreases. Compared with the results obtained with the ISO broth Muller Kauffmann, the experimental broth seems to be more selective with *E. cloacae*.

The significance of these results was evaluated by statistically analyzing the data obtained with the t-test, using GraphPad Prism6 software. Considering the final application and the aim of decreasing the time of analysis, only results after 24 hours are shown (Figure 13). Growth rate of contaminating microorganisms within enrichment broths is expressed in terms of increase in order quantities (log CFU/ml).

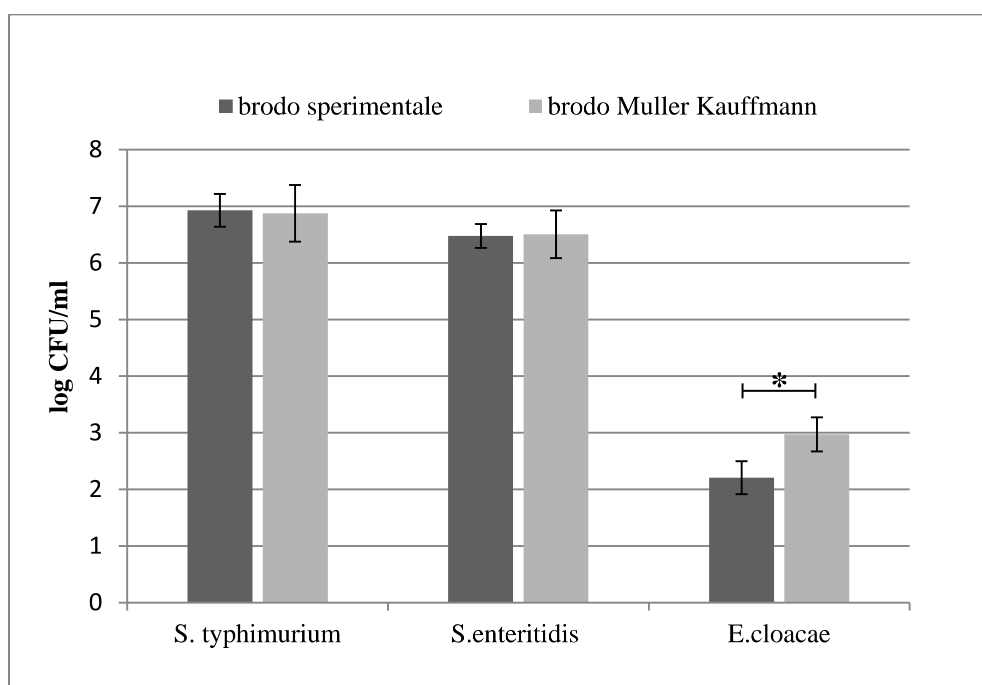


Figure 13. Single Contamination. Growth levels of the inoculated strains individually after 24 hours of enrichment in the experimental broth and broth Muller Kauffmann. Data were analyzed using the t-test, using the GraphPad Prism6 software (* = significant differences). S. typhimurium: p-value = 0.8882 (not significant); S. enteritidis: p-value = 0.9172 (not significant); E. cloacae: p-value = 0.0344 (significant).

This promising result led us to fix the final formulation of the new experimental broth with 0.25 g/l of selective S agent as previously established (see Table 19).

In light of the results obtained above, we decided to evaluate the enrichment and selectivity of the experimental broth, simulating cross-contamination conditions between *Salmonella* spp. Strains. and *E. cloacae*. The artificially contaminated samples were prepared to obtain a cross-contamination condition of about 10^2 CFU / ml for *Salmonella* spp. and 10^6 CFU/ml for *E. cloacae*; 5 ml of the so prepared samples were separately inoculated in 45 ml of experimental broth and 5 ml in the ISO Muller Kauffmann broth. Both broths were incubated at 37 ° C for 48 hours. The concentration of the various contaminating and cross-contaminating strains was evaluated before the enrichment step and the results are reported in Table 23.

Table 23. Concentration of contaminated strains, in cross-contamination condition , before enrichment (see Materials and Methods paragraph 3.4.3.)

Contamination strains	CFU/ml	Cross-contamining strains	CFU/ml
S. typhimurium	1	**E. cloacae**	2.9E10^5
S. enteritidis	2		

Concentration of contaminating and cross-contaminating strains following enrichment in the experimental broth and ISO Muller Kauffmann broth was evaluated after 24 and 48 hours. The results are shown below (Table 24) and refer to an average of three independent experiments in which each sample was analyzed in triplicate.

Table 24. Concentration of the contaminating strains, in cross-contamination condition after 24 and 48 hours of enrichment in the experimental broth and in Muller Kauffmann broth. The results refer to an average of three independent experiments in which each sample was analyzed in triplicate.

	MBS enrichment broth				**Muller Kauffann broth**			
Samples	**CFU/ml *Salmonella* spp.**		**CFU/ml *E. cloacae***		**CFU/ml *Salmonella* spp.**		**CFU/ml *E. cloacae***	
	After 24 h	**After 48 h**	**After 24 h**	**After 48 h**	**After 24 h**	**After 48 h**	**After 24 h**	**After 48 h**
S.typhimurium + E. cloacae	3,4 E10^6	5,3 E10^6	2,3 E10^7	9,1 E10^6	3,5 E10^6	4,9 E10^6	4,5 E10^8	7,2 E10^8
S. enteritidis + E. cloacae	8,6 E10^5	2,1 E10^6	3,9 E 10^7	8,7 E 10^6	1,2 E10^6	4,5 E10^6	3,1 E10^8	3,4 E 10^8

The results show that, under cross-contamination conditions, the experimental broth preserves its enrichment power towards *Salmonella* spp. and partial inhibition of *E.cloacae* growth, both after 24 and after 48 hours. In addition, comparing the results obtained with the ISO broth Muller Kauffmann, the experimental broth seems to be more selective towards *E. cloacae*. The significance of these results was evaluated by statistically analyzing the data obtained with the t-test, using GraphPad Prism6 software. Considering the final application, and the goal of decreasing the time of analysis, only results are shown after 24 hours. The growth rate of inoculated microorganisms within enrichment broths is expressed in terms of increase in size orders (log CFU/ml) (Figure 14).

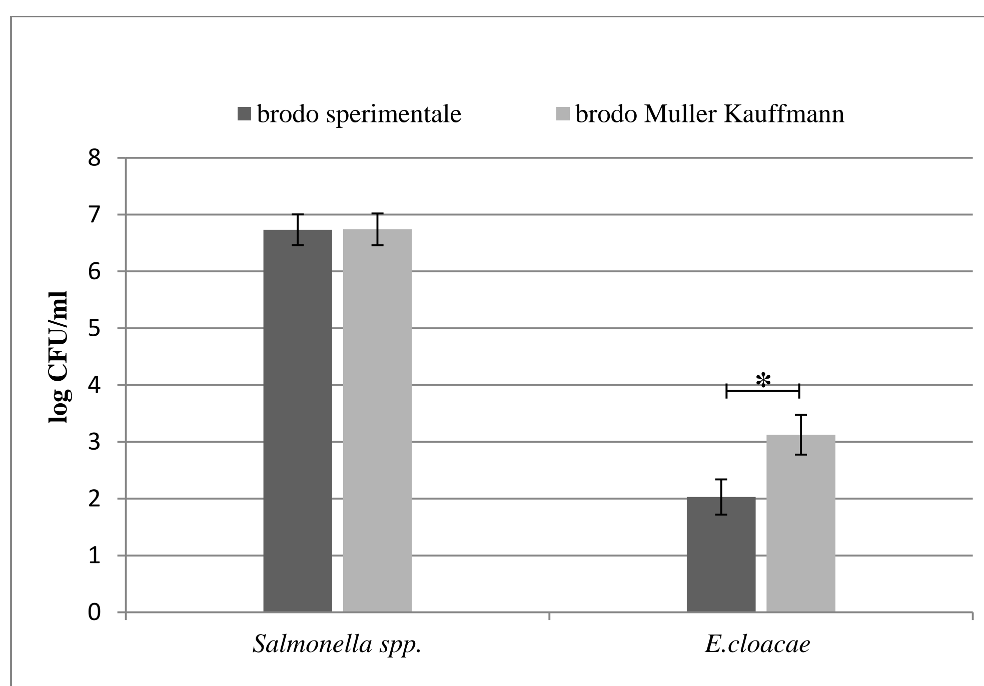


Figure 14.. Cross-contamination. Growth levels of contaminating strains, under conditions of cross-contamination, after 24 hours of enrichment in the experimental broth and broth Muller Kauffmann. The data were analyzed using the t-test, using the GraphPad Prism6 software (* = significant differences). *Salmonella* spp: p-value = 0.9666 (not significant); *E. cloacae*: p-value = 0.0152 (significant).

In order to verify the selectivity of the new experimental broth to other bacterial strains belonging to the *Enterobacteriaceae* family, the artificially contaminated water samples were prepared to obtain a concentration of about 10^6 CFU/ml of the following enterobacteria (selected as representative species of the *Enterobacteriaceae* family): *Escherichia coli* ATCC 25992; *Klebsiella pneumoniae* ATCC 33495; *Citrobacter freundii* ATCC 13316

These bacterial strains' growth was evaluated after 24 and 48 hours of enrichment in the experimental broth and in the Muller Kauffmann broth (Table 25) and the results refer to an average of three independent experiments in which each sample was analyzed in triplicate.

Table 25. Concentration of individually inoculated strains (single contamination) before and after 24 and 48 hours of enrichment in the experimental broth. The results refer to an average of three independent experiments in which each sample was analyzed in triplicate.

Contaminant strains		**MBS enrichment broth**		**Muller Kauffmann Broth**	
		CFU/ml selective media		**CFU/ml selective media**	
	Before enrichment	**After 24 h**	**After 48 h**	**After 24 h**	**After 48 h**
Escherichia Coli	4,6 E10^5	2,8 E10^7	5,9 E10^6	3,2 E10^8	4,2 E10^7
Klebsiella pneumoniae	1,5 E10^5	4,1 E10^5	6,2 E10^4	4,3 E10^6	9,2 E10^5
Citrobacter freundii	2,6 E10^5	5,7 E10^6	2,1 E10^6	1,8 E10^8	8,3 E10^6

Results show that, concerning the experimental broth, the different enterobacteria analyzed show a different replication capability which results in a different inhibitory effect of the broth. After 24 hours, *Klebsiella pneumoniae* growth is partially inhibited. The concentration of *Escherichia coli* increased by 2 orders of magnitude, while that of *Citrobacter freundii* increased by 1 order of magnitude, demonstrating that in all cases the experimental broth is able to reduce the growth of the enterobacteria selected for experimentation. The inhibiting capability is more evident after 48 hours. Compared to the results obtained with the ISO broth Muller Kauffmann, the experimental broth confirms greater selectivity for all the strains. The significance of these results was evaluated by statistically analyzing the data obtained with the t-test, using GraphPad Prism6 software. Considering the final application, and the goal of decreasing the time of analysis, only results are shown after 24 hours. The growth rate of inoculated microorganisms within the enrichment broths is expressed in terms of increase in size orders (log CFU/ml) (Figure 15).

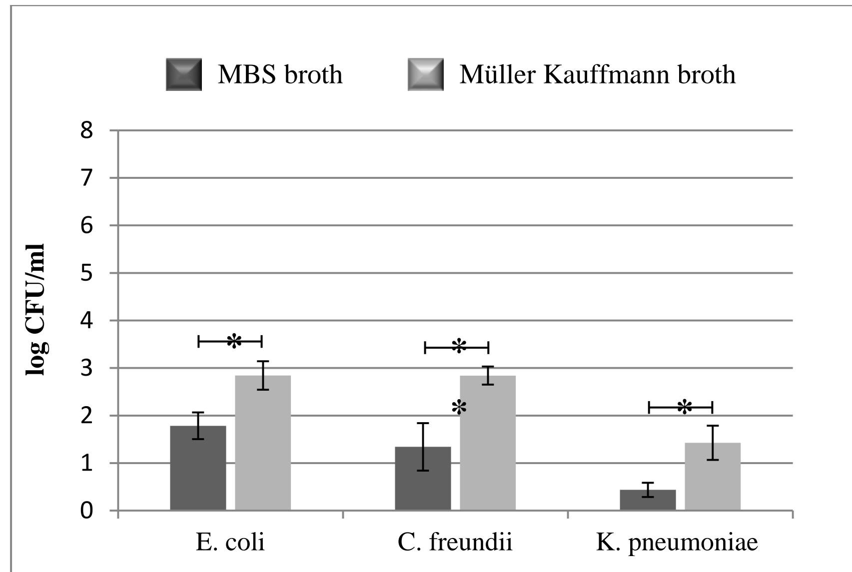


Figure 15. Single contamination (selectivity). Growth levels of the inoculated strains individually after 24 hours of enrichment in the experimental broth and broth Muller Kauffmann. The data were analyzed using the t-test using GraphPad Prism6 software (* = significant differences; ** = highly significant differences). E. coli: p-value = 0.011 (significant); C. freundii: p-value = 0.0083 (highly significant); K. pneumoniae: p-value = 0.0117 (significant).

In light of the results obtained from the formulation of the experimental broth, we decided to evaluate its enrichment and selectivity ability by simulating cross-contamination conditions between strains of *Salmonella* spp. and other enterobacteria, thus recreating the type of contamination that occurs frequently in naturally contaminated samples.

The artificially contaminated water samples were prepared so as to obtain a concentration of about 10^2 CFU/ml of *Salmonella* spp. and about 10^6 CFU / ml of the following enterobacteria: *Escherichia coli* ATCC 25992; *Klebsiella pneumoniae* ATCC 33495; *Citrobacter freundii* ATCC 13316. To facilitate interpretation of the data, the analyzed samples were numbered and reported in Table 26.

Table 26. Analyzed samples.

Contaminant strains	Cross-containant strains	Number of sample
S. typhimurium	*Escherichia coli*	1
	Citrobacter freundii	2
	Klebsiella pneumoniae	3
S. enteritidis	*Escherichia coli*	4
	Citrobacter freundii	5
	Klebsiella pneumoniae	6

The concentration of the various contaminating and cross-contaminating strains was evaluated prior to the enrichment step both in enrichment broth and in Muller Kauffmann broth.

Table 27a, b. Concentration of contaminated strains, in a cross- contamination condition, a) before the enrichment step, and b) after 24 hours in the enrichment in the experimental and Muller Kauffmann broth. The results refer to an average of three independent experiments in which each sample was analyzed in triplicate.

a) **Before enrichment**

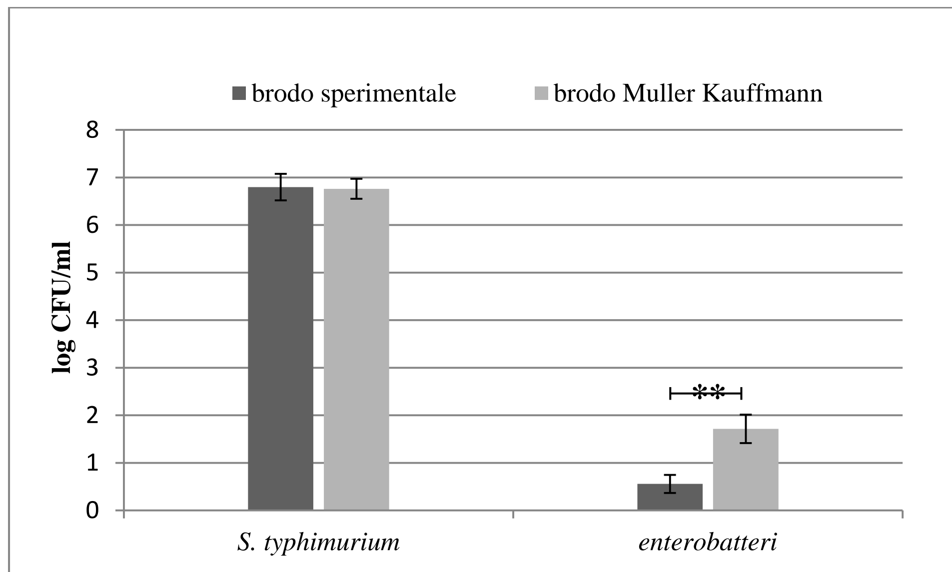
Samples	Contaminant strains	CFU/ml	Cross-contaminant strains	CFU/ml
1	*S. typhimurium*	3	*Escherichia coli*	4,2 $E10^5$
2			*Klebsiella pneumoniae*	3,6 $E10^5$
3			*Citrobacter freundii*	3,8 $E10^5$
4	S. e*nteritidis*	1	*Escherichia coli*	4,2 $E10^5$
5			*Klebsiella pneumoniae*	3,6 $E10^5$
6			*Citrobacter freundii*	3,8 $E10^5$

b)	After enrichment in MBS broth				After enrichment in Muller Kauffmann broth			
		CFU/ml		CFU/ml		CFU/ml		CFU/ml
1	*S. typhimurium*	5,2 $E10^6$	*E. coli*	3,4 $E10^6$	*S. typhimurium*	4,8 $E10^6$	*E.coli*	5,5 $E10^7$
2		7,8 $E10^6$	*K.pneumoniae*	4,7 $E10^5$		6,9 $E10^6$	*K.pneumoniae*	1,8 $E10^6$
3		6,1 $E10^6$	*C.freundii*	1,7 $E10^6$		5,8 E106	*C.freundii*	4,7 $E10^7$
4	S. e*nteritidis*	7,5 $E10^5$	*E.coli*	4,2 $E10^6$	S. e*nteritidis*	7,3 $E10^5$	*E.coli*	7,2 $E10^7$
5		8,1 $E10^5$	*K.pneumoniae*	5,6 $E10^5$		7,9 $E10^5$	*K.pneumoniae*	3,1 $E10^6$
6		6,8 $E10^5$	*C.freundii*	1,1 $E10^6$		7,1 $E10^5$	*C.freundii*	6,3 $E10^7$

It is evident that the ability of the experimental broth to enrich *Salmonella* spp. does not undergo significant variations despite the presence of high initial concentrations of cross-contaminant enterobacteria. The final concentration of *Salmonella* spp. after 24 hours of enrichment always increases of about 6 orders of magnitude while other enterobacterial growth is partially inhibited in comparison to single contamination situations, ie in the absence of cross-contamination. The broth Muller Kauffmann, although capable of promoting *Salmonella* spp. growth, does not prove to be as effective in slowing the growth of cross-contaminant enterobacteria.

The significance of these results was evaluated by statistically analyzing the data obtained with the t-test, using GraphPad Prism6 software. The growth rate of inoculated microorganisms within the enrichment broths is expressed in terms of increase in size orders (log CFU/ml) (Figure 16 a, b).

a)



b)

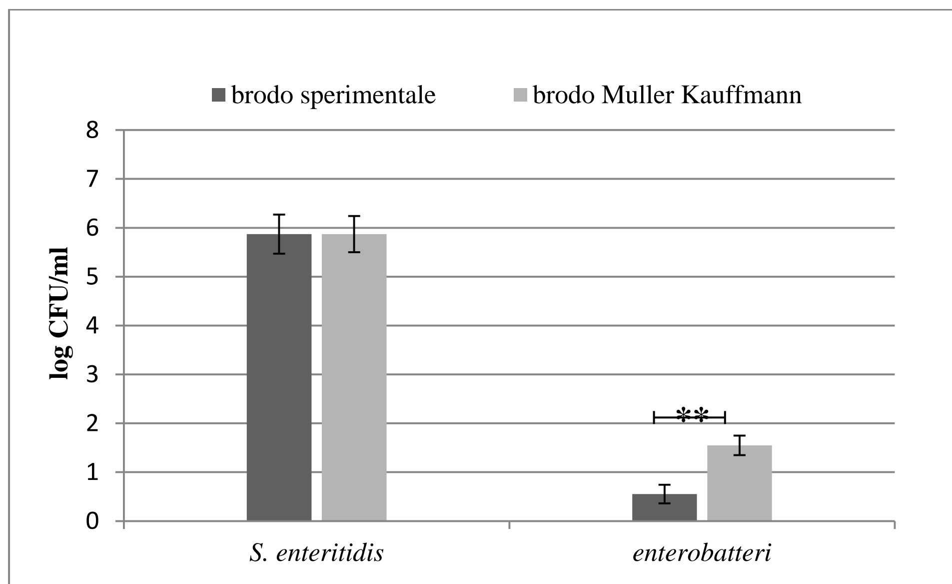


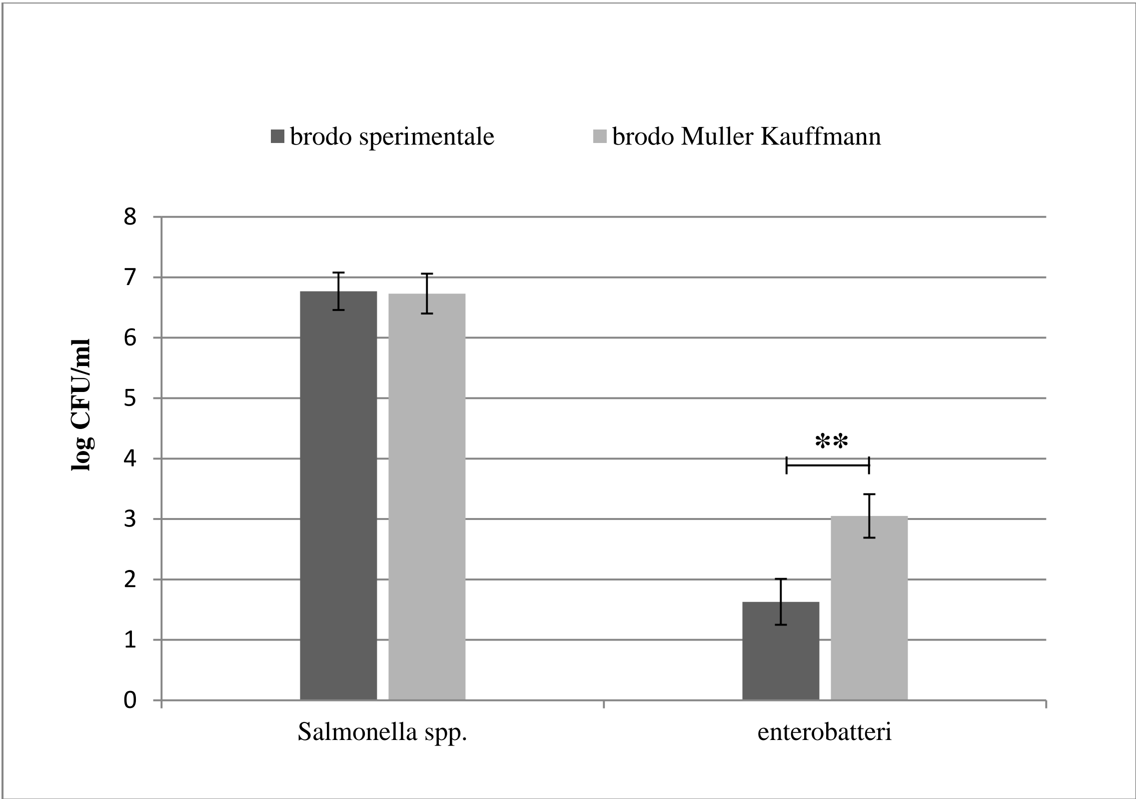
Figure 16. Cross-contamination. Growth levels of contaminating strains, under conditions of cross-contamination after 24 hours of enrichment in the experimental broth and broth Muller Kauffmann. The data were analyzed using the t-test using GraphPad Prism6 software (* = significant differences; ** = highly significant differences).

a) S. typhimurium + Enterobacteria. S. typhimurium: p-value = 0.8478 (not significant); enterobacteria: p-value = 0.0055 (highly significant). b) S. enteritidis + Enterobacteria. S. enteritidis: p-value = 1 (not significant); enterobacteria: p-value = 0.0033 (highly significant)

The results obtained from statistical analysis of data were reported graphically to simplify the interpretation of results and compare the selective enrichment capabilities of the two enrichment broths examined.

Different conditions of single contamination (experimental broths contaminated with a single strain) and cross contamination (cross contamination) are taken into consideration separately. The growth rate of the microorganisms inoculated within the enrichment broths is expressed in terms of increase in size orders (log CFU / ml) and refers to the average of all the results obtained under the experimental conditions under consideration (Figure 17 a, b)

a).



b)

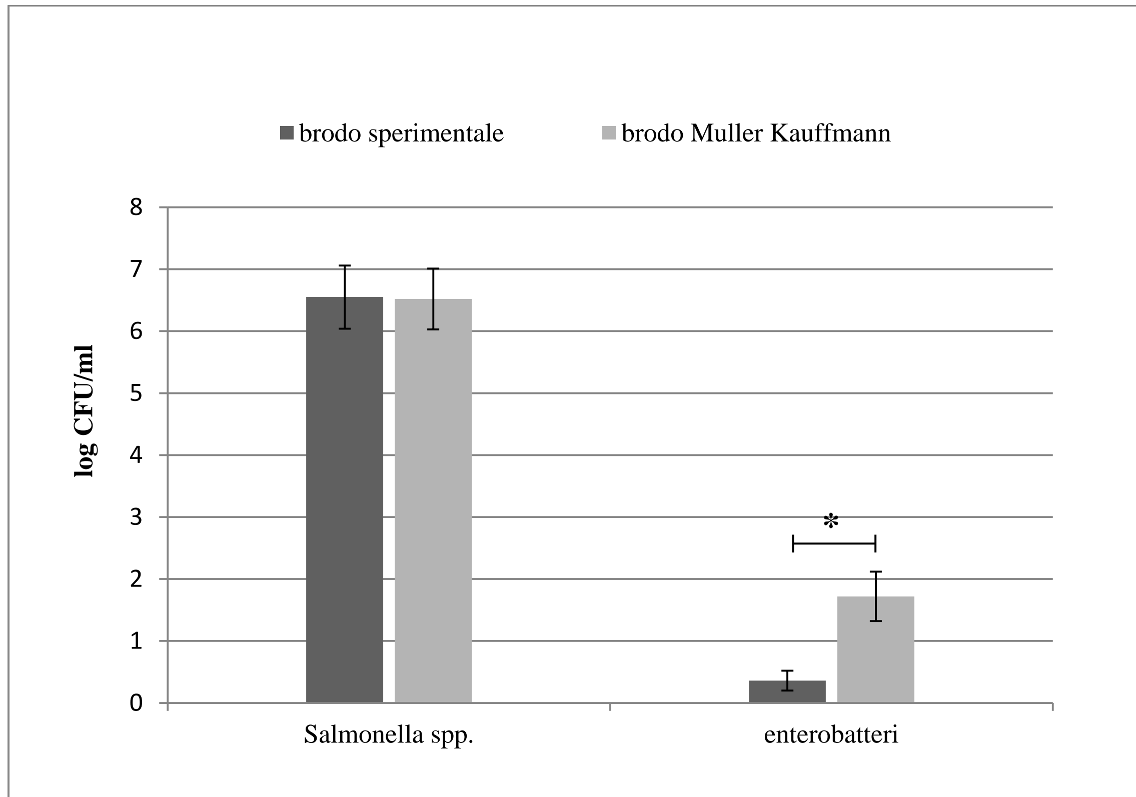


Figure 17. Average growth rates of the strains analyzed after 24 hours of enrichment in the experimental broth and broth Muller Kauffmann. The data were analyzed using the t-test using GraphPad Prism6 software (* = significant differences; ** = highly significant differences).

a) Single contamination. Salmonella spp: p-value = 0.8858 (not significant); enterobacteria: p-value = 0.0093 (highly significant). b) Cross Contamination. *S. enteritidis*: p-value = 0.950 (not significant); enterobacteria: p-value = 0.0197 (significant).

4.2.2. Application of the experimental enrichment broth to the Micro Biological Survey method

The results obtained in the second phase of the research demonstrated an enrichment ability of the experimental broth really comparable to the ISO Muller Kauffmann broth in single contamination conditions, while its selectivity in cross-contaminated conditions was, significantly greater than the Muller Kauffmann broth. In the final phase of the research the experimental broth was applied to MBS analysis, in order to demonstrate that the results obtained could be better compared to the Muller Kauffmann broth.

The new experimental protocol used in this phase of the experiment involves a pre-enrichment of the samples for 24 hours at 37° C, followed by inoculation of 1 ml of enrichment broth in SL vial, then incubated for 72 hours at 37 ° C in the MBS Multireader (MR), which can automatically record the vial's color changing and return the results in terms of hours.

Evaluation of the sensitivity of the SL reagent before and after enrichment. The sensitivity of the SL vials following the introduction of the enrichment analytical procedure and the evaluation of any improvement about the color change were evaluated. Different starting concentrations of *Salmonella* spp. were analyzed in parallel both in the experimental and the Muller Kauffmann broth. Both broths were inoculated with samples artificially contaminated with *Salmonella* spp. (*S. typhimurium* and *S. enteritidis*), prepared as previously described in order to obtain different initial starting concentrations: A) ~ 100 CFU/ml; B) ~ 10 CFU/ml; C) ~ 1 CFU/ml

In order to evaluate the effects of the enrichment passage, SL vials were inoculated with artificially contaminated samples prior to enrichment. The presence of *Salmonella* spp. was evaluated by inoculating MBS SL vials with 1 ml of both broths after 24 hours of enrichment at 37° C and verifying the presence and time needed for color change within 72 hours of incubation in MBS Multireader (MR).

The concentration of *Salmonella* spp. in the sample before enrichment was confirmed by the plate count method (data not shown). The final concentration of *Salmonella* spp. in broths

after the enrichment step was evaluated by the plate count method, to ensure that the color change was actually due *Salmonella* spp. The results obtained are shown in Table 28 refer to an average of three independent experiments in which each sample was analyzed in triplicate.

Table 28. MBS analysis of artificially contaminated samples with *Salmonella* spp. before and after the enrichment step of 24 hours both in the experimental Muller Kauffmann broth (sensitivity of MBS SL vial). The results refer to an average of three independent experiments in which each sample was analyzed in triplicate.

Contaminant strains	**Conditions CFU/ml**	**Before enrichment**	**After 24 h of enrichment**			
			MBS broth		**Müller Kauffmann broth**	
		Hs of color change	**Hs of color change**	**CFU/ml**	**Hs of color change**	**CFU/ml**
S. typhimurium	A	27,45	**8,45**	4,9 $E10^{7}$	16,36	5,1 $E10^{7}$
	B	32,10	**9,30**	4,1 $E10^{7}$	17,25	4,3 $E10^{7}$
	C	36,30	**9,32**	3,7 $E10^{7}$	17,50	3,5 $E10^{7}$
S. e*nteritidis*	A	36,51	**15,55**	6,1 $E10^{6}$	27,30	5,8 $E10^{6}$
	B	48,20	**16,20**	4,9 $E10^{6}$	27,10	5,3 $E10^{6}$
	C	NV	**16,45**	4,7 $E10^{6}$	28,22	4,1 $E10^{6}$

Results evidently show that time frames for color changing of MBS vial shifts significantly decreasing from about 32 hours for *S. typhimurium* and 42 hours for *S. enteritidis* before enrichment at an average of 9 and 16 hours respectively after enrichment step in the experimental broth, even starting from an initial concentration of *Salmonella* spp. in the sample of ~ 1 CFU/ml. This increases the sensitivity of the MBS analysis. This improvement is not so obvious with the Muller Kauffmann broth: after the enrichment, all conditions show a time of color change considerably higher than the experimental broth (17 and 27 hours respectively for *S. typhimurium* and *S. enteritidis* respectively).

Evaluation of the selectivity of the SL reagent before and after enrichment To assess the selectivity of SL vials following the introduction of the analytical enrichment procedure and to evaluate any improvement over the selectivity limit previously found for the SL reagent, the same experiment was repeated by contaminating the sample with different enterobacteria. Samples were prepared as previously described, to obtain a concentration of about 10^6 CFU / ml of the following enterobacteria: *Escherichia coli* ATCC 25992; *Enterobacter cloacae* ATCC 13047; *Klebsiella pneumoniae* ATCC 33495; *Citrobacter freundii* ATCC 13316

To evaluate the effects of the enrichment step, SL vials were inoculated with artificially contaminated samples before the enrichment. The selectivity of the SL vials towards different enterobacteria analyzed after the enrichment in both broths (experimental and Muller Kauffmann) was evaluated by inoculating MBS SL vials with 1 ml broth after 24 hours of enrichment at 37° C and verifying the presence and color change within the 72-hour incubation in the MBS Multireader (MR).

In parallel with the MBS analysis, concentration of the different enterobacteria in the sample before enrichment was confirmed by the plate count method (data not shown). The concentration of the different enterobacteria after enrichment was evaluated by the plate count method, so as to ensure that the lack of color change was not due to the absence of such bacteria. The results obtained are shown in Table 29.

Table 29. MBS analysis of artificially contaminated samples with enterobacteria strains before and after enrichment passage (24 hours) in experimental broth and broth Muller Kauffmann (MBS SL vial selectivity). The results refer to an average of three independent experiments in which each sample was analyzed in triplicate.

Contaminant strains	**Before enrichment**	**After 24 hs of enrichment**			
		MBS broth		**Müller Kauffmann broth**	
	Hs of color change	**Hs of color change**	**CFU/ml**	**Hs of color change**	**CFU/ml**
Escherichia coli	NV	**NV**	4,6 E10^{7}	30,00	4,7 E 10^{8}
Enterobacter cloacae	46,3	**NV**	1,1 E10^{8}	31,10	2,4 E10^{8}
Klebsiella pneumoniae	NV	**NV**	3,3 E10^{5}	NV	6,23 E10^{6}
Citrobacter freundii	NV	**NV**	6,2 E10^{6}	45,50	9,45 E10^{7}

The results confirmed that after the enrichment in the experimental broth, even in presence of a final enterobacteria concentration greater than that of the previously defined selectivity limit of ~ 10^{6} CFU/ml, the Multireader could not detect any color change in the vials within 72 hours. The selectivity is therefore improved compared to the one before enrichment, where false positive result were observed due to the color change of vials inoculated with *E. cloacae* in long time but within 72 hours. The same result was not achieved using the Muller Kauffmann broth: the increased growth of enterobacteria that is not inhibited or slowed after the enrichment step causes a lack in the selectivity of the MBS analysis: the false positives results are in fact increased within 72 hours.

Evaluation of sensitivity of the SL reagent before and after enrichment in case of cross contamination. Previous results demonstrated that the enrichment step in the experimental broth fully satisfies the requirements, greatly improving the sensitivity and selectivity of the MBS method for the detection of *Salmonella* spp. The efficacy of the enrichment phase applied to the MBS method was also evaluated in cases of cross-contamination. The results previously obtained les these experiments to be conducted only with the experimental broth.

In these tests, artificially contaminated samples were prepared to obtain a high concentration of enterobacteria and a low concentration of *Salmonella* spp.: A) *Salmonella* spp. ~100 CFU/ml + *Enterobacter cloacae* ~10^6 CFU/ml; B) *Salmonella* spp. ~10 CFU/ml + *Enterobacter cloacae* ~10^6 CFU/ml; C) *Salmonella* spp. ~1 CFU/ml + *Enterobacter cloacae* ~10^6 CFU/ml. SL vials were inoculated with artificially contaminated samples before enrichment. The sensitivity of SL vials towards *Salmonella* spp., even in presence of high concentrations of cross-contaminant enterobacteria, after the passage in the experimental broth was evaluated inoculating the MBS SL vials with 1 ml of experimental broth after 24 hours of enrichment at 37° C and verifying the presence and time of color change within 72 hours of incubation in MBS Multireader (Table 30).

Table 30. MBS analysis of artificially contaminated samples in cross-contamination condition between *Salmonella* spp. and enterobacterial strains before and after the enrichment phase (24 hours) in experimental broth. The results refer to an average of three independent experiments in which each sample was analyzed in triplicate.

Samples	Conditions	Before	After
		Hs of color change	Hs of color change
S. typhimurium + *E. cloacae*	A	28,25	**11,40**
	B	30,10	**12,20**
	C	27,30	**12,50**
S. enteritidis + *E. cloacae*	A	31,56	**13,20**
	B	46,30	**13,55**
	C	48,50	**14,45**

Concentration of contaminating and cross-contaminating strains was evaluated by the plate counting method, demonstrating that the decrease of hours of color change was due to the actual presence of *Salmonella* spp. and that cross-contaminating enterobacteria were present (data not shown).

The results evidently show that after the enrichment step in the experimental broth the color change in MBS SL vials has always occurred in the presence of *Salmonella* spp. although the result on the plates indicates the presence of other enterobacteria. This data demonstrates that the Multireader was able to discriminate the presence of *Salmonella* spp. even under high concentrations of cross-contaminant enterobacteria.

4.3. Application of the Micro Biological Survey method for the selective detection of *Listeria monocytogenes* in food samples

4.3.1. Optimization of MBS reagent for *Listeria* spp. detection

The purpose of the research was to optimize the MBS reagent for *Listeria* spp. detection, because in the original formulation it was found to be insensitive to bacteria other than Listeria spp. and not sensitive to *L. monocytogenes*, the only species of the *Listeria* genus of medical interest.

The research has been articulated in different phases: in the first one, some changes to the original of MBS recipe for LY vials were made; in the second phase, the reagent was further modified since the results previously obtained were not fully satisfactory in terms of sensitivity and selectivity. During each experimental test, the analysis was carried out using the plate count reference method in parallel to the MBS method in order to verify the behavior (color changing or not) of the vials used.

Firstly analyses confirmed the problem with the original formulation of the reagent prepared according to the MBS method, using the original formulation and subsequently contamining it with serial dilutions of *L. innocua*, *L. monocytogenes,* and other gram positive bacterial strains considered *Listeria* competitors. These analyses show that the LY color change from blue to yellow within the 72 hours occurred only in *L. innocua* dilutions, while vials inoculated with *L. monocytogenes* dilutions have changed color only in the presence of high bacterial concentration (10^4 CFU/ml), thus showing the different sensitivity of the reagent in its original formulation for the two bacterial species of *Listeria* genus. Moreover, presence of false positive results is evident from the color change of vials inoculated with other gram-positive bacteria (Table 31).

Table 31. Original formulation of MBS LY vials.

Bacterial strains	Concentrations (CFU/ml)	Hrs of color change
L. monocytogenes	~ 10^7	17
	~ 10^5	22,8
	~ 10^3	NV
	~ 10^1	NV
	~ 1	NV
L. innocua	~ 10^7	45,3
	~ 10^5	58,1
	~ 10^3	63,2
	~ 10^1	69,4
	~ 1	73
E. coli	~ 10^7	40,9
	~ 10^5	NV
	~ 10^3	NV
	~ 10^1	NV
	~ 1	NV
S. aureus	~ 10^7	20,9
	~ 10^5	25,4
	~ 10^3	37,6
	~ 10^1	NV
	~ 1	NV
E. faecalis	~ 10^7	23,7
	~ 10^5	30,2
	~ 10^3	39
	~ 10^1	NV
	~ 1	NV

The lack of color change in the vials with lower concentrations of *L. monocytogenes* has been attributed to the presence of high amounts of selective agents, such as selective agent SC and selective agent LC, which, while allowing inhibitory growth of other competitor bacteria present in the environment, such as *S. aureus*, *E. faecalis* and *E. coli*, reduce *L.*

monocytogenes growth. This hypothesis came mainly from the analysis of the results obtained from two sets of tests. In the first case two conditions of MBS reagent for the detection for *Listeria* spp.: one using the reagent without the selective agent SC, and the other using the reagent without the LC selective agent; in both cases, rapid growth of *L. innocua* and a fair growth of *L. monocytogenes* were observed (data not shown).

Thus, while the simultaneous presence of the salts at the amounts indicated by the original recipe showed negative effects on the growth of *L. monocytogenes*, and that the elimination of either component had instead improved the conditions for its development , the following four formulations were analyzed in the second set of tests: the MBS reagent in its original formulation, the MBS reagent in its original formulation with a reduced concentration of selective agent LC, the MBS reagent in its original formulation without the selective agent SC, and finally the MBS reagent in its original formulation without SC-selective agent and with a reduced LC concentration. The condition of the reagent without selective agent SC showed the most promising results: only the presence of a selective LC 13 g / l agent allowed growth, and the consequent increase the color change of *L. monocytogenes* vials until low concentrations and, at the same time, guaranteed the selectivity towards *S. aureus*, *E. faecalis* and *E. coli*.

The promising results led the research to verify the sensitivity of the reagent with this formulation (LY reagent without the selective SC agent) using the Multireader, the analysis was carried out in parallel using the plate count reference method using the selective PALCAM agarized medium for the *Listeria* genus. The results obtained by the two methods were used to construct a specific calibration line for the reagent analyzed, showing the linear proportional relationship between the time needed for color change of the vials and the bacterial concentration values obtained by the reference method of plate count (Figures 18A and 18B, Table 32).

Table 32. Results obtained from MBS reactive sensitivity analysis for Listeria spp. in the original formulation without a selective SC agent. The analysis was carried out in parallel using the platelet count method (results expressed as bacterial concentration in CFU / ml logs) and the MBS method (results expressed as hours of shifts in hours). The data reported are the average of the results obtained for each test, performed in duplicate or triplicate; for each value, the standard deviation is returned

Samples	Concentration (CFU/ml)	Reference method (Log CFU/ml)	MBS method (hours)
L. monocytogenes	10^6	5,03	52,3
	10^4	3,03	47,17
	10^2	3,61	60,43
	10^1	1,52	NV
L. innocua	10^6	6,57	14,45
	10^4	4,57	20,17
	10^2	2,57	26,80
	10^1	1,54	29,31

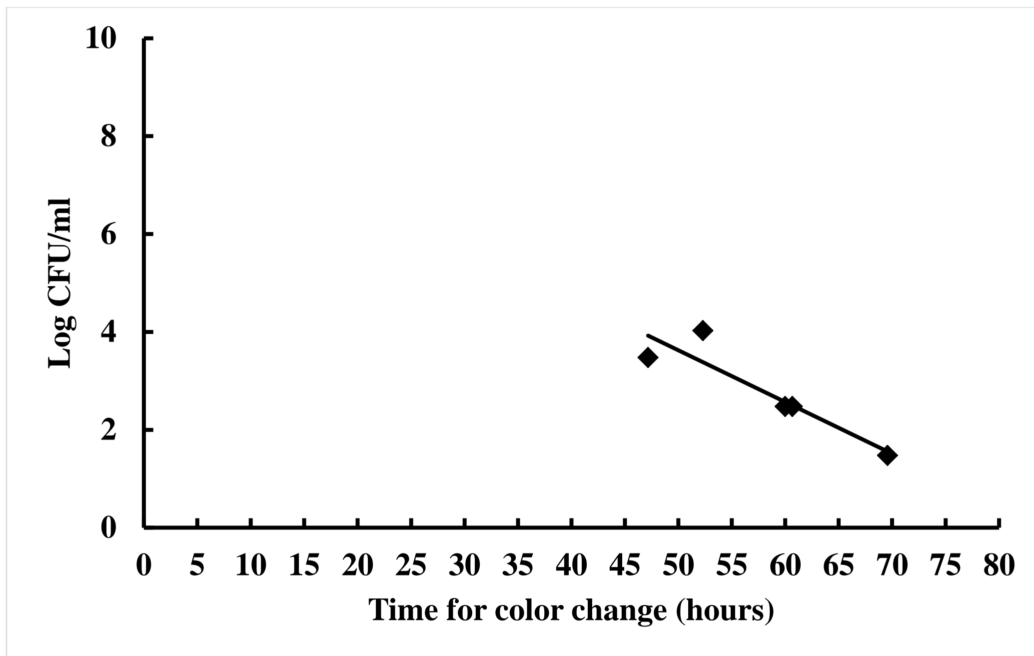


Figure 18A. Linearity: linear regression line (R^2 = 0,96) between the bacterial concentration values (expressed in terms of log CFU/ml) obtained by the plate count reference method and the values of time for color changing (expressed in terms of hours) obtained using the MBS method for the search for *Listeria monocytogenes*. Each point corresponds to the average of the results obtained by analyzing each sample at least in **t**riplicate.

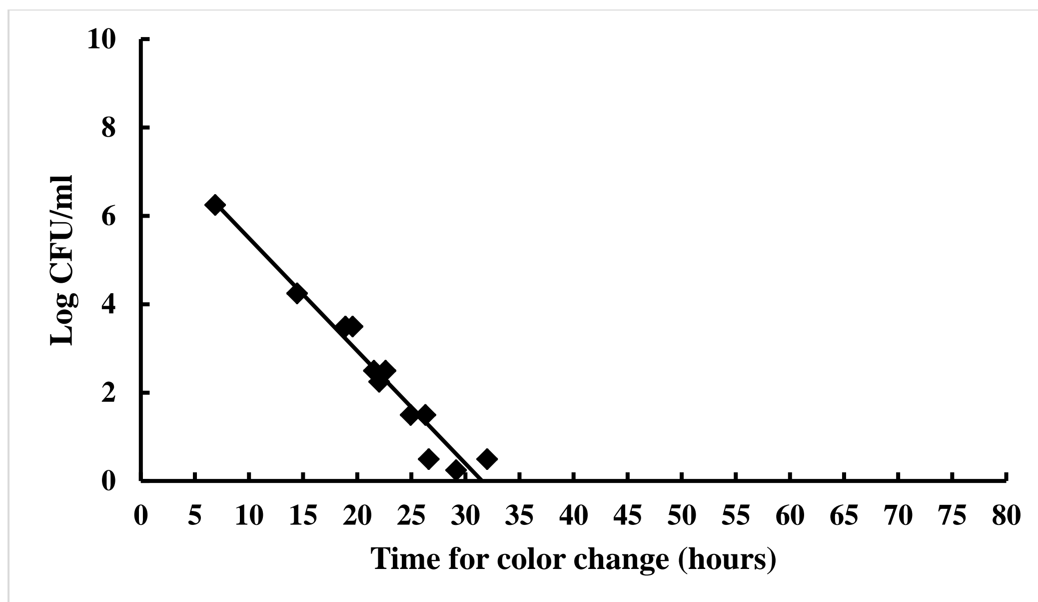


Figure 18B. Linearity: linear regression line (R^2 = 0,93) between the bacterial concentration values (expressed in terms of log CFU ml) obtained by the reference plate reference method and the values of time for color change (expressed in terms of hours) obtained using the MBS method for the search for *Listeria innocua*. Each point corresponds to the average of the results obtained by analyzing each sample at least in duplicate.

Although this formulation seemed encouraging, further analyses had to performed, in order to improve sensitivity towards *L. monocytogenes* and the selectivity towards those more or less pathogenic microorganisms more frequently present in foods compared to the original one.

In addition to optimizing the reagent eliminating, adding and changing concentrations of selective agents, a change in the amount of sterile vaseline oil to be added to each vial as provided by the MBS method has also been considered at this stage.

Generally, sterile vaseline oil is used to create an environment with lower oxygen concentration in a bacterial growth container than the one generally present in the air we breathe, in order to promote the growth of microorganisms. The difficulty of oxygen in reaching the bacterial growth medium is directly proportional to the thickness of the sterile vaseline oil layer on the surface of the culture medium, due to its specific weight less than that of aqueous solutions. According to the oxygen demand, *Listeria* is an aerobic and microaerophilic bacterial genus, or can only use oxygen when it is present at lower oxygen atmospheric concentration (hypoxic conditions) [76]. For this reason, tests were carried out to find out which was the optimal concentration of sterile vaseline oil to be added in each vial, according to the MBS method, to promote growth and thus the detection of *Listeria* spp. The results of these tests indicated a volume of 2 ml vaseline oil as optimal to allow faster growth of *Listeria* spp. and especially *L. monocytogenes*.

Subsequently, the selective agents present in the original recipe MBS recipe for Listeria spp. detection were analyzed in order to examine their efficacy first individually and then in combination.

After verifying that the presence in the reactive MBS of the selective LC was not sufficient to inhibit the growth of other bacteria than Listeria spp., it was confirmed the need to add to the reagent components with specific antibiotic activity which prevented the development of a microflora different from *Listeria* spp..

The selective antibacterial agents already present in the original formulation were first individually analyzed by microplate testing for the determination of the MIC (Minimal Inhibitory Concentration), in order to verify their efficacy. Analysis of the results of these trials suggested to eliminate the selective agents C and N from the original formulation, because they cannot inhibit the growth of numerous bacterial strains present in foods and potentially able to cause foodborne diseases. The selective agent B, on the contrary, showed good inhibitory capacity against various bacterial genres belonging to Gram-negative bacteria.

The research was then carried out analyzing the sensitivity and selectivity of LY reagent modified eliminating the selective agent SC (eliminated due the studies carried out in the first step of the research), the selective agent C and the selective agent N (ineffective results in inhibiting microbial growth in MIC analysis), with various concentrations of the selective agent B. These tests made possible to observe how the growth of *L. monocytogenes* was favored in presence of low concentrations of selective agent B and slowed down as the concentration of this compound increased. However, a concentration of selective B agent has been found to be highly selective for various bacterial strains not belonging to the *Listeria* genus considered, while allowing for a fairly rapid growth of *L. innocua* and especially *of L. monocytogenes*; however, the selectivity of such formulation, determined by the selective LC agent in combination with the selective agent B, has appeared inadequate towards the gram-positive strains such as *S. aureus*.
It was then hypothesized the addition of an antibiotic agent never included before in the MBS LY reagent. Thanks to careful research in literature, the selective bactericidal agent Cp has been identified, which is highly effective against a wide range of gram-positive and gram-negative bacteria and from being tolerated by *Listeria* spp. to a concentration of 2 μg / ml [77;78;79]. This concentration was added to the selective MBS reagent selective agent SC and to other selective antibiotic agents to verify selectivity and sensitivity to *L. monocytogenes* of the reagent containing only selective Cp. This formulation showed excellent selectivity against various collecting bacterial strains other than *Listeria* spp and a discreet yet unsatisfactory sensitivity to *L. monocytogenes*, as demonstrated by the lack of vials color change inoculated with a bacterial concentrations of about 10^3 CFU/ml .
After confirmation of the selectivity of the reactive reagent containing the selective agent LC, selective agent B and selective agent Cp, several tests were performed according to the MBS method (parallel to the reference method) in order to determine concentrations of substances with optimal antibiotic power for the growth of *Listeria* spp. and confirm the selectivity of the new formulation of the reagent against bacterial collection microorganisms not belonging to the *Listeria* genus.
The promising results obtained from these trials allowed the formulation of a MBS LY reagent different from the original one for: absence of selective SC agent, selective N agent and selective C agent; variation of the concentration of selective B agent from 0.01 g / l to 0.05 g / l; addition of the selective Cp agent 1 μg / ml; (Table 33) In addition, an increase in the concentration of 2 ml sterile vaseline was suggested to be added to each MBS vial to recreate a

habitat from the most suitable conditions for the development of *Listeria* spp., disrupting or delaying as far as possible other bacterial genes. This formulation allowed the growth of low concentrations (1-10 CFU/ml) of both *L. innocua* and *L. monocytogenes*, maintaining a good selectivity against non-interest strains.

Tabella 33. Formulation of the new optimized MBS LY reagent.

Components	Quantity (g/l)
Carbon and nitrogen source B	10
Carbon and nitrogen source T	10
Vitamin source Y	5
Buffered system P	9
Buffered system H	1
Selective agent SC	13
Energy source P	2
Selective agent B	0,05
Selective agent Cp	0,001
Redox indicator T	0,5
Redox indicator M	0,004

pH 6,7. Dark blue.

To conclude, the sensitivity of the new formulation of the reagent was analyzed using the Multireader. The data provided by this analysis (Table 32) were used to construct a specific calibration line for the reagent under consideration for the two *Listeria* species analyzed (Figure 19); these charts show the linear relationship of inverse proportionality between the results obtained with the MBS method expressed in terms of time of changing color of the vials and the results obtained with the plate count reference method expressed in terms of bacterial concentration in log CFU/ml. Comparing the calibration lines and the corresponding table developed at the end of the first experimental phase (Figure 18 and Table 32) with those obtained from the tests of the fourth phase of the current work thesis (Figure 19 and Table 34), it is shown how further modification to the MBS LY reagent proposed in the first stage improved sensitivity towards *L. monocytogenes*, allowing detection of lower concentrations of this bacterium (up to 10 CFU/ml), while maintaining a comparable sensitivity to *L. innocua*,

reducing time of analyses from 72 to about 51 hours. Figure 20 shows the accuracy of the two *Listeria* species examined; these graphs show the correspondence between the bacterial concentration values of the assay sample obtained by the plate count reference method and the MBS method, expressed both in terms of log CFU/ml; the line outlined in both cases shows how the results obtained with the MBS method are comparable to those obtained by the reference method (slope, or slope, equal to 1.00). False positive results are considerably reduced, due to the introduction of selective agent Cp and the modification of the concentration of the selective agent B.

Table 34. Results obtained from MBS reactive sensitivity analysis for *Listeria* spp. in the new formulation (see Table 31). The analysis was carried out in parallel using the plate count method (results expressed as bacterial concentration in logs CFU/ ml) and the MBS method (results expressed as hours of color change). The data reported are the average of the results obtained for each test, performed in triplicate.

Samples	Concentration (CFU/ml)	Reference method (Log CFU/ml)	MBS method (hours)
L. monocytogenes	10^6	6,11	31,75
	10^4	4,11	45,2
	10^2	2,11	56,35
	10^1	1,11	71,85
L. innocua	10^6	6,56	16,29
	10^4	4,56	29,6
	10^2	2,37	35,82
	10^1	1,50	51,20

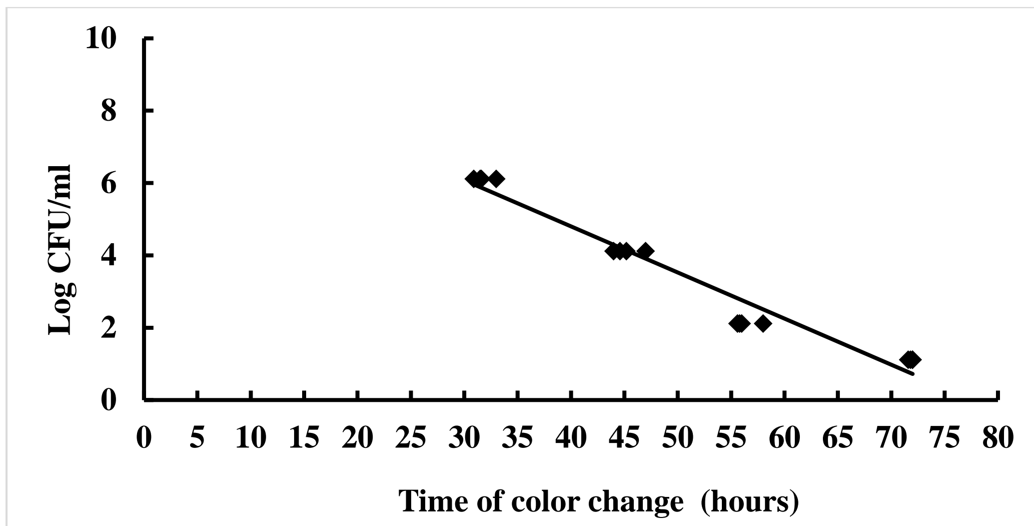


Figure 19A. Linearity: linear regression lines (R2 = 0.96) between the bacterial concentration values (expressed in log CFU/ml) obtained by the plate count reference method and the values of the time of color change (expressed in terms of hours) obtained using the MBS method for the search for *L. monocytogenes*. Each point corresponds to the average of the results obtained by analyzing each sample in triplicate.

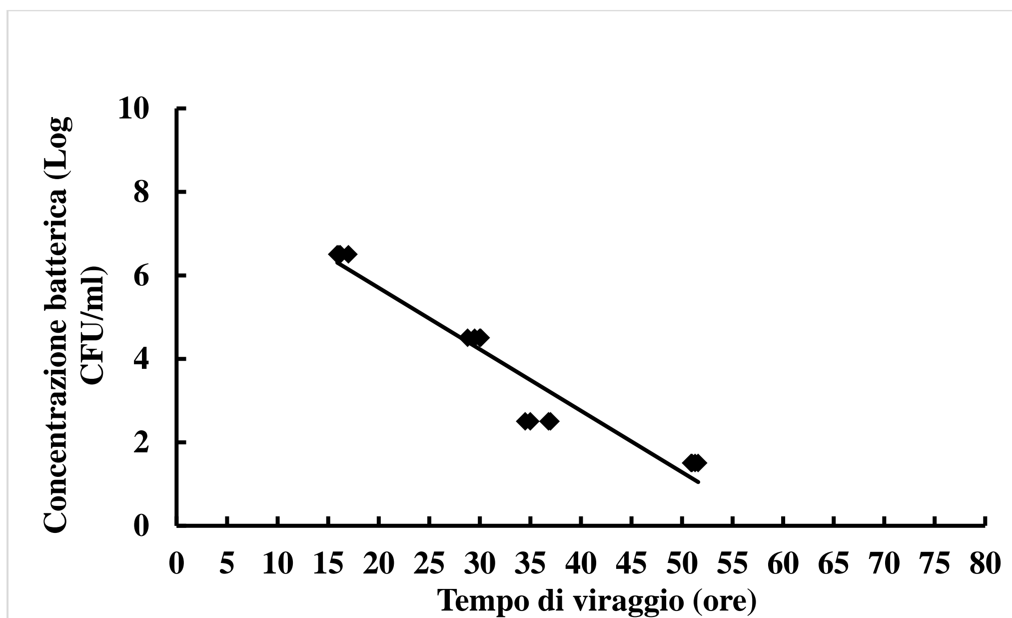


Figure 19B. Linearity: linear regression lines (R2 = 0.93) between the bacterial concentration values (expressed in terms of log CFU/ml) obtained by the reference plate reference method and the values of the time of color change (expressed in terms of hours) obtained using the MBS method for the search for *L. innocua*. Each point corresponds to the average of the results obtained by analyzing each sample in triplicate.

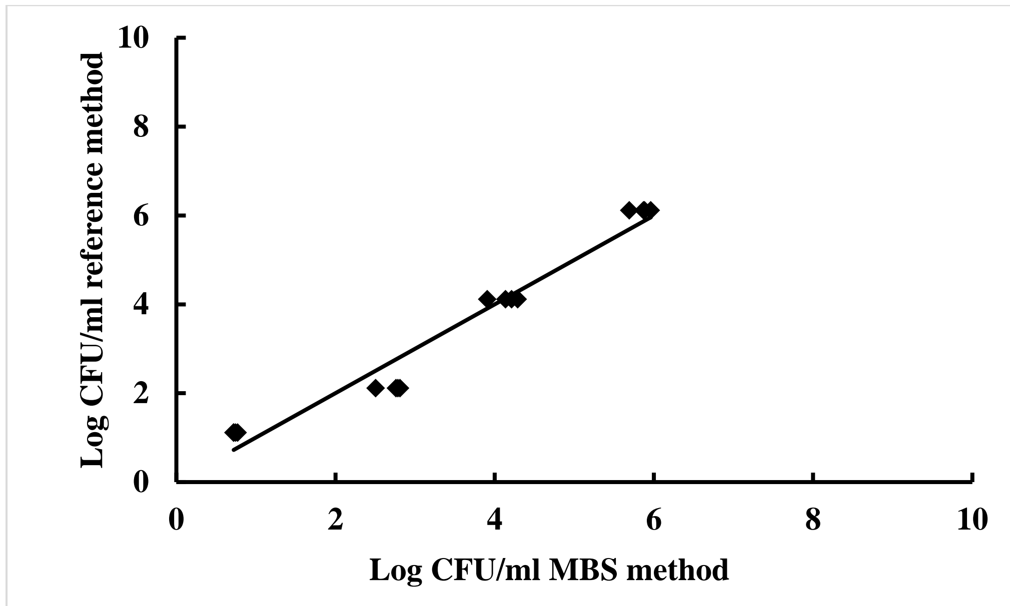


Figure 20A. Accuracy: linear regression line (slope: 1.00; R2 = 0.96) between the bacterial concentration values (expressed in terms of log CFU/ml) obtained by the plate count reference method and the MBS method for the research of *L. monocytogenes*. Each point corresponds to the average of the results obtained by analyzing each sample in triplicate.

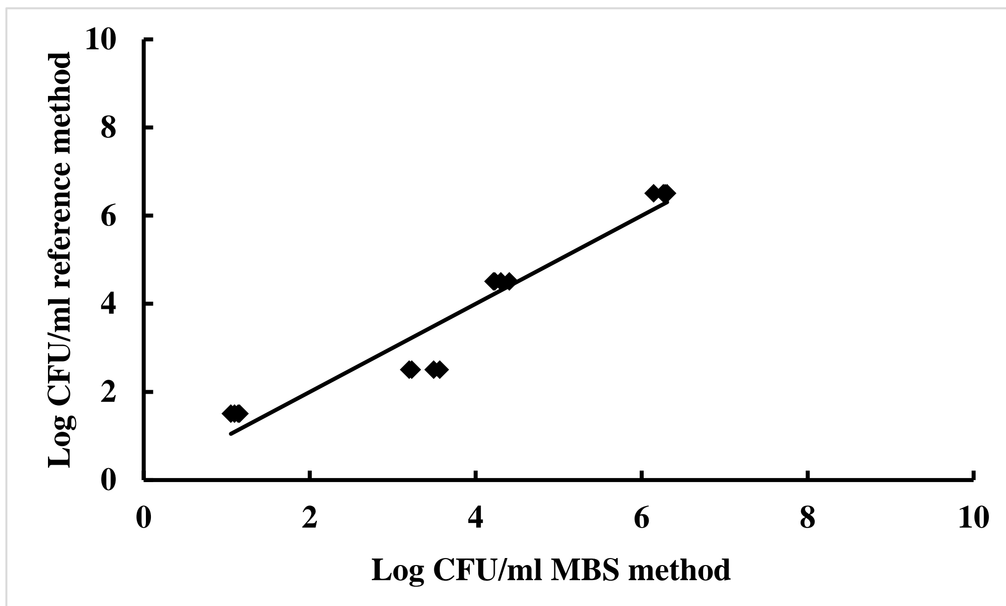


Figure 20B. Accuracy: linear regression line (slope: 1.00, R2 = 0.93) between the bacterial concentration values (expressed in terms of log CFU/ml) obtained by the reference plate reference method and the MBS method for the search for *L innocua*. Each point corresponds to the average of the results obtained by analyzing each sample in triplicate.

4.3.2. Development of experimental enrichment broth

According to official regulations, there are two standards for *Listeria* spp. and *Listeria monocytogenes* detection: UNI EN ISO 11290-1:2005 and 11290-2:2005, respectively for qualitative and quantitative analyses. These regulations contemplate analysis of 1g/ml of food sample for quantitative analysis, with a maximum contamination of 10^2 CFU/g or ml of sample, and the absence of the bacterium in 25 g/ml of sample, for qualitative analysis.

Both the directives are divided in different phases: a semi-selective pre-enrichment phase (~ 24 hrs), in order to revive starving Listeria cells eventually present in the sample (the measure of the sample depends on the kind on quantitative or qualitative analyses); a second phase of selective enrichment in broth or solid medium (~ 24-48 hrs) that promotes the growth of *Listeria* spp. towards other competitor bacteria and the final phase of identification of *Listeria* species with molecular, biochemical and immunological techniques. All these steps are however long, about 4 or 5 days, and expensive to carry out.

This procedure also presents limitations on the possibility of obtaining false positive results in the case of high contamination by different kinds of bacteria metabolically correlated with *Listeria* spp. [57].

The MBS method analytical procedure involves the inoculum of 1 g or 1 ml of the sample (or homogenate) directly into sterile disposable vials containing the specific reagent for the assay to be conducted. Once inoculated, the vials can be incubated at the required temperature for analysis (30, 37 or 44 ° C) in the MBS Multireader (MR). The presence of the microorganisms of interest, in a time inversely proportional to the present bacterial load, induces a color change that can be detected automatically by the MR. The MBS Reagent for *Listeria* spp. (MBS LY) was already optimized in order to reduce false positive results, leading to an increasing of selectivity towards bacterial species biochemically related to *Listeria* spp., with a limit of selectivity for microorganisms belonging to other genre equal to ~ 10^6 CFU / ml, although the maximum time of analysis is still quite long, about 51 hours, because of the fact that *Listeria monocytogenes* is a slow growing bacterium and concentrations of about 10 CFU/ml cause color change of the vials in that time frame.

There is no pre-enrichment step in the original experimental protocol.

The aim of this the research was the development of a selective enrichment broth, to be used preliminarily in MBS analysis, which could meet the characteristics of sensitivity, selectivity and rapidity of this method. The ultimate aim of this research is to optimize the MBS method for *Listeria* spp. detection in food samples of different origin. In the starting stages of the work, the possibility of using directly naturally contaminated food samples was evaluated. However, the difficulty in obtaining such kind of samples lead to the test of the enrichment broth in vitro with artificially contaminated water samples.

Evaluation of the selective enrichment capacity of the different formulations of the experimental broth. In the first phase of the research, based on information found in literature [75], an enrichment broth was formulated with the aim of selectively favoring the growth of *Listeria* spp. and contemporarily preventing competitor's growth. Various tests have been conducted in order to seek nutritional and at the same time selective agents to ensure the best condition for the detection and growth of *Listeria* spp. and slow down or inhibit the growth of metabolically related bacteria.

Artificially contaminated samples were prepared inoculating in 25 ml of sterile water different concentrations of ATCC strains of *Listeria* spp. (*Listeria monocytogenes* ATCC 7644, *Listeria innocua* ATCC 33090); *Staphilococcus aureus* (ATCC 29212); *Enterococcus faecalis* (ATCC 29212).

First formulation of the experimental enrichment broth Taking into account data reported in the literature on the different types of enrichment media for *Salmonella* spp., common components have been highlighted and accordingly a first formulation of the experimental broth was established (Table 35). As the broth could be patented, it is not possible to show the full composition, so the names of the compounds in the MBS broth are indicated by acronyms.

Table 35. First composition of the experimental enrichment broth**.**

Ingredients	g/l
Carbon and nitrogen source B	10
Carbon and nitrogen source T	10
Vitamine source Y	10
Salt N	10
Buffer system K	9
Buffer system H	1
Selective agent L	5
Selective agent A	0,025
Selective agent Cp	0,001

The sterility of the experimental broth was assured by filtering and checked for each experiment.

Water samples were contaminated with single strains through serial dilutions obtained from overnight culture to verify the replication capacity of each strain analyzed in the experimental broth without taking into account cross-contamination situations. A preliminary presumption of contamination strains was performed to determine the correct inoculum to be used to prepare the samples. The artificially contaminated samples were prepared to obtain a concentration of about 10^2 CFU/ml for *Listeria* spp. and about 10^6 CFU / ml for *S. aureus* and *E. faecalis*, in order to simulate the conditions most likely to be observed in natural samples, The samples were inoculated into 225 ml of experimental broth and incubated at 37 ° C for 48 hours.

The starting concentrations of the inoculated strains in the experimental broth were verified using the traditional counting method (PALCAM Agar for *Listeria* spp., Baird Parker Agar for *S. aureus* and Bile Esculine Azide Agar for *E. faecalis*). The enrichment capacity of the experimental broth was evaluated after 24 and 48 hours. The results obtained are shown in the table (Table 36) and refer to an average of three independent experiments in which each sample was analyzed in triplicate.

Table 36. Concentration of contaminated samples before and after enrichment of the first formulation of the enrichment broth. The results refer to an average of three independent experiments in which each sample was analyzed in triplicate.

Contaminants strains	CFU/ml selective medium		
	Before enrichment	**After 24 hours**	**After 48 hours**
L. monocytogenes	2	3,5 $E10^{2}$	2 $E10^{1}$
L. innocua	1	4 $E10^{3}$	2,9 $E10^{2}$
S. aureus	3,4 $E10^{5}$	2,8 $E10^{8}$	6,4 $E10^{7}$
E. faecalis	2,8 $E10^{5}$	3,9 $E10^{6}$	2,6 $E10^{6}$

The results show that this first formulation is not suitable for the purpose because, although there is an enrichment of about 2 orders of magnitude of *Listeria* spp. concentrations after 24 hours, the enrichment broth better favours *S. aureus* and *E. faecalis* growth. For this reason, the formulation was modified in order to improve its enrichment capability towards Listeria species, and contemporarily inhibiting competitor's growth.

Second formulation of the experimental enrichment broth. Previous results led to the hypothesis that high nutrients concentrations and low concentrations of selective agent L could promote growth of *Listeria* spp. competitors; thus several analyses were carried out in order to find another nutrient source to add to the formulation, and enhance the selective agent concentration (data not shown). The results were quite encouraging for the enrichment capability towards *Listeria* species, which enhanced of 1 order of magnitude each, and there was a significant inhibition of *E. faecalis* growth. *S. aureus* was not influenced by these modifications, so a third formulation was proposed.

Third formulation of the experimental enrichment broth. The third formulation of the experimental broth concerned about the reduction of salt concentration and an enhancement of selective agent Cp concentration, in order to inhibit *S. aureus* growth. The results are shown in Table 37.

Table 37. Concentration of contaminated samples before and after enrichment of the third formulation of the enrichment broth. The results refer to an average of three independent experiments in which each sample was analyzed in triplicate.

		MBS broth conditions CFU/ml selective media	
Contaminant strains	**Before enrichment**	**After 24 hours**	**After 48 hours**
L. monocytogenes	3	3,6 E10^4	6,8 E10^4
L. innocua	2	1,8 E10^6	5,2 E10^6
S. aureus	1,2 E10^6	2,4 E10^4	1 E10^4
E. faecalis	2,7 E10^6	3 E10^4	4,7 E10^3

Results demonstrate that this formulation is encouraging in terms of selective growth of *L. monocytogenes* and *L. innocua*, and show a significant inhibition of competitors growth, with a decrease of about 2 order of magnitude per strain. Moreover, an important achievement was reached: after the enrichment step, *L. monocytogenes* growth in plates is detectable in a time frame even lower than 24 hours, despite the previous 48 hours before enrichment.

Evaluation of the sensitivity, selectivity and efficiency of the experimental broth and Fraser broth. The promising results achieved led to the evaluation of selectivity and sensitivity compared to the ISO Fraser broth.

Samples were prepared to obtain a concentration of about 10^2 CFU/ml for *Listeria* spp. and about 10^6 CFU/ml for *S. aureus* and *E. faecalis*, *Lactic Acid Barteria*, *B. cereus*, *B. subtilis, Salmonella enterica subsp. enterica serovar Enteritidis* and *E. coli,* in order to simulate the conditions most likely to be observed in natural samples. The samples were inoculated into 225 ml of experimental broth in the Fraser broth and incubated at 37° C for 48 hours. The enrichment capability was verified using the traditional counting method. The results obtained are shown in table 38.

Table 38. Concentration of contaminated samples before and after enrichment step in the MBS experimental enrichment broth and the Fraser broth. The results refer to an average of three independent experiments in which each sample was analyzed in triplicate.

Contaminant strains		MBS enrichment broth CFU/ml selective media		Fraser Broth CFU/ml selective media	
	Before enrichment	**After 24 h**	**After 48 h**	**After 24 h**	**After 48 h**
L. monocytogenes	4	2,8 E10^3	5,9 E10^4	3,2 E10^2	4,2 E10^3
L. innocua	6	4,1 E10^5	6,2 E10^6	4,3 E10^3	9,2 E10^4
S. aureus	2,6 E10^6	5,7 E10^4	4,9 E10^3	3,6 E10^6	8,3 E10^7
E. faecalis	3,6 E10^6	1,2 E10^4	1,5 E10^3	2,6 E10^5	4,1 E10^5
E. coli	6 E10^6	2 E10^1	3E10^2	1,1 E10^3	2,3 E10^2
S. enteritidis	5,1 E10^6	0	0	4,2 E10^2	5,1 E10^1
B. cereus	7 E10^6	1,01 E10^2	E10^1	E10^3	E10^2
B. subtilis	4,9 E10^6	1,0 E10^1	3 E10^1	2 E10^3	1,2 E10^2
L. casei	3,7 E10^6	0	0	1 E10^1	1,0 E10^1
L. lactis	4,2 E10^6	0	0	2 E10^1	2 E10^1
L. rhamnosus	6,1 E10^6	0	0	1 E10^1	2 E10^1

The analyses highlight a very high sensitivity the MBS towards Listeria species, confirming an enrichment of about 3 order of magnitude for L. monocytogenes and about 5 order of magnitude for L. innocua. The experimental broth also confirms its inhibiting action towards competitor bacterial strains, such as gram positive and gram negative bacteria. Its inhibiting action is particularly evident with Lactic Acid Bacteria. The Fraser broth shows lower abilities of enrichment and inhibition compared to the MBS experimental enrichment broth.

In light of the results obtained above, we decided to evaluate the enrichment and selectivity of the experimental broth, simulating cross-contamination conditions between *Listeria* spp. strains. and *S.aureus*, considered as the main competitor bacterial strain in naturally contaminated samples. The artificially contaminated samples were prepared to obtain a cross-contamination condition of about 10^2 CFU / ml for *Listeria* spp. and 10^6 CFU/ml for *S. aureus*; 5 ml of the so prepared samples were separately inoculated in 45 ml of experimental broth and 5 ml in the ISO Fraser broth. Both broths were incubated at 37° C for 48 hours. Results were verified using the traditional counting method. Results were significantly encouraging for the MBS enrichment broth, with a complete inhibition of *S. aureus* and a

confirm of 3 order of magnitude enrichment for *L. monocytogenes* and 4 order of magnitude for *L. innocua*. The enrichment in the Fraser broth highlighted an enrichment for both strains, lower than the MBS broth (data not shown).

These results encouraged the research to apply this new analytical protocol to the MBS method.

4.3.3. Application of the experimental enrichment broth to the Micro Biological Survey method

The results obtained in the second phase of the research demonstrated an enrichment ability of the experimental broth really comparable to the ISO Fraser broth in single contamination conditions, while its selectivity in cross-contaminated conditions was, significantly greater than the Fraser broth. In the final phase of the research the experimental broth was applied to MBS analysis, in order to demonstrate that the results obtained could be better compared to the Fraser broth.

The new experimental protocol used in this phase of the experiment involves a pre-enrichment of the samples for 24 hours at 37° C, followed by inoculation of 1 ml of enrichment broth in LY vials, then incubated for 72 hours at 37° C in the MBS Multireader (MR), which can automatically record the vial's color changing and return the results in terms of hours.

The inoculum of the sample after the selective enrichment phase in the modified MBS LY vials was not so encouraging in results, prompting to further researches, in order to modify again the MBS LY reagent that could be used to perform qualitative analyses according to the method.

In conclusion, the optimization of the reagent already present led to a decrease in false positive results ratio, and a consistent analysis time reduction, and the selective broth formulated allows the analysis on 25 g or ml of sample with a significant selective enrichment of *Listeria monocytogenes*, generally very difficult to detect following reference standards' procedures,

which is faster than the reference broth. It is however necessary to continue the research to modify again the MBS LY reagent that could be suitable for the analysis including an enrichment step, in accordance to the principles of the method in order to make it also in line with qualitative standards.

5. CONCLUDING REMARKS

Alternative rapid microbiological methods are based on technologies which can be growth-based, viability-based, or surrogate-based cellular markers for a microorganism (e.g. nucleic acid-based, fatty acid-based) and include any microbiological technique or process that increases the speed or efficiency of isolating, culturing, or identifying microorganisms when compared with conventional methods. They have been developed in order to provide more rapid, sensitive, accurate, precise, and reproducible test results when compared with conventional, growth-based methods. Moreover alternative methods should aim to simplify analytical procedures and interpretation of results and help laboratories reduce worker subjectivity, increase labor efficiency and reduce errors. They should require minimal training and equipment, be stand-alone and ensure the most error prone processes. Unfortunately not all alternative methods share these features. Most rapid methods are technically more complex than culture methods and require skilled personnel and equipped laboratories. They require high upfront capital investments, and the cost per test is high compared with that of culture tests.

The MBS method is an alternative colorimetric method, which has proven to be reliable and providing results for food and water analyses, comparable with those obtained with the reference methods required by national and international regulations. Its main features are: easy protocol and interpretation of results, rapidity, user-friendliness and suitability for *in situ* analyses.

The aim of my PhD work was to find new and different applications of the Micro Biological Survey method, in order to make it always more competitive towards other alternative methods and always more in accordance with the reference standards. It has already been demonstrated its suitability in dug wells water monitoring in developing countries [84], allowing the analyses also on 100 ml of water as international regulations require, but it is well known the problem of waterborne diseases ratio and how difficult could be in these countries water disinfection using traditional methods. The tropical plant *Moringa oleifera* is nowadays used in many fields, including water disinfection, due to its purifying activity on bacteria. For this reason I studied the purificating activity of *Moringa oleifera* and prepared an easy protocol of use in order to apply it on field, and at least in developing countries.

Although the obtained results show the effectiveness of this plant in the breakdown of bacterial load even in artificially contaminated and in naturally contaminated samples, and the protocol prepared is very easy to apply in developing countries, its use as an alternative method in drinking water treatment could not be desirable, because this plant alters the orgaloleptic characteristics of colorless and odorless of water, making it unpleasant for human consumption.

Concerning the new applications in food safety control, it has already been demonstrated the effectiveness of the MBS method [82] as a control tool in quantitative analyses, but specific regulations for pathogens detection, in particular for *Salmonella* spp. and *Listeria monocytogenes* detection, require qualitative analyses and the complete absence of these pathogens in 25 grams of sample. The reference standards methods for their detection require different steps, including one or two enrichment phases, selective or not selective.

For this reason, my research focused on the study of a new analytical procedure that, following the principle of the MBS method, allows the analysis of 25 g or ml of samples and assure the same reliability, sensitivity and selectivity of the reference standards', reducing however the time of analysis. I studied the development of two enrichment broths, one for *Salmonella* spp detection, and one for *Listeria* spp. detection, particularly referring *on Listeria monocytogenes*.

The results obtained for the *Salmonella* spp. MBS enrichment broth demonstrated that the broth promotes a selective growth of *Salmonella* spp. towards other bacterial species biochemically related to this genre. The effectiveness of this broth was also demonstrated in artificially cross contaminated samples, in order to repeat naturally contaminated food samples. A consistent reduction of maximum time of analysis was recorded in the MBS procedure using the MBS enrichment broth, from 72 hours to 48 hours approximatively. This maximum time frame accounts the enrichment phase and the analysis with the reaction vials.

The results obtained for *Listeria* MBS enrichment broth demonstrated that the formulated broth promotes a selective growth of *Listeria* spp. species and in particular a very fast growth of *Listeria monocytogenes*, towards other competitor bacterial species. At the same time I optimized the MBS reagent for quantitative detection of *Listeria* spp. (MBS LY) because of the presence of false positive results. Results obtained from these tests demonstrated that the MBS LY was optimized for quantitative analysis with a decrease of false positive results.

A reduction of maximum time of analysis was recorded in the MBS quantitative analyses, from 72 hours to 48 hours approximatively. Although these encouraging results, the inoculum of the sample after the selective enrichment phase in the modified MBS LY vials was not so encouraging in results, prompting to further researches, in order to modify again the MBS LY reagent that could be used to perform qualitative analyses according to the method.

In conclusion, the MBS method has confirmed to be an effective, reliable and precise alternative to traditional microbiological testing, both in laboratory settings and on field trials. It has been possible to add simple analytical protocols to this method that could make it more suitable for its use in developing countries and more in accordance with reference standard methods for pathogen's detection. Despite this encouraging results, it is however necessary to continue the research on *Listeria* spp. detection, and modify again the MBS LY reagent that could be suitable for the analysis including an enrichment step, in accordance to the principles of the method in order to make it also in line with qualitative standards.

6. REFERENCES

[1] Yeni F, Acar S, Polat ÖG, Soyer Y, Alpas H. Rapid and standardized methods for detection of foodborne pathogens and mycotoxins on fresh produce. Food Control. 2014;40:359-367. http://doi.org/10.1016/j.foodcont.2013.12.020.

[2] Forsythe S. The microbiology of safe food. 2nd ed. UK: Blackwell Publishing Ltd.; 2010.

[3] Zhao X, Lin C-W, Wang J, Oh HD. Advances in rapid detection methods for foodborne pathogens. J Microbiol Biotechnol. 2014;24(3):297-312.

[4] Wang Y, Salazar JK. Culture-independent rapid detection methods for bacterial pathogens and toxins in food matrices. Compr Rev Food Sci F. 2016;15:183–205. 10.1111/1541-4337.12175.

[5] Rohde A, Hammerl JA, Appel B, Dieckmann R, Al Dahouk S. FISHing for bacteria in food – A promising tool for the reliable detection of pathogenic bacteria? Food Microbiol. 2015;46:395–407. 10.1016/j.fm.2014.09.002.

[6] Feldsine P, Abeyta C, Andrews WH, AOAC International Methods Committee. Guidelines for Validation of Qualitative and Quantitative Food Microbiological Official Methods of Analysis. J AOAC Int. 2002;85(5):1187-1200.

[7] Food and Drug Administration. Analytical Procedures and Methods Validation for Drugs and Biologics; Guidance for Industry. Federal Register. 2015 July 27; Vol 80,N 143 (document number: 2015-18270).

[8] International Organization for Standardization (ISO). ISO 16140 Microbiology of food and animal feeding stuffs - Protocol for the validation of alternative methods. Geneva, Switzerland: International Organization for Standardization; 2003.

[9] Prest E.I., Hammes F., van Loosdrecht M.C.M. and Vrouwenvelder J.S., 2016, Biological Stability of Drinking Water: Controlling Factors, Methods, and Challenges. *Front. Microbiol.* 7:45. doi: 10.3389/fmicb.2016.00045

[10] Hunter P.R., 2003, Climate change and waterborne and vector-borne disease. *J Appl Microbiol;* 94: 37S-47S (A).

[11] Noosorn N., Niamkamnerd P., 2009. Public participation to minimize waterborne diseases in Yom riverside communities. *J Public Health and Epidemiol* 1 (1), 037-040.

[12] Dragone M., Gioffrè A., Marramao A., 2007, The pathogens responsible for waterborne infections: the biological risk for agricultural laborer. *G Ital Med Lav Ergon;* 29: 740-742.

[13] World Health Organization (WHO) 2014a. Preventing diarrhoea through better water, sanitation and hygiene: Exposures and impacts in low-and middle-income countries. Geneva: World Health Organization; (http://www.who.int/water_sanitation_health/ gbd_poor_water/en/, accessed 16 February 2015).

[14] Yang K., LeJeune J., Alsdorf D., Lu B., Shum C., Liang S., 2012, Global distribution of outbreaks of water-associated infectious diseases. *PLoS Negl Trop Dis;* Vol 6(2): e1483

[15] Barbuti S., Bellelli E., Fara G.M., Giammanco G. (2002), *Igiene*. II ed. Monduzzi Editore. Bologna.

[16] Kotloff K.L., Nataro J.P., Blackwelder W.C., Nasrin D., Farag T.H., Panchalingam S. et al., 2013. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. Lancet. 382: 209-222.

[17] Luby S.P., Halder A.K., Huda T.M., Unicomb L., Islam M.S., Arnold B.F., Johnston R.P., 2015. Microbiological contamination of drinking water associated with subsequent child diarrhea.Am J Trop Med Hyg. 93(5), 904-911.

[18] Maheux A.F., Boudreau D.K., Bisson M.A., Dion-Dupont V., Bouchard S., Nkuranga M., et al., 2014, Molecular method for detection of total coliforms in drinking water samples. *Applied Environmental Microbiology*; 80(14), 4074–4084

[19] Review of Coliforms As Microbial Indicators of Drinking Water Quality. *Australian Drinking Water Guidelines,* 2004

[20] World Health Organization (WHO) Guidelines for Drinking-water Quality, Incorporating 1st and 2nd Addenda, Volume 1, Recommendations. 3rd ed. WHO; Geneva, Switzerland: 2008 87

[21] Figueras M.J, Borrego J.J., 2010, New Perspectives in Monitoring Drinking Water Microbial Quality. Int J Environ Res Public Health.;7:4179-4202. 10.3390/ijerph7124179.

[22] Cabral J.P.S., 2010, Water Microbiology. Bacterial Pathogens and Water. Int J Environ Res Public Health.;7(10):3657-3703. 10.3390/ijerph7103657.

[23] Rice R.W., Baird R.B., Eaton A.D., Clesceri L.S., Standard Methods for the Examination of Water and Wastewater 22nd Edition. American Public Health Association, American Water Works Association, Water Environment Federation

[24] Gensberger E.T., Gössl E.M., Antonielli L., Sessitsch A., and Kostić T., 2015, Effect of different heterotrophic plate count methods on the estimation of the composition of the culturable microbial community. *Peer J,*; 3: e862

[25] Reasoner D.J., 2004, Heterotrophic plate count methodology in the United States. *International Journal of Food Microbiology,*; 92(3): 307-15.

[26] Sanders E. R.., 2012, Aseptic Laboratory Techniques: Plating Methods. *Journal of Visualized Experiments,*; 63, e3064

[27] APHA-AWWA-WEF. 1999 Standard Methods for the Examination of Water and Wastewater 28th ed. American Public Health Association, Washington DC

[28] Feng P., Weagant S., Grant M., 2002, Enumeration of *Escherichia coli* and the coliform bacteria. Bacteriological analytical manual. 8th ed. FDA/Center for Food Safety & Applied Nutrition

[29] Medema G.J., Payment P. Dufour A., Robertson W., Waite M., Hunter P., Kirby R., Anderson Y., 2003, Safe drinking water: an ongoing challenge. In Assessing Microbial Safety of Drinking Water Improving Approaches and Method; WHO & OECD, IWA Publishing: London, UK,; 11–45.

[30] Wuryastuti H., Wasito R., Chalimah S., Andayani S., Indraswati Y., Lestariyadi L., Prapti K., Amien,. 2002, Analysis of coliform bacteria in the well water and its possible biopathologic effects. *Jurnal sain veteriner.*; 18

[31] Odonkor S.T., Ampofo J.K., 2013, *Escherichia coli* as an indicator of bacteriological quality of water: an overview. *Microbiology Research,*; 4:e2

[32] Rompré A., Servais P., Baudart J., de-Roubin M-R., Laurent P.A., 2002, Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. J Microbiol Methods.;49:31–54.

[33] Sutton S., 2010, The Most Probable Number Method and Its Uses in Enumeration, Qualification, and Validation. *Journal of Validation Technology,*. 88

[34] Gronewold A.D., Wolpert R.L., 2008, Modeling the relationship between most probable number (MPN) and colony-forming unit (CFU) estimates of fecal coliform concentration. *Water Research,*; 42(13), 3327–3334

[35] Wohlsen T., Bates J., Vesey G., Robinson W.A., Katouli M., 2006, Evaluation of the methods for enumerating coliform bacteria from water samples using precise reference standards. *Letters of Applied Microbiology,*; 42(4):350-6

[36] Mesquita S. and Noble R.T., 2013, Recent developments in monitoring of microbiological indicators of water quality across a range of water types, water resources planning, development and management. *INTECH*,

[37] Guidance on Waterborne Bacterial Pathogens Water, Air and Climate Change Bureau, Healthy Environments and Consumer Safety Branch. Health Canada, Ottawa, Ontario 2012

[38] Colwell R.R., Grimes D.J., 2000, Nonculturable microorganisms in the environment. ASM Press, Washington, DC, USA

[39] Drinking Water and Health, Volume 2 Safe Drinking Water Committee, Board on Toxicology and Environmental Health Hazards, Assembly of Life Sciences. ISBN: 0-309-55406-3

[40] Bache, D., Gregory, R., 2007. Flocs in Water Treatment. IWA Publishing, London.

[41] Weinberg H.S., 2009, Modern approaches to the analysis of disinfection by-products in drinking water. Philos Transact A Math Phys Eng Sci; 367: 4097-4118.

[42] Broin M., C. Santaella, S. Cuine, K. Kokou, G. Peltier, T. Joel, 2002, Flocculant activity of a recombinant protein from *Moringa oleifera* lam. seed, *Appl. Microbiol. Biol*. 60 114–119.

[43] Silvestro L, Weiser JN, Axelsen PH., 2000. Antibacterial and antimembrane activities of cecropin A in Escherichia coli. *Antimicrob Agents Chemother* 44: 602–607.

[44] Eilert U, Wolters B, Nadrtedt A., 1981. The antibiotic principle of seeds of *Moringa* oleifera and *Moringa* stenopetala. *Planta Med* 42: 55–61.

[45] Ghebremichael K.A., K.R. Gunaratna, H. Henriksson, H. Brumer, G. Dalhammar, 2005, A simple purification and activity assay of the coagulant protein from *Moringa oleifera* seed, *Water Res*. 39 2338–2344.

[46] FAO/WHO, 2006a. Risk assessments, pathogen-commodity combinations: *Salmonella* in eggs and broiler chickens, *Listeria monocytogenes* in ready-to-eat foods, *Vibrio* spp. in seafoods, *Campylobacter* spp. in broiler chickens, *Enterobacter sakazakii* and other microorganisms in powdered infant formulas.

[47] FAO/WHO, 2006b. The use of microbiological risk assessment outputs to develop practical risk management strategies: metrics to improve food safety. A Report of a Joint FAO/WHO Expert Consultation.

[48] Australian Government, 2006. The annual cost of foodborne illness in Australia. Australian Government. Department of Health and Ageing.

[49] Crossley S. and Motarjemi Y., 2011. Food Safety Management Tools, 2nd Edition, International Life Science Institute (ILSI)

[50] ICMSF - International Commission on Microbiological Specifications for Foods, 1988, Microorganisms in Foods 4: Application of the hazard analysis critical control point (HACCP) system to ensure microbiological quality and safety. Blackwell Scientific Publications, London.

[51] Andreoletti O., Budka H., Buncic S., Colin P., Collins J.D., De Koeijer A., Griffin J., Havelaar A., Hope L., Klein G., Kruse H., Magnino S., Martinez López A., McLauchlin J.,

Nguyen-Thé C., Noeckler K., Noerrung B., Prieto Maradona M., Roberts T., Vågsholm I., Vanopdenbosch E., 2007. The EFSA Journal,. 462, 1-29 Microbiological criteria and targets based on risk analysis.

[52] ICMSF - International Commission on Microbiological Specifications for Foods, 1986. Microorganisms in Foods, 2. Sampling for microbiological analysis. Principles and specific applications, 2nd Edition, Blackwell Science, Oxford.

[53] CAC - Codex Alimentarius Commission, 1997. Principles for the establishment and application of microbiological criteria for foods. CAC/GL 21.

[54] Threlfall E. J., 2007. *Salmonella*; Topley & Wilson's Microbiology and microbial infection 10th edition.

[55] REGULATION (EC) No. No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs Official Journal of the European Union. (L 338/1)

[56] UNI EN ISO 6579:2008, Microbiology Of Food And Animal Feeding Stuffs - Horizontal Method For The Detection Of *Salmonella* Spp.

[57] Carrasco E., Morales-Rueda A., García-Gimeno R.M., 2012. Cross-contamination and recontamination by *Salmonella* in foods: A review. Food Research International. 45: 545–556.

[58] Lee K.M., Runyon M., Herrman T.J., Phillips R., Hsieh J., 2015. Review of *Salmonella* detection and identification methods: Aspects of rapid emergency response and food safety. Food Control. 47: 264–276.

[59] McLauchlin J., *Listeria.* 2007. Topley & Wilson's Microbiolgy and microbial infection 10th edition.

[60] Liu D., 2008. Handbook of *Listeria Monocytogenes*. *CRC Press Taylor & Francis Group*.

[61] ISO 11290-1:2017. Microbiology of the food chain -- Horizontal method for the detection and enumeration of Listeria monocytogenes and of Listeria spp. -- Part 1: Detection method

[62] ISO 11290-2:2017. Microbiology of the food chain -- Horizontal method for the detection and enumeration of Listeria monocytogenes and of Listeria spp. -- Part 2: Enumeration method

[63] Bottini G, Losito F, De Ascentis A, Priolisi FR, Mari A, Antonini G., 2011. Validation of the Micro Biological Survey method for Total Viable Count and *E. coli* in food samples. Am J Food Technol.;6(11):951-962. 10.3923/ajft.2012.340.351.

[64] Losito F, Bottini G, De Ascentis A, Priolisi FR, Mari A, Tarsitani G, Antonini G., 2012. Qualitative and quantitative validation of the Micro Biological Survey method for *Listeria* spp., *Salmonella* spp., Enterobacteriaceae and *Staphylococcus aureus* in food samples. Am J Food Technol.;7(6):340-351. 10.3923/ajft.2012.340.351.

[65] Antonini G., Malatesta F. and Sarti P., 2007. Twenty-five Years of Cytochrome Oxidase Research in Rome with Maurizio Brunori. *IUBMB Life,*; 59(8-9),570-577

[66] Berlutti F., Rosso F., Bosso P., et al., 2003. Quantitative evaluation of bacteria adherent to polyelectrolyte HEMA-based hydrogels. *Journal of Biomedical Materials Research, Part A*,; 67(1),18–2

[67] FAO, Food and Nutrition Paper, Assuring Food Safety And Quality: Guidelines For Strengthening National Food Control Systems. ISSN 0254-4725

[68] Wright J.A., Yang H., Walker K., Pedley S., Elliott J., Gundry S.W., 2012. The H2S test versus standard indicator bacteria tests for faecal contamination of water: systematic review and meta-analysis. *Tropical Medicine International Health*,; 17(1):94-105

[69] MacConkey. 1902. Zentralbl. Bakteriol. 29:740

[70] World Health Organization www.who.int

[71] Baird-Parker A.C., 1962. An improved diagnostic and selective medium for isolating coagulase positive Staphylococci. Journal of Applied MIcrobiology, 25: 12-19.

[72] Murray E.G.D., Webb R.A. and Swann M.B.R., 1926. A disease of rabbits characterized by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus Bacterium monocytogenes. The Journal of Pathology and Bacteriology; 29:407–39.

[73] ISO 6887-1:2017. Microbiology of the food chain -- Preparation of test samples, initial suspension and decimal dilutions for microbiological examination -- Part 1: General rules for the preparation of the initial suspension and decimal dilutions

[74] Food and Drug Administration (FDA) www.fda.gov

[75] Bell R.L., Jarvis K.G., Ottesen A.R., McFarland M.A., Brown E.W., 2016. Recent and emerging innovations in *Salmonella* detection: a food and environmental perspective. Microb Biotechnol. 9(3): 279-92.

[76] Madigan M.T., Martinko J.M., Stahl A.A. and Clarck D.P., 2012. Brock Biologia dei microrganismi. microbiologia generale 1. Pearson

[77] Adegoke A.A. and Okoh AI., 2014. Species diversity and antibiotic resistance properties of Staphylococcus of farm animal origin in Nkonkobe Municipality, South Africa. Folia Microbiologica; 59(2):133-40.

[78] Osaili T.M., Al-Nabulsi A.A., Taha M.H., Al-Holy M.A., Alaboudi A.R., Al-Rousan W.M. and Shaker R.R., 2012. Occurrence and antimicrobial susceptibility of Listeria monocytogenes isolated from brined white cheese in Jordan. Journal of Food Science; 77(9): M528-32.

[79] Rakic-Martinez M. ,Drevets D. A., Dutta V., Katic V., and Kathariou S., 2011. Listeria monocytogenes Strains Selected on Ciprofloxacin or the Disinfectant Benzalkonium Chloride Exhibit Reduced Susceptibility to Ciprofloxacin, Gentamicin, Benzalkonium Chloride, and Other Toxic Compounds. Applied and Environmental Microbiology; 77(24): 8714–8721.

[80] Alzubaidy Z.M., Kakey S. I. and Ali J. F., 2014. Antibacterial Effects of Lactic Acid Bacteria Cell Free Extract on the Growth of Local Isolates of Listeria moncytogenes from some Food Sources. Raf Journal of Cell Science; 25(1): 19-30.

[81 Losito F.*, Arienzo A. *, Bottini G., Priolisi F.R., Mari A. and Antonini G., 2014. Microbiological safety and quality of Mozzarella cheese assessed by the microbiological survey method. *Journal of Dairy Science*.;97(1):46-55. *The authors with this symbol participated equally in the present investigation.

[82] Arienzo A.*, Losito F.*, Stalio O. and Antonini G., 2016. Comparison of Uncertainty Between Traditional and Alternative Methods for Food Microbiological Analysis. *American Journal of Food Technology*. *Published online ahead-of-print.*

[83] Arienzo A., Sobze M.S., Wadoum R.E.G., Losito F., Colizzi V. and Antonini G., 2015. Field Application of the Micro Biological Survey Method for a Simple and Effective Assessment of the Microbiological Quality of Water Sources in Developing Countries. *International Journal of Environmental Research and Public Health*; 12, 10314-10328.

[84] Kirschner AKT, Kavka GG, Velimirov B, Mach RL, Sommer R, Farnleitner AH., 2009. Microbiological water quality along the Danube River: Integrating data from two whole- river surveys and a transnational monitoring network. Water Res.;43(15):3673–3684.

[85] Gensberger ET, Gössl EM, Antonielli L, Sessitsch A, Kostić T., 2015. Effect of different heterotrophic plate count methods on the estimation of the composition of the culturable microbial community. Peer J.;3:e862. 10.7717/peerj.862.

[86] Barrell R.A., Hunter P.R., Nichols G., 2000. Microbiological standards for water and their relationship to health risk. *Communicable Disease and Public Health,*; 3(1): 8-13.

[87] European Drinking water Directive. Water Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption Official Journal L 330 , 05/12/1998 P. 0032 – 0054

[88] Maurer JJ., 2011. Rapid detection and limitations of molecular techniques. Annu Rev Food Sci Technol.;2:259–279. 10.1146/annurev.food.080708.100730.

[89] Rasooly A, Herold KE. Biosensors for the analysis of food- and waterborne pathogens and their toxins. J AOAC Int. 2006;89:873-883.

[90] Amann RI, Ludwig W, Schleifer KH., 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev.;59:143–169.

[91] Straub TM, Pepper IL, Gerba CP., 1995. Removal of PCR inhibiting substances in sewage sludge amended soil. Water Sci Technol.;31:311–315. 10.1016/0273- 1223(95)00286-V.

[92] Glynn B., Lahiff S., Wernecke M., Barry T., Smith T.J., Maher M., 2006.Current and emerging molecular diagnostic technologies applicable to bacterial food safety. Int J Dairy Technol.;59:126–139.

[93] Law J.W-F., Ab Mutalib N-S., Chan K-G., Lee L-H., 2015. Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. Front Microbiol.;5:2-19. https://doi.org/10.3389/fmicb.2014.00770.

[94] Shan S, Lai W, Xiong Y, Wei H, Xu H., 2015. Novel strategies to enhance lateral flow immunoassay sensitivity for detecting foodborne pathogens. J Agric Food Chem.;63:745–53. 10.1021/jf5046415.

[95] Faude UC, Hofle MG., 1997. Development and application of monoclonal antibodies for *in situ* detection of indigenous bacterial strains in aquatic ecosystems. Appl Environ Microbiol.;63:4534–4542.

[96] Velasco-Garcia MN, Mottram T., 2003. Biosensor technology addressing agricultural problems. Biosyst Eng.;84(1):1-2. 10.1016/S1537-5110(02)00236-2.

[97] Briancesco R., 2005, Indicatori microbiologici e valutazione della qualità delle acque superficiali. *Ann Ist Super Sanità*; 41: 353-358.

[98] Jasson V, Jacxsens L, Luning P, Rajkovic A, Uyttendaele M., 2010. Alternative microbial methods: an overview and selection criteria. Food Microbiol.;27(6):710-730. 10.1016/j.fm.2010.04.008.

ADDENDUM – PUBLICATIONS DURING THE PHD COURSE

[1]*Stalio O., *Arienzo A., Losito F., Murgia L., Perrone I., Biondi A., Tarsitani G., and Antonini G. **A simplified trend analysis to improve microbilogical quality of products in AgriFood Small and Medium Enterprises** *(SME) (submitted to International Journal of Food Science, 2017)*

[2] Stalio O., Arienzo A., Losito F., Murgia L., Angelini M.C., Pizzato C., Ricciardi Tenore C., Fiori F. and Antonini G., **How to Warrant Microbiological Food Safety in Food Companies: Problems and a Possible Solution**, *(submitted to Food Studies: An Interdisciplinary Journal, 2017)*

[3] Murgia L., Stalio O., Arienzo A., Ferrante V., Cellitti V., Di Somma S., Visca P. and Antonini G., **Management of Urinary Tract Infections: Problems and Possible Solutions.** ISBN: 978-953-51-5636-9 InTechOpen (*Chapter in press*)

[4] Traversetti L, Losito F, Arienzo A, Stalio O., Antonini G, Scalici M (2017) **Integrating running water monitoring tools with the Micro Biological Survey (MBS) method to improve water quality assessment**. PLoS ONE 2017. 12(9): e0185156.

[5] Losito F., Arienzo A., Somma D., Murgia L., Stalio O., Zuppi P., Rossi E., Antonini G., **Field application of the Micro Biological Survey method for the assessment of the microbiological safety of different water sources in Horn of Africa and the evaluation of the effectiveness of *Moringa oleifera* in drinking water purification.** *Journal of Public Health in Africa* 2017; 8:679 doi:10.4081/jphia.2017.679

[6] Arienzo A., Cellitti V., Ferrante V., Losito F., Stalio O., Cristofano F., Marino R., Magrini L., Santino I., Mari A., Visca P., Di Somma S. and Antonini G. **A Pilot Clinical Trial on a New Point-of-care Test for the Diagnosis and Fast Management of Urinary Tract Infections in the Emergency Department.** *International Journal of Clinical & Medical Microbiology* 2016; 1,107

[7] Arienzo A.*, Losito F.*, Stalio O. and Antonini G. (2016), **Comparison of Uncertainty Between Traditional and Alternative Methods for Food Microbiological Analysis**, *American Journal of food Technology*, ISSN 1557-4571, DOI: 10.3923/ajft.2016.29.36h