Departamento de Química

Ano 2016

ANA RITA CHAVES BOTELHO VAZ MENDES

DESENVOLVIMENTO DE *STOCKS* DE REFERÊNCIA LIOFILIZADOS PARA CONTROLO DE QUALIDADE EM ANÁLISES MICROBIOLÓGICAS

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DEVELOPMENT OF LYOPHILIZED REFERENCE STOCK CULTURES FOR QUALITY CONTROL IN MICROBIOLOGICAL ANALYSIS

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Alimentar, realizada sob orientação científica da Professora Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro e coorientação de Ana Paula Tavares Martins Costa, General Manager Iberia for Technical R&D and Quality – Controlvet SA.

Dedico este trabalho à “Natélia”, ao “*Jorge*” e ao “Rubim”. Obrigada por terem acreditado em mim!

O júri

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Agradecimentos

À Professora **Adelaide Almeida**, pelo excelente sentido de oportunidade, sem o qual este trabalho e este estágio não teriam existido.

À Dr.ª **Ana Martins**, pela proposta deste ambicioso projeto e por toda a orientação prestada.

À Eng.ª **Elisa Maia**, a pessoa que mais me ensinou e orientou. Muito obrigada pela sempre pronta ajuda. Foste essencial!

Ao Dr. **Rui Sereno**, por me ter ensinado a trabalhar com o liofilizador e por se ter mostrado sempre disponível para me ajudar.

Às **técnicas de laboratório** da Controlvet, que me ensinaram o trabalho de um laboratório de microbiologia.

À **Controlvet ALS**, por me ter recebido de braços abertos.

Aos meus pais, **Cidália** e **Jorge**. Obrigada pelo esforço e por nunca, nem por um segundo, terem duvidado de mim. Amo-vos!

Ao **Rúben**, amor da minha vida, agradeço, mais do que tudo, pela paciência. Não deve ter sido nada fácil. Amo-te!

Palavras-chave

Culturas *stock* de referência; liofilização; meio crioprotetor, gram-positiva; gram-negativa.

Resumo

No final de 2014 foi publicada a norma *ISO 11133* que obriga à realização de testes de performance em todos os lotes de meio produzidos, recorrendo ao uso de microrganismos de referência específicos, com um nível de inóculo estabelecido.

A liofilização é um processo de secagem de culturas microbianas, e que permite a sua preservação por largos períodos de tempo, sem ser necessária refrigeração.

Os efeitos de três meios crioprotetores diferentes (skim milk + 10% sacarose, nutrient broth nº2 + 20% glicerol, e sacarose 10%) na sobrevivência à liofilização de *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes* e *Salmonella enterica* serogrupo Typhimurium; o acompanhamento da concentração celular de *S. aureus* ao longo do tempo, depois de ter sido liofilizado com skim milk + 10% sacarose; e ainda o estudo da estabilidade de *E. coli* e *S. aureus*, depois de liofilizados e armazenados à temperatura ambiente, foram investigados.

E. coli, *S. aureus* e *L. monocytogenes* foram liofilizadas com skim milk + 10% sacarose; *E. coli* e *S. aureus* foram liofilizadas com nutrient broth nº2 + 20% glicerol; e *Salmonella* Typhimurium e *E. coli* foram liofilizadas com sacarose 10%. Terminadas as liofilizações dos estudos de viabilidade, cada amostra foi reidratada e inoculada em PCA. Para o acompanhamento da concentração celular de *S. aureus*, com intervalos regulares ao longo do tempo, as amostras foram reidratadas e inoculadas em PCA e BP egg yolk. Para estudar a estabilidade de *E. coli* e *S. aureus* as amostras foram reidratadas e inoculadas em PCA e no respetivo meio seletivo, com intervalos regulares ao longo do tempo.

Skim milk + 10% sacarose é o melhor meio protetor dos três usados. Das bactérias gram-positivas testadas, *L. monocytogenes* é a mais resistente, com uma redução na sua viabilidade virtualmente nula; das gram-negativas, a *Salmonella* Typhimurium foi a que obteve melhores resultados, com a redução de 1 Log. Naturalmente, as gram-positivas têm uma melhor capacidade de sobrevivência à liofilização por causa da composição da sua parede celular, rica em peptidoglicanos, e isso foi comprovado nos testes feitos. No estudo da estabilidade de *S. aureus*, a sua concentração celular manteve-se estável ao longo do tempo, acima dos 6 Log ufc/200 µl. Nos estudos em que a estabilidade de *E. coli* e *S. aureus* armazenados à temperatura ambiente foi avaliada, comprovou-se que culturas microbianas liofilizadas necessitam de refrigeração para manter a viabilidade.

Com este trabalho deu-se início à investigação necessária para a elaboração de um protocolo com o intuito de produzir culturas *stock* de referência liofilizadas, com o nível de inóculo necessário, para aplicação futura em análises microbiológicas.

Keywords

Reference stock cultures; lyophilization; cryprotective medium; gram-positive; gram-negative.

Abstract

In late 2014 it was published the standard ISO 11133 which obligates the achievement of performance tests in every batch of media produced, resorting to specific reference microorganisms, with an established inoculum level.

Lyophilization is a drying process applied to microbial cultures that allows its preservation for large periods of time, with no refrigeration needed.

The effects of three different cryoprotective media (skim milk + 10% sucrose, nutrient broth no2 + 20% glycerol, and sucrose 10%) on survival to lyophilization of *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Salmonella enterica* serovar Typhimurium; a monitoring of *S. aureus* cell concentration over time, after being lyophilized with skim milk + 10% sucrose; and also a stability study of *E. coli* and *S. aureus* after lyophilization and storage at room temperature, were investigated.

E. coli, *S. aureus* and *L. monocytogenes* were lyophilized with skim milk + 10% sucrose; *E. coli* and *S. aureus* were lyophilized with nutrient broth no2 + 20% glycerol; and *Salmonella* Typhimurium and *E. coli* were lyophilized with sucrose 10%. Finished every lyophilization of the viability studies, each sample was rehydrated and inoculated in PCA. To monitor *S. aureus* cell concentration, the samples were rehydrated and inoculated in PCA and BP egg yolk, with regular intervals throughout time. For the stability study of *E. coli* and *S. aureus*, also in regular intervals, samples were rehydrated and inoculated in PCA and in the respective selective culture media.

Skim milk + 10% sucrose is the best cryoprotective medium used. From the gram-positive bacteria tested, *L. monocytogenes* is the most resistant, registering a virtually null reduction in viability; from gram-negative, *Salmonella* Typhimurium performed best, with only 1 Log reduction. Naturally, gram-positive bacteria have a better survivability to lyophilization because of their cell wall composition, rich in peptidoglycan, and that was proven with the experiments performed. In *S. aureus* stability study, cellular concentration was kept stable over time, above 6 Log cfu/200µl. In the stability studies in which *E. coli* and *S aureus* were storage at room temperature, it has been proved that lyophilized microbial cultures require lyophilization to maintain viability.

This work initiated the research needed for the elaboration of a protocol intended for the production of lyophilized reference stock cultures, with a specific inoculum level, for future application in microbiological analyses.

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LIST OF ACRONYMS AND ABBREVIATIONS

UA	University of Aveiro
ISO	International Standard Organization
WDCM	World Data Centre for Microorganisms
P_R	Productivity ratio
cfu	Colony forming units
ATCC	American Type Culture Collection
NCTC	National Collection of Type Culture
µl	microliter
TSA	Trypto-casein Soy Agar
ºC	Degrees Celsius
NB2	Nutrient broth no2
ml	mililiter
TS	Tryptone-salt broth
PCA	Plate Count Agar
g	grams
min	minutes
BP egg yolk	Baird-Parker agar with egg yolk tellurite
mBar	milibar

INTRODUCTION

Under the Cooperation Protocol between University of Aveiro (UA) and Controlvet ALS, a project was designed with the goal of developing lyophilized reference stock cultures for quality control in microbiological analysis.

The entire project took place in Controlvet ALS' installations, in Tondela, and every material and equipment required were property of Controlvet ALS.

LEGAL FRAMEWORK

The main objective in food law is to ensure elevated levels of public health, as it is defined in *Regulation (EC) No 178/2002*. Microorganisms are a danger for foodstuffs, being a source of food-borne diseases in humans. Microbiological criteria give the orientation needed for the acceptability or deniability of foodstuffs. This is why it is important to establish the microbiological criteria and the food safety microbiological criteria to set a limit above which foodstuff should be considered unacceptable (*Commission Regulation (EC) No 2073*, 2005).

In 2005, the Commission of the European Communities, regarding the microbiological criteria for foodstuffs, released the Commission Regulation (EC) 2073/2005. In this Regulation, the microbiological criteria for specific microorganisms are established, as well as the analytical reference methods to be applied by the food business operators.

Three important definitions need to be kept in mind, as described in Regulation 2073/2005 (*Commission Regulation (EC) No 2073*, 2005).

- Microbiological criterion: "a criterion defining the acceptability of a product, a batch of foodstuffs or a process, based on the absence, presence or number or microorganisms, and/or on the quantity of their toxins/metabolites, per unit(s) of mass, volume, area or batch;"
- Food safety criterion: "a criterion defining the acceptability of a product or a batch of foodstuff applicable to products placed on the market;"
- Compliance with microbiological criteria: "obtaining satisfactory or acceptable results set in Annex I when testing against the values set for the

criteria through the taking of samples, the conduct of analyses and the implementation of corrective action, in accordance with food law and the instructions given by the competent authority."

A table with several food safety criteria composes the first chapter of the Annex I in the Commission Regulation 2073/2005. In this table, according to food category, there are the microorganisms/their toxins or metabolites that need to be taken into account, the sampling plan, the limits to be set, the analytical reference method and the stage at which the criterion applies. An example is presented in Table 1.

TABLE 1 Example of food safety criterion displayed in Annex I, Commission Regulation 2073/2005. Adapted from *Commission Regulation (EC) No 2073/2005*.

Food category	Microorganisms/their toxins, metabolites	Sampling plan		Limits		Analytical reference method	Stage where the criterion applies
		n[1]	c[2]	m[3]	M[4]		
Ready-to-eat foods intended for infants and ready-to-eat foods for special medical purposes	*Listeria monocytogenes*	10	0	Absence in 25g		EN/ISO 11290-1	Products placed on the market during their shelf-life

As shown in Table 1, for every food category there is a correspondent analytical reference method wherein the methodology to find the microorganism/toxin or metabolite is described. However, if we take a closer look to every analytical reference method mentioned in Commission Regulation 2073/2005 - Annex I, they all refer and redirect to ISO 11133.

The International Standard ISO 11133 edited in 2014 refers to the microbiology of food, animal feed and water, more specifically, the preparation, production, storage and performance testing of culture media.

ISO 11133:2014 defines every requirement needed for the preparation of culture media with the purpose of microbiological analysis of food, animal feed and water. Because many assays depend on the ability of culture media to provide consistent and

[1] Number of units comprising the sample
[2] Number of sample units giving values over m or between m and M
[3] Lower limit
[4] Maximum limit

reproducible results, it is very important to do performance tests on culture media (*ISO 11133*, 2014).

The performance tests done to culture media, evaluate (*ISO 11133*, 2014):

- Productivity of culture medium which is the level of recovery of a target microorganism in a specific culture medium;
- Selectivity of culture medium which is the degree of inhibition of a non-target microorganism in a selective culture medium;
- Specificity of culture medium that demonstrates that the non-target microorganism do not show the same visual characteristics as target microorganisms.

An example of performance test applied to a medium is represented in Table 2.

TABLE 2 An example of a test microorganism, and its performance criteria, for culture media. Adapted from *ISO 11133*, 2014.

Media	Microorganism	ISO	Function	Control Strain	WDCM	Reference media	Method of control	Criteria	Characteristic reaction
Baird-Parker	Coagulase-positive staphylococci	ISO 6888-1	Productivity	*Staphylococcus aureus*	00034[b] 00032	TSA	Quantitative	$P_R \geq 0.5$	Black or grey colonies with clear halo (egg yolk clearing reaction)
			Selectivity	*Escherichia coli*[d]	00012 00013		Qualitative	Total inhibition (0)	
			Specificity	*Staphylococcus saprophyticus* *Staphylococcus epidermidis*	00159[b] 00036		Qualitative		Black or grey colonies without egg yolk clearing reaction

As seen in the example featured in Table 2, for every performance test done to a specific medium, there is a specific control strain to be used. This is specified in ISO 11133:2014 Annex E and Annex F. In these two annexes, the test microorganisms to be used in food and water microbiology, respectively, are displayed.

[b] Strains to be used as a minimum.
[d] Strains free of choice.

Another requirement from *ISO 11133*, 2014, relates to the fact that depending on the function and method of control of the performance tests, there is a specific inoculum level to be used.

Productivity testing is divided in (*ISO 11133*, 2014):

- quantitative testing, in which a level of 100 cfu is required to achieve precision;
- qualitative testing, wherein plate media testing needs an inoculum of 10^3 to 10^4 cfu, and pre-enrichment and enrichment media require ≤100 cfu.

For selectivity testing, the inoculum should contain 10^4 to 10^6 cfu, and for specificity testing, the level of inoculum should be 10^3 to 10^4 cfu (ISO 11133, 2014).

Test microorganisms must be representative of their species and this is why to perform these tests, microorganisms from reference cultures collections should be used (*ISO 11133*, 2014).

The definition presented in ISO 11133:2014 of reference strain (Figure 1) says that it is a microorganism obtained directly from a reference culture collection. However, if the laboratory is going to use a reference strain every time it performs a performance test, then the costs are going to be too elevated. ISO 11133:2014, taking the latter into account, provides a way to overcome this obstacle by producing reference stock cultures and working cultures.

By the book (*ISO 11133*, 2014), a reference stock culture is a set of separate identical cultures obtained by a single subculture from the reference strain either in the laboratory or from a supplier. A working culture (Figure 2) is a subculture from a reference stock culture and is produced for routine use (EURACHEM, 2013).

FIGURE 1 Bought reference strains. Image property of (Microbiologics, 2016).

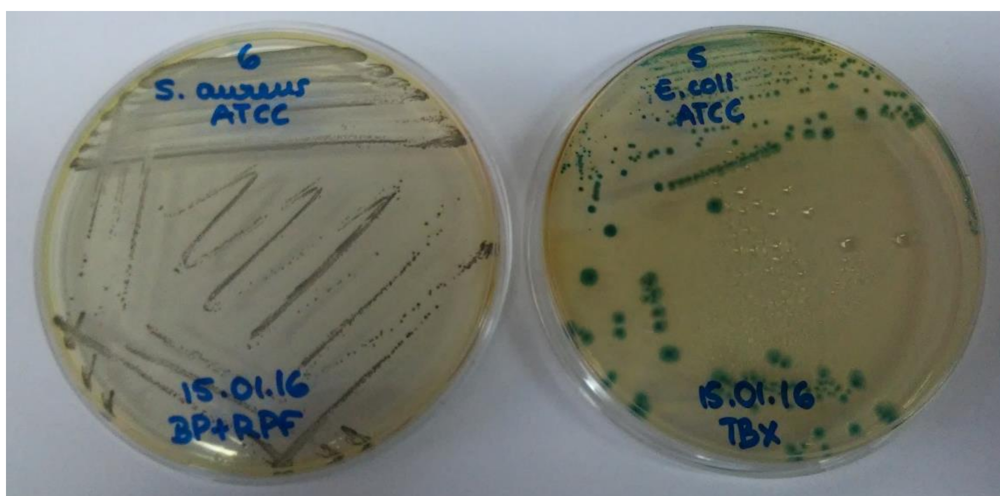

FIGURE 2 Working cultures (*Staphylococcus aureus* and *Escherichia coli*). Property of Controlvet ALS.

Because there is an elevated risk of cross-contamination, alteration of typical characteristics and mutation, reference stock cultures prepared from reference strains should be multiplied and stored deep frozen (-70ºC) or lyophilized (*ISO 11133*, 2014).

A few steps must be followed before storage at -70ºC or lyophilizing. Those are represented in Figure 3.

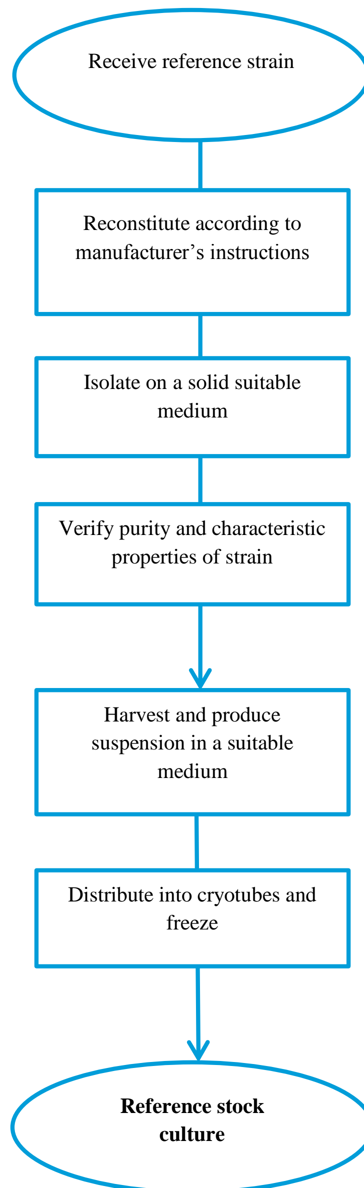

FIGURE 3 Scheme representing the steps to prepare a reference stock starting from a reference strain. Adapted from *ISO 11133*, 2014.

REFERENCE MATERIALS

Reference materials, besides being used as starting point in the development of lyophilized reference stock cultures with the final goal of the execution of performance tests, can be used with other purposes, such as:

- implementation of a new methodology;
- qualification and training of an analyst;
- positive and negative control of confirmation tests;
- positive control of an executing method;
- execution of parallel and duplicate assays, for quality control.

For the implementation of a new methodology, validation is required, which culminates in a series of evaluations (EURACHEM, 2013). Generally, accuracy and relative accuracy are evaluated, and for both, reference materials are used for artificially contaminate samples every time naturally contaminated samples are not available (*EN ISO 16140*, 2002; EURACHEM, 2013).

When qualifying and training a new analyst or technician, as well as in maintaining qualification, the use of reference materials is very common. Once again, in the event of the use of naturally contaminated samples is not possible, these are contaminated with said reference materials so as to fulfill the qualification tests needed (Lightfoot e Maier, 1998).

For the confirmation tests, a positive and negative control should be used. These are obtained from fresh working cultures to facilitate the interpretation and confirmation of the results obtained (*ISO/IEC 17025*, 2005).

ISO/IEC 17025 (2005), in the case of the detection methods, recommends the completion of positive controls for the execution method. For this, a reference strain is submitted to the full method. In the end, the method should be capable to adequately recover the target microorganism.

To ensure a good precision in results, duplicate and parallel assays are performed. These must be executed regularly, and can be done in water or food samples. For both cases, the matrix is contaminated with reference materials, once again (*ISO/IEC 17025*, 2005).

In Controlvet's microbiology laboratory, to achieve every purpose evidenced earlier, reference stock cultures are used. Starting from bought reference cultures (Figure 1) and following the scheme presented in Figure 3, the reference stock cultures are obtained and then maintained at freezing temperatures. Weekly or when necessary, one of the cryotubes is thawed, in order to inoculate a subculture – working culture. Routinely, the working cultures are mostly used for confirmation tests.

Reference cultures can be sold in different shapes and quantities; consequently, prices may vary. Sticks (Figure 1), pills (Figure 4), pellets (Figure 5) or lenticules (Figure 6) are the most common forms commercialized.

FIGURE 4 Reference material presented in a pill. Image property of IELAB, 2016.

FIGURE 5 Reference material in a form of a pellet. Image property of Microbiologics, 2016.

FIGURE 6 Reference material imbedded in the form of lenticules. Property of Controlvet ALS.

Relating to prices, these may vary, depending on the way reference materials are presented, because it is related to their final purpose.

Pills and lenticules, routinely used for the execution of parallel and duplicate assays, are cheaper, around 5€ per unit, wherein they are usually sold in packs of 5 or 10 units. Pellets and sticks, most used as starting point of stock cultures, are more expensive, 20€ per unit. In these formats, the actual reference material, that is, the microorganism, can influence the final price.

The lyophilized reference stock cultures this project aims to develop may one day be used for every application previously mentioned in this chapter. However, the main goal is the development of these lyophilized reference stock cultures for the realization of performance tests in culture media.

By demand of *ISO 11133* edited in 2014, for every batch of media produced, performance tests should be executed. In these, the microorganisms to be used depend, naturally, from the culture media the laboratory uses. In Controlvet's microbiology laboratory in Tondela, taking into account *Commission Regulation (EC) No 2073* (2005), the main analysis conducted are to determine the presence of microrganisms indicative of process hygiene, as well as, the presence of pathogenic microorganisms as is the case of *Listeria monocytogenes* and *Salmonella* (*Commission Regulation (EC) No 2073*, 2005).

Because the latest analysis represent the bulk of the work performed in Controlvet's microbiology laboratory, the choice of the microorganisms to test in this

project lies here. For being important to study both gram-positive and gram-negative bacteria, due to reported differences during lyophilization (Lacasse, 1995; Madigan *et al.*, 2009; Miyamoto-Shinohara *et al.*, 2006; Tortora, Funke e Case, 2010), two microorganisms of each group were chosen:

- Gram-positive:
  - *Staphylococcus aureus*
  - *Listeria monocytogenes*
- Gram-negative
  - *Escherichia coli*
  - *Salmonella enterica* serovar Typhimurium

Listeria monocytogenes is known for causing listeriosis, an infection provoked by the ingestion of contaminated food stuffs (*ID 3*, 2014).

Salmonella species are commonly found in poultry, eggs and even in raw meat and milk. Cross contamination is frequent, leading to severe food poisoning incidents. The mere presence of *Salmonella* species in ready-to-eat foods is considered to be not in accordance (*FNES16 (F13)*, 2014).

Lyophilization

Lyophilization, also known as freeze-drying, is a drying process used for several years with many applications, including the processing of food and development of drugs (Labconco, 2007), or even the restoration of books or other artifacts damaged by water, and the preservation of specimens for future museum display (Labconco, 2010). However, it is the fact of this process' possibility to be used in the preservation and storage of biological samples (Carvalho *et al.*, 2003), that it is one of the most used methodologies in microbial industry (Miyamoto-Shinohara *et al.*, 2006; Morgan *et al.*, 2006) and in microbial culture collections, including the American Type Culture Collection (ATCC) and the National Collection of Type Cultures (NCTC), that makes it so remarkable.

The advantages associated with this methodology include (Barbosa-Cánovas e Vega-Mercado, 1996):

- the easy reconstitution to the original shape and structure of the product/cell, by simply adding a liquid;
- the fact that this same reconstitution is fairly quick;
- the rehydrated product is similar to the original product, in terms of features;
- the actual porosity of products that were lyophilized enables a faster and complete rehydration than with air dried products.

The two biggest disadvantages of lyophilization are the energy cost and the drying time, both very elevated (Barbosa-Cánovas e Vega-Mercado, 1996).

Lyophilized materials loose a small percentage of its original weight (Barbosa-Cánovas e Vega-Mercado, 1996) and do not need refrigeration (Barbosa-Cánovas e Vega-Mercado, 1996; Costa *et al.*, 2000; Labconco, 2010). This also contributes, logistically speaking, for bigger winnings.

The process of lyophilization consists in two main steps: freezing and drying (Barbosa-Cánovas e Vega-Mercado, 1996).

The principal behind lyophilization is the exclusion of a solvent, usually water, from a frozen product, through sublimation (Castro, Teixeira e Kirby, 1997; Labconco, 2010) in which the frozen solvent goes directly to the gaseous phase without passing through the liquid state (Labconco, 2007).

For lyophilization to occur, there are three separate parts of the process that need to be completed. These are prefreezing, primary drying and secondary drying (Labconco, 2010; Morgan *et al.*, 2006).

Prefreezing

Before drying, samples must be frozen in order to sublimation can later occur.

Freezing can be done separately from drying, commonly at -80ºC, prior handling the freeze dryer, or inside the freeze dryer chamber (Figure 7) if the equipment is prepared for such (Morgan *et al.*, 2006).

FIGURE 7 Lyophilizer equipped with a freezing chamber. Equipment property of Controlvet ALS.

How fast or slow a microbial culture is frozen, is a matter of massive importance. A slow cooling translates into the formation of large crystals extracellularly (Ramaswamy e Marcotte, 2006), causing harm to the cells membrane or eventual death due to the lack of ability to repair after desiccation (Morgan *et al.*, 2006). A rapid cooling means that the numerous crystals are formed with a very small size, both intra and extracellularly, not causing a significant injury to the cells (Morgan *et al.*, 2006; Ramaswamy e Marcotte, 2006). Therefore, the aim when freezing, is a rapid cooling to obtain small ice crystals and an amorphous state (Barbosa-Cánovas e Vega-Mercado, 1996).

In Figure 8, a schematic of the difference between slow cooling and fast cooling is illustrated. Ice crystals are represented in black and cells in white. It is clear to see that with slow freezing, cells are deformed due to the increasing size of the ice crystals. As for rapid freezing, the crystals formed are so small in size that they are not even distinguishable in the figure. It is the small size of the ice that allows the cell structure to be maintained practically intact (Ramaswamy e Marcotte, 2006).

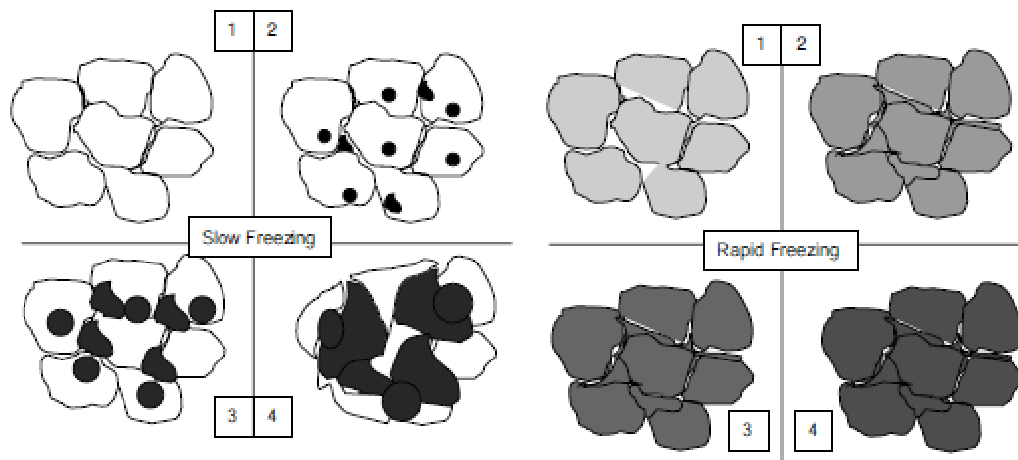

FIGURE 8 Scheme representing the difference of slow and rapid freezing (Ramaswamy e Marcotte, 2006).

When lyophilizing, it is important for samples (microbial cells) to be in a complete frozen state; however, it can be difficult to know when that state is reached or even which temperature is needed for the frozen state to be achieved. This is the point where the knowledge of the term “eutectic temperature” comes in hand. To understand this concept, the notion of eutectic product must first be revised.

The most part of products to be lyophilized are comprised in water, or any other solvent, and a solute. An eutectic product is, basically, a mixture of solutes that freezes at lower temperatures than the solvent (Labconco, 2010). This means that at a certain temperature, the sample may seem completely frozen because its solvent is frozen, but the rest of the components of the sample are not, leading to the erroneous idea that the sample is ready to be lyophilized.

This term can also be applied to cells, since water represents 80 to 90% of the whole cell (Pelczar, Chan e Krieg, 1996). Thereby, the term “eutectic temperature” refers to the temperature at which the frozen state is reached in all constituents of the eutectic sample (Labconco, 2010); thus, when the intention is to lyophilize cells, it is important that the prefreezing at an appropriate temperature happens in order for the eutectic temperature is achieved and the sample becomes correctly and uniformly frozen.

Primary drying

In this second phase, the frozen moisture in the sample is extracted by sublimation via reduction of the dryer chamber’s pressure to below the vapor pressure of the ice within the sample (Barbosa-Cánovas e Vega-Mercado, 1996; Morgan *et al.*, 2006); the result being a dry and intact sample.

In primary drying, there are two very important criteria that require attention: temperature and pressure (Labconco, 2010).

Water molecules naturally migrate from higher pressure points to lower pressure points. Vapor pressure and temperature are two connected components. This is why when drying, for the water molecules to go from the frozen product to the water collector, it is required that the water/cold collector (Figure 9) is at a lower temperature than the frozen sample (Labconco, 2010).

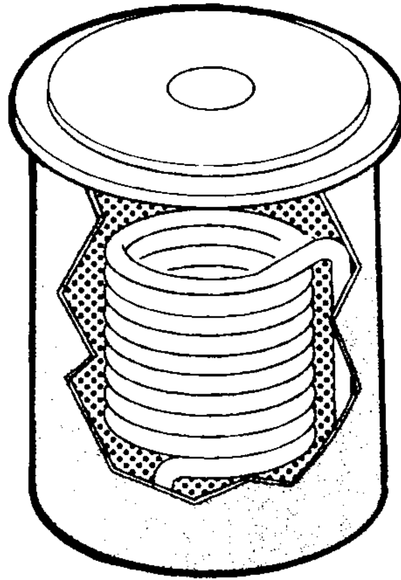

FIGURE 9 Illustration of a collecting system used for the extraction of frozen moisture of the sample (Labconco, 2010).

It is very important for a balance between the temperature that keeps the product integrity and the temperature that maximizes the vapor pressure of the product to exist.

Figure 10 illustrates a phase diagram. As a matter of security, samples are commonly frozen below their eutectic temperature (A). For lyophilization to start, pressure is lowered and the temperature is slightly raised (B) in order for the water molecules can migrate to the water collector. For lyophilization to proceed correctly is essential for the vacuum pump to lower the pressure around the product (C) and for the water collector's temperature (D) to be lower than the product temperature (Labconco, 2010).

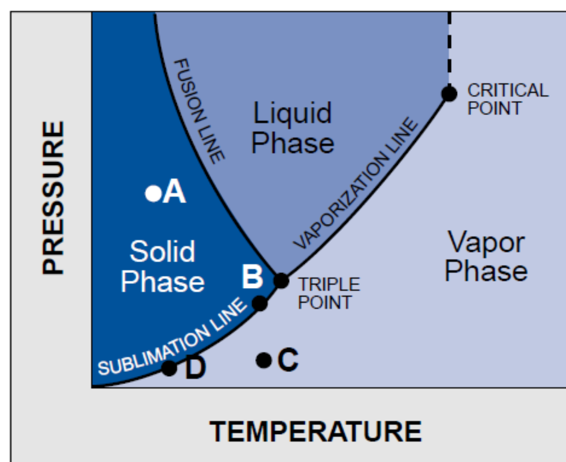

FIGURE 10 Diagram phase (Labconco, 2010).

The initial rate of drying is quite high because the resistance to heat or mass flux is low, but with time, a resistive layer starts to build up around the material, slowing the process down (Barbosa-Cánovas e Vega-Mercado, 1996). Figure 11 illustrates the rate of the drying process in lyophilization.

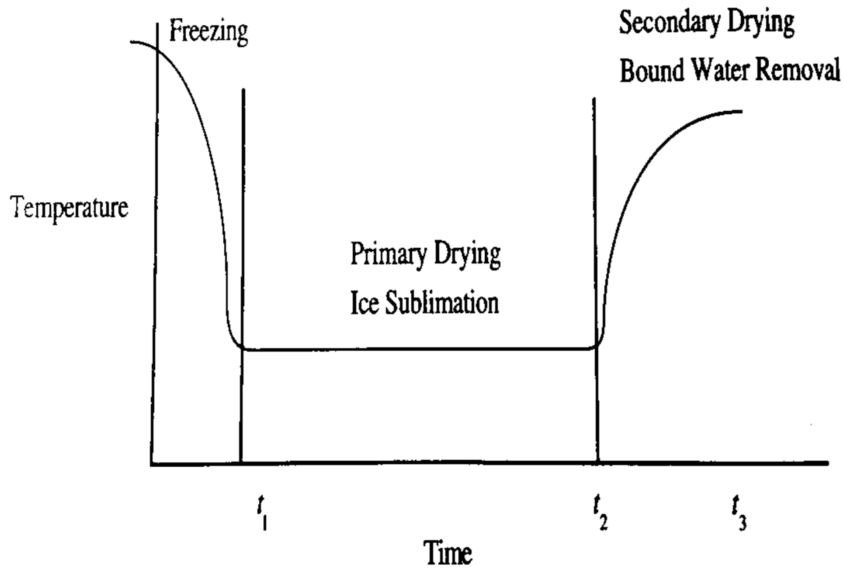

FIGURE 11 Lyophilization stages presented in a graphic time/temperature (Barbosa-Cánovas e Vega-Mercado, 1996).

Secondary drying

The second drying starts when no more unbound water is in the sample -all the ice has sublimated- and the moisture lies in the partially bound water of the drying material (Barbosa-Cánovas e Vega-Mercado, 1996; Labconco, 2010).

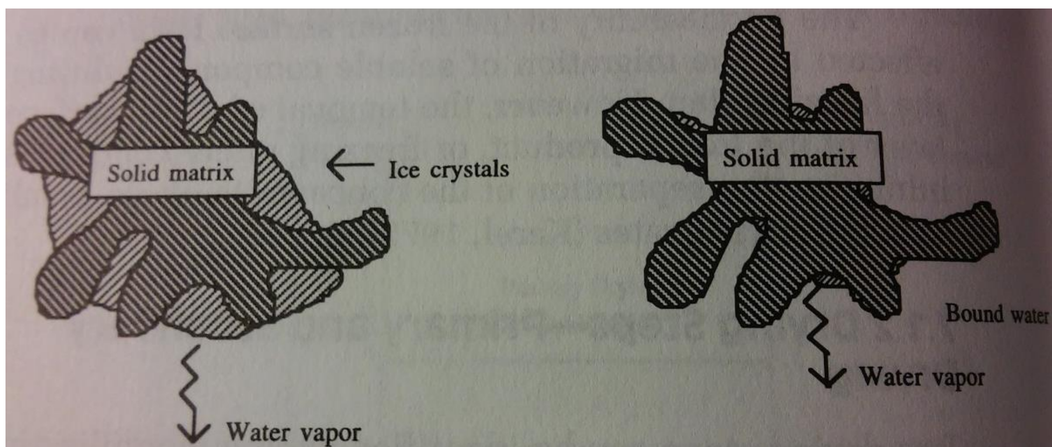

FIGURE 12 Difference between primary and secondary drying, respectively in the figure (Barbosa-Cánovas e Vega-Mercado, 1996).

This process is necessary to reduce the moisture content to optimum values (Labconco, 2010), being those values as low as 2% (Barbosa-Cánovas e Vega-Mercado, 1996).

Secondary drying can take up to 1/3 of the entire lyophilization time (Barbosa-Cánovas e Vega-Mercado, 1996; Labconco, 2010), making this the reason why it is a process with energy costs.

Figure 12 illustrates the difference between primary and secondary drying, for a better understanding of the process itself.

CRYOPROTECTIVE MEDIA

Bacterial cell survival during lyophilization depends on several factors, including the initial cell concentration, the cryoprotective medium, rehydration and storage terms (Costa *et al.*, 2000; Otero, Espeche e Nader-Macías, 2007). Intrinsic factors like genus, species, cell wall composition can affect the way bacteria act during lyophilization, translating in a higher or lower cell concentration after this process (Carvalho *et al.*, 2004; Otero, Espeche e Nader-Macías, 2007).

Of all the above mentioned, probably the one factor with most relevance in the lyophilization process success, is the composition of the cryoprotective medium (Hubálek, 2003). The addition of an adequate cryoprotective increases survival as is showed in the studies performed in *Pantoea agglomerans* by Costa et al. 2000, or in *Enterococcus* spp. by Carvalho et al. 2003.

In 2000's Costa et al. study, different additives were tested as cryoprotectives against injuries endured during lyophilization. Five different groups of additives were examined; the best results were achieved with sugars suspensions. Inside the sugars group, different sugars were tested, being the best results trehalose at 5%, followed by sucrose at 10% concentration. Because of the elevated cost of trehalose, its use becomes limited, thus making it sucrose the best option.

The summary presented by Hubálek in 2003 mentions skim milk, at varying concentrations, sometimes in combination with other substances, being used successfully as a protective agent in lyophilization processes.

Morgan *et al.* (2006) arrives at the conclusion that the most efficient cryoprotectives are indeed a mixture of sugars and proteins; it is therefore possible to combine a protein like skim milk with an inexpensive sugar such as sucrose, to create a good cryoprotective agent.

The cell wall of bacteria

The cell wall of bacteria can be considered a complex structure (Tortora, Funke e Case, 2010) responsible for numerous functions such as the maintenance of the cell shape and rigidity (Madigan *et al.*, 2009; Tortora, Funke e Case, 2010).

The bacteria cell wall is mainly composed of peptidoglycan, a disaccharide that can form a network alone or in composition with other components (Lacasse, 1995; Madigan *et al.*, 2009; Tortora, Funke e Case, 2010).

Bacteria species can be divided in gram-positive and gram-negative, according with the cell wall composition (Madigan *et al.*, 2009). In gram-positive bacteria, peptidoglycan composes almost 90% of the cell wall, making it a homogenous rigid structure (Figure 13 a) and c)) (Lacasse, 1995; Madigan *et al.*, 2009; Tortora, Funke e Case, 2010). As for gram-negative species, the cell wall has a thinner sheet of peptidoglycan (Lacasse, 1995; Madigan *et al.*, 2009; Tortora, Funke e Case, 2010) and is more complex, having also an outer membrane, composed of lipids and polysaccharides, primarily responsible for keeping the structure of the cell (Figure 14 b) and d)) (Madigan *et al.*, 2009).

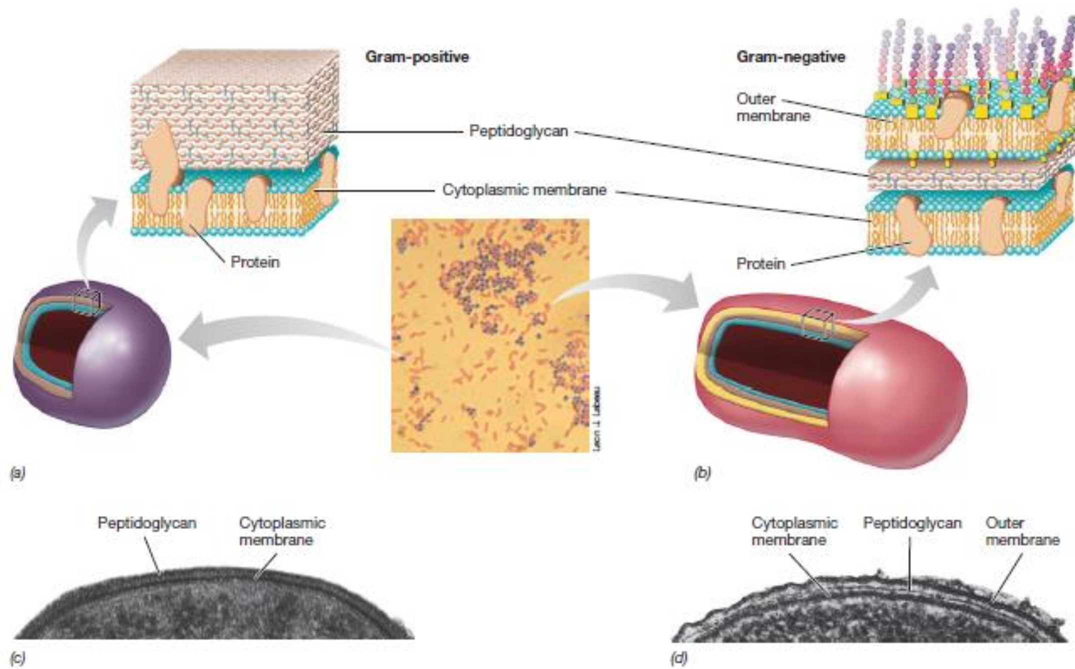

FIGURE 13 Cell walls of bacteria. (a, b) Schematics of gram-positive and gram-negative cell walls. (c, d) Transmission electron micrographs of gram-positive and gram-negative cell walls. Adapted from Madigan *et al.*, 2009.

Gram-positive and gram-negative differences in the cell wall can affect the bacteria's ability to endure in the environment, its nutritional requirements and many other physiological functions (Lacasse, 1995). In Table 3, a summary of the principle differences between gram-positive and gram-negative bacteria is presented.

TABLE 3 Comparison of the main characteristics between gram-positive and gram-negative cell walls. Adapted from Lacasse, 1995; Tortora, Funke e Case, 2010.

Feature	Gram-positive	Gram-negative
Peptidoglycan layer	Thick	Thin
Outer membrane	Absent	Present
Resistance to physical disruption	High	Low
Resistance to drying	High	Low
Nutritional needs	Complex	Simple
Resistance to osmotic shock	High	Low

Gram-negative species, because of their thinner peptidoglycan layer and complex composition (with the presence of the outer membrane), the resistance to physical

disruption and drying is lower, but because of their cell wall complexity, they are more resistant to digestive enzymes and to several antibiotics. Gram-positive bacteria, because of their thick peptidoglycan layer, have a higher resistance to mechanical and osmotic shocks, and elevated resistance to drying, electing lyophilization as one of the best preservation processes (Tortora, Funke e Case, 2010).

SCOPES AND OBJECTIVES

Controlvet ALS Group (Figure 14) started as Controlvet Segurança Alimentar providing test services to the food industry in the field of food microbiology, diagnosis and immunological tests. Along the years, Controlvet became a reference in Portugal. In 2015, Controlvet was acquired by ALS (Australian Laboratory Services), promoting a faster growth in the food sector in the Iberian Peninsula.

FIGURE 14 Controlvet ALS Group logos. Provided by Controlvet ALS.

As mentioned before, in late 2014 the International Standard ISO 11133 was published. This standard says that for every batch of medium produced, a performance test should be done with a determined control microorganism. This presents a problem on the amount of control microorganisms needed once, on a daily-basis, a laboratory of food safety, produces multiple batches.

ISO 11133:2014 also emphasizes the fact that for such performance tests, accordingly to function and method of control, there is an inoculum level to respect, making this a time-costing measure for laboratories, requiring them to do serial dilutions.

The production of lyophilized reference stock cultures is not new, however there are several problems associated with the acquisition of reference stock cultures. They are highly expensive, generally unstable, and the way the transportation occurs can cause changes in the product. Not only will be important and of relevance for Controlvet ALS to cause a great impact on the market, but also, it will allow a bigger economic sustainability to the company.

Due to the fact control microorganisms need to be representative of their species, the use of reference strains for every performance test is economically unviable, and lyophilization is one of the recommended procedures to preserve reference stock cultures, rises the opportunity to create a protocol to produce reference stock cultures with the correct inoculum level.

This project aims, with collaboration of Controlvet ALS, to start the development of a protocol intended for the commercialization of lyophilized reference stock cultures.

Listeria monocytogenes and *Salmonella* Typhimurium are two pathogenic microorganisms whose presence in ready-to-eat foods is considered to be nonconforming, according with *Commission Regulation (EC) No 2073*, 2005. Due to the importance and danger of these microorganisms, a major amount of tests for the detection of these bacteria is performed in food safety laboratories. Reason why, it is important the immediate development of lyophilized reference stock cultures for the realization of performance testes in culture media used for the detection of the referred microorganisms.

In this work, viability studies to the gram-positive bacteria *S. aureus* and *L. monocytogenes* and to the gram-negative bacteria *E. coli* and *Salmonella* Typhimurium, are performed with different cryoprotective agents – skim milk + 10% sucrose, nutrient broth no2 + 20% glycerol and sucrose 10%. A stability study with skim milk + 10% sucrose as a protective agent is done to the gram-positive bacteria *Staphylococcus aureus*, as well as, three stability studies with skim milk + 10% sucrose as a cryoprotectant is conducted with *Escherichia coli* and *Staphylococcus aureus*.

MATERIALS AND METHODS

VIABILITY STUDIES

Viability studies are meant to understand the behavior of the different cells to lyophilization, while changing some variables such as cryoprotective medium or bacterial species.

In total, seven viability studies were conducted, with three different cryoprotective agents: skim milk + 10% sucrose, nutrient broth no2 + 20% glycerol and a suspension of sucrose at 10%.

E. coli and *S. aureus* were submitted to lyophilization with skim milk + 10% sucrose as a cryoprotective agent. Lyophilization was repeated with nutrient broth no2 + 20% glycerol as a cryoprotective. *E. coli* and *Salmonella enterica* serovar Typhimurium, two gram-negative bacteria, were subjected to lyophilization with a solution of sucrose at 10% and *Listeria monocytogenes*, a gram-positive bacteria, was lyophilized with skim milk + 10% sucrose as a cryoprotective agent.

Microorganisms

All the microorganisms used in the study were obtained from the reference stock culture stored at -20ºC in Controlvet ALS's Laboratory in Tondela. Originally, the references strains were obtained from a culture collection, as referenced in Table 4.

TABLE 4 Microorganisms used in viability studies.

Microorganism	Culture Collection Number	WDCM number
Escherichia coli	ATCC 25922	00013
Staphylococcus aureus	ATCC 6538	00032
Listeria monocytogenes serovar 4b	ATCC 13932	00021
Salmonella enterica serovar Typhimurium	ATCC 14028	00031

Cell culture preparation

Culture preparation was the same for the different microorganisms, in the viability studies.

After thawing the reference stock culture, with a 10 µl loop, a TSA plate was inoculated in order to get isolated colonies. The TSA plate was left to incubate at 37ºC, overnight. Past that, 1 colony was put in a test tube with nutrient broth no2 (NB2) (Biokar, Allonne, FR), an enrichment media intended for microbial growth, at 37ºC, overnight, with occasional shaking for a better cell spreading. Finally, 1 ml of the broth with the microorganism of interest was suspended in 9 ml of the cryoprotective agent to be tested. This suspension, after vortex, was distributed in sterilized cryotubes, suitable to the lyophilization process.

Cell concentration calculation

Before lyophilizing, is essential to know the initial cell concentration. To do so, tenfold dilutions were made using triptone-salt broth (TS) (Biokar, Allonne, FR), a diluent. Dilutions made, 0.1 ml of each dilution was inoculated in PCA (Biokar, Allonne, FR) by incorporation. The plates were left to incubate at 37ºC, overnight, and the number of cfu was counted. In order to know how many cfu were in the cryotube before lyophilizing, the following equation was used.

EQUATION 1 Equation used to calculate initial cell concentration.

$$N_i = \frac{V_c \times n \times D}{V_i}$$

N_i= initial cell concentration

V_c= volume of suspension in the cryotube

n= number of cfu counted in the Petri dish

D= dilution in which the cfu count was made

V_i= volume of suspension inoculated

Cryoprotective media preparation

The suspensions of cryoprotectives were prepared with distilled water.

For the skim milk + 10% sucrose suspension, to 50 ml of distilled water, 1 g of skim milk powder (Molico, Nestlé) and 5g of sucrose were added. To produce the NB2 + 20% glycerol, 6.25 g of NB2 (Biokar, Allonne, FR) were added to 200 ml of distilled water, followed by 50 ml of glycerol. To prepare the 10% sucrose solution, 5 g of sucrose were added to 50 ml of distilled water.

After correct homogenization of the suspensions, they all were sterilized at 121°C for 15 min.

Lyophilization

For each viability test, 6 cryotubes were filled with 200 µl of the bacterial suspension with the cryoprotective agent. Of the 6 vials, 3 were used as samples.

For a proper lyophilization, is important to leave a gap between the vial and the lid (Figure 15), in order for sublimation to occur inside the cryotube.

FIGURE 15 Example of a cryotube with a gap between the lid and the vial for sublimation to occur inside it.

The cryotubes were placed in the freezing chamber of the lyophilizer and the refrigeration mode was activated manually. The samples were left to freeze for 2h, reaching the temperature of -53ºC. Past the 2h refrigeration, vacuum was switched on, and the samples were left 24h at 0.045 mBar to lyophilize.

After the 24h in vacuum, this was switched off. To properly close the cryotubes, the samples were set in the clear stoppering chamber (Figure 16), the vacuum was again activated and once it reached 0.060 mBar, the cryotubes were mechanically closed. Once the gap between the lid and the vial was shut, the lyophilizer was turned off.

FIGURE 16 Clear stoppering chamber where cryotubes are closed in vacuum. Property of Controlvet ALS.

To ensure higher levels of security, both to the operator and to the sample – to minimize possible entry to the vial of humidity – a metallic cap was put over the vial's lid and sealed (Figure 17).

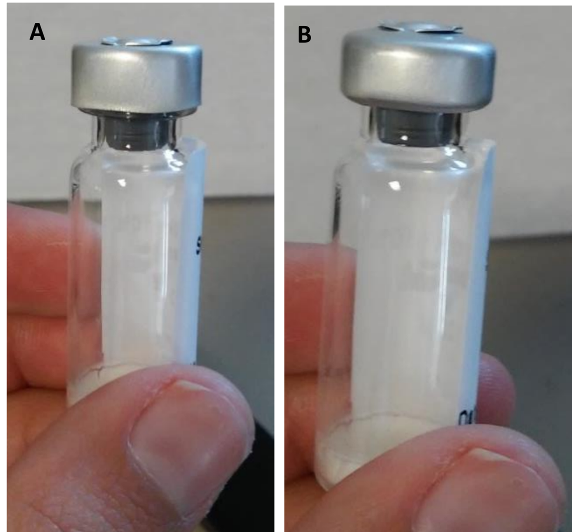

FIGURE 17 A) Cryotube with unsealed metallic cap. B) Cryotube with already sealed metallic cap.

Rehydration and final cell concentration

After lyophilization, the samples were immediately rehydrated to their original volume with NB2 and homogenized in the vortex.

To record the final cell concentration, a series of tenfold dilutions were made and inoculated in PCA medium by incorporation. These plates were then incubated at 37ºC, overnight.

To calculate the final cell concentration, after lyophilization, Equation 2 was used.

EQUATION 2 Equation applied to calculate the cell concentration, after lyophilization.

$$N_f = \frac{V_c \times n \times D}{V_i}$$

N_f= final cell concentration

V_c= volume of suspension in the cryotube

n= number of cfu counted in the Petri dish

D= dilution in which the cfu count was made

V_i= volume of suspension inoculated

Stability studies

The stability study aims to do a description over time of the cells' concentration after lyophilization.

The first study was conducted with skim milk + 10% sucrose as cryoprotective agent and *S. aureus* (Table 4) as the microorganism test. The last two studies were performed in *E. coli* and *S. aureus* (Table 4) with three separate lyophilization processes, both with skim milk + 10% sucrose as protective agent.

All the stability studies had the same cell culture preparation, cryoprotective media preparation and lyophilization as described previously in the Viability studies. Regarding storage, in the first study the lyophilized cultures were placed at 2 to 8°C, while for the last two studies the cryotubes were left at room temperature.

Rehydrations

For the first study, every two weeks following lyophilization, a rehydration to the original volume with NB2 of three samples was made, as well as tenfold dilutions were plated in PCA and BP egg yolk (Biokar, Allonne, FR), a selective medium intended for the detection and enumeration of *S. aureus*.

In the last three stability studies, every two weeks, three samples were rehydrated with NB2 to their original volume. Tenfold dilutions were made and plated in PCA and BP egg yolk in the case of *S. aureus*, and in PCA and TBX (Biokar, Allonne, FR), in the case of *E. coli*.

To know the cell concentration at each time, in every stability study, Equation 2 was used.

Statistical analysis

A comparison between the same species of bacteria in different cryoprotective mediums, and between different species in the same cryoprotective agent was made with the respective mean and standard deviation. For pre-lyophilization, data was retrieved once, while for post lyophilization, data was collected three times.

To verify if there were any significant differences between the same species of bacteria in different cryoprotective mediums, and between different species in the same cryoprotective agent, the t-test was applied to the difference of cell concentration before and after lyophilization. With a P-value below 0.05, the null hypotheses (no significant differences between the same species of bacteria in different cryoprotective mediums, and no significant differences between different species in the same cryoprotective agent) were rejected, favoring the alternative hypotheses (there is significant differences between the same species of bacteria in different cryoprotective mediums, and there is significant differences between different species in the same cryoprotective agent).

For the first stability study, to track *S. aureus* concentration after lyophilization, for every t (time analyzed), mean and standard deviation were collected for both PCA and BP egg yolk. To verify if there were any significant differences between culture media (PCA vs. BP egg yolk) the t test was, once again, applied. If the P-value was below 0.05 the null hypothesis (no significant differences between culture media) would be rejected; otherwise the null hypothesis would be maintained (meaning there is significance in the difference between culture media).

For the last three stability studies also cell concentration over time was analyzed. Mean and standard deviation at each time, for both selective and non-selective media, in *S. aureus* and *E. coli*, were collected.

Student t-test is a hypothesis test that compares medium values of two normal populations. The t-test statistics allows you to take an objective decision on the hypothesis you are investigating (Pestana e Velosa, 2010).

RESULTS

VIABILITY STUDIES

Comparison of cryoprotective agents

The results of cell concentration for the different cryoprotective agents, before and after lyophilization, are presented in Figures 18 to 20.

In Figure 18, cell concentration before and after lyophilization is presented for *E. coli*, *S. aureus* and *L. monocytogenes* with skim milk + 10% sucrose as a cryoprotective agent. Log cfu/200 µl for *E. coli*, *S. aureus* and *L. monocytogenes* with skim milk + 10% sucrose before and after lyophilization are 6.61, 6.60, 6.69 and 5.65 ± 0.20, 6.21 ± 0.72, 6.74 ± 0.08, respectively. The difference between *E. coli* and *S. aureus*, as well as, between *S. aureus* and *L. monocytogenes* is not statistically significant ($P > 0.05$), while the difference between *E. coli* and *L. monocytogenes* has statistical significance ($P < 0.05$).

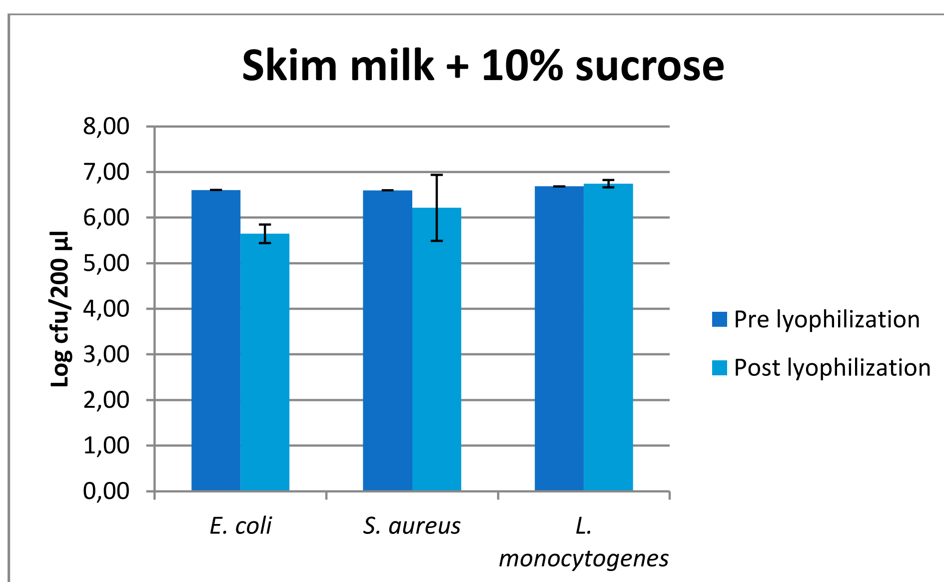

FIGURE 18 Comparison in Log cfu/200 µl, of cell concentration before and after lyophilization, with skim milk+10% sucrose as cryoprotective.

In Figure 19, cell concentration before and after lyophilization is presented for *E. coli* and *S. aureus*, with the cryoprotective agent NB2 + 20% glycerol. Log cfu/200µl

for *E. coli* and *S. aureus*, before and after lyophilization, are 6.99, 6.75 and 3.97 ± 0.58, 6.07 ± 0.04, respectively. The difference between *E. coli* and *S. aureus* is statistically significant (P < 0.05).

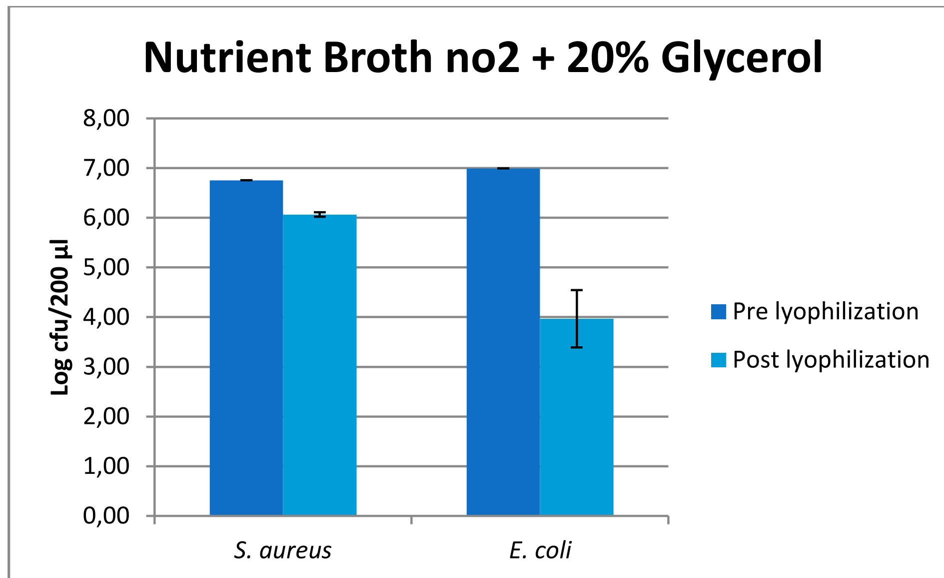

FIGURE 19 Comparison of *S. aureus* and *E. coli* cell concentration, before and after lyophilization, with nutrient broth no2 + 20% glycerol as a cryoprotective agent.

In Figure 20, the cell concentration pre lyophilization and post lyophilization is presented for *Salmonella* Typhimurium and *E. coli*, with the cryoprotective medium, sucrose 10%. Log cfu/200 µl for *Salmonella* Typhimurium and *E. coli*, before and after lyophilization, are 6.82, 6.87 and 5.76 ± 0.03, 4.52 ± 0.34, respectively. The difference between *Salmonella* Typhimurium and *E. coli* is also statistically significant (P < 0.05).

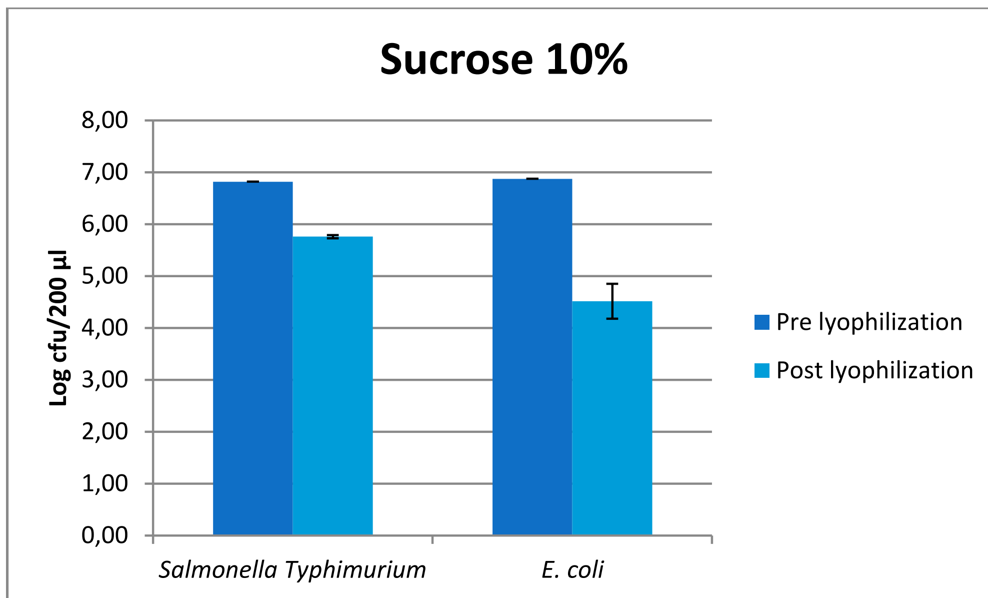

FIGURE 20 Cell concentration, pre and post lyophilization of *Salmonella* Typhimurium and *E. coli*, with the solution of sucrose 10% as cryoprotective medium.

Comparison of E. coli and S. aureus in different cryoprotective agents

The results of the viability studies of *E. coli* and *S. aureus* in the different cryoprotective media are summarized in Figures 21 and 22.

In Figure 21, a comparison of the different cryoprotective agents in which *E. coli* was lyophilized is displayed. Comparing the difference between pre and post lyophilization, the different results of *E. coli* lyophilized with skim milk + 10% sucrose and NB2 + 20% glycerol, as well as, skim milk + 10% sucrose and sucrose 10%, are statistically significant ($P<0.05$). On the other hand, the results of *E. coli* lyophilized with NB2 + 20% glycerol and the results of *E. coli* lyophilized with sucrose 10%, are not statistically significant ($P>0.05$).

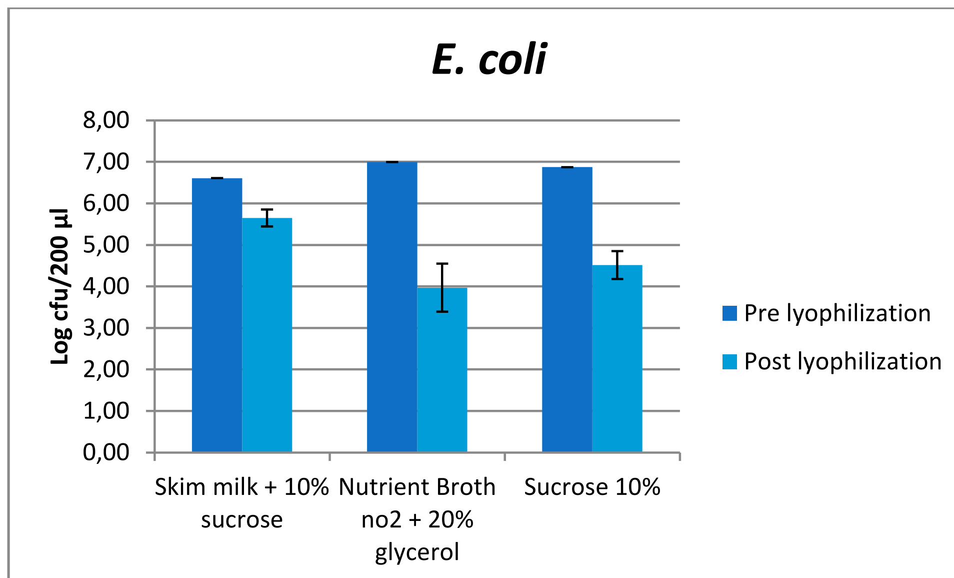

FIGURE 21 Cell concentration, before and after lyophilization, of *E. coli* in skim milk + 10% sucrose, nutrient broth no2 + 20% glycerol and sucrose 10%.

In Figure 22, data relating *S. aureus* lyophilization in skim milk + 10% sucrose and in NB2 + 20% glycerol are presented. Once again it is the difference between pre and post lyophilization cell concentration that is used for comparison. The results of lyophilization with skim milk + 10% sucrose and with NB2 + 20% glycerol, are not statistically significant (P>0.05).

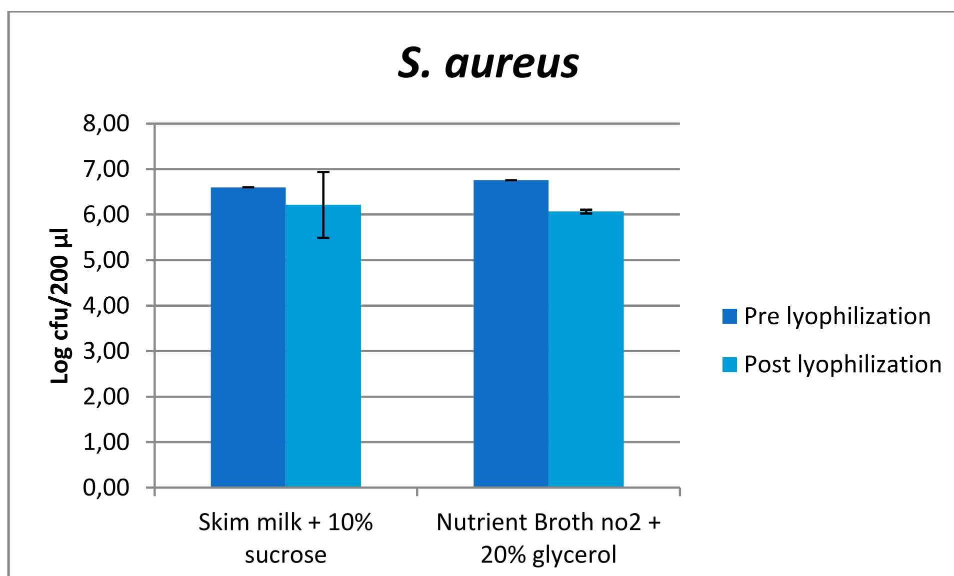

FIGURE 22 Comparison of *S. aureus* cell concentration, before and after lyophilization, in skim milk + 10% sucrose and in nutrient broth no2 + 20% glycerol.

STABILITY STUDIES

The averages of three determinations and respective standard deviation of the first stability study performed in *S. aureus* after lyophilization, in which the cell concentration is registered over time, is presented in Figure 23. For each reconstitution, tenfold dilutions were plated in PCA and BP egg yolk, in parallel. A comparison between the results obtained in PCA and in BP egg yolk was made, being those differences not statistically significant (P>0.05).

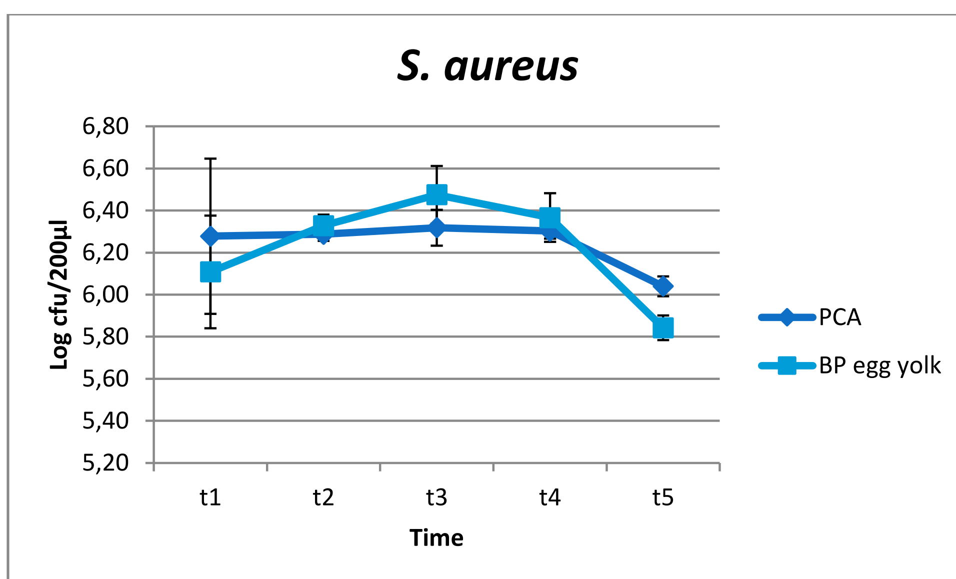

FIGURE 23 *S. aureus* cell concentration over time, inoculated in parallel, in PCA and BP egg yolk.

For the last two stability studies, the mean and standard deviation of three determinations was registered over time, for both *E. coli* (Figures 24 and 25) and *S. aureus* (Figure 26 and 27).

In all the three lyophilizations performed with *E. coli* as test microorganism, the reconstitutions executed involved the concretization of tenfold dilutions which were then plated in PCA and TBX. At all times, in the three studies, mean and standard deviation were calculated and are presented in Figure 25 for PCA and Figure 26 for TBX data. For the three studies, the starting cell concentration prior to lyophilization (pre lyoph.), is also shown.

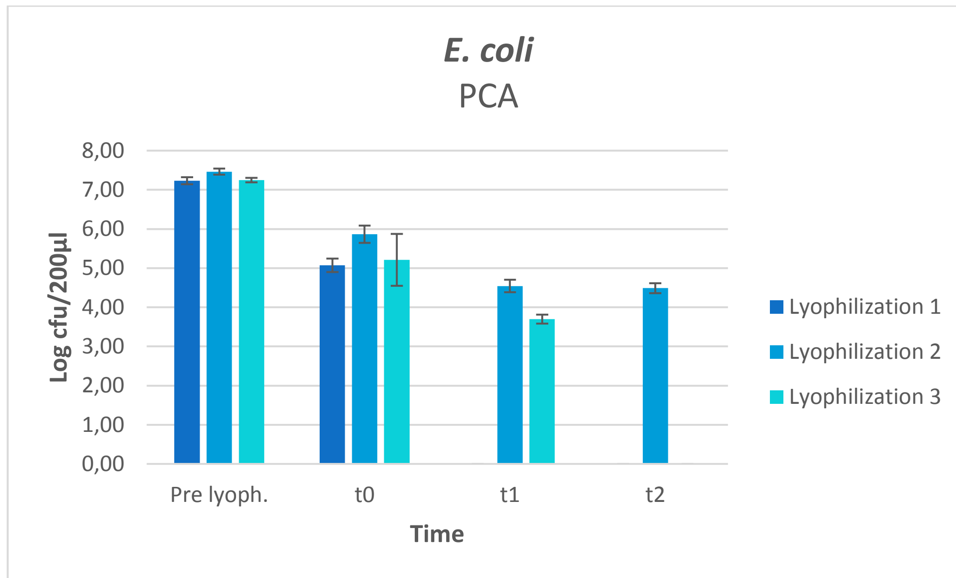

FIGURE 24 *E. coli* cell concentration over time, inoculated in PCA.

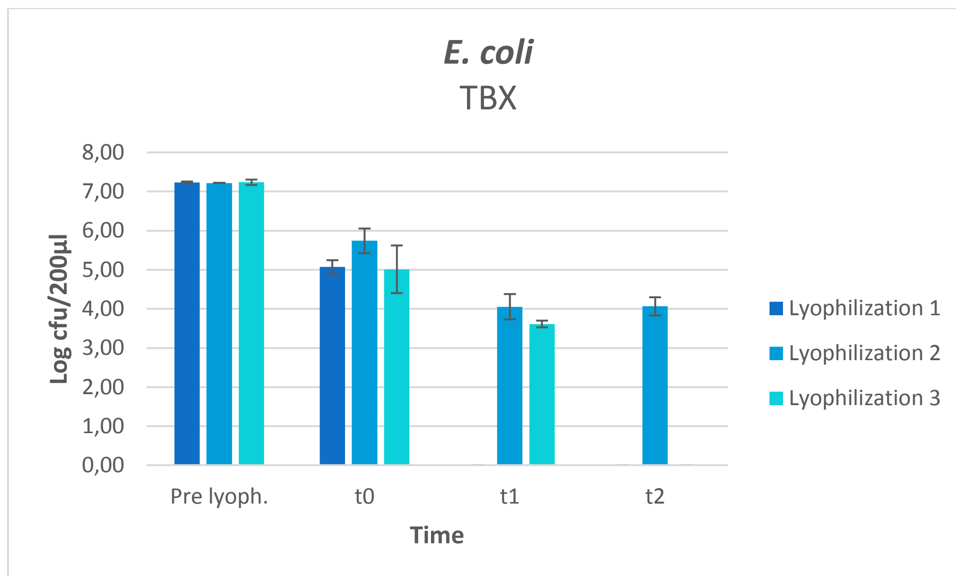

FIGURE 25 *E. coli* cell concentration over time, inoculated in TBX.

In *S. aureus* case, the reconstitutions over time were also made for all three studies. The tenfold dilutions needed were conducted and inoculated in in parallel in PCA and BP egg yolk. Mean and standard deviation were collected in every reconstitution for the three studies performed with *S. aureus*, for PCA (Figure 26) and BP egg yolk (Figure 27). Once again, cell concentration before lyophilization for the three *S. aureus* studies in the two media, is also figured in the graphics.

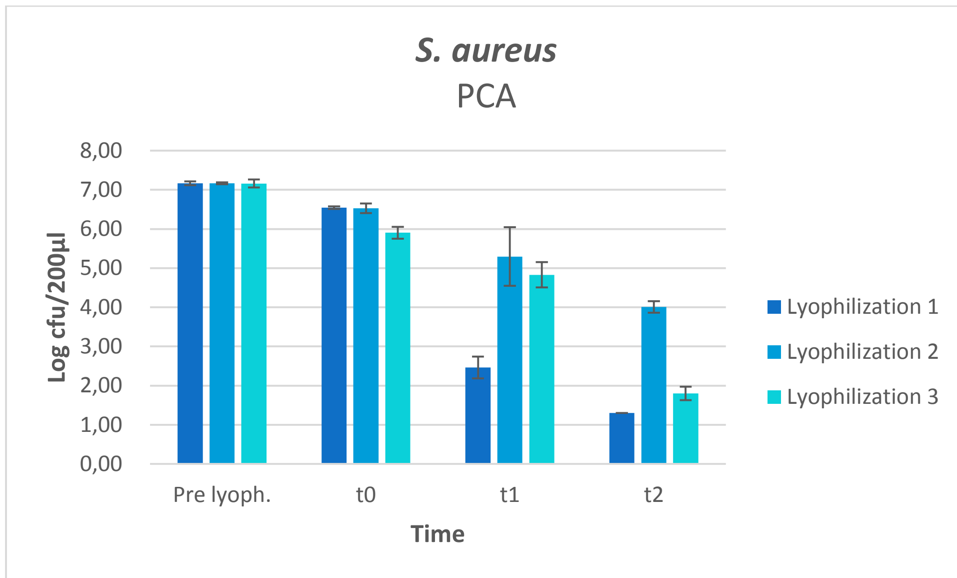

FIGURE 26 *S. aureus* cell concentration over time, inoculated in PCA.

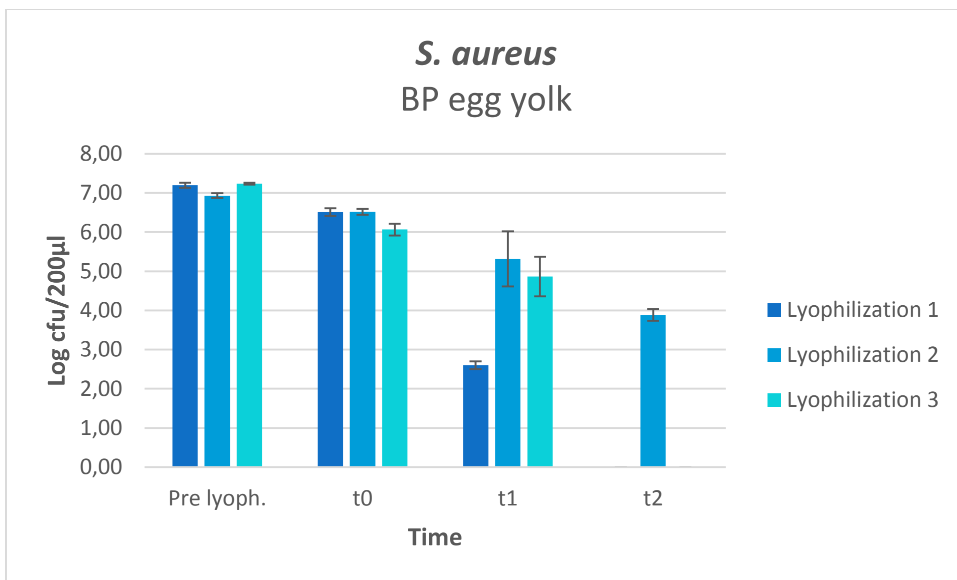

FIGURE 27 *S. aureus* cell concentration over time, inoculated in BP egg yolk.

Discussion

Stability studies

S. aureus is a gram-positive bacteria (Madigan *et al.*, 2009). This group is known for its greater ability to survive lyophilization (Conrad *et al.*, 2000; Miyamoto-Shinohara *et al.*, 2006) mainly because of their cell wall composition – rich in peptidoglycan layers (Lacasse, 1995; Madigan *et al.*, 2009; Tortora, Funke e Case, 2010). Because positive results were expected, *S. aureus* cell concentration, after being lyophilized with skim milk + 10% sucrose was studied, to try to understand its behavior after lyophilization over large periods of time.

Relating storage, it is noteworthy that microbial cultures were left at refrigeration temperatures. Although lyophilization is a process known for the production of products which storage can be done at room temperature (Barbosa-Cánovas e Vega-Mercado, 1996; Costa *et al.*, 2000; Labconco, 2010), several authors suggest that in the particular case of lyophilized microbial cultures, these must be kept at refrigeration temperatures (Carvalho *et al.*, 2004; Miyamoto-Shinohara *et al.*, 2006; Morgan *et al.*, 2006) to ensure maximum viability during storage.

With lyophilization, *S. aureus* concentration was expected to suffer a decrease in viability (Carvalho *et al.*, 2003; Miyamoto-Shinohara *et al.*, 2006) followed by a stabilization during storage (Carvalho *et al.*, 2003; Conrad *et al.*, 2000; Miyamoto-Shinohara *et al.*, 2006). In this experiment, cell concentration remained stable, around 6 Log cfu/200 µl, confirming the expectations predicted in literature.

In all data collections, inoculations were made in two different culture media: PCA and BP egg yolk. This procedure was due to ensure that if there were any kind of contamination, BP egg yolk's data would be reliable. Although in every reconstitution, sterility tests have been conducted and ISO 11133:2014 states that selective media compared with non-selective media have a $P_R \geq 0.50$ (i.e. the culture medium has a recoverability of cfu greater than or equal to 0.50), and non-selective media compared with non-selective media have a $P_R \geq 0.70$ (i.e. the culture medium has a recoverability of cfu greater than or equal to 0.70), the decision to compare PCA and BP egg yolk data using the t-test, was made. Therefore, PCA and BP egg yolk's difference in medium

productivity is not considered statistically significant (P>0.05), which means that the differences registered in both media, are not related to the recoverability/productivity of media, but most probably with the intrinsic variability of the method. In true, there are several sources of uncertainty incremented in a method, being the main sources, the operator (especially if said operator has little practice), time, equipment, culture media and reagents used (*ISO/TS 19036*, 2006).

Because the results obtained in the first stability study were promising, the idea to initiate a more complete study that would allow evaluating cell concentration after lyophilization, over time, of two bacteria, *S. aureus* (gram-positive) and *E. coli* (gram-negative), emerged.

The choice of these two microorganisms lies in the need to compare bacteria of the two gram groups. Gram-positive bacteria, as stated above, have a higher capacity of survival to the lyophilization process (Conrad *et al.*, 2000; Miyamoto-Shinohara *et al.*, 2006) due to the cell wall composition, high in peptidoglycan layers (Lacasse, 1995; Madigan *et al.*, 2009; Tortora, Funke e Case, 2010). Gram-negative bacteria, on the other hand, have a reduced peptidoglycan layer (Lacasse, 1995; Madigan *et al.*, 2009; Tortora, Funke e Case, 2010), conferring, theoretically, a lower resistance to lyophilization (Miyamoto-Shinohara *et al.*, 2006).

This study consisted in performing three independent lyophilizations, with one week apart from each other. The cultures were lyophilized in the same conditions as the previous stability study, with skim milk + 10% sucrose as the cryoprotective agent. After lyophilization, the cryotubes were left at room temperature.

Lyophilization is a process known for its advantage of, products and microbial cultures that underwent lyophilization, do not need refrigeration (Costa *et al.*, 2000; Labconco, 2010). Carvalho *et al.* (2003) confirms the latest information in his study when cultures are left at room temperature during storage.

Taking a first look at *S. aureus*, it was expected that, like the previous study, there was a decrease in cell concentration with the lyophilization process, followed by a stabilization of said cell concentration. However, the latest description was not observed. Effectively, there was an initial minor decrease in cell concentration, justified by the lyophilization, but during storage, cell concentration decreased significantly

(Figure 26). The accentuated decline in cell concentration during storage can only be explained by the fact that the microbial cultures were kept at room temperature and not at refrigeration temperatures like the first stability study.

In truth, there are a few studies reporting the need to keep lyophilized microbial cultures at refrigeration temperatures as is the case of Carvalho *et al.* (2004), Miyamoto-Shinohara *et al.*, (2006) and Morgan *et al.* (2006). Also interlaboratory studies, commonly presented as lyophilized cultures, come with instructions to storage the lyophilized microbial cultures between 2ºC and 8ºC (*Standard scheme*, 2015).

Most likely, the fact that lyophilized cultures were storage at room temperature explains the accentuated decline in viability, up until 6 Log.

On the other hand, comparing the three lyophilizations it is possible to note:

- in the first lyophilization, cell concentration decreased between 6.5 and 5.2 Log, depending on culture media;
- in the second study/lyophilization, both the selective and non-selective culture media registered a decline in biomass of only 2.5 Log;
- the third study, registered a generalized decline of 6 Log.

Given that the three studies, performed in exactly the same conditions, have had so disparate results, reveals some level of imprecision linked to the method.

Turning now the attention to the studies performed with *E. coli*, a gram-negative, it was expected this bacteria had less survivability to lyophilization, when compared to *S. aureus*, a gram-positive (Miyamoto-Shinohara *et al.*, 2006). The expectation was confirmed (Table 7), having been noticed a reduction of 2 Log, comparatively with 0.5 – 1 Log observed with *S. aureus*.

Once again, during storage, there was a clear decrease in cell concentration, mainly in studies 1 and 3, having the counts reached 0 cfu. These results should also be associated with the fact that storage happened at room temperature.

It is also evident that the three lyophilizations, although in the same conditions, produced different results; especially the second study. Evaluating the graphic of Figure 25 and the data of Table 7, becomes clear that the lyophilization method used in this studies is probably still imprecise.

VIABILITY STUDIES

E. coli and S. aureus in skim milk + 10% sucrose

After the first data collection of *S. aureus* stability study, the idea of comparing the surviving ability to lyophilization between a gram-positive, *S. aureus*, and a gram-negative, *E. coli*, emerged. Thus, the viability study of *S. aureus* and *E. coli* with skim milk + 10% sucrose proceeded.

Analyzing Figure 19 it is seen that in the case of *E. coli*, there is a significant difference in cell concentration before lyophilization and after lyophilization having had an average reduction of 1 Log in cell concentration. In the case of *S. aureus*, the difference between before and after lyophilization is not significant when the standard deviation after lyophilization is taken into account.

These results were expected. In the study carried out by Miyamoto-Shinohara *et al.* (2006), a comparison of the survival of different gram-positive and gram-negative species to lyophilization, was conducted. In these experiments, gram-positive species revealed a better capacity of survival to lyophilization by showing more elevated values of cell concentration after lyophilization, when compared with gram-negative species.

The higher survivability of gram-positive is due to the composition of its cell wall (Miyamoto-Shinohara *et al.*, 2006; Tortora, Funke e Case, 2010). The fact that gram-positive cell walls are rich in peptidoglycan layers (Lacasse, 1995; Madigan *et al.*, 2009; Tortora, Funke e Case, 2010), allows them to endure high stress processes such as lyophilization.

A comparative study amongst *E. coli* and *S. aureus* was made, to know if with the cryoprotective media used, skim milk + 10% sucrose, there were any statistically significant differences in the decreasing viability. The results were both surprising and positive as there is no statistically significant differences ($P>0.05$), meaning that skim milk + 10% sucrose might be a very good option as a cryoprotective agent for lyophilization in future experiments with both gram-positive and gram-negative bacteria.

E. coli and S. aureus in nutrient broth no2 + 20% glycerol

Completed the study with *S. aureus* and *E. coli* in skim milk + 10% sucrose, the opportunity to repeat the viability study of *S. aureus* and *E. coli* but this time with NB2 + 20% glycerol as cryoprotective medium, emerged. This cryoprotective agent is used in Controlvet ALS, and other laboratories, to preserve microbial cultures at freezing temperatures.

From Figure 20, it is very clear that with NB2 + 20% glycerol, *S. aureus* viability, but mainly *E. coli*'s, suffers a marked decrease with lyophilization. *S. aureus* concentration declines practically 1 Log while *E. coli* viability decreases approximately 3 Log.

With NB2 + 20% glycerol as a cryoprotective medium, the expected differences between gram-positive and gram-negative bacteria in terms of survival to lyophilization (Miyamoto-Shinohara *et al.*, 2006), become more apparent. This statement gains conviction when the t-test result states that the difference between *E. coli* and *S. aureus* lyophilized with NB2 + 20% glycerol is statistically significant ($P<0.05$).

Comparing skim milk + 10% sucrose with NB2 + 20% glycerol, becomes clear that *E. coli* (Figure 19) has better chances of surviving to lyophilization if skim milk + 10% sucrose is used as cryoprotective medium ($P<0.05$). On the other hand, in the case of *S. aureus* (Figure 20), the differences in viability between the two cryoprotective media are not significant ($P>0.05$), reason why in future, any of the two cryoprotectives could be used.

L. monocytogenes in skim milk + 10% sucrose

In parallel with *E. coli* and *S. aureus* viability study in NB2 + 20% glycerol, a viability study in *L. monocytogenes* with skim milk + 10% sucrose was carried out. The reason for this study lies in the need to compare the results obtained with *S. aureus* (lyophilized with skim milk + 10% sucrose), with another gram-positive bacteria, *L. monocytogenes* (*ID 3*, 2014), a pathogenic species considered to be a risk to public health when limits are exceeded (*Commission Regulation (EC) No 2073*, 2005, *FNES22 (F19)*, 2014).

By lyophilizing, *L. monocytogenes* has not suffered any lost in viability counts. This should be explained, once again, by cell wall composition – abundant in peptidoglycan layers (Lacasse, 1995; Madigan *et al.*, 2009; Tortora, Funke e Case, 2010).

Observing Figure 19, to compare *L. monocytogenes* with *S. aureus* is inevitable once both species belong to the same group of bacteria and both were lyophilized with the same cryoprotective. The differences observed are of no significance ($P>0.05$).

A comparison between *L. monocytogenes* and *E. coli*, both lyophilized with skim milk + 10% sucrose, by way of a t-test concludes that the differences stated between the two bacteria are statistically significant ($P<0.05$). The same assessment among *E. coli* and *S. aureus* dictated that there were no significant differences.

From here one can extrapolate that among the cells lyophilized in skim milk + 10% sucrose, from the gram-positives; *L. monocytogenes* is most probably the one with better or more resistance mechanisms to lyophilization, and amongst gram-positives and gram-negatives, the latest are the least resistant to the lyophilization process.

E. coli and Salmonella Typhimurium in sucrose 10%

Concomitantly with the viability studies of *E. coli* and *S. aureus* in NB2 + 20% glycerol and the study of *L. monocytogenes* in skim milk + 10% sucrose, two more studies were performed with a solution of sucrose at 10% as protective media and *E. coli* and *Salmonella* Typhimurium as objects of study.

The constant mention in literature of the use of sugars as protective agents in lyophilization (Carvalho *et al.*, 2004; Costa *et al.*, 2000; Hubálek, 2003; Leslie *et al.*, 1995; Morgan *et al.*, 2006) raised the interest in experimenting a sugar solution as cryoprotective media in a lyophilization. Sucrose 10% choice, in particular, was inspired by the results obtained by Costa *et al.* (2000).

The choice of the bacteria was based in the raw results of *E. coli*'s viability study with skim milk + 10% sucrose. The apparent worse survivability of *E. coli* in skim milk + 10% sucrose was essential for the decision to test the new protective media only in gram-negative species. The additional choice of *Salmonella* Typhimurium relates to the fact that this is a highly pathogenic bacteria, considered to be a hazard to public health

(*Commission Regulation (EC) No 2073*, 2005, *FNES16 (F13)*, 2014) reason why its control at the level of food safety needs to be very strict (*Commission Regulation (EC) No 2073*, 2005).

Assessing Figure 21, after lyophilization with sucrose 10%, *E. coli* suffers a decrease in its cell concentration of 2.5 Log. *Salmonella* Typhimurium viability, on the other hand, decreases approximately 1 Log. An immediate comparison between the two species allows reaching the conclusion that although both *E. coli* and *Salmonella* Typhimurium are gram-negative bacteria, the latest seems to resist better to lyophilization. This information is confirmed by the result of the t-test, giving to the differences demonstrated between *E. coli* and *Salmonella* Typhimurium a statistical significance ($P<0.05$).

At last, the comparison of the results of *E. coli* lyophilized with sucrose 10% with the results of *E. coli* lyophilized in skim milk + 10% sucrose and in NB2 + 20% glycerol is inevitable (Figure 22). From the three protective agents, skim milk + 10% sucrose is definitely the one that allows less lost in *E. coli* viability when the bacteria is lyophilized and this may be due to the ability of the proteins contained in skim milk to form a protective coat around the cells (Carvalho *et al.*, 2004), helping skim milk + 10% sucrose to become the most efficient cryoprotective media (Hubálek, 2003; Morgan *et al.*, 2006); it also helps the fact that sugars such as sucrose, have the ability to preserve protein structure, and consequently, their function, enabling the proteins present in skim milk to create the protective coat.

CONCLUSION

This project aims to initiate the development of a protocol for the production of lyophilized reference stock cultures. This work, is based on *ISO 11133*, 2014 that states that performance tests should be done to every batch of culture media, using specific control microorganisms for every medium, with an adequate inoculum level.

It was first assessed the behavior of *S. aureus*, after lyophilization with skim milk + 10% sucrose, over time (stability study). The cellular concentration was always kept stable, around 6 Log cfu/200 µl, thus giving the first evidence that the lyophilization process truly allows the preservation of microbial cultures over extensive periods of time.

Taking into account the good results obtained with *S. aureus* stability study, the survivability during storage of *E. coli* and *S. aureus* after three separate lyophilizations was evaluated.

After lyophilization and during storage, cultures were left at room temperature, unlike the previous stability study. The weak results obtained allow to reach the conclusion that in the particular case of lyophilized microbial cultures, is essential to storage them at refrigeration temperatures, to guarantee their viability.

Be emphasized that different results were noted amongst the different lyophilizations, although the exact same conditions were applied. A possible explanation for these unexpected results relates with the errors associated with the method and technology.

In the impossibility to evaluate cultures stability over time, it can be highlighted the survivability of *S. aureus* to lyophilization, comparatively with *E. coli*, revealing losses in cell concentration around 1 Log, while *E. coli* had a loss in biomass rounding 2 Log. These results are easily explained by the cell wall composition of both bacteria.

To know the survivability of different bacteria to lyophilization, some variables such as protective media or bacterial species were played with, and multiple viability studies were done. From those studies, it was possible to draw some interesting conclusions.

With cryoprotective media NB2 + 20% glycerol, it became clear what already had been mentioned in literature: there are significant differences when lyophilizating gram-positive and gram-negative cells. Because of cell wall composition poor in peptidoglycan, gram-negative cells are not as resistant to lyophilization, suffering heavy losses in their viability, as seen in this study.

From NB2 + 20% glycerol viability studies, one can draw another conclusion. NB2 + 20% glycerol, as protective medium, is very weak. This can be stated when a comparison with other cryoprotective media is made.

The solution of sucrose at 10% was applied as cryoprotective to the gram-negative bacteria, *E. coli* and *Salmonella* Typhimurium. Although both bacteria are from the same group, *Salmonella* Typhimurium has demonstrated a better capacity to resist lyophilization, which should be due to the presence of more cellular protection mechanisms.

E. coli, *S. aureus* and *L. monocytogenes* were lyophilized with skim milk + 10% sucrose. As expected, *E. coli* was the one with most cell concentration loss; while the gram-positive bacteria lyophilization was more successful.

L. monocytogenes compared with *S. aureus*, had a virtually null viability decrease, what should be justified, once again, by the presence of different resistance mechanisms, probably absent in *S. aureus*.

When doing a comparison between all three the protective media used in this work, skim milk + 10% sucrose is definitely the one elected the best cryoprotective media.

In a future continuation of this work, new viability studies could be performed to new microorganisms, using skim milk + 10% sucrose as protective medium; as well as, new stability studies should be done to *L. monocytogenes* and *Salmonella* Typhimurium. Given the contradiction of results due to the imprecision in the method and technology, new stability studies should be performed with *S. aureus* and *E. coli* in order to verify the viability of this project.

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ANNEXES

ANNEX I, COMMISSION REGULATION (EC) NO 2073/2005

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ANNEX I

Microbiological criteria for foodstuffs

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Chapter 1. Food safety criteria

Food category	Micro-organisms/their toxins, metabolites	Sampling-plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies
		n	c	m	M		
1.1. Ready-to-eat foods intended for infants and ready-to-eat foods for special medical purposes (4)	*Listeria monocytogenes*	10	0	Absence in 25 g		EN/ISO 11290-1	Products placed on the market during their shelf-life
1.2. Ready-to-eat foods able to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes	*Listeria monocytogenes*	5	0	100 cfu/g (5)		EN/ISO 11290-2 (6)	Products placed on the market during their shelf-life
		5	0	Absence in 25 g (7)		EN/ISO 11290-1	Before the food has left the immediate control of the food business operator, who has produced it
1.3. Ready-to-eat foods unable to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes (4) (8)	*Listeria monocytogenes*	5	0	100 cfu/g		EN/ISO 11290-2 (6)	Products placed on the market during their shelf-life
1.4. Minced meat and meat preparations intended to be eaten raw	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.5. Minced meat and meat preparations made from poultry meat intended to be eaten cooked	*Salmonella*	5	0	From 1.1.2006 Absence in 10 g From 1.1.2010 Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.6. Minced meat and meat preparations made from other species than poultry intended to be eaten cooked	*Salmonella*	5	0	Absence in 10 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.7. Mechanically separated meat (MSM) (9)	*Salmonella*	5	0	Absence in 10 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.8. Meat products intended to be eaten raw, excluding products where the manufacturing process or the composition of the product will eliminate the salmonella risk	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life

Food category	Micro-organisms/their toxins, metabolites	Sampling-plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies
		n	c	m	M		
1.9. Meat products made from poultry meat intended to be eaten cooked	*Salmonella*	5	0	From 1.1.2006 Absence in 10 g From 1.1.2010 Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.10. Gelatine and collagen	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.11. Cheeses, butter and cream made from raw milk or milk that has undergone a lower heat treatment than pasteurisation (10)	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.12. Milk powder and whey powder (10)	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.13. Ice cream (11), excluding products where the manufacturing process or the composition of the product will eliminate the salmonella risk	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.14. Egg products, excluding products where the manufacturing process or the composition of the product will eliminate the salmonella risk	*Salmonella*	5	0	Absence in 25g		EN/ISO 6579	Products placed on the market during their shelf-life
1.15. Ready-to-eat foods containing raw egg, excluding products where the manufacturing process or the composition of the product will eliminate the salmonella risk	*Salmonella*	5	0	Absence in 25 g or ml		EN/ISO 6579	Products placed on the market during their shelf-life
1.16. Cooked crustaceans and molluscan shellfish	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.17. Live bivalve molluscs and live echinoderms, tunicates and gastropods	*Salmonella*	5	0	Absence in 25g		EN/ISO 6579	Products placed on the market during their shelf-life

Food category	Micro-organisms/their toxins, metabolites	Sampling-plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies
		n	c	m	M		
1.18. Sprouted seeds (ready-to-eat) (12)	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.19. Pre-cut fruit and vegetables (ready-to-eat)	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.20. Unpasteurised fruit and vegetable juices (ready-to-eat)	*Salmonella*	5	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.21. Cheeses, milk powder and whey powder, as referred to in the coagulase-positive staphylococci criteria in Chapter 2.2 of this Annex	Staphylococcal entero-toxins	5	0	Not detected in 25g		European screening method of the CRL for Milk (13)	Products placed on the market during their shelf-life
1.22. Dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age, as referred to in the Enterobacter-iaceae criterion in Chapter 2.2 of this Annex	*Salmonella*	30	0	Absence in 25 g		EN/ISO 6579	Products placed on the market during their shelf-life
1.23. Dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age, as referred to in the Enterobacter-iaceae criterion in Chapter 2.2 of this Annex	*Enterobacter sakazakii*	30	0	Absence in 10 g		ISO/DTS 22964	Products placed on the market during their shelf-life
1.24. Live bivalve molluscs and live echino-derms, tunicates and gastropods	*E.coli* (14)	1 (15)	0	230 MPN/100g of flesh and intra-valvular liquid		ISO TS 16649-3	Products placed on the market during their shelf-life
1.25. Fishery products from fish species asso-ciated with a high amount of histidine (16)	Histamine	9 (17)	2	100 mg/kg	200 mg/kg	HPLC (18)	Products placed on the market during their shelf-life

Food category	Micro-organisms/their toxins, metabolites	Sampling-plan [1]		Limits [2]		Analytical reference method [3]	Stage where the criterion applies
		n	c	m	M		
1.26. Fishery products which have undergone enzyme maturation treatment in brine, manufactured from fish species associated with a high amount of histidine [16]	Histamine	9	2	200 mg/kg	400 mg/kg	HPLC [18]	Products placed on the market during their shelf-life

[1] n = number of units comprising the sample; c = number of sample units giving values over m or between m and M.

[2] For points 1.1-1.24 m=M.

[3] The most recent edition of the standard shall be used.

[4] Regular testing against the criterion is not useful in normal circumstances for the following ready-to-eat foods:

- those which have received heat treatment or other processing effective to eliminate *L. monocytogenes*, when recontamination is not possible after this treatment (e.g. products heat treated in their final package),
- fresh, uncut and unprocessed vegetables and fruits, excluding sprouted seeds,
- bread, biscuits and similar products,
- bottled or packed waters, soft drinks, beer, cider, wine, spirits and similar products,
- sugar, honey and confectionery, including cocoa and chocolate products,
- live bivalve molluscs.

[5] This criterion applies if the manufacturer is able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit 100 cfu/g throughout the shelf-life. The operator may fix intermediate limits during the process that should be low enough to guarantee that the limit of 100 cfu/g is not exceeded at the end of the shelf-life.

[6] 1 ml of inoculum is plated on a Petri dish of 140 mm diameter or on three Petri dishes of 90 mm diameter.

[7] This criterion applies to products before they have left the immediate control of the producing food business operator, when he is not able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit of 100 cfu/g throughout the shelf-life.

[8] Products with pH ≤ 4,4 or a_w ≤ 0,92, products with pH ≤ 5,0 and a_w ≤ 0,94, products with a shelf-life of less than five days are automatically considered to belong to this category. Other categories of products can also belong to this category, subject to scientific justification.

[9] This criterion applies to mechanically separated meat (MSM) produced with the techniques referred to in Chapter III, paragraph 3, in section V of Annex III to Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin.

[10] Excluding products when the manufacturer can demonstrate to the satisfaction of the competent authorities that, due to the ripening time and a_w of the product where appropriate, there is no salmonella risk.

[11] Only ice creams containing milk ingredients.

[12] Preliminary testing of the batch of seeds before starting the sprouting process or the sampling to be carried out at the stage where the highest probability of finding *Salmonella* is expected.

[13] Reference: Hennekinne et al., J. AOAC Internat. Vol. 86, No 2, 2003.

[14] *E. coli* is used here as an indicator of faecal contamination.

[15] A pooled sample comprising a minimum of 10 individual animals.

[16] Particularly fish species of the families: *Scombridae, Clupeidae, Engraulidae, Coryfenidae, Pomatomidae, Scombresosidae.*

[17] Single samples may be taken at retail level. In such a case the presumption laid down in Article 14(6) of Regulation (EC) No 178/2002, according to which the whole batch should be deemed unsafe, shall not apply.

[18] References: 1. Malle P., Valle M., Bouquelet S. Assay of biogenic amines involved in fish decomposition. J. AOAC Internat. 1996, 79, 43-49.
2. Duflos G., Dervin C., Malle P., Bouquelet S. Relevance of matrix effect in determination of biogenic amines in plaice (*Pleuronectes platessa*) and whiting (*Merlangus merlangus*). J. AOAC Internat. 1999, 82, 1097-1101.

Interpretation of the test results

The limits given refer to each sample unit tested, excluding live bivalve molluscs and live echinoderms, tunicates and gastropods in relation to testing *E. coli*, where the limit refers to a pooled sample.

The test results demonstrate the microbiological quality of the batch tested [1].

L. monocytogenes in ready-to-eat foods intended for infants and for special medical purposes:

— satisfactory, if all the values observed indicate the absence of the bacterium,

— unsatisfactory, if the presence of the bacterium is detected in any of the sample units.

L. monocytogenes in ready-to-eat foods able to support the growth of *L. monocytogenes* before the food has left the immediate control of the producing food business operator when he is not able to demonstrate that the product will not exceed the limit of 100 cfu/g throughout the shelf-life:

— satisfactory, if all the values observed indicate the absence of the bacterium,

— unsatisfactory, if the presence of the bacterium is detected in any of the sample units.

L. monocytogenes in other ready-to-eat foods and *E. coli* in live bivalve molluscs:

— satisfactory, if all the values observed are ≤ the limit,

— unsatisfactory, if any of the values are > the limit.

Salmonella in different food categories:

— satisfactory, if all the values observed indicate the absence of the bacterium,

— unsatisfactory, if the presence of the bacterium is detected in any of the sample units.

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[1] The test results can be used also for demonstrating the effectiveness of the HACCP or good hygiene procedure of the process.

Staphylococcal enterotoxins in dairy products:

— satisfactory, if in all the sample units the enterotoxins are not detected,

— unsatisfactory, if the enterotoxins are detected in any of the sample units.

Enterobacter sakazakii in dried infant formulae and dried dietary foods for special medical purposes intended for infants below 6 months of age:

— satisfactory, if all the values observed indicate the absence of the bacterium,

— unsatisfactory, if the presence of the bacterium is detected in any of the sample units.

Histamine in fishery products from fish species associated with a high amount of histidine:

— satisfactory, if the following requirements are fulfilled:

1. the mean value observed is ≤ m
2. a maximum of c/n values observed are between m and M
3. no values observed exceed the limit of M,

— unsatisfactory, if the mean value observed exceeds m or more than c/n values are between m and M or one or more of the values observed are >M.

Chapter 2. Process hygiene criteria

2.1. Meat and products thereof

Food category	Micro-organisms	Sampling plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.1.1. Carcases of cattle, sheep, goats and horses (4)	Aerobic colony count			3,5 log cfu/cm^2 daily mean log	5,0 log cfu/cm^2 daily mean log	ISO 4833	Carcases after dressing but before chilling	Improvements in slaughter hygiene and review of process controls
	Enterobacteriaceae			1,5 log cfu/cm^2 daily mean log	2,5 log cfu/cm^2 daily mean log	ISO 21528-2	Carcases after dressing but before chilling	Improvements in slaughter hygiene and review of process controls
2.1.2. Carcases of pigs (4)	Aerobic colony count			4,0 log cfu/cm^2 daily mean log	5,0 log cfu/cm^2 daily mean log	ISO 4833	Carcases after dressing but before chilling	Improvements in slaughter hygiene and review of process controls
	Enterobacteriaceae			2,0 log cfu/cm^2 daily mean log	3,0 log cfu/cm^2 daily mean log	ISO 21528-2	Carcases after dressing but before chilling	Improvements in slaughter hygiene and review of process controls
2.1.3. Carcases of cattle, sheep, goats and horses	*Salmonella*	50 (5)	2 (6)	Absence in the area tested per carcase		EN/ISO 6579	Carcases after dressing but before chilling	Improvements in slaughter hygiene, review of process controls and of origin of animals
2.1.4. Carcases of pig	*Salmonella*	50 (5)	5 (6)	Absence in the area tested per carcase		EN/ISO 6579	Carcases after dressing but before chilling	Improvements in slaughter hygiene and review of process controls, origin of animals and of the biosecurity measures in the farms of origin
2.1.5. Poultry carcases of broilers and turkeys	*Salmonella*	50 (5)	7 (6)	Absence in 25 g of a pooled sample of neck skin		EN/ISO 6579	Carcases after chilling	Improvements in slaughter hygiene and review of process controls, origin of animals and biosecurity measures in the farms of origin

Food category	Micro-organisms	Sampling plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.1.6. Minced meat	Aerobic colony count (7)	5	2	$5x10^5$ cfu/g	$5x10^6$ cfu/g	ISO 4833	End of the manufacturing process	Improvements in production hygiene and improvements in selection and/or origin of raw materials
	E.coli (8)	5	2	50 cfu/g	500 cfu/g	ISO 16649-1 or 2	End of the manufacturing process	Improvements in production hygiene and improvements in selection and/or origin of raw materials
2.1.7. Mechanically separated meat (MSM) (9)	Aerobic colony count	5	2	$5x10^5$ cfu/g	$5x10^6$ cfu/g	ISO 4833	End of the manufacturing process	Improvements in production hygiene and improvements in selection and/or origin of raw materials
	E.coli (8)	5	2	50 cfu/g	500 cfu/g	ISO 16649-1 or 2	End of the manufacturing process	Improvements in production hygiene and improvements in selection and/or origin of raw materials
2.1.8. Meat preparations	*E.coli* (8)	5	2	500 cfu/g or cm^2	5 000 cfu/g or cm^2	ISO 16649-1 or 2	End of the manufacturing process	Improvements in production hygiene and improvements in selection and/or origin of raw materials

(1) n = number of units comprising the sample; c = number of sample units giving values between m and M.
(2) For points 2.1.3 — 2.1.5 m=M.
(3) The most recent edition of the standard shall be used.
(4) The limits (m and M) apply only to samples taken by the destructive method. The daily mean log is calculated by first taking a log value of each individual test result and then calculating the mean of these log values.
(5) The 50 samples are derived from 10 consecutive sampling sessions in accordance with the sampling rules and frequencies laid down in this Regulation.
(6) The number of samples where the presence of salmonella is detected. The c value is subject to review in order to take into account the progress made in reducing the salmonella prevalence. Member States or regions having low salmonella prevalence may use lower c values even before the review.
(7) This criterion does not apply to minced meat produced at retail level when the shelf-life of the product is less then 24 hours.
(8) *E. coli* is used here as an indicator of faecal contamination.
(9) These criteria apply to mechanically separated meat (MSM) produced with the techniques referred to in Chapter III, paragraph 3, in section V of Annex III of Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin.

Interpretation of the test results

The limits given refer to each sample unit tested, excluding testing of carcases where the limits refer to pooled samples.

The test results demonstrate the microbiological quality of the process tested.

Enterobacteriaceae and aerobic colony count in carcases of cattle, sheep, goats, horses and pigs:

— satisfactory, if the daily mean log is < m,

— acceptable, if the daily mean log is between m and M,

— unsatisfactory, if the daily mean log is >M.

Salmonella in carcases:

— satisfactory, if the presence of *Salmonella* is detected in a maximum of c/n samples,

— unsatisfactory, if the presence of *Salmonella* is detected in more than c/n samples.

After each sampling session, the results of the last ten sampling sessions are assessed in order to obtain the n number of samples.

E. coli and aerobic colony count in minced meat, meat preparations and mechanically separated meat (MSM):

— satisfactory, if all the values observed are < m,

— acceptable, if a maximum of c/n values are between m and M, and the rest of the values observed are < m,

— unsatisfactory, if one or more of the values observed are >M or more than c/n values are between m and M.

2.2. Milk and dairy products

Food category	Micro-organisms	Sampling plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.2.1. Pasteurised milk and other pasteurised liquid dairy products (4)	Enterobacteriaceae	5	2	<1 cfu/ml	5 cfu/ml	ISO 21528-1	End of the manufacturing process	Check on the efficiency of heat- treatment and prevention of recontamination as well as the quality of raw materials
2.2.2. Cheeses made from milk or whey that has undergone heat treatment	*E.coli* (5)	5	2	100 cfu/g	1 000 cfu/g	ISO 16649- 1 or 2	At the time during the manufacturing process when the *E. coli* count is expected to be highest (6)	Improvements in production hygiene and selection of raw materials
2.2.3. Cheeses made from raw milk	Coagulase-positive staphylococci	5	2	10^4 cfu/g	10^5 cfu/g	EN/ISO 6888-2	At the time during the manufacturing process when the number of staphylococci is expected to be highest	Improvements in production hygiene and selection of raw materials. If values >10^5 cfu/g are detected, the cheese batch has to be tested for staphylococcal enterotoxins.
2.2.4. Cheeses made from milk that has undergone a lower heat treatment than pasteurisation (7) and ripened cheeses made from milk or whey that has undergone pasteurisation or a stronger heat treatment (7)	Coagulase-positive staphylococci	5	2	100 cfu/g	1 000 cfu/g	EN/ISO 6888-1 or 2		
2.2.5. Unripened soft cheeses (fresh cheeses) made from milk or whey that has undergone pasteurisation or a stronger heat treatment (7)	Coagulase-positive staphylococci	5	2	10 cfu/g	100 cfu/g	EN/ISO 6888-1 or 2	End of the manufacturing process	Improvements in production hygiene. If values > 10^5 cfu/g are detected, the cheese batch has to be tested for staphylococcal enterotoxins.
2.2.6. Butter and cream made from raw milk or milk that has undergone a lower heat treatment than pasteurisation	*E.coli* (5)	5	2	10 cfu/g	100 cfu/g	ISO 16649- 1 or 2	End of the manufacturing process	Improvements in production hygiene and selection of raw materials

Food category	Micro-organisms	Sampling plan (1)		Limits (2)		Analytical reference method (3)	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.2.7. Milk powder and whey powder (4)	Enterobacteriaceae	5	0	10 cfu/g		ISO 21528- 1	End of the manufacturing process	Check on the efficiency of heat treatment and prevention of recontamination
	Coagulase-positive staphylococci	5	2	10 cfu/g	100 cfu/g	EN/ISO 6888-1 or 2	End of the manufacturing process	Improvements in production hygiene. If values > 10^5 cfu/g are detected, the batch has to be tested for staphylococcal enterotoxins.
2.2.8. Ice cream (8) and frozen dairy desserts	Enterobacteriaceae	5	2	10 cfu/g	100 cfu/g	ISO 21528- 2	End of the manufacturing process	Improvements in production hygiene
2.2.9. Dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age	Enterobacteriaceae	10	0	Absence in 10 g		ISO 21528- 1	End of the manufacturing process	Improvements in production hygiene to minimise contamination. If Enterobacteriaceae are detected in any of the sample units, the batch has to be tested for *E. sakazakii* and *Salmonella*

(1) n = number of units comprising the sample; c = number of sample units giving values between m and M.
(2) For point 2.2.7 m=M.
(3) The most recent edition of the standard shall be used.
(4) The criterion does not apply to products intended for further processing in the food industry.
(5) *E. coli* is used here as an indicator for the level of hygiene.
(6) For cheeses which are not able to support the growth of *E. coli*, the *E. coli* count is usually the highest at the beginning of the ripening period, and for cheeses which are able to support the growth of *E. coli*, it is normally at the end of the ripening period.
(7) Excluding cheeses where the manufacturer can demonstrate, to the satisfaction of the competent authorities, that the product does not pose a risk of staphylococcal enterotoxins.
(8) Only ice creams containing milk ingredients.

Interpretation of the test results

The limits given refer to each sample unit tested.

The test results demonstrate the microbiological quality of the process tested.

Enterobacteriaceae in dried infant formulae and dried dietary foods for special medical purposes intended for infants below six months of age:

— satisfactory, if all the values observed indicate the absence of the bacterium,

— unsatisfactory, if the presence of the bacterium is detected in any of the sample units

E. coli, enterobacteriaceae (other food categories) and coagulase-positive staphylococci:

— satisfactory, if all the values observed are < m,

— acceptable, if a maximum of c/n values are between m and M, and the rest of the values observed are < m,

— unsatisfactory, if one or more of the values observed are >M or more than c/n values are between m and M.

2.3. Egg products

Food category	Micro-organisms	Sampling plan [1]		Limits		Analytical reference method [2]	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.3.1. Egg products	Enterobacteriaceae	5	2	10 cfu/g or ml	100 cfu/g or ml	ISO 21528-2	End of the manufacturing process	Checks on the efficiency of the heat treatment and prevention of recontamination

(1) n = number of units comprising the sample; c = number of sample units giving values between m and M.
(2) The most recent edition of the standard shall be used.

Interpretation of the test results

The limits given refer to each sample unit tested.

The test results demonstrate the microbiological quality of the process tested.

Enterobacteriaceae in egg products:

— satisfactory, if all the values observed are < m,

— acceptable, if a maximum of c/n values are between m and M, and the rest of the values observed are ≤ m,

— unsatisfactory, if one or more of the values observed are >M or more than c/n values are between m and M.

2.4. Fishery products

Food category	Micro-organisms	Sampling plan (¹)		Limits		Analytical reference method (²)	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.4.1. Shelled and shucked products of cooked crustaceans and molluscan shellfish	*E.coli*	5	2	1 cfu/g	10 cfu/g	ISO TS 16649-3	End of the manufacturing process	Improvements in production hygiene
	Coagulase-positive staphylococci	5	2	100 cfu/g	1 000 cfu/g	EN/ISO 6888-1 or 2	End of the manufacturing process	Improvements in production hygiene

(¹) n = number of units comprising the sample; c = number of sample units giving values between m and M.
(²) The most recent edition of the standard shall be used.

Interpretation of the test results

The limits given refer to each sample unit tested.

The test results demonstrate the microbiological quality of the process tested.

E. coli in shelled and shucked products of cooked crustaceans and molluscan shellfish:

— satisfactory, if all the values observed are ≤ m,

— acceptable, if a maximum of c/n values are between m and M, and the rest of the values observed are ≤ m,

— unsatisfactory, if one or more of the values observed are >M or more than c/n values are between m and M.

Coagulase-positive staphylococci in shelled and cooked crustaceans and molluscan shellfish:

— satisfactory, if all the values observed are < m,

— acceptable, if a maximum of c/n values are between m and M, and the rest of the values observed are < m,

— unsatisfactory, if one or more of the values observed are >M or more than c/n values are between m and M.

2.5. Vegetables, fruits and products thereof

Food category	Micro-organisms	Sampling plan [1]		Limits		Analytical reference method [2]	Stage where the criterion applies	Action in case of unsatisfactory results
		n	c	m	M			
2.5.1. Pre-cut fruit and vegetables (ready-to-eat)	*E.coli*	5	2	100 cfu/g	1 000 cfu/g	ISO 16649- 1 or 2	Manufacturing process	Improvements in production hygiene, selection of raw materials
2.5.2. Unpasteurised fruit and vegetable juices (ready-to-eat)	*E.coli*	5	2	100 cfu/g	1 000 cfu/g	ISO 16649- 1 or 2	Manufacturing process	Improvements in production hygiene, selection of raw materials

(1) n = number of units comprising the sample; c = number of sample units giving values between m and M.
(2) The most recent edition of the standard shall be used.

Interpretation of the test results

The limits given refer to each sample unit tested.

The test results demonstrate the microbiological quality of the process tested.

E. coli in pre-cut fruit and vegetables (ready-to-eat) and in unpasteurised fruit and vegetable juices (ready-to-eat):

— satisfactory, if all the values observed are ≤ m,

— acceptable, if a maximum of c/n values are between m and M, and the rest of the values observed are ≤ m,

— unsatisfactory, if one or more of the values observed are >M or more than c/n values are between m and M.

Chapter 3. Rules for sampling and preparation of test samples

3.1. General rules for sampling and preparation of test samples

In the absence of more specific rules on sampling and preparation of test samples, the relevant standards of the ISO (International Organisation for Standardisation) and the guidelines of the Codex Alimentarius shall be used as reference methods.

3.2. Bacteriological sampling in slaughterhouses and at premises producing minced meat and meat preparations

Sampling rules for carcases of cattle, pigs, sheep, goats and horses

The destructive and non-destructive sampling methods, the selection of the sampling sites and the rules for storage and transport of samples are described in standard ISO 17604.

Five carcases shall be sampled at random during each sampling session. Sample sites should be selected taking into account the slaughter technology used in each plant.

When sampling for analyses of enterobacteriaceae and aerobic colony counts, four sites of each carcase shall be sampled. Four tissue samples representing a total of 20 cm^2 shall be obtained by the destructive method. When using the non-destructive method for this purpose, the sampling area shall cover a minimum of 100 cm^2 (50 cm^2 for small ruminant carcases) per sampling site.

When sampling for *Salmonella* analyses, an abrasive sponge sampling method shall be used. The sampling area shall cover a minimum of 100 cm^2 per site selected.

When samples are taken from the different sampling sites on the carcase, they shall be pooled before examination.

Sampling rules for poultry carcases

For the *Salmonella* analyses, a minimum of 15 carcases shall be sampled at random during each sampling session and after chilling. A piece of approximately 10 g from neck skin shall be obtained from each carcase. On each occasion the neck skin samples from three carcases shall be pooled before examination in order to form 5 x 25 g final samples.

Guidelines for sampling

More detailed guidelines on the sampling of carcases, in particular concerning the sampling sites, may be included in the guides to good practice referred to in Article 7 of Regulation (EC) No 852/2004.

Sampling frequencies for carcases, minced meat, meat preparations and mechanically separated meat

However, when justified on the basis of a risk analysis and consequently authorised by the competent authority, small slaughterhouses and establishments producing minced meat and meat preparations in small quantities may be exempted from these sampling frequencies.

In the case of sampling for *Salmonella* analyses of minced meat, meat preparations and carcases, the frequency can be reduced to fortnightly if satisfactory results have been obtained for 30 consecutive weeks. The salmonella sampling frequency may also be reduced if there is a national or regional salmonella control programme in place and if this programme includes testing that replaces the described sampling. The sampling frequency may be further reduced if the national or regional salmonella control programme demonstrates that the salmonella prevalence is low in animals purchased by the slaughterhouse.

As regards the sampling of minced meat and meat preparations for *E. coli* and aerobic colony count analyses and the sampling of carcases for enterobacteriaceae and aerobic colony count analyses, the frequency may be reduced to fortnightly testing if satisfactory results are obtained for six consecutive weeks.

The food business operators of slaughterhouses or establishments producing minced meat, meat preparations or mechanically separated meat shall take samples for microbiological analysis at least once a week. The day of sampling shall be changed each week to ensure that each day of the week is covered.

Annex E
(normative)

Test microorganisms and performance criteria for culture media commonly used in food microbiology

This annex gives information on the culture medium, culture conditions, test microorganisms, culture collection number of test organisms and the expected reactions when performance testing of culture media is carried out.

Specific strains have been selected for testing in order to ensure consistency between laboratories and to facilitate the demonstration of differences between media (batch to batch, between manufacturers). These strains have been fully evaluated to ensure their suitability and consistency in performance.

Where more than one strain is listed for each aspect of performance testing (productivity, selectivity, specificity), the minimum strains to be used have been indicated by the letter b. Commercial or non-commercial suppliers are expected to use additional strains e.g. those shown in Table E.1 to further ensure the quality of the culture media they supply.

Table E.1 has been established taking into account the control strains used in the European Pharmacopoeia (EP) and the recommendations for culture media for food microbiology from the Working Party of the International Committee on Food Microbiology and Hygiene (ICFMH). These criteria shall be included in specific International Standards when prepared or revised in the future. A validated batch of culture medium is one which has shown satisfactory performance. The strain numbers specified in Table E.1 are those from the catalogue of universal strain identifiers compiled by the World Data Centre for Microorganisms (WDCM).[20] This catalogue contains details of the reference strains represented by each WDCM number and contact details of the culture collections. All cited media are described within European and International Standards.

If strain variability is encountered, investigate the effect of the culture medium (e.g. by obtaining the same medium from a different manufacturer), and obtain an additional reference culture from the culture collection in which it was originally deposited. Users are requested to give relevant feedback on strain variability to WG 5, *Culture media*, of ISO/TC 34/SC 9 through the secretariat of ISO/TC 34/SC 9.

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The footnotes used in Table E.1 are the following:

a Full names of media abbreviated terms are given in Table E.2.

b Strains to be used as a minimum.

c Make reference to the reference strain catalogue available on http://www.wfcc.info for information on culture collection strain numbers and contact details.

d Strain free of choice; one of the strains has to be used as a minimum.

e L: liquid medium, S: solid medium, SS: semi-solid medium.

f Growth/turbidity is categorized as: 0 — no growth/no turbidity; 1 — weak growth/slight turbidity; 2 — growth/good turbidity (see 7.4.1.2, 8.4.1).

g *Escherichia coli* WDCM 00013 is given by the specific standard.

h *Escherichia coli* WDCM 00013 is a strong β-d-glucuronidase producer and WDCM 00202 is a weak β-d-glucuronidase producer.

i Some national restrictions and directions may require the use of a different serovar. Make reference to national requirements relating to the choice of *Salmonella* serovars.

j In case of both quantitative and qualitative use for the medium, only results of the quantitative tests are required (see Table E.1).

k More details for quality control of MSRV medium including final concentration of the inoculum and criteria are given in ISO 6579.

l If nutrient agar is used for two or three of these different applications: perform the *Salmonella* growth test as a minimum (if laboratory tests for this organism).

m If BPW is used for two or three of these different applications: perform the *Salmonella* enrichment test as a minimum (if laboratory tests for this organism).

n Choose the strain(s) according to the method for which TSA is used as a reference medium.

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Table E.1 — Test microorganisms and performance criteria for culture media commonly used in food microbiology

Selective media for enumeration of microorganisms											
Media[a]	Type[e]	Microorganism	International Standard	Function	Incubation	Control strain	WDCM number[c]	Reference media	Method of control	Criteria	Characteristic reaction
Agar Listeria according to Ottaviani and Agosti	S	*Listeria monocytogenes*	ISO 11290-2	Productivity	(44 ± 4) h/ (37 ± 1) °C	*Listeria monocytogenes* 4b *Listeria monocytogenes* 1/2a	00021[b] 00109	TSA	Quantitative	$P_R \geq 0,5$	Blue green colonies with opaque halo
				Selectivity		*Escherichia coli*[d] *Enterococcus faecalis*[d]	00012 or 00013 00009 or 00087	—	Qualitative	Total inhibition (0)	—
				Specificity		*Listeria innocua*	00017	—	Qualitative	—	Blue green colonies without opaque halo
Baird-Parker	S	Coagulase-positive staphylococci	ISO 6888-1	Productivity	(24 ± 2) h to (48 ± 2) h/ (37 ± °1) °C	*Staphylococcus aureus*	00034[b] 00032	TSA	Quantitative	$P_R \geq 0,5$	Black or grey colonies with clear halo (egg yolk clearing reaction)
				Selectivity	(48 ± 2) h/ (37 ± 1) °C	*Escherichia coli*[d]	00012 00013	—	Qualitative	Total inhibition (0)	—
				Specificity	(24 ± 2) h to (48 ± 2) h/ (37 ± 1) °C	*Staphylococcus saprophyticus* *Staphylococcus epidermidis*	00159[b] 00036	—	Qualitative	—	Black or grey colonies without egg yolk clearing reaction
BGBLB	L	Coliforms	ISO 4831	Productivity	(24 ± 2) h to (48 ± 2) h/ (30 ± 1) °C	*Escherichia coli* *Citrobacter freundii*	00012[b] 00013 00006	—	Qualitative	Turbidity (2)[f] and gas in Durham tube	Gas production and turbidity
				Selectivity		*Enterococcus faecalis*[d]	00009 00087	—	Qualitative	Partial inhibition without gas production	—
CFC	S	*Pseudomonas spp.*	ISO 13720	Productivity	(44 ± 4) h/ (25 ± 1) °C	*Pseudomonas fluorescens* *Pseudomonas fragi*	00115[b] 00116	TSA	Quantitative	$P_R \geq 0,5$	—
				Selectivity		*Escherichia coli*[d]	00012 00013	—	Qualitative	Total inhibition (0)	—

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Table E.1 *(continued)*

DG18	S	Yeasts and moulds	ISO 21527-2	Productivity	5 d/ (25 ± 1) °C	*Saccharomyces cerevisiae*	00058[b]	SDA	Quantitative	$P_R \geq 0,5$	Characteristic colony/propagules according to each species
						Wallemia sebi	00182[b]				
						Aspergillus restrictus	00183				
						Eurotium rubrum	00184				
				Selectivity		*Escherichia coli*	00012 or 00013[g]	—	Qualitative	No growth	—
						Bacillus subtilis subsp. spizizenii	00003				
DRBC	S	Yeasts and moulds	ISO 21527-1	Productivity	5 days/ (25 ± 1) °C	*Saccharomyces cerevisiae*	00058[b]	SDA	Quantitative	$P_R \geq 0,5$	Characteristic colony/propagules according to each species
						Aspergillus brasiliensis	00053[b]				
						Candida albicans	00054				
						Mucor racemosus	00181				
				Selectivity		*Escherichia coli*	00012 or 00013[g]	—	Qualitative	No growth	—
						Bacillus subtilis subsp. spizizenii	00003				
EC	L	*Escherichia coli*	ISO 7251	Productivity	(24 ± 2) h to (48 ± 2) h/ (44 ± 1) °C	*Escherichia coli*	00012[b] 00013	—	Qualitative	Turbidity (2)[f] and gas in Durham tube	Gas production and turbidity
				Selectivity		*Pseudomonas£ aeruginosa*	00025	—	Qualitative	No growth	—
IS ("TS")	S	Sulfite-reducing bacteria	ISO 15213	Productivity	(24 ± 3) h to (48 ± 2) h/ (37 ± 1) °C anaerobic atmosphere	*Clostridium perfringens*	00007[b] 00080	TSA or other non-selective medium for anaerobes	Quantitative	$P_R \geq 0,5$	Black colonies
				Specificity		*Escherichia coli*[d]	00012 00013	—	Qualitative	—	No blackening

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Table E.1 *(continued)*

LST	L	Coliforms	ISO 4831	Productivity	(24 ± 2) h to (48 ± 2) h/ (30 ± 1) °C	*Escherichia coli*	00012[b] 00013	—	Qualitative	Turbidity (2)[f] and gas in Durham tube	Gas production and turbidity
						Citrobacter freundii	00006				
				Selectivity		*Enterococcus faecalis*[d]	00009 00087	—	Qualitative	No growth	—
		Escherichia coli	ISO 7251	Productivity	(24 ± 2) h to (48 ± 2) h/ (37 ± 1) °C	*Escherichia coli*	00012[b] 00013	—	Qualitative	Turbidity (2)[fi] and gas in Durham tube	Gas production and turbidity
				Selectivity		*Enterococcus faecalis*[d]	00009 00087	—	Qualitative	No growth	—
mCCDA	S	*Campylobacter*	ISO 10272-2	Productivity	(44 ± 4) h/ (41,5 ± 1) °C microaerobic atmosphere	*Campylobacter jejuni*[d] *Campylobacter coli*[d]	00156 00005 00004	Blood agar	Quantitative	$P_R \geq 0,5$	Greyish, flat and moist, sometimes with metallic sheen
				Selectivity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	—	—
						Staphylococcus aureus	00034	—	Qualitative	—	—
MRS	S	Lactic acid bacteria	ISO 15214	Productivity	(72 ± 3) h/ (30 ± 1) °C	*Lactobacillus sakei*	00015[b]	Media batch MRS already validated	Quantitative	$P_R \geq 0,7$	Characteristic colonies according to each species
						Lactococcus lactis	00016[b]				
						Pediococcus pentosaceus	00158				
				Selectivity	(72 ± 3) h/ (30 ± 1) °C	*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total inhibition (0)	—
						Bacillus cereus	00001				
MYP	S	*Bacillus cereus*	ISO 7932	Productivity	(24 ± 3) h to (44 ± 4) h/ (30 ± 1) °C	*Bacillus cereus*	00001	TSA	Quantitative	$P_R \geq 0,5$	Pink colonies with precipitation halo
				Selectivity	(44 ± 4) h/ (30 ± 1) °C	*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total inhibition (0)	—
				Specificity		*Bacillus subtilis subsp. spizizenii*	00003	—	Qualitative	—	Yellow colonies without precipitation halo

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Table E.1 *(continued)*

RPFA	S	Coagulase-positive staphylococci	ISO 6888-2	Productivity	(24 ± 2) h to (48 ± 2) h/ (37 ± 1) °C	*Staphylococcus aureus*	00034[b] 00032	TSA	Quantitative	$P_R \geq 0,5$	Black or grey colonies with opacity halo
				Selectivity	(48 ± 2) h/ (37 ± 1) °C	*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total inhibition (0)	—
				Specificity	(24 ± 2) h to (48 ± 2) h/ (37 ± 1) °C	*Staphylococcus saprophyticus* *Staphylococcus epidermidis*	00159[b] 00036	—	Qualitative	—	Black or grey colonies without opacity halo
PPA	S	*Pseudomonas spp.*	ISO/ TS 11059	Productivity	(48 ± 2) h/ (25 ± 1) °C	*Pseudomonas fluorescens* *Pseudomonas aeruginosa*	00115[b] 00025	TSA	Quantitative	$P_R \geq 0,5$	—
				Selectivity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total inhibition (0)	—
TBX	S	β-D-Glucuronidase-positive *Escherichia coli*	ISO 16649-1 and ISO 16649-2	Productivity	(21 ± 3) h/ (44 ± 1) °C	*Escherichia coli*[h]	00012[d] 00013[d] 00202[b]	TSA	Quantitative	$P_R \geq 0,5$	Blue colonies
				Selectivity		*Enterococcus faecalis*[d]	00009 00087		Qualitative	Total inhibition (0)	—
				Specificity		*Citrobacter freundii* *Pseudomonas aeruginosa*	00006[b] 00025		Qualitative	—	White to green-beige colonies
TSC (SC)	S	*Clostridium perfringens*	ISO 7937	Productivity	(20 ± 2) h/ (37 ± 1) °C anaerobic atmosphere	*Clostridium perfringens*	00007[b] 00080	TSA or other non-selective medium for anaerobes	Quantitative	$P_R \geq 0,5$	Black colonies
				Selectivity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total inhibition (0)	—
						Pseudomonas aeruginosa	00025				
VRBG	S	*Enterobacteriaceae*	ISO 21528-2	Productivity	(24 ± 2) h/ (37 ± 1) °C	*Escherichia coli* *Salmonella* Typhimurium[d,j] *Salmonella* Enteritidis[d,j]	00012[b] 00013 00031 00030	TSA	Quantitative	$P_R \geq 0,5$	Pink to red colonies with or without precipitation halo
				Selectivity		*Enterococcus faecalis*[d]	00009 00087	—	Qualitative	Total inhibition (0)	—

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Table E.1 *(continued)*

VRBL	S	Coliforms	ISO 4832	Productiv-ity	(24 ± 2) h/ (30 ± 1) °C	*Escherichia coli*	00012[b] 00013	TSA	Quantita-tive	$P_R \geq 0,5$	Purplish-red colonies with or without precipitation halo
				Selectivity		*Enterococcus faecalis*[d]	00009 00087	—	Qualitative	Total inhibi-tion (0)	—
				Specificity		*Pseudomonas aeruginosa*	00025		Qualitative	—	Colourless to beige colonies
Non-selective media for enumeration of microorganisms											
Media[a]	**Type[e]**	**Micro-organisms**	**Interna-tional Standard**	**Function**	**Incubation**	**Control strains**	**WDCM num-bers[c]**	**Reference media**	**Method of control**	**Criteria**	**Characteristic reactions**
PCA MPCA	S	Colony count	ISO 4833	Productiv-ity	(72 ± 3) h/ (30 ± 1) °C	*Bacillus subtilis subsp. spizizenii*	00003[b]	TSA	Quantita-tive	$P_R \geq 0,7$	—
						Escherichia coli	00012[b] 00013				
						Staphylococcus aureus	00034				
Selective enrichment media											
Media[a]	**Type[e]**	**Micro-organisms**	**Interna-tional Standard**	**Function**	**Incubation**	**Control strains**	**WDCM num-bers[c]**	**Reference media**	**Method of control**	**Criteria**	**Characteristic reactions of tar-get microorganism**
Bolton	L	*Campylobacter*	ISO 10272-1	Productiv-ity	(5 ± 1) h/ (37 ± 1) °C then (44 ± 4) h/ (41,5 ± 1) °C microaerobic atmosphere	*Campylobacter jejuni*[d] *Campylobacter coli*	00156 or 00005 00004	—	Qualitative	> 10 colonies on mCCDA	Greyish, flat and moist, sometimes with metallic sheen
						+ *Escherichia coli*[d]	00012 00013				
						+ *Proteus mirabilis*	00023				
				Selectivity		*Escherichia coli*[d]	00012 00013	—	Qualitative	Total inhibi-tion (0) on TSA	—
						Proteus mirabilis	00023				

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Table E.1 *(continued)*

EE	L	*Enterobacteriaceae*	ISO 21528-1	Productivity	(24 ± 2) h/ (37 ± 1) °C	*Escherichia coli*	00012[b] 00013	—	Qualitative	> 10 colonies on VRBG	Pink to red colonies with or without precipitation halo
						+ *Enterococcus faecalis*[d]	00009 or 00087				
						Salmonella Typhimurium[i] *Salmonella* Enteritidis[i]	00031 or 00030				
						+ *Enterococcus faecalis*[d]	00009 or 00087				
				Selectivity		*Enterococcus faecalis*[d]	00009 or 00087	—	Qualitative	Total inhibition (0) on TSA	—
Fraser	L	*Listeria monocytogenes*	ISO 11290-1	Productivity	(48 ± 2) h/ (37 ± 1) °C	*Listeria monocytogenes* 4b	00021[b]	—	Qualitative	> 10 colonies on Agar Listeria according to Ottaviani and Agosti	Blue green colonies with opaque halo
						+ *Escherichia coli*[d]	00012 or 00013				
						+ *Enterococcus faecalis*[d]	00009 or 00087				
						Listeria monocytogenes 1/2a	00109				
						+ *Escherichia coli*[d]	00012 or 00013				
						+ *Enterococcus faecalis*[d]	00009 or 00087				
				Selectivity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total inhibition (0) on TSA	—
						Enterococcus faecalis[d]	00009 or 00087	—	Qualitative	< 100 colonies on TSA	—
Giolitti Cantoni	L	Coagulase-positive staphylococci	ISO 6888-3	Productivity	(24 ± 2) h to (48 ± 2) h/ (37 ± 1) °C	*Staphylococcus aureus*	00034[b]	—	Qualitative	> 10 colonies on Baird Parker or RPFA	Characteristic colonies according to each medium (see ISO 6888-1 for Baird Parker and ISO 6888-2 for RPFA)
						+ *Escherichia coli*[d]	00012 or 00013				
						Staphylococcus aureus	00032				
						+ *Escherichia coli*[d]	00012 or 00013				
				Selectivity	(48 ± 2) h/ (37 ± 1) °C	*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total inhibition (0) on TSA	—

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Table E.1 *(continued)*

Half-Fraser	L	*Listeria monocytogenes*	ISO 11290-1	Productivity	(24 ± 2) h/ (30 ± 1) °C	*Listeria monocytogenes* 4b	00021[b]	—	Qualitative	> 10 colonies on Agar Listeria according to Ottaviani and Agosti	Blue green colonies with opaque halo
						+ *Escherichia coli*[d]	00012 or 00013				
						+ *Enterococcus faecalis*[d]	00009 or 00087				
						Listeria monocytogenes 1/2a	00109				
						+ *Escherichia coli*[d]	00012 or 00013				
						+ *Enterococcus faecalis*[d]	00009 or 00087				
				Selectivity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total inhibition (0) on TSA	—
						Enterococcus faecalis[d]	00009 or 00087	—	Qualitative	< 100 colonies on TSA	—
ITC	L	*Yersinia enterocolitica*	ISO 10273	Productivity	(44 ± 4) h/ (25 ± 1) °C	*Yersinia enterocolitica*	00038[b]	—	Qualitative	> 10 colonies on CIN or SSDC	Characteristic colonies according to each medium (see ISO 10273)
						+ *Escherichia coli*[d]	00012 or 00013				
						+ *Pseudomonas aeruginosa*	00025				
				Selectivity		*Pseudomonas aeruginosa*	00025	—	Qualitative	Total inhibition (0) on TSA	—
						Proteus mirabilis	00023				
MKTTn	L	*Salmonella*	ISO 6579	Productivity	(24 ± 3) h/ (37 ± 1) °C	*Salmonella* Enteritidis[d,i] *Salmonella* Typhimurium[d,i]	00030 00031	—	Qualitative	> 10 colonies on XLD or other medium of choice	Characteristic colonies according to each medium (see ISO 6579)
						+ *Escherichia coli*[d]	00012 or 00013				
						+ *Pseudomonas aeruginosa*	00025				
				Selectivity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Partial inhibition ≤ 100 colonies on TSA	—
						Enterococcus faecalis[d]	00009 or 00087	—	Qualitative	< 10 colonies on TSA	—

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Table E.1 *(continued)*

MSRV[k]	SS	*Salmonella*	ISO 6579	Productivity	2 × (24 ± 3) h/ (41,5 ± 1) °C	*Salmonella* Enteritidis[d,i]	00030	—	Qualitative	Grey-white, turbid zone extending out from inoculated drop(s). After 24–48 h, the turbid zone(s) will be (almost) fully migrated over the plate.	Possible extra: characteristic colonies after subculturing on XLD[k]
						Salmonella Typhimurium[d,i]	00031				
				Selectivity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Possible growth at the place of the inoculated drop(s) without a turbid zone.	—
						Enterococcus faecalis[d]	00009 or 00087	—	Qualitative	No growth	—
MMG	L	β-D-Glucuronidase-positive *E. coli*	ISO 16649-3	Productivity	(24 ± 2) h/ (37 ± 1) °C	*Escherichia coli*	00012[b] 00013	—	Qualitative	Acid production	Colour change to yellow
				Selectivity		*Enterococcus faecalis*[d]	00009 or 00087	—	Qualitative	No growth	—
PSB	L	*Yersinia enterocolitica*	ISO 10273	Productivity	3 to 5 days/ (25 ± 1) °C	*Yersinia enterocolitica*	00038[b]	—	Qualitative	> 10 colonies on CIN or SSDC	Characteristic colonies according to each medium (see ISO 10273)
						+ *Escherichia coli*[d]	00012 or 00013				
						+ *Pseudomonas aeruginosa*	00025				
						Yersinia enterocolitica	00160				
						+ *Escherichia coli*[d]	00012 or 00013				
						+ *Pseudomonas aeruginosa*	00025				
				Selectivity		*Pseudomonas aeruginosa*	00025	—	Qualitative	Total inhibition (0) on TSA	—
						Proteus mirabilis	00023				

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Table E.1 *(continued)*

Media[a]	Type[e]	Micro-organisms	International Standard	Function	Incubation	Control strains	WDCM numbers[c]	Reference media	Method of control	Criteria	Characteristic reactions
RVS	L	*Salmonella*	ISO 6579	Productivity	(24 ± 3) h/ (41,5 ± 1) °C	*Salmonella* Enteritidis[d,i] *Salmonella* Typhimurium[d,i]	00030 00031	—	Qualitative	> 10 colonies on XLD or other medium of choice	Characteristic colonies according to each medium (see ISO 6579)
						+ *Escherichia coli*	00012 or 00013				
						+ *Pseudomonas aeruginosa*	00025				
				Selectivity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Partial inhibition ≤ 100 colonies on TSA	—
						Enterococcus faecalis[d]	00009 or 00087	—	Qualitative	< 10 colonies on TSA	—
TSPB	L	*Bacillus cereus*	ISO 21871	Productivity	(48 ± 4) h/ (30 ± 1) °C	*Bacillus cereus*	00001	—	Qualitative	> 10 colonies on PEMBA or MYP	Characteristic colonies according to each medium (see ISO 21871)
				Selectivity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total inhibition (0) on TSA	—
Non-selective liquid media											
Media[a]	**Type[e]**	**Micro-organisms**	**International Standard**	**Function**	**Incubation**	**Control strains**	**WDCM numbers[c]**	**Reference media**	**Method of control**	**Criteria**	**Characteristic reactions**
BHI	L	Coagulase-positive staphylococci	ISO 6888-1 ISO 6888-3	Productivity	(24 ± 2) h/ (37 ± 1) °C	*Staphylococcus aureus*	00034	—	Qualitative	Turbidity (1–2)[f]	—
Brucella	L	*Campylobacter*	ISO 10272 (all parts)	Productivity	2 to 5 days/ (41,5 ± 1) °C microaerobic atmosphere	*Campylobacter jejuni*[d]	00156 00005	—	Qualitative	Turbidity (1–2)[f]	—
						Campylobacter coli[d]	00004				
Diluents for special purposes e.g. BPW with bromo-cresol-purple	L	Dilution liquids	ISO 6887 (all parts)	Diluent	45 min – 1 h/ 20 °C to 25 °C	*Escherichia coli*[d]	00012 or 00013	TSA	Quantitative	±30 % colonies/ T_0 (±30 % of original count)	—
						Staphylococcus aureus	00034[b]				

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Table E.1 *(continued)*

Quarter-strength Ringer's Peptone solution Peptone-salt Phosphate buffer solution	L	Dilution liquids	ISO 6887 (all parts)	Diluent	45 min – 1 h/ 20 °C to 25 °C	*Escherichia coli*[d] *Staphylococcus aureus*	00012 or 00013 00034[b]	TSA	Quantitative	±30 % colonies/ T_0 (±30 % of original count)	—
Thioglycollate	L	*Clostridium perfringens*	ISO 7937	Productivity	(21 ± 3) h/ (37 ± 1) °C	*Clostridium perfringens*	00007	—	Qualitative	Turbidity (1–2)[f]	—
TSYEB	L	*Listeria monocytogenes*	ISO 11290 (all parts)	Productivity	(21 ± 3) h/ (25 ± 1) °C	*Listeria monocytogenes* 4b *Listeria monocytogenes* 1/2a	00021[b] 00109	—	Qualitative	Turbidity (1–2)[f]	—
Selective isolation media											
Media[a]	**Type**[e]	**Micro-organisms**	**International Standard**	**Function**	**Incubation**	**Control strains**	**WDCM numbers**[c]	**Reference media**	**Method of control**	**Criteria**	**Characteristic reactions**
Agar Listeria according to Ottaviani and Agosti[j]	S	*Listeria monocytogenes*	ISO 11290-1	Productivity	(44 ± 4) h/ (37 ± 1) °C	*Listeria monocytogenes* 4b *Listeria monocytogenes* 1/2a	00021[b] 00109	—	Qualitative	Good growth (2)	Blue green colonies with opaque halo
				Selectivity		*Escherichia coli*[d] *Enterococcus faecalis*[d]	00012 or 00013 00009 or 00087	—	Qualitative	Total inhibition (0)	—
				Specificity		*Listeria innocua*	00017	—	Qualitative	—	Blue green colonies without opaque halo
mCCDA[j]	S	*Campylobacter*	ISO 10272 (all parts)	Productivity	(44 ± 4) h/ (41,5 ± 1) °C microaerobic atmosphere	*Campylobacter jejuni*[d] *Campylobacter coli*[d]	00156 00005 00004	—	Qualitative	Good growth (2)	Greyish, flat and moist, sometimes with metallic sheen
				Selectivity		*Escherichia coli*[d]	000012 or 00013	—	Qualitative	Total or partial inhibition (0 – 1)	No characteristic colonies
						Staphylococcus aureus	00034	—	Qualitative	Total inhibition (0)	—

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Table E.1 *(continued)*

CT-SMAC	S	*Escherichia coli* O157	ISO 16654	Productiv-ity	(21 ± 3) h/ (37 ± 1) °C	*Escherichia coli* O157:H7	00014 (non-toxigenic strain)	—	Qualitative	Good growth (2)	Transparent colonies with a pale yellowish-brown appearance and a diameter ~1 mm
				Selectivity		*Staphylococcus aureus*[d]	00032 or 00034	—	Qualitative	Total inhibi-tion (0)	—
						Escherichia coli[d]	00012 or 00013	—	Qualitative	Partial inhi-bition (1)	Growth of some pink colonies
CIN SSDC	S	*Yersinia entero-colitica*	ISO 10273	Productiv-ity	(21 ± 3) h/ (30 ± 1) °C	*Yersinia enterocolitica*	00038[b] 00160	—	Qualitative	Good growth (2)	Characteristic colonies according to each medium (see ISO 10273)
				Selectivity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total or partial inhi-bition (0 – 1)	No characteristic colonies
						Staphylococcus aureus	00034	—	Qualitative	Total inhibi-tion (0)	—
CPC mCPC	S	*Vibrio spp.* other than *Vibrio parahaemolyt-icus/V. cholerae*	ISO/ TS 21872-2	Productiv-ity	(24 ± 3) h/ (37 ± 1) °C	*Vibrio vulnificus*	00187[b]	—	Qualitative	Good growth (2)	Yellow colonies surrounded by a yellow coloration in the medium
						Vibrio cholerae non-O1/non-O139	00203[b]	—	Qualitative	Good growth (2)	Purple colonies surrounded by a purple coloration in the medium
				Selectivity		*Escherichia coli*[d]	00012 or 00013 or 00090	—	Qualitative	Total inhibi-tion (0)	—
MYP[j]	S	*Bacillus cereus*	ISO 21871	Productiv-ity	(21 ± 3) h to 48 h/ (30 ± 1) °C	*Bacillus cereus*	00001	—	Qualitative	Good growth (2)	Pink colonies with precipitation halo
				Selectivity	(44 ± 4) h/ (30 ± 1) °C	*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total inhibi-tion (0)	—
				Specificity		*Bacillus subtilis subsp. spizizenii*	00003	—	Qualitative	—	Yellow colonies without precipita-tion halo
PEMBA	S	*Bacillus cereus*	ISO 21871	Productiv-ity	(21 ± 3) h to (44 ± 4) h/ (37 ± 1) °C	*Bacillus cereus*	00001	—	Qualitative	Good growth (2)[i]	Turquoise-blue colonies with precipitation halo
				Selectivity	(44 ± 4) h/ (37 ± 1) °C	*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total inhibi-tion (0)	—
				Specificity		*Bacillus subtilis subsp. spizizenii*	00003	—	Qualitative	—	White colonies without precipita-tion halo

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Table E.1 *(continued)*

Media[a]	Type[e]	Micro-organisms	International Standard	Function	Incubation	Control strains	WDCM numbers[c]	Reference media	Method of control	Criteria	Characteristic reactions
SDS	S	*Vibrio spp.* other than *Vibrio parahaemolyticus/V. cholerae*	ISO/ TS 21872-2	Productivity	(24 ± 3) h/ (37 ± 1) °C	*Vibrio vulnificus*	00187[b]	—	Qualitative	Good growth (2)	Purple/green colonies with an opaque halo
						Vibrio cholerae non-O1/non-O139	00203[b]	—	Qualitative	Good growth (2)	Yellow colonies with an opaque halo
				Selectivity		*Escherichia coli*[d]	00012 or 00013 or 00090	—	Qualitative	Total inhibition (0)	—
TBX[j]	S	β-D-Glucuronidase-positive *Escherichia coli*	ISO 16649-3	Productivity	(21 ± 3) h/ (44 ± 1) °C	*Escherichia coli*[h]	00012[d] 00013[d] 00202[b]	—	Qualitative	Good growth (2)	Blue colonies
				Selectivity		*Enterococcus faecalis*[d]	00009 or 00087	—	Qualitative	Total inhibition (0)	—
				Specificity		*Citrobacter freundii* *Pseudomonas aeruginosa*	00006[b] 00025	—	Qualitative	—	White to green-beige colonies
VRBG[j]	S	*Enterobacteriaceae*	ISO 21528-1	Productivity	(24 ± 2) h/ (37 ± 1) °C	*Escherichia coli* *Salmonella* Typhimurium[i] *Salmonella* Typhimurium[i]	00012[b] 00013 00031 or 00032	—	Qualitative	Good growth (2)	Pink to red colonies with or without precipitation halo
				Selectivity		*Enterococcus faecalis*[d]	00009 or 00087	—	Qualitative	Total inhibition (0)	—
XLD	S	*Salmonella*	ISO 6579	Productivity	(24 ± 3) h/ (37 ± 1) °C	*Salmonella* Typhimurium[d,i] *Salmonella* Enteritidis[d,i]	00031 00030	—	Qualitative	Good growth (2)	Colonies with black centre and a lightly transparent zone of reddish colour due to the colour change of the medium
				Selectivity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Growth or partial inhibition (0 – 1)	Yellow colonies
						Enterococcus faecalis[d]	00009 or 00087	—	Qualitative	Total inhibition (0)	—
Non-selective isolation media											

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Table E.1 *(continued)*

Media[a]	Type[e]	Micro-organisms	International Standard	Function	Incubation	Control strains	WDCM numbers[c]	Reference media	Method of control	Criteria	Characteristic reactions
Nutrient agar[l]	S	*Entero-bacteriaceae*	ISO 21528 (all parts)	Productivity	(24 ± 2) h/ (37 ± 1) °C	*Escherichia coli*	00012[b] 00013	—	Qualitative	Good growth (2)	—
		Salmonella	ISO 6579		(24 ± 2) h/ (37 ± 1) °C	*Salmonella* Typhimurium[d,i] *Salmonella* Enteritidis[d,i]	00030 00031				
		Yersinia entero-colitica	ISO 10273		(24 ± 2) h/ (30 ± 1) °C	*Yersinia enterocolitica*	00038[b] 00160				
TSYEA	S	*Listeria mono-cytogenes*	ISO 11290 (all parts)	Productivity	(21 ± 3) h/ (37 ± 1) °C	*Listeria monocytogenes* 4[b] *Listeria monocytogenes* 1/2a	00021b 00109	—	Qualitative	Good growth (2)	—
Multipurpose media											
Media[a]	**Type[e]**	**Micro-organisms**	**International Standard**	**Function**	**Incubation**	**Control strains**	**WDCM numbers[c]**	**Reference media**	**Method of control**	**Criteria**	**Characteristic reactions**
BPW[m]	L	Diluent for all enumerations of microorganisms	ISO 6887 (all parts), ISO 6887-5	Dilution	45 min – 1 h/ 20 °C to 25 °C	*Escherichia coli* *Staphylococcus aureus*	00012[b] 00013 00034[b]	TSA	Quantitative	±30 % colonies/ T_0 (±30 % of original count)	—
		Diluent for *Listeria monocytogenes* enumeration	ISO 11290-2	Dilution	(1 h ± 5°min) / (20 ± 2) °C	*Listeria monocytogenes* 4b *Listeria monocytogenes* 1/2a	00021[b] 00109	TSA	Quantitative	±30 % colonies/ T_0 (±30 % of original count)	—
		Pre-enrichment for *Salmonella* detection	ISO 6579	Productivity	(18 ± 2) h/ (37 ± 1) °C	*Salmonella* Typhimurium[d,i] *Salmonella* Enteritidis[d,i]	00031 00030	—	Qualitative	Turbidity (1–2)[f]	—
		Pre-enrichment for *Entero-bacteriaceae* detection	ISO 21528-1	Productivity	(18 ± 2) h/ (37 ± 1) °C	*Escherichia coli* *Salmonella* Typhimurium[i] *Salmonella* Enteritidis[i]	00012[b] 00013 00031 or 00030	—	Qualitative	Turbidity (1–2)[f]	—
Reference media for enumeration of microorganisms											
Media[a]	**Type[e]**	**Micro-organisms**	**International Standard**	**Function**	**Incubation**	**Control strains**	**WDCM numbers[c]**	**Reference media**	**Method of control**	**Criteria**	**Characteristic reactions**
Blood agar	S	*Campylobacter*	ISO 10272-2	Productivity	(44 ± 4) h/ (41,5 ± 1) °C	*Campylobacter jejuni*[d] *Campylobacter coli*[d]	00156 00005 00004	Media batch blood agar already validated	Quantitative	$P_R \geq 0,7$	—

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Table E.1 *(continued)*

TSA[n]	S	Colony count	—	Productivity	As specified in the method in which TSA is used as reference medium	*Bacillus cereus*	00001	Media batch TSA already validated	Quantitative	$P_R \geq 0{,}7$	Characteristic colony according to each species
						Bacillus subtilis subsp. spizizenii	00003				
						Escherichia coli	00012				
						Escherichia coli O157:H7	00014 (non-toxigenic strain)				
						Listeria monocytogenes 4b	00021				
						Staphylococcus aureus	00034				
SDA	S	Colony count	—	Productivity	As specified in the method in which SDA is used as reference medium	*Saccharomyces cerevisiae*	00058[b]	Media batch SDA already validated	Quantitative	$P_R \geq 0{,}7$	Characteristic colony/propagules/germs according to each species
						Aspergillus brasiliensis					

[a] Full names of media abbreviated terms are given in Table E.2.

[b] Strains to be used as a minimum.

[c] Make reference to the reference strain catalogue available on http://www.wfcc.info for information on culture collection strain numbers and contact details.

[d] Strain free of choice; one of the strains has to be used as a minimum.

[e] L: liquid medium, S: solid medium, SS: semi-solid medium.

[f] Growth/turbidity is categorized as: 0 — no growth/no turbidity; 1 — weak growth/slight turbidity; 2 — growth/good turbidity (see 7.4.1.2, 8.4.1).

[g] *Escherichia coli* WDCM 00013 is given by the specific standard.

[h] *Escherichia coli* WDCM 00013 is a strong β-d-glucuronidase producer and WDCM 00202 is a weak β-d-glucuronidase producer.

[i] Some national restrictions and directions may require the use of a different serovar. Make reference to national requirements relating to the choice of *Salmonella* serovars.

[j] In case of both quantitative and qualitative use for the medium, only results of the quantitative tests are required (see Table E.1).

[k] More details for quality control of MSRV medium including final concentration of the inoculum and criteria are given in ISO 6579.

[l] If nutrient agar is used for two or three of these different applications: perform the *Salmonella* growth test as a minimum (if laboratory tests for this organism).

[m] If BPW is used for two or three of these different applications: perform the *Salmonella* enrichment test as a minimum (if laboratory tests for this organism).

[n] Choose the strain(s) according to the method for which TSA is used as a reference medium.

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Table E.2 — Abbreviated terms for media used in Table E.1

Abbreviated media term	Full name of the media	International Standard
Baird –Parker	Baird-Parker agar	ISO 6888-1
BGBLB	Brilliant green lactose bile broth	ISO 4831
BHI	Brain heart infusion broth	ISO 6888-1 and ISO 6888-3
Bolton	Bolton broth	ISO 10272-1
BPW	Buffered peptone water	ISO 6887 (all parts) ISO 6579 ISO 11290-2 ISO 21528-1
Brucella	Brucella broth	ISO 10272 (all parts)
CFC	Cephalothin fucidin cetrimide agar	ISO 13720
CIN	Cefsulodin, Irgasan novobiocin agar	ISO 10273
CPC	Cellobiose polymyxin B colistin agar	ISO/TS 21872-2
CT-SMAC	Cefixime tellurite sorbitol MacConkey agar	ISO 16654
DG18	Dichloran glycerol agar	ISO 21527-2
DRBC	Dichloran-rose bengal chloramphenicol agar	ISO 21527-1
EC	EC broth	ISO 7251
EE	Buffered brilliant green bile glucose broth	ISO 21528-1
Fraser	Fraser broth	ISO 11290-1
Half-Fraser	Half Fraser broth	ISO 11290-1
IS ("TS")	Iron sulfite agar ("Tryptose sulfite agar")	ISO 15213
ITC	Irgasan, ticarcillin chlorate broth	ISO 10273
LST	Lauryl sulfate broth, lauryl tryptose broth	ISO 4831 and ISO 7251
mCCDA	Modified charcoal cefoperazone deoxycholate agar	ISO 10272 (all parts)
mCPC	Modified cellobiose polymyxin B colistin agar	ISO/TS 21872-2
MKTTn	Muller-Kauffmann tetrathionate novobiocin broth	ISO 6579
MMG	Minerals-modified glutamate medium	ISO 16649-3
MPCA	Plate count agar with skimmed milk/ milk plate count agar	ISO 4833
MRS	MRS medium (de Man, Rogosa and Sharpe)	ISO 15214
MSRV	Modified semi-solid Rappaport-Vassiliadis medium	ISO 6579
MYP	Mannitol egg yolk polymyxin agar	ISO 7932
PCA	Plate count agar	ISO 4833
PEMBA	Polymyxin pyruvate egg yolk mannitol bromothymol blue agar	ISO 21871
PPA	Penicillin and pimaricin agar	ISO/TS 11059
PSB	Peptone, sorbitol and bile salts broth	ISO 10273
RPFA	Rabbit plasma fibrinogen agar	ISO 6888-2

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Table E.2 *(continued)*

Abbreviated media term	Full name of the media	International Standard
RVS	Rappaport-Vassiliadis soya peptone broth	ISO 6579
SDA	Sabouraud dextrose agar	—
SDS	Sodium dodecyl sulfate polymyxin sucrose agar	ISO/TS 21872-2
SSDC	Salmonella Shigella deoxycholate calcium agar	ISO 10273
TBX	Tryptone bile X-glucuronide agar	ISO 16649 (all parts)
TCBS	Thiosulfate citrate bile salts sucrose agar	ISO/TS 21872-1
Thioglycollate	Fluid thioglycollate medium	ISO 7937
TSA	Tryptone soya agar	—
TSC/SC	Sulfite cycloserine agar/ tryptose sulphite cycloserine agar without egg yolk	ISO 7937
TSPB	Tryptone soya polymyxin broth	ISO 21871
TSYEA	Tryptone soya yeast extract agar	ISO 11290 (all parts)
TSYEB	Tryptone soya yeast extract broth	ISO 11290 (all parts)
VRBG	Violet red bile glucose agar	ISO 21528 (all parts)
VRBL	Violet red bile lactose agar	ISO 4832
XLD	Xylose lysine deoxycholate agar	ISO 6579

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ANNEX F, ISO 11133:2014

Annex F
(normative)

Test microorganisms and performance criteria for culture media commonly used in water microbiology

Specific strains have been selected for testing in order to ensure consistency between laboratories and to facilitate the demonstration of differences between media (batch to batch, between manufacturers). The strains specified in Table F.1 have been fully evaluated to ensure their suitability and consistency in performance.

Where more than one strain is listed for each aspect of performance testing (productivity, selectivity, specificity), the minimum strains to be used have been indicated by the letter b. Commercial or non-commercial suppliers are expected to use additional strains e.g. those shown in Table F.1 to further ensure the quality of the culture media they supply.

These criteria shall be included in specific standards when prepared or revised in the future. A validated batch of media is one which has shown satisfactory performance. The strain numbers specified in Table F.1 are those from the catalogue of universal strain identifiers compiled by the World Data Centre for Microorganisms (WDCM).[20] This catalogue contains details of the reference strains represented by each WDCM number and contact details of the culture collections. All cited media are described within EN and ISO standards.

If strain variability is encountered, investigate the effect of the culture medium (e.g. by obtaining the same medium from a different manufacturer), and obtain an additional reference culture from the culture collection in which it was originally deposited. Users are requested to feed back relevant information on strain variability to WG 5, *Culture media*, of ISO/TC 34/SC 9 through the secretariat of ISO/TC 34/SC 9.

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The footnotes used in Table F.1 are the following:

a	Full names of media abbreviated terms are given in Table F.2.

b	Strains to be used as a minimum.

c	Make reference to the reference strain catalogue available on http://www.wfcc.info for information on culture collection strain numbers and contact details.

d	Strain free of choice; one of the strains has to be used as a minimum.

e	L: liquid medium, S: solid medium, SS: semi-solid medium.

f	More details for quality control of *Legionella* media including storage of the control strains are given in ISO 11731.

g	More details for quality control and quality criteria of MUG/EC medium are given in ISO 9308-3:1998, Annex E; selectivity is not specified in the standard.

h	More details for quality control and quality criteria of MUD/SF medium are given in ISO 7899-1:1998, Annex E.

i	Some national restrictions and directions may require the use of a different serovar. Make reference to national requirements relating to the choice of *Salmonella* serovars.

j	Growth/turbidity is categorized as: 0 — no growth/turbidity; 1 — weak growth/turbidity; 2 — good growth/turbidity (see 7.4.1.2, 8.4.1).

k	If BPW is used for two of these different applications: perform the *Salmonella* enrichment test as a minimum (if laboratory tests for this organism).

l	Choose the strain(s) according to the method for which TSA is used as a reference medium.

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Table F.1 — Test microorganisms and performance criteria for culture media commonly used in water microbiology

Selective media for enumeration of microorganisms by comparing with a non-selective reference medium											
Media[a]	Type[e]	Micro-organisms	International Standard	Function	Incubation	Control strains	WDCM numbers[c]	Reference media	Method of control	Criteria	Characteristic reactions
Colilert	L	*Escherichia coli* /coliform bacteria	ISO 9308-2	Productivity	(20 ± 2)[e]h/ (36 ± 2) °C	*Escherichia coli*	00013[b] 00090	TSA	Quantitative	$P_R \geq 0,5$	Yellow colour and fluorescence for *E. coli*
						Klebsiella pneumoniae	00206	TSA	Quantitative	$P_R \geq 0,5$	Yellow colour equal or greater than the comparator for coliform bacteria
				Selectivity		*Pseudomonas aeruginosa*[d]	00207 or 00025	—	Qualitative	Total inhibition (0)	Less yellow than the comparator
GVPC[f]	S	*Legionella*	ISO 11731 and ISO 11731-2	Productivity	2-5 days/ (36 ± 2) °C	*Legionella pneumophila*	00107[b] 00180	BCYE	Quantitative	$P_R \geq 0,5$	White-grey-blue-purple colonies with an entire edge and exhibiting a characteristic ground-glass appearance
					5-10 days/ (36 ± 2) °C	*Legionella anisa*	00106				
				Selectivity	3 days/ (36 ± 2) °C	*Enterococcus faecalis*[d]	00009 or 00087	—	Qualitative	Total inhibition (0)	—
						Pseudomonas aeruginosa[d]	00026 or 00025	—	Qualitative	Total or partial inhibition (0-1)	—
						Escherichia coli[d]	00012 or 00013				
Lactose TTC	S	*Escherichia coli* / coliform bacteria	ISO 9308-1	Productivity	(21 ± 3) h/ (36 ± 2) °C	*Escherichia coli*	00179[b] 00012 00013	TSA	Quantitative	$P_R \geq 0,5$	Yellow colour in the medium under the membrane
						Enterobacter aerogenes	00175				
						Citrobacter freundii	00006				
				Selectivity		*Enterococcus faecalis*[d]	00009 or 00087	—	Qualitative	Total inhibition (0)	—
				Specificity		*Pseudomonas aeruginosa*[d]	00025 or 00026	—	Qualitative	—	Red colonies, blue colour in the medium
mCP	S	*Clostridium perfringens*	Council Directive 98/83/EC	Productivity	(21 ± 3) h/ (44 ± 1) °C anaerobic atmosphere	*Clostridium perfringens*	00007[b] 00080 00174	TSA or other non-selective medium for anaerobes	Quantitative	$P_R \geq 0,5$	Yellow colonies; Phophatase test positive
				Specificity		*Clostridium bifermentans*	00079	—	Qualitative	—	Blue colonies; Phosphatase test negative
				Selectivity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total inhibition (0)	—

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Table F.1 *(continued)*

Media[a]	Type[e]	Micro-organisms	International Standard	Function	Incubation	Control strains	WDCM numbers[c]	Reference media	Method of control	Criteria	Characteristic reactions
Pseudo-monas CN	S	*Pseudomonas aeruginosa*	ISO 16266	Productivity	(44 ± 4) h/ (36 ± 2) °C	*Pseudomonas aeruginosa*	00024[b] 00025 00026	TSA	Quantitative	$P_R \geq 0,5$	Blue-green colonies with fluorescence under UV light (360 ± 20 nm)
				Selectivity		*Escherichia coli*[d] *Enterococcus faecalis*[d]	00012 or 00013 00009 or 00087	—	Qualitative	Total inhibition (0)	—
Slanetz and Bartley	S	Intestinal enterococci	ISO 7899-2	Productivity	(44 ± 4) h/ (36 ± 2) °C	*Enterococcus faecalis* *Enterococcus faecium*[d]	00009[b] 00087 00176 00177 00178	TSA	Quantitative	$P_R \geq 0,5$	Red-maroon-pink colonies
				Selectivity		*Escherichia coli*[d] *Staphylococcus aureus*[d]	00012 or 00013 00032 or 00034	—	Qualitative	Total inhibition (0)	—
Sulfite Iron Tryptose Sulfite (TS)	S	Sulfite-reducing anaerobes (clostridia)	ISO 6461-2	Productivity	(44 ± 4) h/ (37 ± 1) °C anaerobic atmosphere	*Clostridium perfringens*	00007[b] 00080	TSA or Blood agar or other non-selective medium for anaerobes	Quantitative	$P_R \geq 0,5$	Black colonies
				Specificity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	—	No blackening
TSC	S	*Clostridium perfringens*	ISO 14189	Productivity	(21 ± 3) h/ (44 ± 1) °C anaerobic atmosphere	*Clostridium perfringens*	00007[b] 00080 00174	TSA or Blood agar or other non-selective medium for anaerobes	Quantitative	$P_R \geq 0,5$	Black colonies
				Selectivity		*Bacillus subtilis subsp. spizizenii*	00003	—	Qualitative	Total inhibition (0)	—
Selective media for enumeration of microorganisms by comparing with a previously accepted batch (for use in special cases)											

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Table F.1 *(continued)*

Colilert	L	*Escherichia coli* /coliform bacteria	ISO 9308-2	Productivity	(20 ± 2)h/ (36±2) °C	*Escherichia coli*	00013[b] 00090	previously validated batch Colilert	Quantitative	$P_R \geq 0{,}7$	Yellow colour and fluorescence for *E. coli*
						Klebsiella pneumoniae	00206	previously validated batch Colilert	Quantitative	$P_R \geq 0{,}7$	Yellow colour equal or greater than the comparator for coliform bacteria
				Selectivity		*Pseudomonas aeruginosa*[d]	00207 or 00025	—	Qualitative	Total inhibition (0)	Less yellow than the comparator
GVPC[f]	S	*Legionella*	ISO 11731 and ISO 11731-2	Productivity	2-5 days/ (36 ± 2) °C	*Legionella pneumophila*	00107[b] 00180	Media batch GVPC already validated	Quantitative	$P_R \geq 0{,}7$	White-grey-blue-purple colonies with an entire edge and exhibiting a characteristic ground-glass appearance
					5-10 days/ (36 ± 2) °C	*Legionella anisa*	00106				
				Selectivity	3 days/ (36 ± 2) °C	*Enterococcus faecalis*[d]	00009 or 00087	—	Qualitative	Total inhibition (0)	—
						Pseudomonas aeruginosa[d] *Escherichia coli*[d]	00026 or 00025 00012 or 00013	—	Qualitative	Total or partial inhibition (0 - 1)	—
Lactose TTC	S	*Escherichia coli* / coliform bacteria	ISO 9308-1	Productivity	(21 ± 3) h/ (36 ± 2) °C	*Escherichia coli* *Enterobacter aerogenes* *Citrobacter freundii*	00179[b] 00012 00013 00175 00006	Media batch Lactose TTC already validated	Quantitative	$P_R \geq 0{,}7$	Yellow colour in the medium under the membrane
				Selectivity		*Enterococcus faecalis*[d]	00009 or 00087	—	Qualitative	Total inhibition (0)	—
				Specificity		*Pseudomonas aeruginosa*[d]	00025 or 00026	—	Qualitative	—	Red colonies, blue colour in the medium
mCP	S	*Clostridium perfringens*	Council Directive 98/83/EC	Productivity	(21 ± 3) h/ (44 ± 1) °C anaerobic atmosphere	*Clostridium perfringens*	00007[b] 00080 00174	Media batch mCP already validated	Quantitative	$P_R \geq 0{,}7$	Yellow colonies; Phophatase test positive
				Specificity		*Clostridium bifermentans*	00079	—	Qualitative	—	Blue colonies; Phosphatase test negative
				Selectivity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Total inhibition (0)	—

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Table F.1 *(continued)*

Pseudo-monas CN	S	*Pseudomonas aeruginosa*	ISO 16266	Productivity	(44 ± 4) h/ (36 ± 2) °C	*Pseudomonas aeruginosa*	00024[b] 00025 00026	Media batch Pseudomonas CN already validated	Quantitative	$P_R \geq 0,7$	Blue-green colonies with fluorescence under UV light (360 ± 20 nm)
				Selectivity		*Escherichia coli*[d] *Enterococcus faecalis*[d]	00012 or 00013 00009 or 00087	—	Qualitative	Total inhibition (0)	—
Slanetz and Bartley	S	Intestinal enterococci	ISO 7899-2	Productivity	(44 ± 4) h/ (36 ± 2) °C	*Enterococcus faecalis* *Enterococcus faecium*[d]	00009[b] 00087 00176 00177 00178	Media batch Slanetz and Bartley already validated	Quantitative	$P_R \geq 0,7$	Red-maroon-pink colonies
				Selectivity		*Escherichia coli*[d] *Staphylococcus aureus*[d]	00012 or 00013 00032 or 00034	—	Qualitative	Total inhibition (0)	—
Sulfite Iron Tryptose Sulfite (TS)	S	Sulfite-reducing anaerobes (clostridia)	ISO 6461-2	Productivity	(44 ± 4) h/ (37 ± 1) °C anaerobic atmosphere	*Clostridium perfringens*	00007[b] 00080	Media batch Sulfite iron or TS already validated	Quantitative	$P_R \geq 0,7$	Black colonies
				Specificity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	—	No blackening
TSC	S	*Clostridium perfringens*	ISO 14189	Productivity	(21 ± 3) h/ (44 ± 1) °C anaerobic atmosphere	*Clostridium perfringens*	00007[b] 00080 00174	Media batch TSC already validated	Quantitative	$P_R \geq 0,7$	Black colonies
				Selectivity		*Bacillus subtilis subsp. spizizenii*	00003	—	Qualitative	Total inhibition (0)	—
Non-selective media for enumeration of microorganisms											
Media[a]	**Type[e]**	**Micro-organisms**	**International Standard**	**Function**	**Incubation**	**Control strains**	**WDCM numbers[c]**	**Reference media**	**Method of control**	**Criteria**	**Characteristic reactions**
YEA	S	Total flora	ISO 6222	Productivity	(44 ± 4) h/ (36 ± 2) °C	*Escherichia coli*[d] *Bacillus subtilis subsp. spizizenii*	00012 or 00013 00003	Media batch YEA already validated	Quantitative	$P_R \geq 0,7$	—
Selective enrichment media											

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Table F.1 *(continued)*

Media[a]	Type[e]	Micro-organisms	International Standard	Function	Incubation	Control strains	WDCM numbers[c]	Reference media	Method of control	Criteria	Characteristic reactions
Bolton Preston	L	*Campylobacter*	ISO 17995	Productivity	(44 ± 4) h/ (37 ± 1) °C microaerobic atmosphere	*Campylobacter jejuni*[d] *Campylobacter coli*[d] + *Escherichia coli*[d] + *Proteus mirabilis*	00156 00005 00004 00012 or 00013 00023	—	Qualitative	> 10 colonies on mCCDA	Small, flat or convex colonies with a glossy surface
				Selectivity		*Escherichia coli*[d] *Proteus mirabilis*	00012 or 00013 00023	—	Qualitative	Total inhibition on TSA (0)	—
MUG/EC[g]	L	*Escherichia coli* / coliform bacteria	ISO 9308-3	Productivity	48 h/ (44 ± 0,5) °C	*Escherichia coli*	00179	Details for method of control and quality criteria of MUG/EC medium are given in ISO 9308-3:1998, Annex E.			
MUD/SF[h]	L	Intestinal enterococci	ISO 7899-1	Productivity	(44 ± 4) h/ (44 ± 0,5) °C	*Enterococcus faecalis* *Enterococcus hirae* *Enterococcus faecium*	00176 00089 00178	Details for method of control and quality criteria of MUD/SF medium are given in ISO 7899-1:1998, Annex E.			
				Selectivity		*Aerococcus viridans* *Lactococcus lactis* *Staphylococcus epidermidis*	00061 00016 00132				
RVS	L	*Salmonella*	ISO 19250	Productivity	(24 ± 3) h/ (41,5 ± 1) °C	*Salmonella* Enteritidis[d,i] *Salmonella* Typhimurium[d,i] + *Escherichia coli*[d] + *Pseudomonas aeruginosa*	00030 00031 00012 or 00013 00025	—	Qualitative	> 10 colonies on XLD or other medium of choice	Characteristic colonies according to each medium (see standard)
				Selectivity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Partial inhibition ≤ 100 colonies on TSA	—
						Enterococcus faecalis[d]	00009 or 00087	—	Qualitative	< 10 colonies on TSA	—
Non-selective liquid media											

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Table F.1 *(continued)*

Media[a]	Type[e]	Micro-organisms	International Standard	Function	Incubation	Control strains	WDCM numbers[c]	Reference media	Method of control	Criteria	Characteristic reactions
DRCM	L	Sulfite-reducing anaerobes (clostridia)	ISO 6461-1	Productivity	(44 ± 4) h/ (36 ± 1) °C anaerobic atmosphere	*Clostridium perfringens*	00007[b] 00080	—	Qualitative	Turbidity (1-2)[j]	Blackening
				Specificity		*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Turbidity (0-1)[j]	No blackening
Saline salt Peptone diluent Peptone salt solution Ringer's solution (1/4 strength) Phosphate buffer solution	L	Dilution liquids	ISO 8199	Diluent	45 min – 1 h/ 20 °C – 25 °C	*Escherichia coli*[d]	00012 or 00013	TSA	Quantitative	+/- 30 % colonies/ T_0 (+/- 30 % of original count)	—
						Staphylococcus aureus	00034				
Selective isolation media											
Media[a]	**Type[e]**	**Micro-organisms**	**International Standard**	**Function**	**Incubation**	**Control strains**	**WDCM numbers[c]**	**Reference media**	**Method of control**	**Criteria**	**Characteristic reactions**
mCCDA	S	*Campylobacter*	ISO 17995	Productivity	(44 ± 4) h/ (41,5 ± 1) °C microaerobic atmosphere	*Campylobacter jejuni*[d]	00156 or 00005	—	Qualitative	Good growth (2)	Small, flat or convex colonies with a glossy surface
						Campylobacter coli[d]	00004				
				Selectivity		*Escherichia coli*[d]	00012 or 00013 or 00179 or 00090	—	Qualitative	Total or partial inhibition (0–1)	No characteristic colonies
						Staphylococcus aureus[d]	00032 or 00034	—	Qualitative	Total inhibition (0)	—

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Table F.1 *(continued)*

XLD	S	*Salmonella*	ISO 19250	Productivity		*Salmonella* Typhimurium[d,i]	00031	—	Qualitative	Good growth (2)	Colonies with black centre and a lightly transparent zone of reddish colour due to the colour change of the medium
						Salmonella Enteritidis[d,i]	00030				
				Selectivity	(24 ± 3) h/ (36 ± 2) °C	*Escherichia coli*[d]	00012 or 00013	—	Qualitative	Growth or partial inhibition (0–1)	Yellow colonies
						Enterococcus faecalis[d]	00009 or 00087	—	Qualitative	Total inhibition (0)	—
Multipurpose media											
Media[a]	**Type[e]**	**Micro-organisms**	**International Standard**	**Function**	**Incubation**	**Control strains**	**WDCM numbers[c]**	**Reference media**	**Method of control**	**Criteria**	**Characteristic reactions**
BPW[k]	L	Diluent for enumerations of all microorganisms	ISO 6887	Dilution	45 min – 1 h/ 20 °C to 25 °C	*Escherichia coli*[d]	00012 or 00013	TSA	Quantitative	+/- 30 % colonies/T_0 (+/- 30 % of original count)	—
						Staphylococcus aureus	00034				
		Pre-enrichment for *Salmonella* detection	ISO 19250	Productivity	(18 ± 2) h/ (36 ± 2) °C	*Salmonella* Typhimurium[d,i]	00031	—	Qualitative	Turbidity (1-2)[j]	—
						Salmonella Enteritidis[d,i]	00030				
Reference media for enumeration of microorganisms											
Media[a]	**Type[e]**	**Micro-organisms**	**International Standard**	**Function**	**Incubation**	**Control strains**	**WDCM numbers[c]**	**Reference media**	**Method of control**	**Criteria**	**Characteristic reactions**
BCYE	S	Colony count	ISO 11731 and ISO 11731-2	Productivity	2-5 days / (36 ± 2) °C	*Legionella pneumophila*	00107[b]	Media batch BCYE already validated	Quantitative	$P_R \geq 0,7$	White-grey-blue-purple colonies with an entire edge and exhibiting a characteristic ground-glass appearance

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Table F.1 *(continued)*

TSA[l]	S	Colony count	—	Productivity	As specified in the method in which TSA is used as reference medium	*Escherichia coli*[d] *Clostridium perfringens* *Pseudomonas aeruginosa* *Enterococcus faecalis*	00012 00013 00090 00179 00007 00024 00087	Media batch TSA already validated	Quantitative	$P_R \geq 0,7$	Characteristic colony according to each species

[a] Full names of media abbreviated terms are given in Table F.2.

[b] Strains to be used as a minimum.

[c] Make reference to the reference strain catalogue available on http://www.wfcc.info for information on culture collection strain numbers and contact details.

[d] Strain free of choice; one of the strains has to be used as a minimum.

[e] L: liquid medium, S: solid medium, SS: semi-solid medium.

[f] More details for quality control of *Legionella* media including storage of the control strains are given in ISO 11731.

[g] More details for quality control and quality criteria of MUG/EC medium are given in ISO 9308-3:1998, Annex E; selectivity is not specified in the standard.

[h] More details for quality control and quality criteria of MUD/SF medium are given in ISO 7899-1:1998, Annex E.

[i] Some national restrictions and directions may require the use of a different serovar. Make reference to national requirements relating to the choice of *Salmonella* serovars.

[j] Growth/turbidity is categorized as: 0 — no growth/turbidity; 1 — weak growth/turbidity; 2 — good growth/turbidity (see 7.4.1.2, 8.4.1).

[k] If BPW is used for two of these different applications: perform the *Salmonella* enrichment test as a minimum (if laboratory tests for this organism).

[l] Choose the strain(s) according to the method for which TSA is used as a reference medium.

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Table F.2 — Abbreviated terms for media used in Table F.1

Media abbreviated term	Full name of the media	International Standard
BCYE	Buffered charcoal yeast extract agar medium	ISO 11731 and ISO 11731-2
Bolton	Bolton broth	ISO 17995
BPW	Buffered peptone water	ISO 6887 ISO 19250
DRCM	Differential reinforced clostridial medium	ISO 6461-1
GVPC	Buffered charcoal yeast extract agar with glycine, vancomycin, polymyxin B, cycloheximide	ISO 11731 and ISO 11731-2
Lactose TTC	Lactose triphenyltetrazolium chloride agar with sodium heptadecylsulfate	ISO 9308-1
mCCDA	Modified charcoal cefoperazone deoxycholate agar	ISO 17995
mCP	Membrane clostridium perfringens agar	Council Directive 98/83/EC
MUD/SF	4-methylumbelliferyl-α-D glucoside /SF medium	ISO 7899-1
MUG/EC	4-methylumbelliferyl-β-D glucuronide /EC medium	ISO 9308-3
Preston	Preston broth	ISO 17995
Pseudomonas CN	Pseudomonas cetrimide nalidixic acid agar	ISO 16266
RVS	Rappaport-Vassiliadis soya peptone broth	ISO 19250
Slanetz and Bartley	Slanetz and Bartley medium	ISO 7899-2
Sulfite Iron	Iron Sulfite agar	ISO 6461-2
Tryptose Sulfite (TS)	Tryptose sulphite agar	ISO 6461-2
TSA	Tryptone soya agar	—
TSC	Tryptose sulphite cycloserine agar (without egg yolk)	ISO 14189
XLD	Xylose lysine deoxycholate agar	ISO 19250
YEA	Yeast extract agar	ISO 6222

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