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RESEARCH ARTICLE OPEN ACCESS

Comparison of Preservation Methods of *Staphylococcus aureus* and *Escherichia coli* Bacteria

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Abstract

One of the most important problems faced by microbiologists is to preserve bacterial isolates in the best state to study and further diagnosis. The current study aims to provide a summary of experimental results to maintain two species of bacteria alive after being stored by using some additives. This study found that the best temperature to preserve *Staphylococcus aureus* was -20°C for a year, while for *Escherichia coli* it was the same temperature except in using Glycerol (G) 100% and Food oil (FO) methods. The optimum method to preserve *S. aureus* was by using Normal Saline (NS), while Distilled Water (DW) was the optimum method to preserve *E. coli* at temperatures (4, 25 and -20)°C for a year, the phenotypic patterns for examining bacteria were maintained except in NS at 4°C for *S. aureus* after a year ago. Glycerol was used alone at concentrations (100, 50, 30 and 15)%, and another group used G+NS in the same volumes, good results were achieved when it used alone or with NS to preserve bacteria for six months at 4°C except for methods of G100% and (G100% + NS) for examining bacteria. FO has never been used as preservation liquid, it is successful to survive *S. aureus* at -20°C for a year, and when it was added to NS, *E. coli* survived for a year at three temperatures (4, 25 and -20)°C, while *S. aureus* didn't survive for a year when FO+NS method used at room temperature. The precipitation method was used for bacterial suspension, then added the preserving liquid, but the results were not effective compared to the First method.

Keywords: Preservation of bacteria, *Staphylococcus aureus*, *Escherichia coli*, Distilled water, Normal saline, Food oil, Glycerol.

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INTRODUCTION

Preservation of bacteria is very important in the bacteriological laboratory. After obtaining bacterial isolates, they should be stored to protect them from contamination, so that they can be studied, diagnosed and tested later. It is difficult to keep microorganisms alive and pure, without any changes in physiological or genetic status when they are cultured on solid media and the accumulation of substances resulting from microbial activity in culture media. Over time, nutrient cultured media lose the proper moisture for growth, their basic substances run out, and the activity of bacteria decreases[1,2]. In general, mutations occur when bacteria are re-culturing on solid media[3]. Preservation methods are based on the nature of the bacteria, the duration of the storage, and the facilities in the laboratory[4]. Numerous methods have been used to preserve bacteria over the years[5], and the most common and successful cryopreservation and lyophilization[3]. Cryoprotectants such as glycerol and dimethyl sulfoxide (DMSO) are currently used as additives in preserving microbes. These protective materials adsorbed at the surface of microorganisms and envelop the cells, thus providing a cover for the formation of ice crystals over long-term preservation. Glycerol work as membrane permeability and aids glazing by exchanging water in cells and making hydrogen bonds with water molecules for a protective effect[6]. The optimal method to preserve microorganisms has not been clearly defined and it differs from one species to another. The preservation liquid which used includes distilled water, Normal saline, tryptic soy broth, and nutrient broth, and it can all be used with or without cryopreservants[7,8]. We need to develop new strategies aimed at ensuring greater stability and storage of the functions of microorganisms[9], at the same time low cost. The study aimed to find an effective and economical for preserving bacteria by using different temperatures and minimizing space at the time of preservation.

MATERIALS AND METHODS

This study lasted from April / 2018 to June / 2019.

Bacterial isolates

Two pathogenic strains provided from Lab. of Microbiology / College of Veterinary Medicine / University of Mosul were tested, one of them is *S. aureus* as an example of gram-positive bacteria, and the other is *E. coli* as an example of gram-negative bacteria.

Culture method

5 – 7 colonies from each of the two species of bacteria which is tested and grown on Brain Heart Infusion Agar (BHIA) (Lab M. a Neogen company, UK) transferred to a micro-centrifuge tube (Mct.) (Vol. 1.5 ml., Dia. 10.6×39.5 mm. Citotest®, Barcopharma company) containing 1 ml. of preservation liquid[1,8].

Preservation methods

Preservation liquid consists of one of these Substances: Distilled water, Normal saline, and Glycerol and Food oil, Table (1).

The Mct. tubes which contain the preservation liquid were maintained at

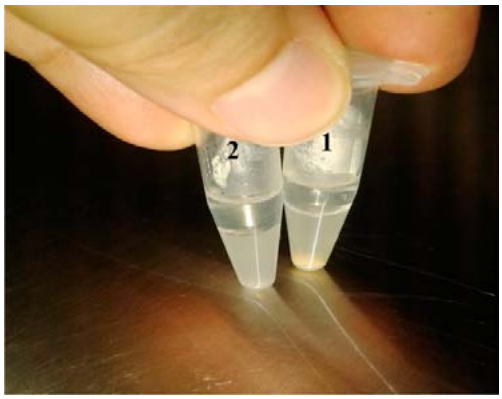

Fig. 1. FO+NS method, 1: *S. aureus*, 2: *E. coli*

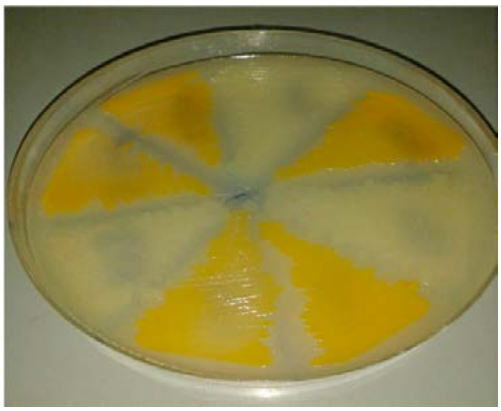

Fig. 2. Golden endo-pigmentation of *S. aureus* on the Milk agar, after one year of preservation

temperatures of 25°C, 4°C (Refrigerator, Arcelik company, Turkey), and -20°C (Freezer, Arcelik company, Turkey). Room temperature ranges from 20 - 30 at rate 25°C.

The preservation methods were also tested by Precipitation, in this method bacteria were cultured in McCartney bottles containing nutrient broth (LAB M. a Neogen company, UK), then distributed into Mct. tubes and precipitated by the centrifuge (NF048, Nuve, Turkey) at 3000 ppm. for 3 minutes, then the glycerol and food oil (Preservation liquid) were added after discarding the supernatant, by using the concentration and volume of glycerol and food oil as in Table (1). Mct. tubes stored at three temperatures (25, 4 and -20)°C.

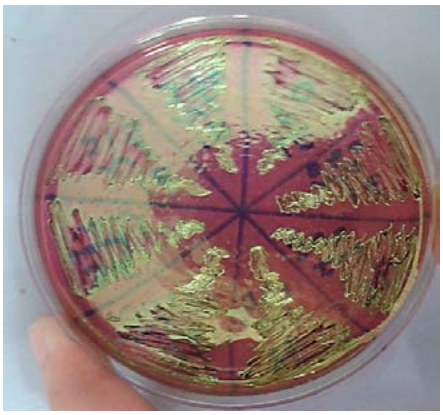

Fig. 3. Metallic sheen of *E. coli* on the EMB medium, after one year of preservation

Viability test

To determine the viability of bacteria, 1 Mct. of preserved bacteria was examined after 1, 2 weeks, 1, 3, 6 and 12 months respectively at three temperatures. Bacteria in a preserving liquid were re-cultured on the BHIA and incubated at 37°C for 24 hours. For testing of phenotypic properties of the preserved bacteria, they were inoculated on Milk agar (MA), which was prepared as nutrient agar and 1.5% skim milk, to detect golden endo-pigmentation produced by *S. aureus*, *E. coli* was cultured on Eosin Methylene Blue (EMB) (Lab M. a Neogen company, UK) to detect the Metallic sheen phenomenon[8,10].

RESULTS

Fig. 4, shows that the best method to maintain the viability of *S. aureus* at 25°C were by

Table 1. The Substances used as preservation liquid

Substance	Abbreviation	Conc. %	Sterilization	Manufacture company
Distilled water	DW	------	Autoclave	------
Normal saline	NS	0.9 (Sodium Chloride)	Autoclave	POLIFARMA, Ergone, Tokirdag, Turkey
Glycerol	G	100 50 30 15	Filtration	Scharlau, Scharlab S.L., Gato, Pereze, Spain
Glycerol with Normal saline	G+NS	Same volumes of them (0.5 ml.) G100+NS G50+NS G30+NS G15+NS	Filtration	------
Food oil	FO	-----	Filtration	Aldar oil, Sun Flower oil, Etihad food industries company, Babylon, Iraq
Food oil with Normal saline	FO+NS	Same volumes of them (0.5 ml.)	Filtration	------

using DW and NS after one year of preservation, while all preservation methods except G100%, G50%, NS+G100% or NS+G50%, maintained these bacteria alive for 3 months at this temperature.

Fig. 5, shows that the best method to maintain the viability of *S. aureus* for a year at 4°C was by using NS+G50% or NS+FO methods, while all preservation methods except FO, G100% and NS+G100%, maintained *S. aureus* alive for 6 months.

The best temperature for maintaining *S. aureus* alive was -20°C for one year, in all preservation methods used in the study, as shown in Fig. 6.

These methods maintained the phenotypic pattern of the endo-pigmentation production (golden pigmentation) and observed after *S. aureus* cultivation in milk agar at 37°C for 24 hours, except by using normal saline alone at 4°C which bacteria survived for a year, but golden pigmentation disappeared in this medium at the end of preservation period, Fig. 2.

Fig. 7, shows that the best method to maintain the viability of *E. coli* at 25°C was by using DW and NS+FO for one year of preservation, while by using all preservation methods except G100% and NS+G100%, maintained these bacteria alive for 3 months at this temperature.

Fig. 8, shows that the best method to maintain the viability of *E. coli* for a year at 4°C by using DW, NS, NS+G50%, NS+G30% or NS+FO methods, while all preservation methods except G100% and NS+G100%, maintained *E. coli* alive for 6 months.

The best temperature for maintaining *E. coli* alive was -20°C in one year, in all preservation methods used in the study except for the FO and G100% methods, bacteria can't grow after one year of preservation at this temperature, as shown in Fig. 9.

All methods used in this study maintained the phenotypic pattern of the metallic sheen production and observed after *E. coli* cultivation in EMB medium, at 37°C for 24 hours, Fig. 3.

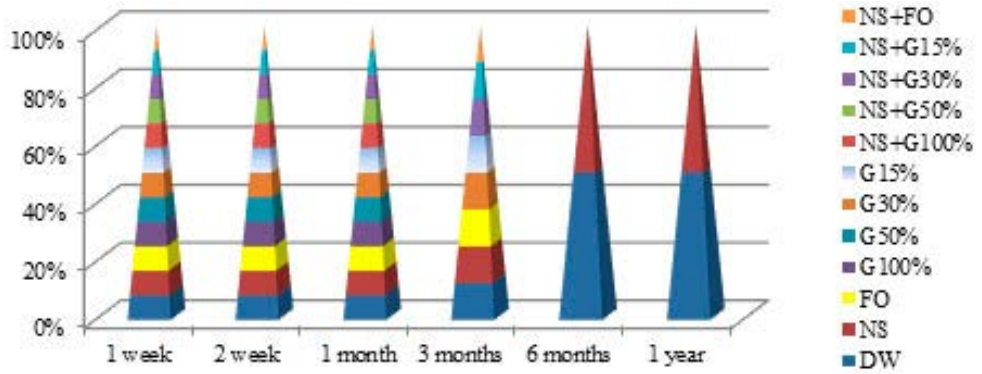

Fig. 4. Viability of *Staphylococcus aureus* at 25°C using different preservation liquid.

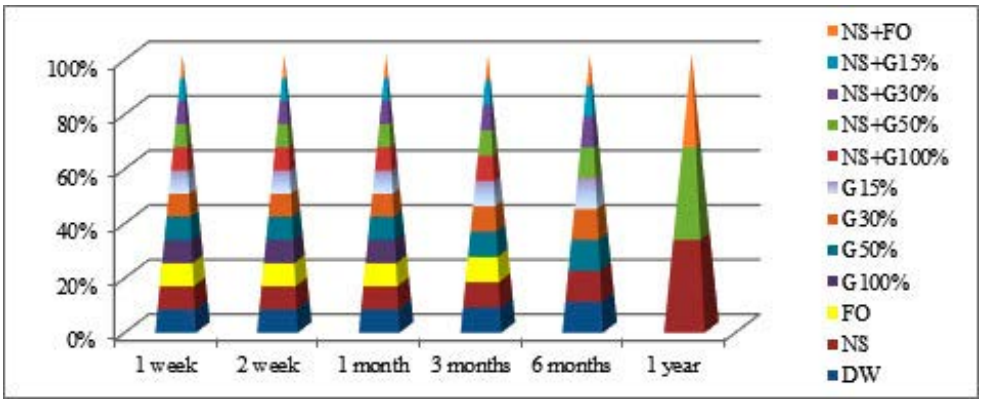

Fig. 5. Viability of *Staphylococcus aureus* at 4°C using different preservation liquid.

In the Precipitation method, *S. aureus* couldn't survive for one year at 25°C in all preservation liquid, used FO alone maintained these bacteria alive for 6 months at this temperature. At 4°C *S. aureus* survived for a year by using FO while using G50% or G15% maintained *S. aureus* alive for 3 months. The optimum preservation method is -20°C by using G30% or G15% or FO methods, the bacteria survived for a year, while by using G100% or G50% maintained *S. aureus* alive for 6 months. In all preservation methods used, *E. coli* was maintained alive for a month at 25°C. At 4°C *E. coli* survived for a year by using G30% or G15%, while using FO maintained *E. coli* alive for 3 months. The best temperature for preservation of *E. coli* by using Precipitation method is -20°C, the bacteria

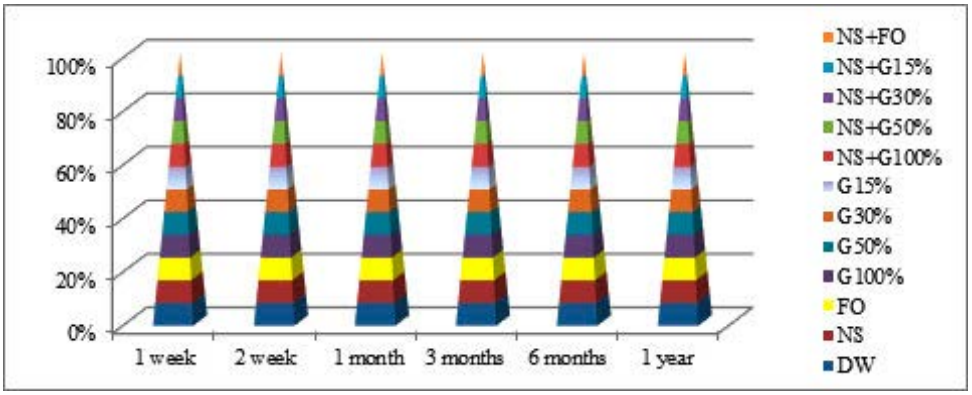

Fig. 6. Viability of *Staphylococcus aureus* at -20°C using different preservation liquid.

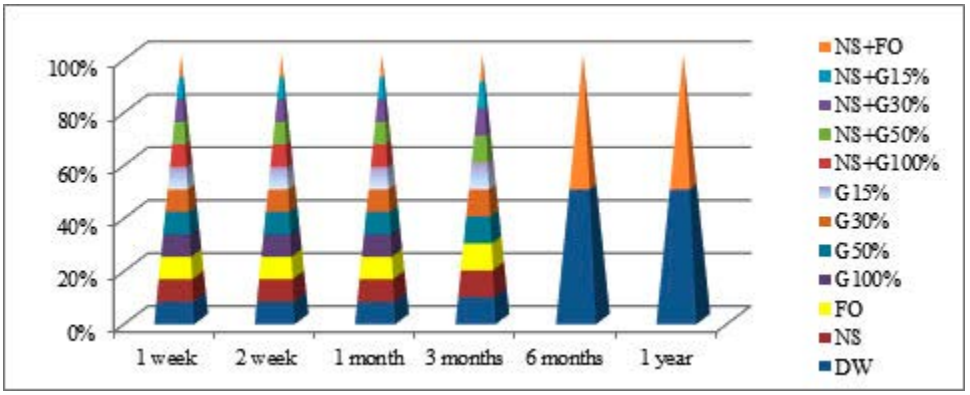

Fig. 7. Viability of *Escherichia coli* at 25°C using different preservation liquid.

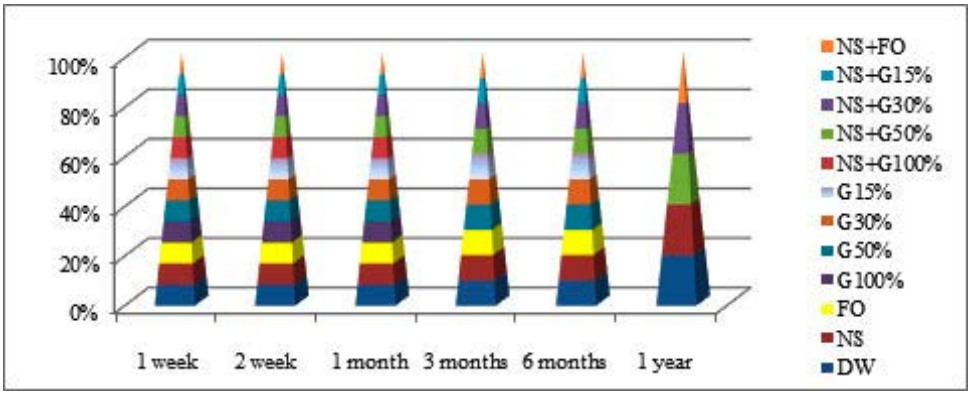

Fig. 8. Viability of *Escherichia coli* at 4°C using different preservation liquid.

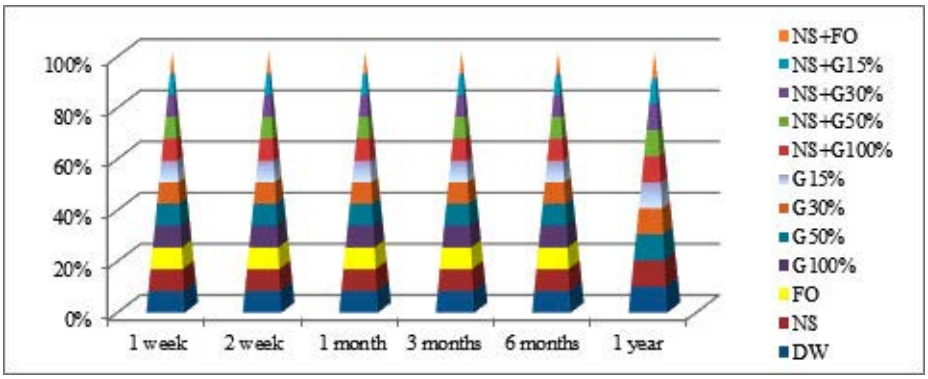

Fig. 9. Viability of *Escherichia coli* at -20**°**C using different preservation liquid.

survived for a year in G30% or G15% or FO, while by using G100% or G50% maintained *E. coli* alive for 6 months.

DISCUSSION

It is important to develop an easy and convenient technique for maintaining the viability, function, morphology and genetic traits of pure isolates to allow maximum restoration after storage[11]. The present study found that the best temperature of preservation for *S. aureus* and *E. coli* was -20°C for a year, in all preservation methods used in our study except G100% and FO methods in *E. coli*, Fig. 6 and 9. To date, cryopreservation is considered the best method for storing bacteria, and the best survival rates are recorded using this method[1]. In the cryopreservation method, bacteria are stored at a frozen temperature to minimize or prevent metabolism and physical change[12].

The current study found that *S. aureus* bacteria survived for one year in normal saline, while *E. coli* survived in sterile DW in the same period at (4, 25 and -20)°C. This is confirmed by researchers from Wyndmoor, PA, USA[8], they were observed that bacteria could remain alive in water and PBS for 2 years at room temperature range between (20-25)°C, and showed that G+ bacteria can be maintained them in PBS better than DW, while the study[4] conducted at University of California, USA too, confirmed that the bacteria they studied could survive in DW more than 20 years. The present study does not agree with researchers[8], that *S. aureus* was unable to give golden endo-pigmentation at 4°C by NS preserving method. This may be due to a lack of continuous supply or fluctuations in electricity during the period under study, due to the city's conditions after liberation. Researchers from the USA (Liao *et al.*) and Australia (Banning *et al.*)[8,13], reported that *E. coli* survived in sterile water and groundwater for several months respectively. This is the conclusion from the current study using DW as a preservative, the tested bacteria survived until the end of the study period. In another study by Ji and Jin of China[14], found that the best option for bacterial preservation, is to use DW alone without any addition at 4°C, which agrees with the present study on *E. coli*, but it is not comparable to our study of *S. aureus*, where it could not live for a year in DW at 4°C, Fig. 5 and 8. Current findings contrast with those of researchers in the last century, where they pointed out that most microorganisms die if suspended with DW, except *Pseudomonas solanacearum*, they remained alive for 10 years in the water at room temperature while dying when stored in the refrigerator[15]. Despite the simplicity of using DW as a preservation liquid, it has not been widely used by microbiologists, due to insufficient data and studies related to it to verify the feasibility of this method or not[8].

The present study is the first to use food oil alone or with normal saline as a preservation liquid for the conservation of bacteria, where no other studies have preceded. The FO+NS proved effectiveness in the preservation of *E. coli* at three temperatures used, Fig. 7-9, but *S. aureus* could not survive at 25°C Fig. (4), while using FO alone preserve *S. aureus* for a year at -20°C, Fig. 6, this may be due to the different composition of the cell wall in G+ve from G-ve bacteria. The use of FO alone or with NS maintained the phenotypic patterns of preserved bacteria. Many studies

successfully used liquid paraffin or mineral oil, which prolongs the microbial growth life, prevents evaporation and reduces the rate of metabolism of the medium by reducing oxygen. This is a simple and economical method for maintaining pure cultures of bacteria. In this method, the oil or paraffin is poured over the slant of culture and stored at room temperature. This condition helps pure culture to remain in a dormant state and the culture can be preserved from months to years[16]. There was a recorded report that Pseudomonas, Bacillus, and *E. coli* remained alive below the oil for 3 years and that their phenotypic characteristics did not change[14]. In a study conducted by Yamasato *et al.*, from Japan[17], they observed that 75-85% of the strains tested, remained viable under liquid paraffin at 10°C for 2 years and at -53°C for 3.5 years.

The researcher classified the Cryoprotectants as penetrating or non-penetrating the cell, Glycerol belongs to the latter one[3]. It is one of the most important types of cell-conserving substances[18], that act to surround the cells, delay the freezing of intracellular substances in these cells and reduce the solution effects[1]. It prevents ice crystallization and as a result, bacteria remain alive longer[3], it is a strong substance that prevents water from entering the cell[19]. Frozen culture is the most commonly used preservation method[20]. We can maintain Microorganisms by freezing broth cultures at -5 to -20°C for 1-2 years in suitable containers[21]. Chen and Jin's study from China[22] indicated that cryopreservation is easier and cheaper compared to lyophilization methods, and if freezing processes are successful, the bacterial mass survives for up to 35 years.

In this study, glycerol was used in many concentrations alone or with NS and found that these concentrations were good for preserving bacteria at -20°C, this does not correlate with the researchers' study from India[3], they may be due to the concentration of glycerol used in their study by 10-15% or without normal saline. An expanded study[17] indicated that the use of a slanted agar method then covered aerobic bacteria growth by 10% glycerol, maintained the bacteria at -53°C for 16 months. In another study[12], the researchers reported that the bacteria kept in 20% glycerol including Staphylococcus that managed to survive for 3 months and there are no changes in biochemical properties, this is the result of the current study when using glycerol by 15% and 30% for the same period at 25°C.

Our study contradicts with Laurin *et al.*, from Canada[23], where they found that the use of 15% glycerol at -20°C was sufficient to keep the bacteria for more than a week, while in this study, glycerol managed to keep the bacteria alive for a year at this temperature except for G100% method for preserving *E. coli*, Fig. 6 and 9.

In this study the Precipitation method was also used, the optimum temperature of preservation in this method is -20°C as well, but at a concentration of G50% and G100% in both *S. aureus* and *E. coli*, the bacteria couldn't survive for a year, the preservation using FO at 4°C gave a result of *S. aureus* and remained alive for a year, while using G30% and G15%, *E. coli* also survived for a year at 4°C.

The difference in the result between the previous method and the Precipitation method in the use of FO or glycerol as a preservative, maybe due to the mechanical deposition of the suspended bacteria and then the oil is added, while in the first method the bacterial inoculation was added to the preservation liquid, so the FO or glycerol surrounding from all direction and maintaining it.

According to the data collected indicate that the preservation liquid consisting of one or both of these substances DW, NS, G, and FO, will provide protection against loss of viability and may increase the long-term preservation when maintained at -20°C.

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FUNDING

None.

DATA AVAILABILITY

All datasets created or investigated during this study are involved in the manuscript and/or the Supplementary Files.

ETHICS STATEMENT

This article does not contain any studies

with human participants or animals performed by the author.

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