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Bacterial growth and competition status of *Escherichia coli* and *Staphylococcus aureus* in different semen media at two temperature degrees

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Abstract

Bacterial growth and competition of (Escherichia coli and Staphylococcus aureus (were assessed in different semen preservation media at two temperature degrees. Five media were prepared (Tris solution, Tris solutions supplemented with: glucose, fructose and sucrose, and Tris solution with egg-yolk. Radiation by gamma ray (1KGy) was used to sterilize these media. The species were incubated at 103 CFU/mL for 3 hours at 4 and 37°C. The storage of the two species in egg yolk medium at 37°C had a clear significant effect on increasing bacterial loads compared to 4°C, whereas it reached approximately 150 and 300% for S.aureus and E.coli respectively. When S.aureus was co-incubated with E.coli in different semen media, a strong ability of S.aureus to dominate E.coli growth was noticed at 4°C. Surprisingly it was completely able to stop E.coli growth at 37°C. It was obvious, that each bacterium is unique and has specific requirements. The competition between E.coli and S.aureus in semen media could be related to the initial load of contamination and the time of incubation but most importantly to the media composition and temperature degree.

Keywords: Semen media, *E.coli*, *S.aureus*, Competition, Gamma radiation

1. Introduction

It is well known that semen media or semen diluents have been used to protect and maintain spermatozoa during semen preservation as well as to increase ejaculate volume [1]. Good semen medium must have nutrient for spermatozoa metabolism; protect sperms from cold shock and also it must preserve membrane integrity, viability and motility of spermatozoa without decline in fertility [2-4]. It should be pointed out that semen media largely differ in their compositions, methods of production, temperature as well as duration of storage [1,5]. In this respect sugars are one of the most important compositions included in these media which were vital for respiration, energy and osmotic balance [6-7]. Glucose and fructose are two monosaccharides commonly found in mammalian seminal, while sucrose is a disaccharide, which is composed by both glucose and fructose [8].

These three sugars types were the most used sugars in different semen media and extenders [9-10]. In addition to sugars, egg-yolk could be added to the based medium for semen storage at ambient temperature or at chilling, where it may increase spermatozoa fertilizing ability and it appears to prevent sperm cell damage during cooling and freezing [11-12].

Many factors may directly affect the quality and the storage of spermatozoa in semen media, being one of the most important factors is the bacterial contamination. Bacterial contamination is a major concern for most semen production laboratories due to its adverse effects on semen quality and consequently on fertility [13]. Such contamination can lead to infertility problems especially if bacteria are present in high concentrations [14].

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Indeed, high levels of bacterial contamination are associated with a high incidence of sperm-to-sperm agglutination, damaged acrosomes, poor sperm motility and less rate of viability [15-16], thus having a reduced shelf life of the diluted semen [14].

The most frequently isolated microorganism in both human and animals with genital tract infections or semen contaminations was *Escherichia coli* (*E.coli*). This Gram-negative bacterium species reduces sperm motility through sperm adhesion and agglutination [17-18] and it also can impair acrosomal function, as demonstrated at the ultra-structural level [19]. Moreover, it has also been demonstrated that *E.coli* can start the apoptotic process, DNA fragmentation and alterations in membrane symmetry [20]. Another important bacterium species which may also present in semen samples is *Staphylococcus aureus* (*S. aureus*). This Gram-positive bacterium is an important zoonotic pathogen, which can infect both human and animals [21]. In Iran, Golshani *et al*., [22] noted that 35% of infertile men showed a contamination with at least one pathogen such as *E.coli*, *Staphylococci* species and most importantly *S.aureus*. Furthermore, in an in vitro study performed by Villegas *et al*., [20], a single incubation with *E.coli* and S.aureus induced apoptosis in human sperm with possible putative mechanisms including a direct cytotoxic activity of bacterial toxins.

Regarding the literature and despite the existence of several studies which clearly noted the isolation of both *E.coli* and *S.aureus* from semen samples and their direct effects on spermatozoa [18,21], no information is available on the growth rates of these two very important pathogen species in different semen media following an experimental contamination and the competition status between these two species during the incubation at different temperature degrees. For that, the objective of this work is to investigate the bacterial growth and the competition status of *E.coli* and *S.aureus* incubated in different semen media at two temperature degrees.

2. Material and methods

2.1. Chemicals and based semen medium preparation

Chemicals for the semen media of this study were purchased from Roth (Carl Roth Gmbh-Karlsruhe-Germany).

Tris based medium was prepared as a 300 mOsm/Kg solution contained the following: 2.44g tris (hydroxymethyl) aminomethane, 1.36g citric acid monohydrate 100 mL of distilled water and held constant at pH 7.

2.2. Bacterial species

Two species of bacteria were chosen: Gram-negative (*E.coli*) and Gram positive (*S.aureus*). All bacteria were supplied from Syrian Atomic Energy Commission (Dep. of Molecular Biology and Biotechnology). Ten ml of nutrient broth (in sterile tube) were inoculated by one colony (colony from each species/tube). All tubes were incubated overnight at 37°C. Each species was cultured on a special culture medium with an Eosin Methylene Blue (EMB) medium (Himedia Labs, India) for the first specie and Plate Count Agar (PCA) medium (Himedia Labs, India) for the second one. The plates were incubated at 37°C for 48h. Once the purity of the two strains was achieved, liquid dilutions with 10^3 CFU/mL were prepared.

2.3. Preparation of mother cultures with bacterial inoculums

Five semen main solutions were prepared; (1) Tris based solution (100 mL), (2) Tris based solution (100 mL) + Glucose (1g/L), (3) Tris based solution (100 mL) + Sucrose (1g/L), (4) Tris based solution (100 mL) + Fructose (1g/L), (5) Tris based solution (80 mL) + 20% Egg-Yolk. Each main solution was divided into two groups: the first group was added to10^3 CFU/mL of activated bacterial inoculums, and the second group was sterilized by one of the approved sterilization methods, and then the bacterial inoculum was added at the same concentration as the first one. The total bacterial counts as colony forming units (CFU) were determined by a spread plate method [23], the plates were incubated at 37°C for 48h.Growth was compared through the bacterial load for each strain separately in mixed solutions, and then by comparing the growth strength competition between the two strains on a common media (PCA).

2.4. Sterilization

Sterilization of semen solutions was carried out using gamma rays emitted from a source ^{60}CO located in the irradiation station/Dep. of Radiation Technology - AECS (dose rate was 15KGy/h). In order to determine the optimal several dose economically and commercially, several irradiation doses (50-100-500-1000-5000 Gy) were applied.

2.5. Pre-incubation storage

The effect of the pre- incubation period and temperature on bacterial growth was studied for both strains, through direct inoculation on solid media in the Petri dish and by comparing the time of the incubation of inoculums in dilution solutions for a period of 3 hours at two temperatures (4°C in the refrigerator and 37°C in the incubator). Then, they were cultured on Petri dishes with the before mentioned specific media.

2.6. Competition bacterial during incubation storage

The compaction in bacterial growth (at the same time) was realised in this work between the *E.coli* and *S.aureus* after pre- incubation period at two temperature degrees. The initial load of each strain (10^3 CFU/mL) was incubated in liquid semen media together for 3 h of at 4 and 37°C, and then this mixed bacteria was inoculated on solid media (PCA) in the Petri dishin triplicate for each parameter in this experiment (≈ 300 Petri dish).

2.7. Statistical analysis

Data were subjected to the analysis of variance test (ANOVA) using the SUPERANOVA computer package (Abacus Concepts Inc, Berkeley, CA, USA; 1998). A separation test on treatment means was conducted using Fisher's least significant differences (LSD) methods at 95% confidence level (Snedecor and Cochran, 1988).

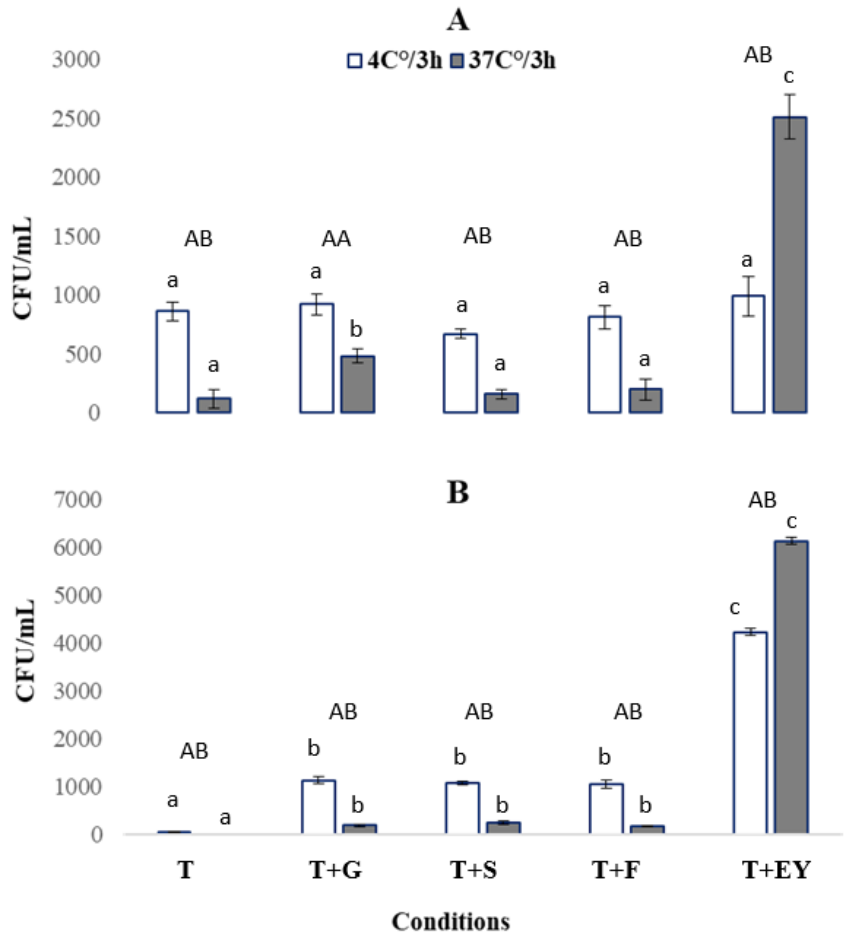

Figure 1.Bacterial load of tow bacteria species of *S.aureus* (**A**) and *E.coli* (**B**) after 3 h of storage at two temperature degrees (4 and 37°C) in different semen media including: (T) Tris based solution, (T+G) Tris + Glucose, (T+S) Tris + Sucrose, (T+F) Tris + Fructose, (T+EY) Tris + Egg-Yolk.

Different letters (A-B) with in different temperature degrees for each bacteria strain and for each medium alone denote significant difference ($P < 0.05$).
Different letters (a-c) with in different semen media for each bacteria strain and for each temperature degree denote significant difference ($P < 0.05$).

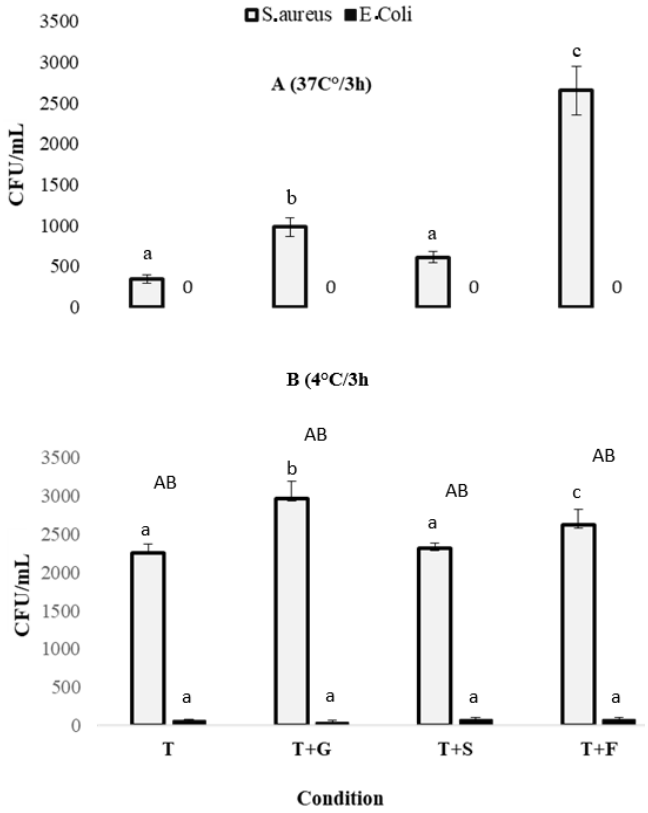

Figure 2. Bacterial load in different semen media at two temperature degrees (A: 37°C; B: 4°C) after 3 h of storage in competition experiences of the two species of (*S.aureus* and *E.coli*); (T) Tris based solution, (T+G) Tris + Glucose, (T+S) Tris + Sucrose, (T+F) Tris + Fructose.

Different letters (A-B) in each medium with in bacteria strains for each temperature degree denote significant difference ($P < 0.05$).
Different letters (a-c) in each bacteria strain with in semen media and for each temperature degree denote significant difference ($P < 0.05$).

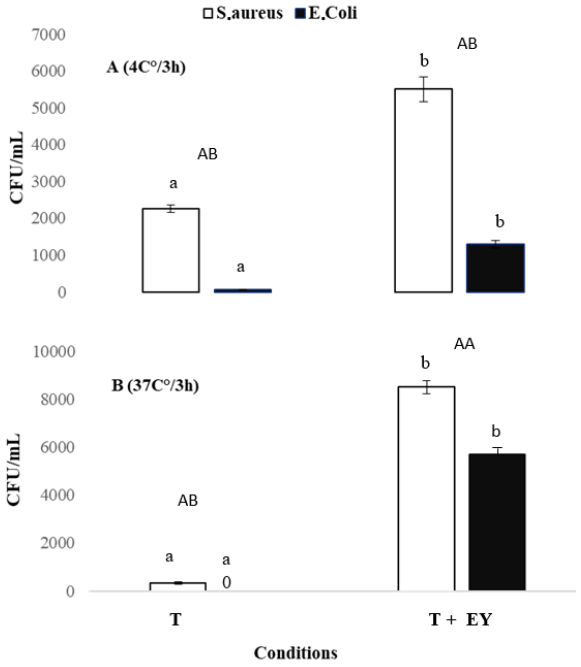

Figure 3. Bacterial load in semen media after 3 h of storage at two temperature degrees (A: 4 and B: 37°C) in competition experiences of the two species of (*S.aureus* and *E.coli*). (T) Tris based solution, (T+EY) Tris + Egg-Yolk

Different letters (a-c) in each bacteria strain with in semen media and for each temperature degree denote significant difference ($P < 0.05$).
Different letters (A-B) in each medium with in bacteria strains for each temperature degree denote significant difference ($P < 0.05$).

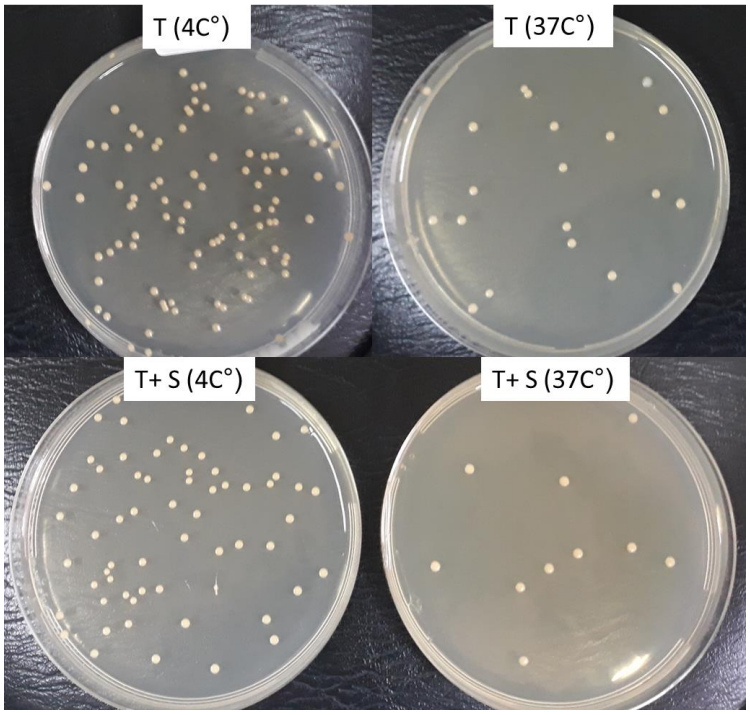

Photo1. Growth of *S.aureus* on plat count agar after 3 h storage in different semen media at two temperature degrees (4 and 37°C); (T) Tris based solution, (T+S) Tris + Sucrose.

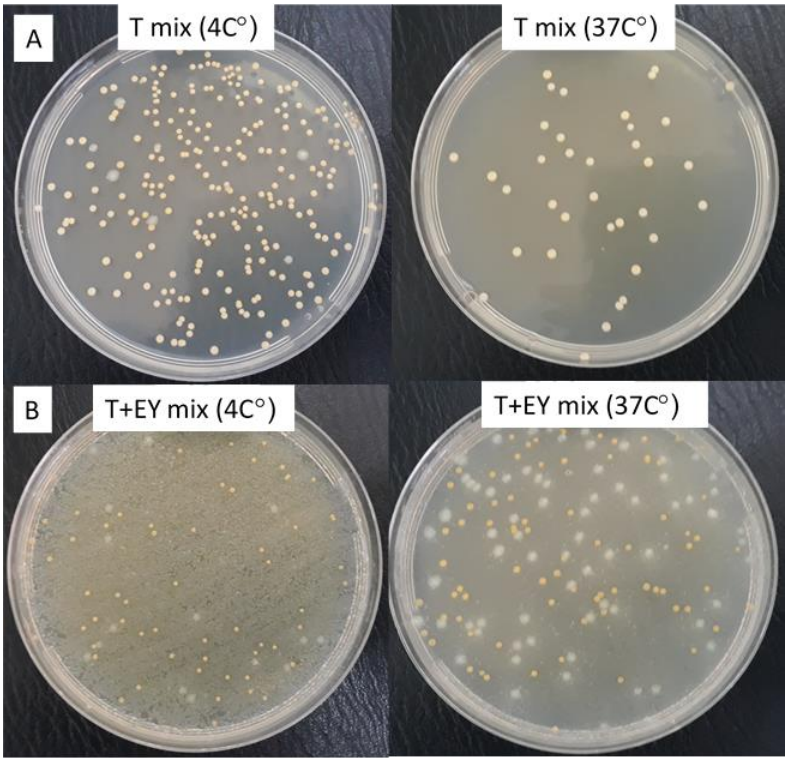

Photo 2. Growth of *S.aureus* and *E.coli* on plat count agar after 3 h storage of the two species together in different semen media at two temperature degrees (4 and 37°C); (T) Tris based solution, (T+EY) Tris + Egg-Yolk**.**

4. Discussion

In the present work, we focused on two important bacterial species of (*E.coli* and *S.aureus*), considering their frequent presence in the ejaculates and the environment, their growth rates in nutrient media and also the severity of causing diseases [24]. To our knowledge this is the first study which shows both the bacterial growth and the competition status of these two species in different semen media at different temperature degrees. On the other hand, it is well known that spermatozoa can transfer different bacteria load to semen media. This transmission process will be clearly related to the spermatozoa concentration and also the amount of bacterial contamination of spermatozoa. In this study, and in contrast with what was done previously in literature [25], the bacteria growth was studied within the semen media without the presence of spermatozoa itself. This approach was very important for us, in order to understand the evolution of bacterial growth experimentally within the semen media apart of the presence of the spermatozoa. However, our study was the first in achieving such experimental approach. Anyhow and as expected, the principal results of this study were the clear differences between the different media in sustaining the growth of these two bacteria strains for the two temperature degrees.

Normally culture bacteria medium is essentially composed of water, agar, nutrients and some additives that will be specific to sustain the life and the growth of each microorganism [26]. The compositions of semen preservation media are to some extent similar to culture bacteria media. Indeed, the semen media were primarily designed to nurture and prolong sperm viability; however, these same properties are what make them a potential base in where bacteria can multiply vigorously [27]. Furthermore, the nutrients such as sugars and the conditions of storage of semen media (including temperature and pH) allowed the development of bacteria, such as *E.coli* [28]. In our study it was obvious that the sugars and the egg-yolk were the major sources that may sustain the growth compared to the tris based medium. In this respect, Tris diluents supplemented with sugars and egg-yolk have become more universal diluents suitable for storage of semen at refrigerated or ambient temperature and also in frozen state [29]. Our results clearly showed that egg yolk had largely sustains the growth rates of the two bacteria species whatever of temperature degree was compared to Tris diluents supplemented with sugars. Indeed, the use of clarified or fresh egg-yolk in semen media represented a potential risk of microbiological contamination in artificial insemination doses, damaging the fertility of the spermatozoa [30]. It should be noted that in this study we added the whole yolk to the tris medium without any clarification process. A significant difference between the clarified yolk and whole yolk concerning bacteria load was previously noted by Ugbamaja *et al*., [31]. Anyhow, the amount and the nature of nutrients available in any given medium determine the numbers and the size of the colonies which was bigger in semen media containing egg yolk versus the control and this was also confirmed by the observations of [32].

It is well known that sugars are growth substrates for pathogens such *as E.coli* and *S.aureus* [33-34]. In this respect, glucose supplementation was crucial for increasing the biomass formation of *E.coli* in LB Lennox medium [35]. Moreover, at high concentration of supplementation (6g/L), glucose triggered an overflow metabolism through the central carbon pathway that led to acetate accumulation and low pH which reduced biomass formation [35]. For that, the use of glucose or any other sugars types in bacteria media required the use of high capacity buffer system for modulating the potentially large pH fluctuation that could arise during bacterial growth. It should be pointed out that sugars including glucose, fructose and sucrose exhibited significant antimicrobial effects at high concentrations, but at low concentrations they act as substrates for the bacteria which results in enhanced microbial growth instead of inhibition [34]. Anyhow, supplementation of Lennox medium with 2g/L glucose, with or without a buffer did not significantly alter the carbon and nitrogen balance of this medium. In our study, only 1 g/L of the three sugars was added to the based solution, which is the normal used concentration for spermatozoa preservation and Tris was used to preserve the pH degree of the different media. It must be noted here that all semen media of the present study had always a stable pH degree at (pH =7).

In this study, the incubation of the two bacteria species was conducted at 37 and 4°C. These two degrees could be the most important temperature degrees for any semen samples; as the first is the body temperature or the temperature of *in vitro* incubator, while the second is a chilling degree. It

must be stressed out that semen preservation methods, artificial insemination (AI) process and *in vitro* fertilization (IVF) techniques largely depend upon these two vital degrees. On the other hand, temperature is a very important physical factor which immediately affects bacteria life and growth. In this respect, Jones *et al*., [36] observed that *E.coli* synthesized a set of proteins in response to a temperature shift from 37 to 10°C. In the case of *E.coli* incubated at 10°C, this temperature is quite the minimum for growth, which induces deep changes in morphology and loss in viability [37]. Moreover, the lower minimum temperature obtained from some cold-resistant mutants of *E.coli* was 7.5°C [38] and later 7°C [37]. In the present study, egg yolk medium sustained the growth of *E.coli* and *S.aureus* at 4 °C, but it highly and significantly support their growth at 37°C. Surprisingly, both *E.coli* and *S.aureus* were significantly inhabited at 37°C compared to the 4°C in Tris based medium and also in Tris media supplemented with sugars. The exact raison of such results is not clear, especially that sugars are well known as growth substrates for bacteria. Despite of glucose is considered the best carbon source for *E.coli*, there were some *E.coli* strains grow slower on glucose than on other sugars, namely when a single amino acid [39]. Few strains of *E.coli*, can utilize sucrose for metabolism [40], contrary to *E.coli* K12 cannot utilize sucrose, but, wild-type *E.coli* strain EC3132 contains chromosomal genes for sucrose metabolism [40-41]. However, it should be stressed out that the incubation period of this study (for 3 hours) is relatively short and longer incubation period may be needed to determine if the pattern of such results will remain the same for the two temperature degrees. In fact, we chose this time point as the first three hours is the most important time window for the spermatozoa to begin the fertilization process *in vitro* and *in vivo*.

One of the most important experiments conducted in the present investigation was the assessment of the competition status in between *E.coli* and *S.aureus* incubated in different semen media. The load of *S.aureus* was not only influenced by the incubation temperature and the constituents of incubation media, but also by the presence of *E.coli* [43]. *E.coli* growth was clearly inhibited in presence of *S.aureus* in the Tris based solutions supplemented with sugars whatever the temperature degree was. In contrast to our study, the competition for nutrient between *E.coli* and *S.aureus* in dairy contaminants led to *S.aureus* growth inhibition, even when its growth was positively stimulated by increasing incubation temperature [43]. In the previous study, the pH decrease together with competition for nutrient led to *S.aureus* growth inhibition. Anyhow, the differences in the contents of incubation media, incubation time, incubation temperature and also the initial concentration of the two bacteria strains between Medvedova study and ours may explain the differences in the growth pattern for the *E.coli* and *S.aureus* between the two studies.

In order to reduce the risk of bacteria contaminants in the different semen media, bacterial control strategies were needed. In this respect, antibiotics were used in semen diluents to control and to eliminate bacteria present in the both semen and media, but such elimination cannot be guaranteed since bacteria have developed antibiotic resistance [44]. Bresciani *et al*., [45] observed a clear resistance to gentamicin for *E.coli* (50%) and *S.aureus* (100%). As we previously observed high rates of bacterial contamination in the present semen media (data not shown) and as it was not possible to experimentally assess any growth of *E.coli* and *S.aureus* in semen media supplemented with antibiotics. Thus, we used gamma radiation to sterilise our media before the conduction of the present experiments. Other sterilising methods such as UV-radiation and ultrasound treatments were largely less affected than gamma radiation whatever the used dose was (data not shown).The positive effects of gamma radiation in sterilizing different products (food, raw materials for the food industry, medical herbs and care products) are well documented in literature [46-48]. However, it is not known whether such sterilizing method could compromise the quality of preserved spermatozoa in these sterilized media. Anyhow, such treatment which is totally experimental for semen media need more profound researches' in order to determine its effects on the spermatozoa and the contents of these media.

5. Conclusions

The present data are extremely vital for a better understanding about the factors that may influence the growth of *E.coli* and *S.aureus* in different semen media. It is obvious from our results that each bacterium is unique and has specific requirements which may differ at different temperature degrees. Egg-yolk must be substitute in semen media to

minimize the bacterial contamination. The pattern of bacteria competition in semen media may be largely related to the time of incubation and the initial load of the contamination but most importantly to media composition, temperature degree and to the bacteria species. Gamma radiation seemed to be a promising method to sterilize semen media. For that, further investigations are needed to study the growth responses of these two pathogens on other temperatures degrees, other contaminated concentrations and other semen media and or extenders.

Compliance with Ethics Requirements. Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human or animal subjects (if exist) respect the specific regulation and standards.

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