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Competition Status of *Salmonella sp.*, *Shigella sp.*, and *Escherichia coli* in Tris Egg Yolk Semen Preservation Medium at Different Experimental Conditions

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Abstract

Competition is a very important fact of microorganisms' life, with the major goal to gain an advantage in terms of scarce nutrients and limited space. In this study, bacterial competition of *Salmonella* sp., *Shigella* sp., and *Escherichia coli* were assessed in Tris- egg-yolk (TEY) semen preservation medium at two temperature degrees and three periods of incubation (3 h at 37°C, 1 and 7 days at 4°C). The three pathogen species were incubated with an initial bacterial load of 10^3 CFU/mL. After three hours of incubation at 37°C, there were significant differences ($P < 0.05$) between the three species; however, *Salmonella sp.* had the highest CFU/ml compared to the two other pathogen species in bilateral and triple competition. Also after one day of storage at 4°C, a clear dominance of *Salmonella* sp. was noted, while in contrast, no significant difference ($P > 0.05$) between *E.coli* and *Shigella sp.,* was observed in the bilateral and triple competition cases. However, the maximum dominance of *Salmonella sp.* was observed after 7 days at 4°C, where the bacterial load percentages between all species for the triple competition were 74±5.6/12±5.9/14±6.9 for *Salmonella* sp., *E. coli,* and *Shigella* sp., respectively. In conclusion, regardless of the temperature degrees and the time point of incubation, *Salmonella* was always able to dominate and compete very effectively compared with the two other bacterial pathogens in the TEY medium. Moreover, 4°C gave clear preference to the survival of *salmonella* with its clear vitality percentage in this media compared to both *E. coli* and *Shigella sp*.

Keywords: bacterial competition, semen media, tris egg-yolk, *Salmonella sp*., *E.coli*, *Shigella sp*.

Резюме

Конкуренцията е много важен факт от живота на микроорганизмите, като основната ѝ цел е да се спечели предимство по отношение на оскъдните хранителни вещества и ограниченото пространство. В това проучване е проучена бактериалната конкуренция на *Salmonella sp*., *Shigella sp*. и *Escherichia coli* в среда за съхранение на сперма от Tris-яйчен жълтък (TEY) при две температурни степени и три периода на инкубация (3 часа при 37°C, 1 и 7 дни при 4°C). Трите вида патогени са инкубирани при първоначално бактериално натоварване от 103 CFU/mL. След три часа инкубация при 37°C са установени значителни разлики ($P < 0.05$) между трите вида; *Salmonella sp*. обаче показва най-високи CFU/ml в сравнение с другите два вида патогени при двустранна и тройна конкуренция. Също така, след еднодневно съхранение при 4°C се наблюдава ясно доминиране на *Salmonella sp*., докато *E. coli* и *Shigella sp*. не се наблюдава значителна разлика ($P > 0.05$). Максималната доминация на *Salmonella sp*. се наблюдава след 7 дни при 4°C, където процентите на бактериално натоварване между всички видове за тройната конкуренция са 74±5.6/12±5.9/14±6.9 съответно за *Salmonella sp*., *E. coli* и *Shigella sp*. В заключение, независимо от температурните градуси и времевия момент на инкубация, *Salmonella* винаги е в състояние да доминира и да се конкурира много ефективно в сравнение с другите два бактериални патогена в среда TEY. Нещо повече, температурата 4°C дава ясно предимство на оцеляването на *Salmonella* с нейния ясен процент жизненост в тази среда в сравнение с *E. coli* и *Shigella sp*.

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Introduction

It is well known that semen media are used for preserving spermatozoa to enable the fertilization process (Bustani and Baiee, 2021). In this respect, different types of semen media such as Tris-based, Tris skim milk, and egg yolk media, have been previously used for the artificial insemination process (Layek *et al.,* 2016). Generally, these media can maintain sperm metabolic processes, control the pH level, and reduce chilling and cryogenic damage (Alomar *et al.,* 2018; Malik *et al.,* 2018) due to several protective ingredients such as egg yolk which permit the survival of spermatozoa outside and inside the reproductive tract (Bustani and Baiee, 2021). It must be noted that the Tris-egg-yolk (TEY) medium appeared superior to other media tested under *in vitro* and *in vivo* conditions (Kumi-Diaka *et al.,* 1994). However, despite the egg yolk being the most important part of this medium, it must be stressed that egg contents especially egg yolk are a good medium for the survival and the growth of bacteria (Abboudi *et al.,* 2021).

Different bacteria species may contaminate both egg and egg yolk. In this respect, *Salmonella* and *Shigella* are usually derived from fecal contamination of the eggshell, and they may be able to contaminate egg contents by migration through the eggshell and membranes (Nnagbo and Nkwoemeka, 2018). It must be pointed out that, *salmonella* is a major pathogen of the gastrointestinal tract (Doble *et al.,* 2012), which was isolated from a large number of animal species (Scherer and Miller, 2001), and constantly released into the environment from infected humans and farm animals (Baudart *et al.,* 2000). On the other hand, recognized worldwide as the most common cause of dysentery, *Shigella* was named because many of its members live in the intestines of humans and animals (Yassine *et al.,* 2022). In addition to both *Salmonella* and *Shigella*, *Escherichia coli* is one of the most frequently isolated microorganisms in humans and animals with genital tract infections or semen contaminations. In this regard, bacterial contamination was a major concern for semen media and semen laboratories due to its adverse effects on spermatozoa quality and consequently on the reproduction status via the viability and the fertility of sperm (Althouse *et al.,* 2000; Goldberg, 2013; Morrell and Wallgren, 2014; Sannat *et al.,* 2015). *E. coli* reduced sperm motility through sperm adhesion (Diemer *et al.,* 2000; 2003) and started to damage DNA and modify the structure of the membrane (Villegas *et al.,* 2005).

Competition between species has been shown to have a key role in shaping species distributions (Celiker and Gore, 2012) and modification of character dislocation. Moreover, the two main necessary pillars for microbial survival are nutrients and space, where nutrients are essential for microbial growth and metabolic functions (Ghoul and Mitri, 2016). On the other hand, the classical example of interference competition is the production of a range of antimicrobials and antibiotics (Riley and Gordon, 1999). However, the study of competition status between different bacterial species is a very important field to determine the necessary needs to control bacterial growth and also to determine the best-requested protocols to prepare sperm preservation media.

Regarding the literature and despite the existence of several studies that clearly noted the isolation of *Salmonella*, *Shigella,* and *E. coli* from different environments (Baudart *et al.,* 2000; Nnagbo and Nkwoemek, 2018), we are not aware of any reports specifically focusing on the competitive behavior between these important three species during the incubation in semen media containing egg yolk. For that, our aim was to carry out the first experimental study to assess the bacterial growth and the competition status of *Salmonella sp.*, *Shigella sp.*, and *E. coli* incubated in TEY semen medium at two temperature degrees and at different periods of *in vitro* incubation.

Material and Methods

Chemicals and based semen medium preparation

The 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 m Osm/Kg solution containing the following: 2.44g tris (hydroxymethyl) aminomethane, 1.36g citric acid monohydrate in 100 mL of distilled water and held constant at pH 7. The Tris egg yolk (TEY) medium was prepared from 80 ml of the based medium and 20 ml of egg yolk was added to complete the final volume of this medium.

Bacterial species

Three species of Gram-negative bacteria were chosen: *Salmonella* sp., *Shigella* sp., and *E. coli*. Ten ml of nutrient broth (in a 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 *Salmonella*-*Shigella* agar (SS Agar) (Himedia Labs, India). The plates were incubated at 37°C for 48h. Once the purity of all species was achieved, liquid dilutions were prepared in sterile distilled water to have 10^3CFU/mL.

Preparation of mother cultures with bacterial inoculums

Two main semen solutions were prepared; (1) Tris-based solution (100 mL), (2) Tris-based solution (80 mL)+20% egg-yolk (TEY). Before the bacteria incubation, all solutions were sterilized using gamma rays emitted from a source ^{60}CO located in the irradiation station/Dep. of Radiation Technology –AECS at dose 1KGy (dose rate was 15KGy/h; Abboudi *et al.,* 2021). The different semen solutions were inoculated by 10^3 CFU/mL before incubation at different times according to each experiment.

Experiment design; bacteria growth and bacteria competition

In the present study three experiments were conducted. In the first experiment, the bacterial growth in Tris-based and TEY was assessed at 37°C for 3 hours (h) separately for each species. In the second experiment, the bacteria competition between the three pathogens in TEY medium at 37°C for 3 h was assessed. In the third experiment, the bacteria competition between the three pathogens in TEY medium at 4°C for 1 and 7 days, respectively was conducted. In the second and the third experiments, the bilateral competition was realized between *Salmonella* sp.,/*E. coli*, *Salmonella* sp.,/*Shigella* sp., *Shigella* sp.,/*E. coli* where 50%/50% of 10^3CFU/ml, and between all species in triple competition *Salmonella* sp.,/*Shigella* sp.,/*E. coli* where 33.3%/33.3%/33.3 of 10^3CFU/ml. For the three experiments and after the different incubation periods in TEY medium, the total bacterial counts (microbial load) as colony forming units (CFU) were determined by a spread plate method (AL-Bachir, 2019) on SS Agar medium (Himedia Labs, India), the plates (Petri dishes) were incubated at 37°C for 48 h in triplicate.

Statistical analysis

Data were subjected to the analysis of variance test (ANOVA) using the SUPERANOVA computer package. A separation test on treatment means was conducted using Fisher's least significant differences (LSD) methods at a 95% confidence level.

Results

Bacterial growth in Tris-based and TEY medium

The three species (*Salmonella* sp., *Shigella* sp., and *E.coli*) separately inoculated in different semen media were able to grow during 3 h of storage conditions (Table 1). The results presented in Table 1 showed that there are no significant differences in bacterial load after incubation in Tris-based between 0h and 3 h at 37°C before the growth on the SS Agar medium. The separate incubation of each species in the TEY medium for 3 h at 37°C caused a significant increase of about 100 times compared to the initial bacterial load at 0h (Table 1).

Competition status between Salmonella, Shigella, and E.coli in TEY semen medium

Photo 1 shows the distribution of the three species after growth on SS Agar medium in the bilateral and triple competition experiments; *Salmonella* sp., colonies appear in black, *E. coli* in pink, and *Shigella* sp., colonies appear in light grey.

The case of bilateral and triple competition between *Salmonella* sp., *Shigella* sp., and *E. coli*

Table 1. Bacterial load of *Salmonella sp., Shigella* sp*.,* and *Escherichia coli* at 0 h and after 3 h of storage at 37°C in Tris based and Tris- egg yolk (TEY) semen media.

Bacteria	Condition				
Salmonella	Time of incubation (h)	0	3	0	3
	Semen media	Tris based	Tris based	TEY	TEY
	CFU/mL	$5.4x10^{3a}$ $\pm0.7x10^2$	$3.3x10^{3a}$ $\pm0.2x10^2$	$1.3x10^{3a}$ $\pm0.6x10^2$	$1.5x10^{5b}$ $\pm5.5x10^3$
Shigella	Time of incubation (h)	0	3	0	3
	Semen media	Tris based	Tris based	TEY	TEY
	CFU/mL	$1.3x10^{3a}$ $\pm0.7x10^2$	$3.9x10^{3a}$ $\pm0.7x10^2$	$1.3x10^{3a}$ $\pm0.4x10^2$	$1.3x10^{5b}$ $\pm4x10^3$
E. coli	Time of incubation (h)	0	3	0	3
	Semen media	Tris based	Tris based	TEY	TEY
	CFU/mL	$2.3x10^{3a}$ $\pm0.8x10^2$	$0.9x10^{3a}$ $\pm0.7x10^2$	$1.4x10^{3a}$ $\pm0.8x10^2$	$3.4x10^{5b}$ $\pm8x10^3$

Values (Mean ±Std. dev), different letters (a-b) in different semen media for each bacteria species and at different time of incubation denote significant difference (P < 0.05).

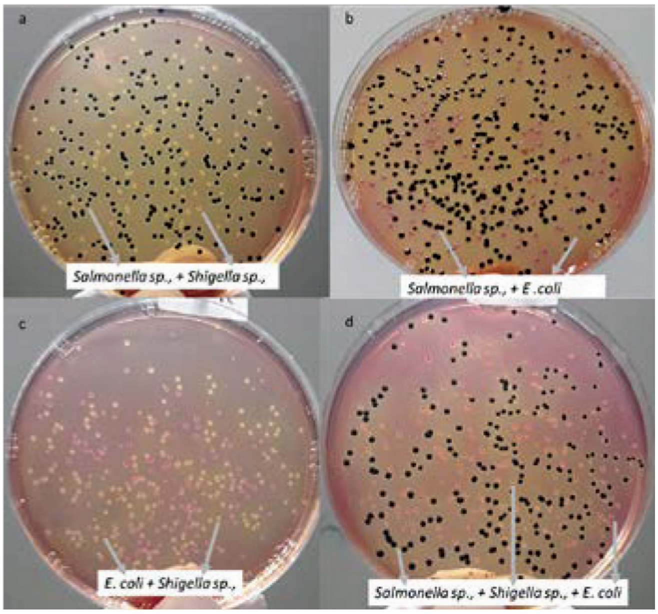

Photo 1. Growth of *Salmonella sp.*, *E. coli,* and *Shigella sp.*, on SS agar medium after storage (1day) of the species together in TEY semen medium at 4°C in the competition experiments. (a) The competition between *Salmonella* sp., and *Shigella* sp., (b) the competition between *Salmonella* sp., and *E. coli*, (c) the competition between *E. coli and Shigella* sp., (d) the competition between *Salmonella* sp., *Shigella* sp., and *E. coli*.

species in TEY semen medium after 3 h of incubation at 37°C was shown in Fig. 1. Figure 1A presents the results of the bilateral competition between each two species with significant differences, where it shows the strength of *Salmonella* sp., followed by *E. coli.* The bacterial load as percentages between *Salmonella* sp.,/*E. coli*, *Salmonella* sp.,/*Shigella* sp., and *E. coli*/*Shigella* sp., were 60±1.4/40±5.5, 68±2.6/32±3.3 and 83±2.1/17±2.1, respectively. Figure 1B presents the results of the triple competition between all species with significant differences. Figure 1B clearly shows the dominance of *Salmonella* sp., in terms of bacterial numbers, followed by *E. coli* and *Shigella* sp. Bacterial load percentages between all species after 3 h of incubation at 37°C were 50±1.6/40±0.9/10±2.0, respectively.

Figure 2 presents the bilateral and triple competition between the three species in TEY media after 1 day of incubation at 4°C. It clearly shows the dominance of *Salmonella* sp. with significant differences with other species, while there are no significant differences between *E. coli* and *Shigella* sp, nor the case of binary competition (Fig. 2A) nor in the case of triple competition (Fig. 2B). The bacterial load percentages between *Salmonella* sp.,/*E. coli*, *Salmonella* sp.,/*Shigella* sp.,

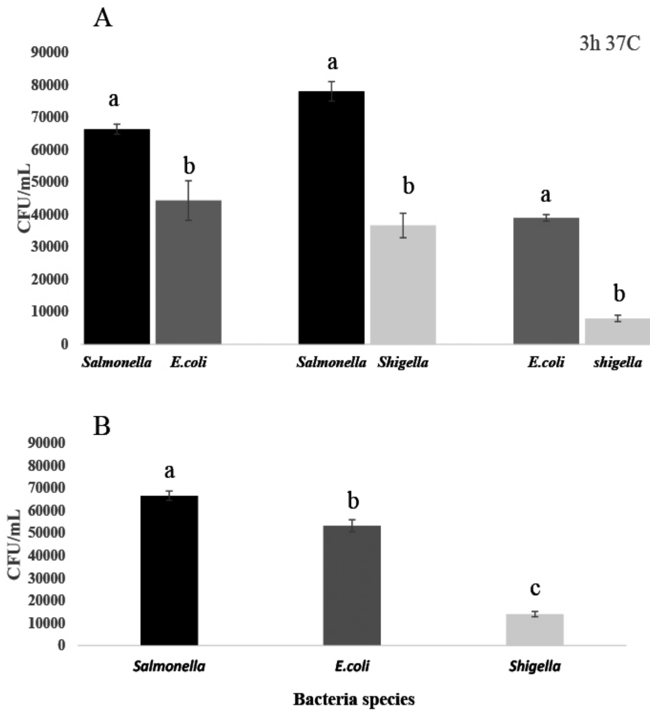

Fig. 1. Bacterial load of *Salmonella sp., E. coli* and *Shigella* sp., after 3 h of storage at 37°C in TEY semen medium: (A) the bilateral competition between each tow species, (B) the triple competition between all species.

Values (Mean ±Std.dev), different letters (a-c) in bacteria species in bilateral and also in triple competition denote significant difference (P < 0.05)

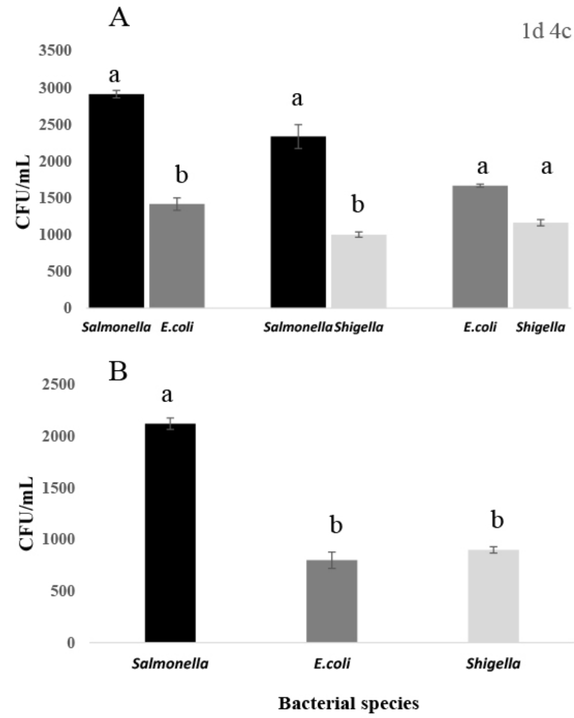

Fig. 2. Bacterial load of *Salmonella sp.*, *E. coli* and *Shigella* sp., after 1 day of storage at 4°C in TEY semen medium: (A) the bilateral competition between each tow species, (B) the triple competition between all species.

Values (Mean ±Std.dev), different letters (a-c) with in bacteria species in bilateral and also in triple competition denote significant difference (P < 0.05).

and *E. coli*/*Shigella* sp., after 1 day of incubation at 4°C were 67±0.9/33±2.0, 70±4.8/30±1.1 and 50±0.9/50±1.8, respectively (Fig. 2A). *Salmonella* sp., followed the same trend in the previous triple competition in terms of dominance (Fig. 2B) with significant differences between *E. coli* and *Shigella* sp., the bacterial load percentages between all species after 1 day of incubation at 4°C were 56±1.4/23±0.7/21±2.1for *Salmonella sp.*, *Shigella sp.,* and *E. coli*, respectively.

Figure 3 presents the bilateral and triple competition between species in TEY semen media after 7 days of incubation at 4°C. The dominance of *Salmonella* sp., with significant differences with other species, was clear in this case.

The bacterial load of *Salmonella* sp., was similar to the incubation for 1 day at 4°C, while there are no significant differences between *E. coli* and *Shigella sp*., neither the case of binary competition (Fig. 3A) nor in the case of triple competition (Fig. 3B). While there was a significant decrease in the bacterial load for each of *E. coli* and *Shigella* sp., after 7 days compared to incubation during one day at 4°C. The bacterial load percentages between

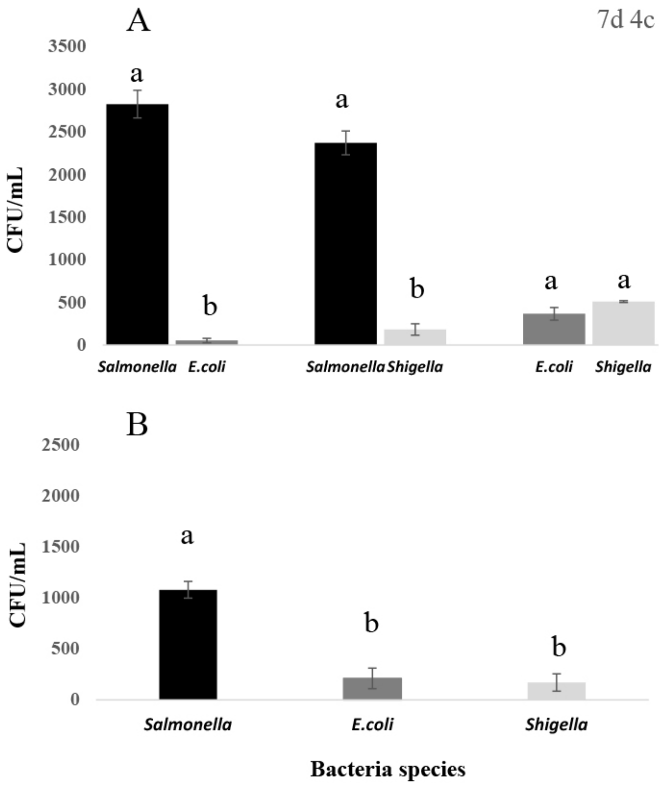

Fig. 3. Bacterial load of *Salmonella sp.*, *E. coli* and *Shigella* sp., after 7 days of storage at 4°C in TEY semen medium: (A) the bilateral competition between each tow species, (B) the triple competition between all species.

Values (Mean ±Std.dev), different letters (a-c) with in bacteria species in bilateral and also in triple competition denote significant difference ($P < 0.05$).

Salmonella sp.,/*E. coli*, *Salmonella* sp.,/*Shigella* sp., and *E. coli*/*Shigella* sp., after 7 days of incubation at 4°C were 98±5.6/2±0.9, 93±5.5/7±2.6 and 42±8.4/58±1.1, respectively)Fig. 3A). The bacterial load percentages between all species after 7 days of incubation at 4°C were 74±5.6/12±5.9/14±6.9 for *Salmonella sp.*, *E. coli,* and *Shigella sp.*, respectively (Fig. 3B).

Discussion

To our knowledge, this is the first study that shows the competition status between *Salmonella*, *Shigella,* and *E. coli* species in semen media at different experimental conditions. It must be stressed that these three bacteria species especially *Salmonella* are the major sources of egg-contaminated products, and they are widely distributed in the environment and food processing places (Saier, 2015).

Different Tris media were used in our studies for several years, whereas TEY medium was employed to compare sperm motility values and quality characteristics of fresh, chilled, and frozen small ruminants' spermatozoa (Alomar *et al.,* 2018; Alomar, 2022 a,b). So any bacterial contamination of this important medium will undoubtedly negatively affect the findings of the different quality analyses, as well as harm the sperm samples preserved within it. In our previous work, we noted that egg yolk in Tris medium was the major source that may sustain the growth of *E. coli* and *Staphylococcus aureus* compared to Tris-based medium and also to Tris media supplemented with different sugar substrates (Abboudi *et al.,* 2021). In the present study, the main focus of the first experiment was to show the differences that result from bacterial growth in TEY medium compared to Tris-based medium. Our data clearly showed that the TEY medium was considered very suitable for the growth of the three tested bacteria, and the incubation temperature (37°C) was able to increase the bacterial growth. The 37°C is the body temperature whether in humans or animals and it is also the temperature degree of *in vitro* incubators. In agreement with our present results, we reported that egg yolk medium sustained *E. coli* and *S. aureus* growth at 4°C, but this medium significantly supported their growth at 37°C (Abboudi *et al.,* 2021). In fact, the life and the growth of different bacteria species were always directly affected by temperature (Jones *et al.,* 1987; Nedwell, 1999).

In the second and third experiments, our goal was to define the competition status between the three species at 37°C and also at 4°C (the preservation temperature of different semen media in the refrigerator). We clearly noticed the superiority of *Salmonella sp*., in the bilateral and triple competition at 37°C, while on the contrary, *Shigella sp*., showed that it was the weakest in the competition. However, regardless of the temperature degrees or the time points of incubation, *Salmonella* was always able to dominate and compete very effectively compared with the two other bacterial pathogens. Several explanations could be hypothesized to explain why *Salmonella* dominates other species in TEY media. For example, the differences between *Salmonella* and both *E. coli* and *Shigella* in terms of survival outside animal hosts could result from genes that are specific to each of these species. However, the distinct lifestyles of these micro-organisms may be attributed to genetic mechanisms, such as disparate regulation or allelic differences between homologous genes (Winfield and Groisman, 2003). Moreover, the nature of *Salmonella* may facilitate a cyclic lifestyle consisting of passage through a host into the environment (TEY semen preservation medium in our present case) and back into a new host. Thus, unlike *E. coli,* which does not appear to survive in nonhost environments for extended periods, long-term survival of *Salmonella* in the secondary habitat may ensure its passage to the next host. In this respect, *Salmonella* survived for 10 to 15 days in a septic system, whereas *E. coli* had a negative growth rate in this environment (Gordon *et al.,* 2002). Moreover, in marine environments, the presence of *Salmonella* was independent of water temperature and did not vary seasonally (Alonso *et al.,* 1992). Generally, and compared to *E. coli*, *Salmonella* appears to withstand a variety of stresses associated with environmental fluctuations (Winfield and Groisman, 2003). In contrast to the clear dominance of *salmonella* on days 1 and 7 of incubation at 4°C, we recorded approximately similar percentages for *E. coli* and *Shigella sp.,* in bacterial load, especially in case of triple competition with *Salmonella.* It seems that this temperature degree (4°C) does not help these two pathogens to be in their optimal competitive status even with a medium that largely sustains their growth. Indeed, at temperatures below their optimum for growth, microorganisms will become increasingly unable to sequester substrates from their environment because of lowered affinity, exacerbating the near-starvation conditions in many natural environments (Nedwell, 1999). We were interested in this particular temperature degree (4°C) and in these incubation times points (3 h, 1, and 7 days) because they represent the conservation degree for semen media and also the time window by which semen laboratories conserve TEY medium.

In addition to the reason that led to the dominance of *salmonella* in semen media containing egg yolk, one of the most important questions is: what are the strategies that help *Salmonella* to dominate? A study in soil bacteria found that there are two major strategies: investing in to efficient growth or in aggressive phenotypes such as antibiotics (Schlatter and Kinkel, 2015), which are choices that may depend on environmental conditions such as population density. In this study, 10^3 CFU/mL of the three species were used in each experiment, and this concentration somehow could not be considered high-density contamination. However, we are inclined to adopt the first strategy in terms of the efficient growth of *Salmonella*, as TEY medium seems to be most suitable for its growth, as well as the two incubation temperatures, especially the 37°C. Anyhow, it is not possible to reject the existence of the aggressive phenotype strategy, but we need to determine the levels of antibiotics within the medium to prove such a hypothesis.

Importantly, in the present work, the assessment of both the bacterial growth and the bacterial competition was conducted within the semen medium without the presence of spermatozoa itself. Such experimental design was important for us to obtain accurate results on the effects of TEY medium that do not interfere with the effects of the different bacteria species that usually accompany the spermatozoa (Moretti *et al.,* 2009).

Finally, we must state three major limitations of our present pilot study here: the first limitation is that in our experimental set-up, we started with an initial cellular ratio of 1:1 or 1:1:1 for the two/three used bacteria species. It must be stressed out that in natural environments, such distribution is rather unlikely; moreover, pathogens are usually outnumbered by far by non-pathogenic bacterial cells, and in this case, they may affect the competition status. The second limitation was that the three used pathogens were in sp. form, however, the use of specific species from these pathogens (eg. O157:H7 for *E. coli*, dysentery for *Shigella*) may reveal a more accurate and clear picture of the competition status. The third limitation was the use of only two temperature degrees of incubation (37 and 4°C). Anyhow, other degrees such as the room temperature degree (20-25°C) may help both *Shigella* sp*.,* and *E. coli* to be more resistant and competitive to *Salmonella*. Despite these significant limitations, our experimental approach here clearly showed the general competition behavior of these three pathogens in this important semen medium. However, the present experiments are initial and pilot experiments and further studies may be conducted using another cell ratio, other specific pathogens, and other experimental conditions.

Conclusions

The present data are extremely vital for a better understanding of the competition status between *Salmonella sp.*, *Shigella sp.,* and *E. coli* in the media supplemented with egg yolk. *Salmonella* was the dominant pathogen in the TEY medium regardless of the temperature degree or the time of incubation. There is extreme importance in evaluating the level and conditions of contaminations in Egg Yolk media to minimize the potential risk of micro-organism contaminations. Anyhow, good handling practices should always be observed in the laboratories. However, future studies of social behavior in micro-organisms would benefit greatly from experimental analyses of the fitness of competition status leading to better management of semen media preparation.

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