Influence of buffer systems on the interaction between a random DNA library and *Salmonella typhimurium* cells.

Influencia de sistemas buffer en la interacción entre una biblioteca de ADN aleatorio y células de **Salmonella typhimurium.**

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Highlights

- The buffer system used does not affect the interaction between DNA sequences and *S. typhimurium cells*.
- Quantification by qPCR enables the identification of changes in the interaction between aptamers and their target molecules.
- $MgCl_2$ influences the binding between potential aptamers and *S. typhimurium cells*.

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ABSTRACT

Introduction. *Salmonella enterica* subsp. enterica ser. Typhimurium (*S. typhimurium*) is a gram-negative bacterium of significant public health concern due to its ability to be transmitted through food and is the cause of several diseases collectively known as salmonellosis. The development of rapid and specific detection systems for this microorganism is crucial to strengthen food quality control programs and the prevention of infectious outbreaks. This need underscores the importance of studying novel diagnostic approaches, such as those based on aptamers, which are typically generated by SELEX technique. The parameters for this technique are defined by individual research groups and directly affect recognition capabilities of the aptamers for their targets. **Objective.** In this study, we investigated the influence of three different buffer systems and varying concentrations of magnesium chloride ($MgCl_2$) on the binding capacity of a single-stranded random DNA library to *S. typhimurium* cells. **Materials and Methods.** *S. typhimurium* cells were incubated with random DNA sequences in the presence of phosphate-buffered saline (PBS), Tris-buffered saline (TBS), or Tris (TK) buffers with varying concentrations of magnesium chloride ($MgCl_2$). Unbound or weakly bound sequences were separated by centrifugation and quantified by qPCR. Statistical differences between experimental conditions were analyzed using analysis of variance (ANOVA). **Results.** The selection of buffer system did not significantly affect the recovery of DNA sequences complexed to *S. typhimurium* cells at a constant $MgCl_2$ concentration. However, changes in $MgCl_2$ concentration markedly influenced the amount of sequences bound to *S. typhimurium* cells. **Conclusions.** The interaction between DNA sequences and *S. typhimurium* cells was maximized at a $MgCl_2$ concentration of 2.5 mM in TBS buffer.

RESUMEN

Introducción. *Salmonella entérica* subsp. entérica ser. Typhimurium (*S. typhimurium*) es una bacteria gramnegativa de gran relevancia para la salud pública debido a su capacidad de transmitirse a través de alimentos, causando diversas enfermedades conocidas colectivamente como salmonelosis. El desarrollo de sistemas de detección rápidos y específicos para este microorganismo es crucial para fortalecer los programas de control de calidad alimentaria y prevenir brotes infecciosos. Esta necesidad resalta la importancia de estudiar nuevos enfoques de diagnóstico, como aquellos basados en aptámeros, que suelen generarse mediante la técnica SELEX. Los parámetros de esta técnica son definidos por cada grupo de investigación y afectan directamente las capacidades de reconocimiento de los aptámeros hacia sus objetivos. **Objetivos.** En este estudio, evaluamos la influencia de tres sistemas tampón diferentes y de concentraciones variables de cloruro de magnesio ($MgCl_2$) en la capacidad de unión de una biblioteca aleatoria de ADN monocatenario a células de *S. typhimurium*. **Materiales y Métodos**. Las células de S. *typhimurium* se incubaron con secuencias de ADN aleatorias en presencia de tampones buffer salino fosfato (PBS), Tris buffer salino (TBS) o Tris (TK), con diferentes concentraciones de $MgCl_2$. Las secuencias no unidas o débilmente unidas se separaron por centrifugación y se cuantificaron mediante qPCR. Las diferencias estadísticas entre las condiciones experimentales se analizaron utilizando ANOVA. **Resultados.** El sistema tampón utilizado no afectó significativamente la recuperación de secuencias de ADN unidas a las células de *S. typhimurium* a una concentración constante de $MgCl_2$. Sin embargo, las variaciones en la concentración de $MgCl_2$ influyeron significativamente en el número de secuencias unidas a las células de *S. typhimurium*. **Conclusiones.** La interacción entre las secuencias de ADN y las células de *S. typhimurium* aumentó a una concentración de $MgCl_2$ de 2.5 mM en el tampón TBS.

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INTRODUCTION

Salmonella typhimurium is an enteric pathogen responsible for the foodborne disease known as salmonellosis. Conventional detection systems for this microorganism are based on microbiological analysis, serological methods, and molecular fingerprinting techniques [1]. However, despite the availability of various diagnostic systems, there is still a need to develop tools that increase sensitivity and specificity for the rapid detection of these enterobacteria. As an alternative, the use of short, artificial oligonucleotide sequences has been described. DNA and RNA possess structural information that enables them to specifically recognize and interact with metal ions, small organic molecules, proteins, and cells [2,3]. This understanding began to solidify with the studies conducted by Craig Tuerk and Larry Gold in 1990, in which they randomly modified RNA sequences to evaluate their binding capacity to T4 DNA polymerase [4]. They developed a method called Systematic Evolution of Ligands by EXponential Enrichment (SELEX), in which known RNA sequences containing a random segment were exposed to a target molecule to determine the sequence with the lowest dissociation constant (K_d) [3]. In the same year, Andrew D. Ellington and Jack W. Szostak coined the term "aptamer" to define RNA sequences that specifically "fit" with their target [5]. Since then, several SELEX variants have been used to design DNA and RNA aptamers capable of specifically recognizing carbohydrates, lipids, proteins, drugs, and cells [6,7,8].

Given current technologies for the specific, cost-effective, and rapid synthesis of synthetic DNA sequences, aptamers are emerging as an alternative to antibodies, reducing production times and avoiding the variability and limitations associated with the use of animals or cells [3]. The design of aptamers requires consideration of the properties of the nucleotide chain, the selected target molecule, and the medium in which their interactions occur. On the one hand, the pleomorphic nature of DNA, RNA, or analogous sequences is determined by the nucleotide sequence and the chemical properties of each base. Conversely, the target molecule or cell also has structural conformations defined by characteristics such as chiral sites, intrinsic biological variability, solubility, and biochemical properties influenced by the solution in which it resides. Finally, the medium in which the aptamer-target recognition takes place provides properties such as ionic strength, pH, and the presence of specific ions that influence the charge of dissociable groups, the stability of weak interactions such as hydrogen bonds and van der Waals forces, and the number and type of interactions that can form [9].

The Mg^{2+} ion is known to stabilize hairpin and duplex structures in aptamers, contributing to the global structural stability and facilitating binding interactions. In summary, the environment of the buffer system environment and magnesium play a key role in the formation of the aptamer structure formation and its binding behavior to the target [10].

Although various studies have reported the development of aptamers for the detection of *Salmonella* sp. cells [11,12,13], they use different buffers for the interaction between sequences and their target, often using a fixed $MgCl_2$ concentration or omitting it from the system. Therefore, in this study, we investigated the influence

of three different buffers and varying $MgCl_2$ concentrations to identify optimal binding conditions for the Cell-SELEX technique using *Salmonella typhimurium* cells.

MATERIALS AND METHODS

Culture of *Salmonella typhimurium*

A commercial strain of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC 14028), obtained from the strain collection of the Universidad de Santander, was used. The strain was mantained at -20°C. For all experiments, the microorganism was reactivated in tryptic soy broth (TSB) at 37°C for 24 hours. Subcultivation was then performed in 10 mL of TSB, from which 1 mL was taken to wash the cells twice by centrifugation at 13,000 rpm, 25°C for 1 minute with PBS (pH 7.2) to remove residual medium that could interfere with the aptamer-cell interaction. The cell concentration was adjusted in 0.9% saline solution using spectrophotometry at 625 nm to achieve an absorbance of 0.08. Under these conditions, colony forming units (CFUs) were established using serial dilutions and drop plating on TSB agar (**Table S1 of the supplemental material 1.**). For each experiment, cell concentration was verified by surface plating on TSB agar in triplicate.

Random DNA library

The random DNA library (RDL) reported by Savory in 2014 was used, consisting of 66 nucleotides arranged in three segments: a central random segment of 24 nucleotides (nt) flanked by two constant segments of 18 nucleotides each at the 5' and 3' ends. The random segment was separated from the constant segments by three thymines at each end (5'-ACGGCTCGCACTCTCTGATTT[N_{24}]TTTACTCCTGCGTGCTTCTCA-3') [14]. The library was synthesized by Macrogen (South Korea) and reconstituted in nuclease-free water to a final concentration of 100 µM, corresponding to 6.022×10^{13} sequences/µL (**Section 2 in the supplemental material**). The theoretical diversity was estimated to be 2.8×10^{14} different combinations (4^{24}).

Interaction tests in different buffer systems

Three buffer systems were evaluated: modified TBS without $MgCl_2$ (10 mM Tris-Cl, 150 mM NaCl, 5 mM KCl) [14]; PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) [15]; and TK (20 mM Tris-Cl, 50 mM KCl). All buffers were used at pH 7.4. Reactions were performed in a final volume of 1 mL, consisting of 5 pmol of the library (3×10^{12} sequences) and 4.5×10^5 CFU/mL, resulting in a bacterium-to-sequence ratio of 1:6.6×10^6, which facilitates interaction between all possible sequences and *S. typhimurium* cells. Reactions were incubated at room temperature with constant shaking at 140 rpm for 60 minutes.

After incubation, the solution was centrifuged at 13,000 rpm for 10 minutes, obtaining two phases: i) the supernatant containing non-interacting RDL sequences, referred to as fraction 1 (F1), and ii) the cell-containing pellet, which is expected to contain RDL sequences bound to *S. typhimurium* cells. From the supernatant, 950 µL was removed, and the cells were washed twice with 1 mL of the appropiate buffer to eliminate unbound or weakly bound sequences. The cells were then resuspended in the corresponding buffer supplemented with 1 M NaCl to release cell-bound sequences. This suspension was centrifuged at 13,000

rpm for 2 minutes, and 100 µL were collected for precipitation with 0.3 M sodium acetate and 300 µL of cold absolute ethanol.

Sequences were centrifuged at 12,000 rpm for 10 minutes and washed twice with cold 70% ethanol. The ethanol was removed, and remaining ethanol was allowed to evaporate for 15 minutes. Finally, 20 µL of nuclease-free water were added to resuspend the recovered RDL sequences, referred to as fraction 2 (F2). RDL in both F1 and F2 were stored at -20°C until quantification by qPCR. Exposure of *S. typhimurium* cells to RDL was performed in duplicate, with the microorganism reactivated for each experiment.

Influence of $MgCl_2$

The binding reaction between *S. typhimurium* cells and RDL was performed as previously described. All buffer systems (TBS, PBS, and TK) were supplemented with 1, 2.5, or 5 mM $MgCl_2$. Fractions F1 and F2 from these reactions were quantified by qPCR, and the results from F2 were used to determine the sequence recovery percentage.

Quantification of sequences by qPCR

Fractions 1 and 2 were diluted 1:100 in nuclease-free water prior quantification. The BrightGreen 2X qPCR MasterMix system (abm Inc) was used according to the manufacturer's instructions. Each reaction included 1 µM of each oligonucleotide [14], 2 µL of the fractions or standards (ranging from 10^5 to 10^8 sequences/µL), in a final volume of 10 µL. The amplification protocol included an enzyme activation cycle at 95°C for 3 minutes, followed by 40 cycles of 95.0°C for 15 seconds, and hybridization at 50.0°C for 40 seconds. A dissociation curve from 70 to 90°C with 0.1°C resolution and 5-second intervals between each step was programmed to detect potential changes in the dissociation pattern of each fraction analyzed.

Each fraction was amplified in triplicate. Amplification results were analyzed using the Bio-Rad CFX Maestro software, and the quantity of aptamers was expressed as the number of sequences per microliter based on the standard curve. For Fraction 1, the total sequences in the supernatant were calculated considering the sample volume and dilution used.

One-way ANOVA followed by Bonferroni's post hoc test was performed to assess the significance of differences between conditions. Statistical analysis was performed using OriginPro (Learning Edition 2024).

RESULTS

Evaluation of Buffer Systems

The sequences from the library binding to *S. typhimurium* cells was performed in three steps, as shown in Figure 1. First, bacterial cells were exposed to the sequences in the presence of TBS, PBS, and TK buffers without the addition of $MgCl_2$ **(Figure 1A)**. Unbound sequences were then separated from the cells by centrifugation. Sequences remaining in the supernatant were designated as fraction 1 (F1), while those bound

to *S. typhimurium* cells in the pellet were termed as fraction 2 (F2) **(Figure 1B)**. Finally, the number of sequences in F1 and F2 was quantified by qPCR **(Figure 1C)**.

A higher number of unbound sequences (F1) was detected compared to the number of sequences recovered from *S. typhimurium* cells (F2) **(Figure 2A)**. This result is consistent with the cell to RDL ratio used in the experiments. Regarding the buffer system, no significant differences were observed in the number of sequences recovered for each system ($p > 0.05$). However, lower data dispersion was observed for F2 in the TBS system.

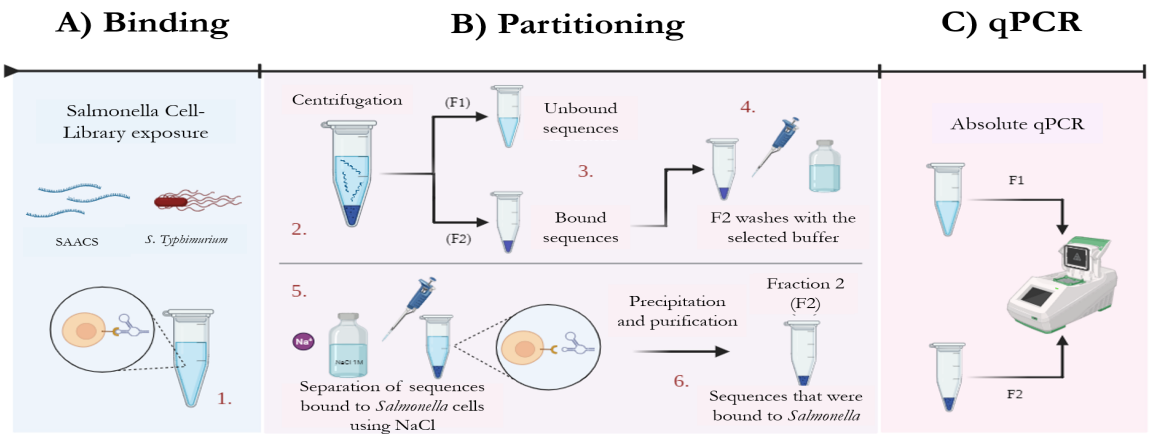

Figure 1. General scheme of the Cell-SELEX procedure. (A) The cells were exposed to each selected buffer system and different concentrations of $MgCl_2$, together with RDL sequences. After incubation, two fractions were separated by centrifugation **(B)**: the sequences remaining in the supernatant (F1) and those bound to *S. typhimurium* cells (F2). The bound sequences were separated by the addition of 1 M NaCl followed by precipitation with absolute ethanol to remove excess salt. Finally, both fractions were used as samples for qPCR analysis **(C)**.

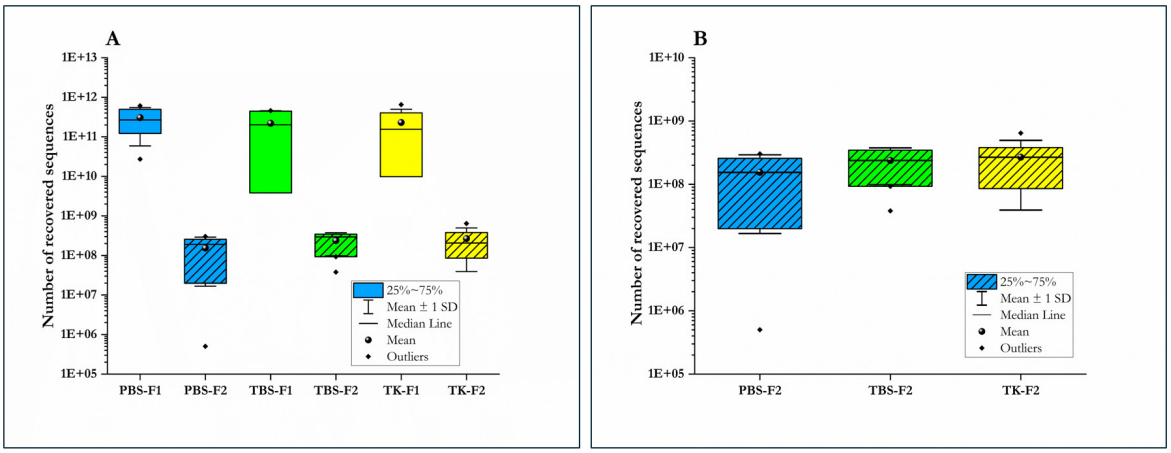

Figure 2. Number of recovered sequences (plotted on a log_{10} scale) after the interaction between RDL and *S. typhimurium* cells using PBS, TBS, and TK buffers. (A) Quantification of sequences in fractions F1 and F2. **(B)** Comparison of sequences recovered exclusively in fraction F2 for each buffer.

Evaluation of the interaction between RDL and *S. typhimurium* cells at different $MgCl_2$ concentrations

Although no significant differences were found in the number of sequences recovered from *S. typhimurium* cells depending on the buffer system, the TBS buffer was selected for experiments with different $MgCl_2$ concentrations due to the lower data scatter observed in the previous phase **(Figure 2A)**. Since the reported composition of this buffer includes 1 mM $MgCl_2$, for this experiment, the buffer was prepared without this salt and subsequently supplemented with 1, 2.5, or 5 mM $MgCl_2$.

As shown in the buffer evaluation experiments, F1 yielded a higher number of sequences compared to F2 **(Figure 3A)**. In contrast, fewer sequences were recovered when the incubation was performed without $MgCl_2$. Notably, the presence or absence of $MgCl_2$ in TBS did not result in significant differences in the number of copies recovered **(Figure 3)**.

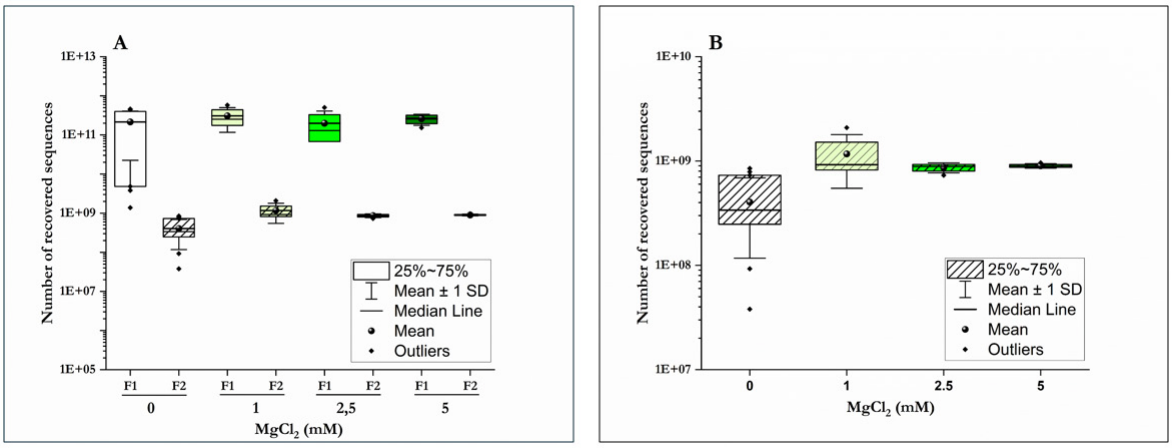

Figure 3. Number of sequences recovered (expressed as Log_{10}) from *S. typhimurium* cells using TBS buffer without or supplemented with $MgCl_2$ (1, 2.5, or 5 mM). (A) Number of sequences recovered for F1 and F2 fractions under each treatment. **(B)** Number of sequences recovered exclusively for fraction 2. No significant differences were observed in the number of sequences recovered among the different $MgCl_2$ concentrations used.

Based on the total number of sequences recovered in fractions F1 and F2 for each condition (TBS with or without $MgCl_2$ supplementation), the percentage of sequences bound to the total exposed cells was calculated **(Figure 4)**. On average, the recovery slightly exceeded 1% when using the unsupplemented TK buffer **(Figure 4A)** or TBS buffer supplemented with 2.5 mM $MgCl_2$ **(Figure 4B)**. No significant differences were observed in the number of sequences bound to *S. typhimurium* between PBS, TBS, or TK buffers. Similarly, no significant differences were observed between $MgCl_2$ concentrations when using TBS buffer supplemented with this ion **(Figure 4B)**. However, it is worth noting that $MgCl_2$ supplementation reduced the data scatter, with this effect being more pronounced at a concentration of 5 mM **(Figure 4B)**.

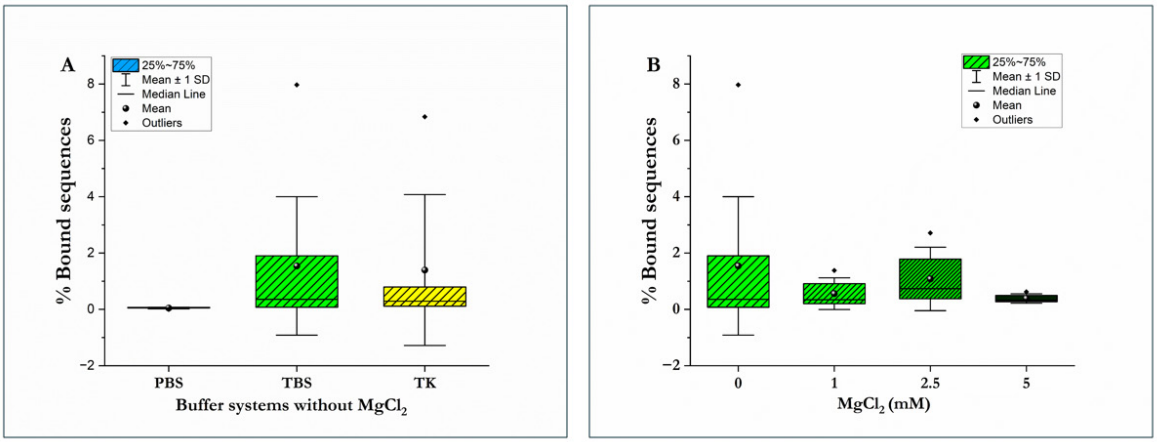

Figure 4. Percentage of sequences bound to *S. typhimurium* using different buffer systems (A) or $MgCl_2$ concentrations (B).

Although no significant differences were observed in the number of sequences recovered in the fraction F2 using the TBS buffer, regardless of $MgCl_2$ supplementation, the inclusion of F2 fractions obtained with unsupplemented PBS and TK systems showed that the TBS buffer supplemented with $MgCl_2$ recovered a higher number of sequences compared to the unsupplemented PBS and TK systems **(Figure 5)**.

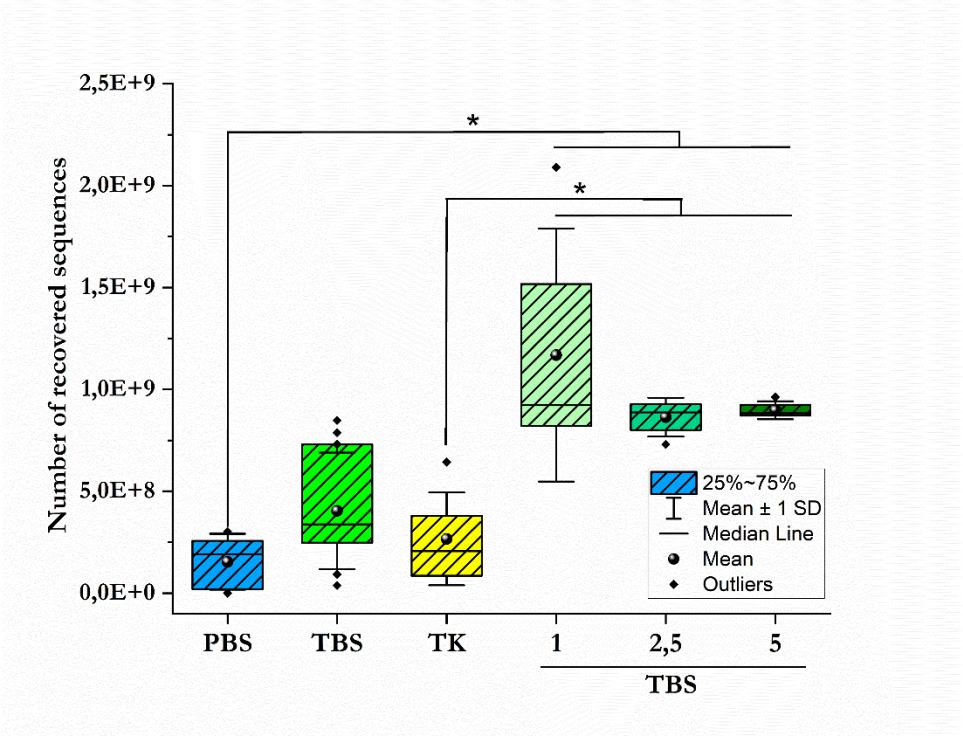

Figure 5. Comparison of fractions recovered from *Salmonella typhimurium* cells using different buffer systems with or without $MgCl_2$ supplementation

Finally, the dissociation curves revealed no differences in the patterns observed for F1 and F2 fractions, regardless of whether the buffers were supplemented with $MgCl_2$. However, a more pronounced dissociation was detected between 80°C and 82°C across all conditions evaluated (**Supplementary Data 5**).

DISCUSSION

S. typhimurium is one of the most important foodborne pathogens worldwide [16]. It is the major cause of human salmonellosis, which is transmitted by survival and multiplication of the bacterium in poultry farms, birds, and eggs [17]. This underscores the importance of developing novel detection techniques and thoroughly investigating the factors that influence these methods to enable continuous improvement in the detection of disease caused by enteropathogens.

In this study, we used a previously reported RDL library for the development of aptamers *E. coli* [14]. This library, designed with a potential of 2.81×10^{14} different sequences, allows the recognition of a wide range of targets. It has been described that the greater the structural diversity within a library, the higher the probability of containing aptamers capable of interacting with the desired target, which ultimately determines the success or failure of the selection process [10]. Examination of the reported conditions for RDL-target interactions reveals that each system is tailored to enhance specificity and optimize the library's ability to adopt specific conformations that enable target interaction [1,18].

Among these variable conditions are the buffers used, with PBS and TBS standing out for their ability to maintain pH, osmolarity, and ionic strength [19,20]. In addition to these systems, we evaluated the TK buffer, a non-conventional, simple buffer composition typically used for DNA-interacting polymerases. TK buffer also contains a higher concentration of Tris (2-amino-2-hydroxymethyl-propane-1,3-diol) concentration, which has been reported to increase RDL binding affinity to adenosine analog targets like ATP [22]. For PBS and TBS buffers, ionic strength (0.161 and 0.160 mol/L, respectively) and osmolarity (313.4 and 333 mOsm, respectively) were similar, whereas TK buffer had lower ionic strength (0.06 mol/L) and osmolarity (140 mOsm) **(Supplementary Table C3.1)**.

Despite these differences, no variation in RDL binding to *S. typhimurium* cells were observed among the three buffers. This result suggests that, in this model, buffer components, ionic strength, and osmolarity do not exert a detectable effect on aptamer recovery as assessed by qPCR. Further studies are required to further investigate how the chemical properties of buffer systems might modulate the structure of aptamers or the membrane conformation of *S. typhimurium* cells.

Ionic strength, particularly the presence of metal cations such as Mg^{2+}, Na^{+}, and K^{+}, has been reported to affect aptamer stability and binding affinity [22,23]. In this study, we found that a significantly higher number of potential aptamers bound to *S. typhimurium* cells in the presence of $MgCl_2$. This result is consistent with the findings of Zhang et al., who reported that the optimal Mg^{2+} concentration for aptamer-bacteria complex formation was 2 mM in a Tris-EDTA buffer system at pH 8.0. They also found that the binding capacity

decreased at concentrations above 2 mM [24]. Similarly, Cai et al. (2018) reported that aptamers binding to tetracycline exhibited high affinity and specificity at $MgCl_2$ concentrations of 3 mM or greater, whereas binding did not occur in the absence of this ion, a finding consistent with our study [25]. Another study reported that 3 mM Mg^{2+} was optimal for stabilizing the structure of a thrombin-binding aptamer, suggesting that higher Mg^{2+} concentrations reduce conformational flexibility, thereby increase aptamer rigidity [26].

Our results also showed that at 5 mM $MgCl_2$, the number of RDL recovered was lower, although not significantly different. It has been described that increasing metal cation concentrations can decrease the binding affinity of aptamers by neutralizing the negative charge of DNA, thereby reducing potential electrostatic interactions with the target. Conversely, lower concentrations typically increase binding affinity, except in some cases, such as Na^+, where binding affinity varies depending on the target [22,23]. This trend was consistent with our findings regarding the number of RDL per bacterial cell **(Figure 4B)**. Although the lowest data dispersion was observed at 5 mM $MgCl_2$, the binding efficiency decreased as this concentration increased **(Figure 3B)**. In this specific case, $MgCl_2$ may have stabilized the secondary structure of the aptamers by neutralizing the phosphate backbone's negative charge, thereby increasing their ability to bind to negatively charged targets, such as the lipid membrane of *Salmonella* [27].

Previous studies have reported changes in dissociation pattern of amplicons obtained by the SELEX technique, specially from the fourth round of selection when an 80-nt library with a 40-nt variable region was used [28]. In our study, a 66-nt library with a 24-nt variable region was used, resulting in a higher proportion of invariant sequences (42 nt or 63% of the total sequence). This may make it more difficult to detect changes in dissociation patterns.

In contrast to other studies focusing of the development of specific aptamers for *Salmonella* cell recognition, this study employed a lower initial sequence count (5 pmol), which limits the exposure of all possible combinations within the original library to bacterial cells. Therefore, we recommend considering the combinatorial diversity of the library when determining the number of sequences that will interact with the target cells [18].

CONCLUSIONS

No significant differences were observed in the number of sequences recovered after exposing RDL to different buffer systems with *S. typhimurium* cells. However, the results obtained with the TBS buffer showed less variability in sequence recovery. Additionally, supplementing the buffer system with $MgCl_2$ increased the binding of RDL to *S. typhimurium* cells.

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