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Surprising Effects of Rocking Motion on *Leishmania tarentolae* Behavior in Culture and Implications for Cell Stress

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Abstract: *Leishmania* are an understudied genus of parasitic protozoans causing significant health problems for people, particularly in tropical climates. To better understand the growth of *Leishmania* and potential drug sensitivity implications, the effects of motion on cells grown in vitro were probed. Using a stock *Leishmania tarentolae* cell culture, cells were placed in 10 mL of a Brain–Heart Infusion medium in either a non-moving (static) environment or on a flat platform of one of two lab rockers (set at a minimal speed) in a dark environment for 13 days. Also, the addition of 0.5 M of L-Proline was evaluated. Microscopy, cell clumping, cell viability, and secreted acid phosphatase (SAP) activity data were collected. Results show that a constant slow rocking motion changed cell growth, clumping behavior, and detectable SAP activity relative to the no-motion cultures, but this change was dependent on which rocker was used, indicating a complexity in the growth of these cells in culture. Thus, continuous motion affects the stresses placed on the cells during a growth curve under some conditions. The implications of this study lead to questions about the effects of motion on the efficacy of pharmaceutical testing in vitro. Further study of the effects of motion on *Leishmania* is important.

Keywords: *Leishmania tarentolae*; rocking motion; cell viability; proline; secreted acid phosphatase

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1. Introduction

Leishmania, which can result in leishmaniasis infections in humans and domestic animals, are an understudied genus of protozoan parasites that cause significant health problems, particularly in tropical climates and marginalized communities. Leishmaniasis is caused via the transfer of the parasitic protozoa to a human or domestic animal from the bite of an infected sandfly. The infections generally manifest in humans in three ways: cutaneous leishmaniasis (skin sores), mucocutaneous leishmaniasis (sores in the mouth, throat, and nose), and visceral leishmaniasis (infection of the internal organs by the parasite) [1].

Treatments for leishmaniasis include pentavalent antimonial drugs, amphotericin B, miltefosine, and pentamidine. However, these drugs come with side effects, and there are reports of drug resistances developing [2]. In addition, the treatment of leishmaniasis patients, especially those who also suffer with co-infections of HIV or tuberculosis, can be difficult [3].

Research on *Leishmania* is essential to improve our understanding of the disease and develop new and effective treatments. To better understand the growth and lifecycle of *Leishmania*, as well as the potential implications for drug sensitivity, the effects of rocking motion on cell growth and viability in vitro were investigated. These results were compared to those of the static cultures (no motion). *Leishmania tarentolae* was used as a model because it is not considered to be pathogenic to humans and has been successful in screening antileishmanial drugs [4].

Assessment of *Leishmania tarentolae* cell viability as a function of days in culture, under three different condition variables (Static, Rocker-1, and Rocker-2), was conducted

along with monitoring the effects of the added L-Proline. The addition of L-Proline, which is an amino acid found in the collagen of skin and other tissues, was of particular interest because cutaneous infections represent the most common clinical presentations of *Leishmania* diseases [5].

Cell cultures were tracked through the lag, log, stationary, and senescence phases of cultures for 13 days. Microscopy was performed daily, and the ability of the cells to form rosettes (clumps) was monitored. After the 13-day growth period, all cultures were tested for the detection of secreted acid phosphatase (SAP). SAP activity is of interest since the enzyme's activity has been implicated in the success of *Leishmania* infections [6]. Implications of this study raise questions about the impact of motion on the efficacy of in vitro pharmaceutical testing.

2. Results

In the early days of the cultures' growth, an interesting observation was made regarding the difference between the Static and Rocked cultures. The cells in the Static culture exhibited a diffuse pattern, which was evenly distributed. In contrast, cells in the Rocker-1 and Rocker-2 cultures congregated in a line parallel to the rocking axis of the gel rocker. This observation held true for all the static and rocked flasks in the experiment. Figure 1 shows the observable 'patterns' seen by the eye in the flasks grown in static and rocked conditions. The rocked culture image was taken without removing the flasks from the rocker platform. Pictures were taken using a Canon Rebel T1i camera.

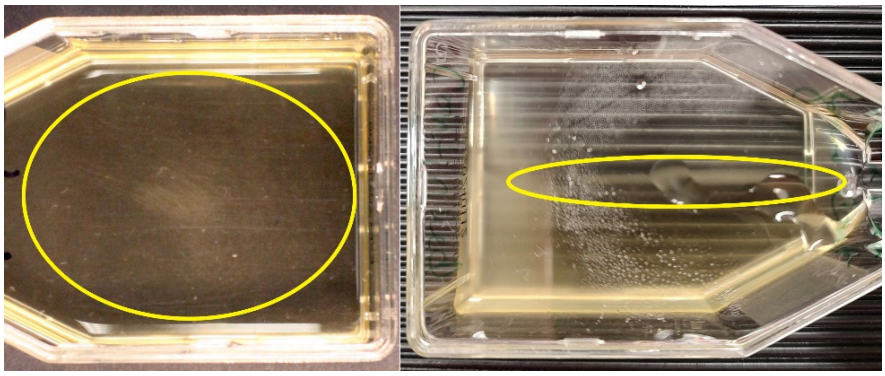

Figure 1. Distinct patterns of cell distribution were observed throughout the growth period. The yellow circles highlight the location and distribution of the cells. The image on the left displays a Static Control culture, where cells exhibited a diffuse distribution pattern. In contrast, the image on the right shows Rocker-1 Control cells congregating on a line parallel to the rocking axis of the gel rocker. The pattern observed for Rocker-1 cultures was also observed for Rocker-2 cultures.

2.1. Cell Viability

2.1.1. Growth Curve

The MTT spectrophotometric assay, utilizing 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheny ltetrazolium bromide (MTT reagent), was used to evaluate cell viability and generate growth curves for each culture. Notable differences in the shapes of the growth curves were observed over the course of the 13-day growth period. Growth curves are shown in Figure 2.

Of particular significance was the wide range of results observed in the growth curves due to varying culture conditions. Each condition [Static (+/− Proline), Rocker-1 (+/− Proline), and Rocker-2 (+/− Proline)] resulted in a distinct response from the cells. This contrast was most pronounced in the cases of Rocker-1 and Rocker-2. The growth curves vary distinctly, despite sharing identical speed and tilt settings. Rocker-2 (+/− Proline) growth curves had a longer lag phase. This resulted in the stationary phase being shifted to the right. As a result, the Rocker-2 cultures (+/− Proline) entered in the logarithmic phase while the Static and Rocker-1 cultures progressed in the senescence phase. Additionally,

both the Rocker-1 and Rocker-2 cultures (+/− Proline) demonstrated longer stationary phases in comparison to the Static cultures (+/− Proline).

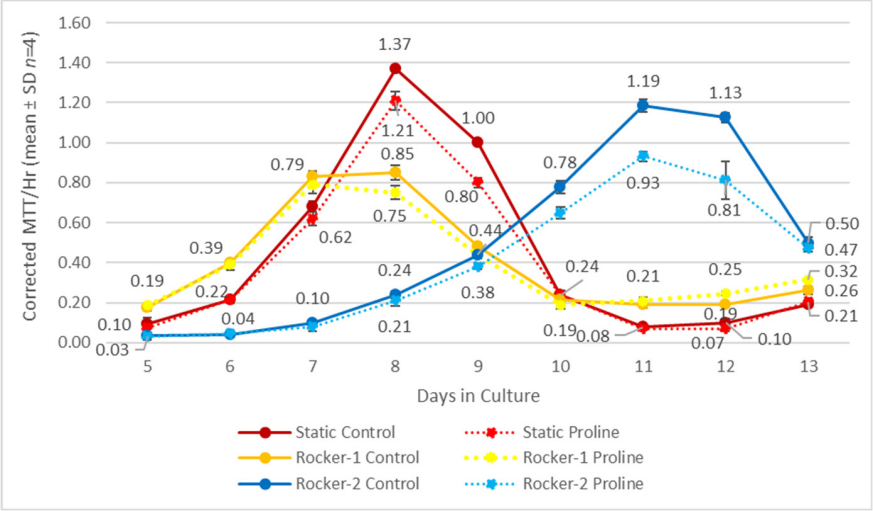

Figure 2. Results of the MTT viability assays over the course of the growth cycle. Corrected Mean and SD values (n = 4) are given for the 13-day growth period for the Static or Rocked conditions (+/− Proline). In some cases, the SD is smaller than the size of the mean symbol used.

Another observation was the consistency of growth trends between the Control and Proline cultures across the three different conditions. All Proline cultures exhibit reduced MTT viability relative to their respective Control cultures. However, Rocker-2 Proline had a more pronounced negative impact (approximately a 20–30% reduction) during the stationary phase, contrasting with the more modest decreases observed in the Static (12% reduction) and Rocker-1 (3% reduction) cultures.

2.1.2. Area under the Curve for Culture Growth Comparison

To better compare the overall cell viability throughout the growth curves of the cultures, we calculated the Area Under the Curve for each condition using the following equation:

$$\text{Area Under the Curve} = \Sigma\,[(\text{day y} - \text{day x}) \times (\text{MTT Day x} + \text{MTT day y})/2]$$

A comparison of the Area Under the Curve of the six different treatments is illustrated in Figure 3. Calculations show that Rocker-2 Control demonstrated the largest area, indicating that it had the highest amount of viable cell detection over the 13-day growth period. In contrast, Rocker-1 Proline showed the smallest area and therefore the lowest amount of viable cell detection. When comparing the Area Under the Curve of the six cultures, we found that Rocker-2 Control demonstrated greater cell viability than both the Static Control and Rocker-1 Control. Specifically, Rocker-2 Control had an 8% increase in cell viability over Static Control cells and a 20% increase in over Rocker-1 Control. We conclude that Rocker-2 Control provided the best conditions for promoting *Leishmania* cell growth, despite these cultures taking longer to reach the stationary phase.

The addition of L-Proline had a negative effect on cell viability across the three treatments. Among the three Control cultures (no Proline), the Total Area trend was 4.81 > 3.87 > 3.38, with the ranking Rocker-2 > Static > Rocker-1, respectively. This trend indicated an overall approximate 20% change from the highest value (Rocker-2) to the lowest value (Rocker-1). This trend was not observed in the three Proline cultures. The trend between the Proline treatments was 3.37 > 3.36 > 3.27 with the ranking Static Control > Rocker-2 > Rocker-1, respectively. This implied an approximate 3% change from the

highest value (Static) to the lowest value (Rocker-1). We conclude that while L-Proline led to reduced apparent cell viability, the static or motion conditions had no additional effect.

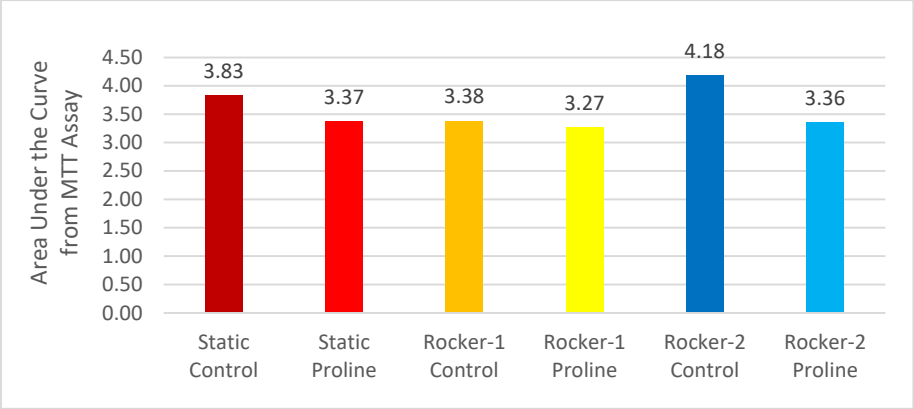

Figure 3. A comparison of the Area Under the Curve from the MTT Viability assays for the six treatment conditions. Area Under the Curve from the MTT assay is the total area underneath the growth curve for each treatment during the 13-day growth period.

2.2. Clumping

Researchers (Lovannisci et al., 2010 [7]) have reported an association of the individual cells forming rosettes, which subsequently associated into larger clumps (clusters) in response to the culture conditions, especially temperature and the expression of polysialic acid (PSA) and de-N-acetylneuraminic acid [7]. These authors also hypothesized that rosettes may initiate mating in *Leishmania*. Thus, clump count can be associated with the adaptation of the cells to their environment, especially in response to stress.

2.2.1. Counting Clumps

Cultures were inspected via light microscopy (at 250× total magnification) daily over 13 days for all six conditions. At the time of inspection, four photographs of random areas of each flask were taken. The photographs were then opened in Adobe Photoshop where the Count Tool was used to determine the number of clumps in each photograph. Only clumps passing a minimum threshold were counted. This threshold was determined before the experiment began. A typical counting area (via microscopy) is shown in Figure 4.

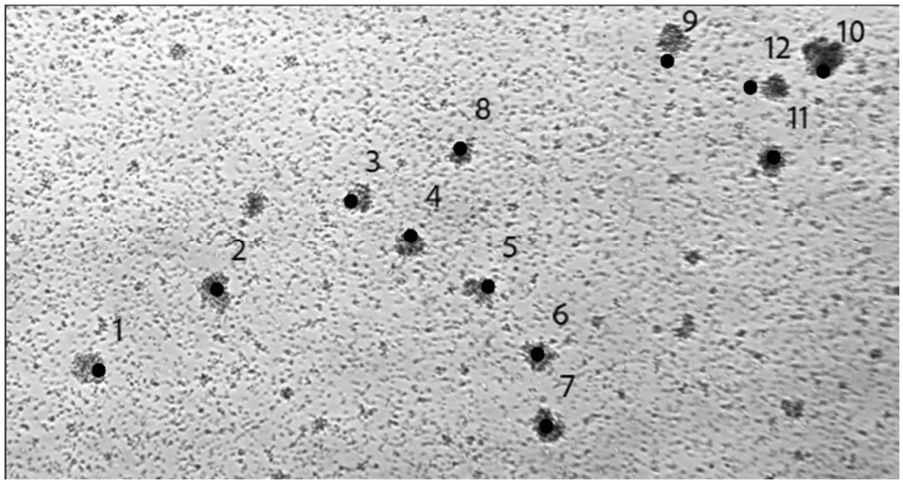

Figure 4. A typical example of clump counting. The Count Tool in Photoshop was used to mark and count the clumps. Only clumps surpassing a minimum threshold were labeled with markers and tally numbers (1–12). Notice the clump just above Clump 2 that was not included in the count because it did not pass the minimum threshold (250× magnification).

2.2.2. Clumping Results

Changes in the average number of clumps per day over the length of the experiment are shown in Figure 5. All cultures were slow to develop noticeable amounts of clumps until Day 9. At that time, the Static cultures and Rocker-1 Proline began to exhibit more detectable clumping. In contrast, the Rocker-2 cultures did not exhibit an increase in detectable clumping until Day 11. Rocker-1 Control produced the greatest number of clumps on a given day with a total of 24 (Day 11) and continued to have a high number of clumps per day for the remainder of the experiment. Rocker-1 Proline produced the second highest number of clumps on a given day with 15 clumps (Day 11). The Rocker-2 cultures produced low numbers of clumps per day as compared to the Static and Rocker-1 cultures. Rocker-2 Control peaked first with seven clumps (Day 12) and Rocker-2 Proline peaked with six (Day 12).

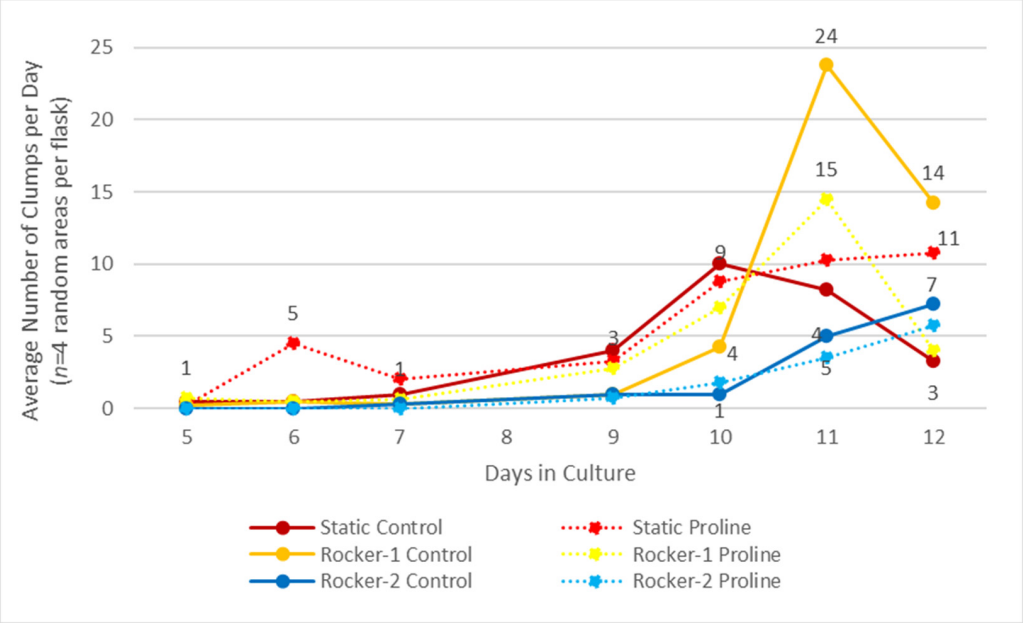

Figure 5. Changes in the Average Number of Clumps per Day. To calculate the Average Number of Clumps per Day, four random areas of a flask were photographed during microscopy. The number of clumps in each image were counted and then averaged together with the number of clumps from the other three images from the same flask.

The overall total clump count generated by a culture is also of interest (Figure 6). To compute this, the clump count from each random area of the flask was obtained. These counts were then cumulatively combined with the data from each day of the culture's growth. The Total Clumps produced by each culture varied greatly. The Total Clump trend was 177 > 157 > 120 > 109 > 58 > 47 with the ranking Rocker-1 Control > Static Proline > Rocker-1 Proline > Static Control > Rocker-2 Control > Rocker-2 Proline, respectively. Interestingly, Rocker-2 Control developed the second smallest number of total clumps (58) while producing the largest Area Under the Curve (4.18) MTT response.

Comparing the ratios for the Total Clumps Produced to the Area Under the Curve from the MTT assay substantiates the inconsistencies (Figure 7). The calculated ratios for the Static and Rocker-1 cultures vary considerably (Static Control = 28, Static Proline = 47, Rocker-1 Control = 52, and Rocker-1 Proline = 37). Only the Rocker-2 cultures demonstrate similarity with both cultures having ratios of 14. The Rocker-2 cultures produced more cells with fewer clumps than any other cultures.

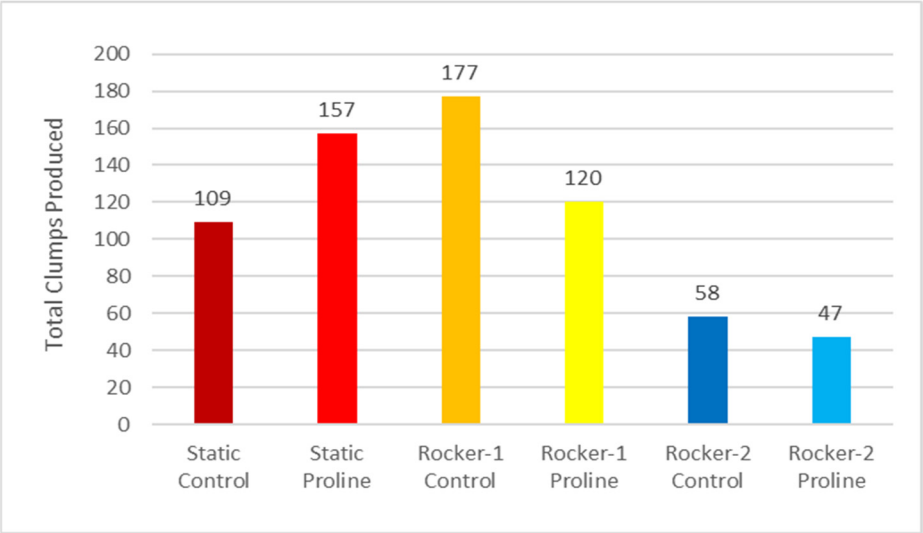

Figure 6. Total Clumps Produced is calculated from the daily totals of each culture.

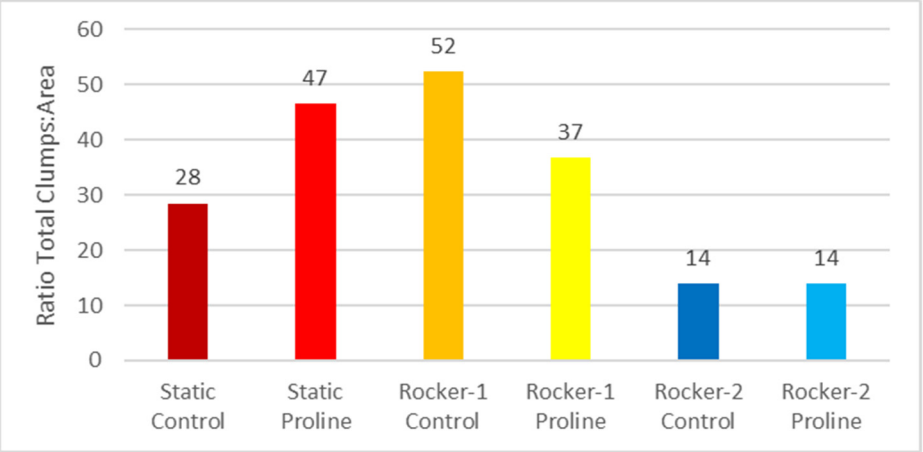

Figure 7. Ratio of the Total Clumps Produced to the calculated Area Under the Curve from the MTT assay.

2.3. Secreted Acid Phosphatase

Secreted Acid Phosphatase (SAP) was measured at the conclusion of the 13-day experiment (Figure 8) and the data are reported as correct SAP activity detected per 24hr of incubation with the substrate. ANOVA testing revealed that Rocker-2 Control SAP differed significantly (decreased by 20%) from Static Control SAP ($p < 0.05$). Also, Rocker-2 Proline SAP exhibited significant differences from all other cultures. Despite Rocker-2 Control having more total viable cells grown in the culture (Figure 3), a lower amount of SAP was detected compared to Static Control. Rocker-2 Proline exhibited markedly reduced SAP levels compared to all other cultures. Notably, the addition of L-Proline demonstrated an apparent adverse impact on SAP production in both the Static and Rocker-2 cultures. Specifically, Static Proline displayed 17% lower SAP than Static Control, while Rocker-2 Proline exhibited a substantial 65% decrease in detectable SAP compared to Rocker-2 Control. Detectable SAP levels from Rocker-1 Control and Proline flasks showed no significant differences.

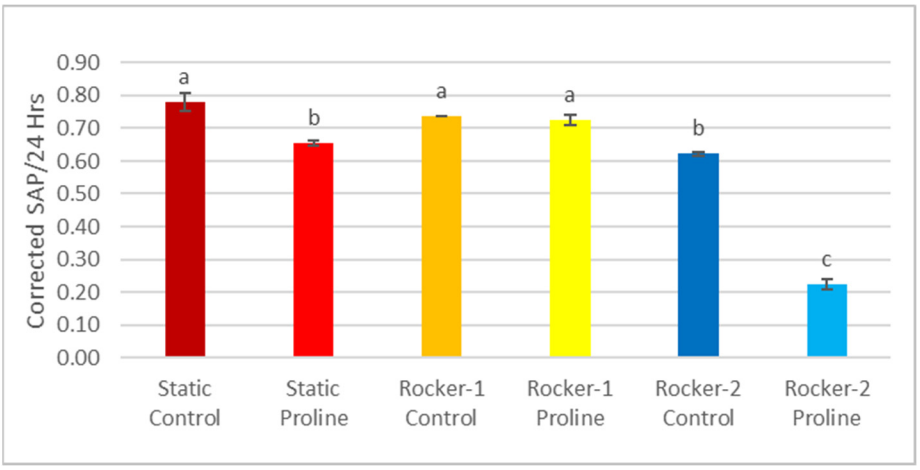

Figure 8. Corrected mean ± SD for SAP/24-h incubations with phosphatase substrate (pNPP) for all six cultures. Values that are significantly different (ANOVA, $p < 0.05$) are indicated by letter a, b, and c.

Area under the Curve and SAP

The ratios for the Area Under the Curve to SAP are higher for both the Rocker-2 Control and Rocker-2 Proline cultures than for Static (+/− Proline) and Rocker-1 (+/− Proline) as shown in Figure 9. The ratio for Rocker-2 Control is 1.25 times greater than the Static and Rocker-1 cultures, while Rocker-2 Proline is three times greater. This implies that despite producing as many or more viable cells than the other cultures, the Rocker-2 cultures generated less SAP. This observation is of interest since Apuzzo et al. (2021) [8] reported that some compounds can directly modulate detectable SAP activity (in vitro) and thus impact parasite infectivity [8].

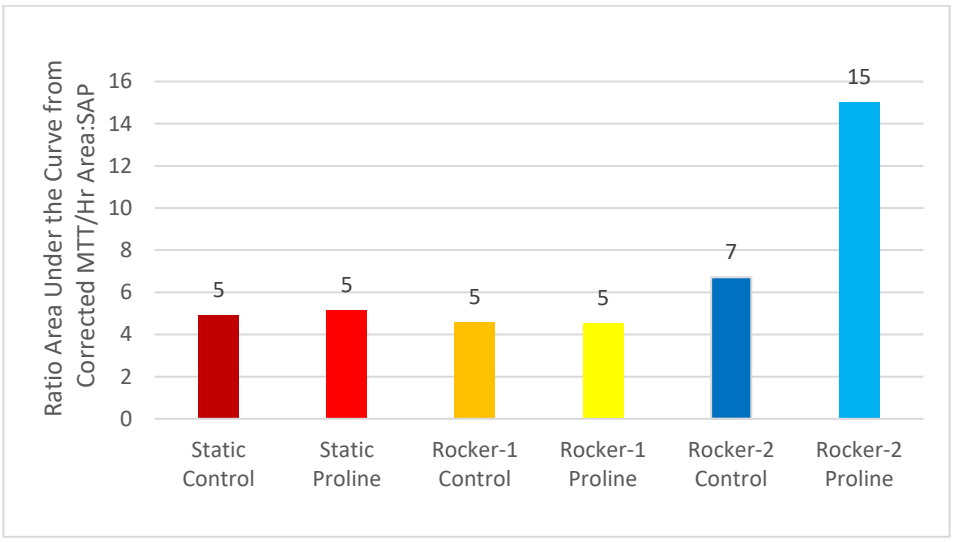

Figure 9. The Ratio of the Area Under the Curve from Corrected MTT/Hr to Secreted Acid Phosphatase.

3. Discussion

The previous literature searches yielded no prior research on the impact of rocking motion compared to static conditions on *Leishmania* cell viability, clumping, and SAP detection. The previous literature searches have reported studies investigating the effects of modifying the growth medium on the *Leishmania* cultures [9], as well as the identification of optimal methods for cultivating large *Leishmania* cultures [10,11]. Therefore, we sought to perform an experiment that would show the effects of movement on *Leishmania* cells

grown in culture. Adding movement to a culture may better mimic the natural growth conditions of these obligate parasites in their insect or other animal hosts and thus give insight into various cell stresses in different environments.

This study yielded several significant findings. Firstly, growing cells on a moving rocker caused the cells to congregate along a line parallel with the axis of the rocker. Second, all six conditions demonstrated comparable cell viability at the experiment's conclusion, although Rocker-2's results displayed a delayed response. Third, the addition of L-Proline decreased cell viability for all cultures. Fourth, a striking outcome was the remarkable lack of clumps produced by the cultures on Rocker-2, even though they generated similar amounts of viable cells (if not more) than the Static and Rocker-1 cultures. Lastly, all the cultures produced somewhat similar amounts of SAP except for Rocker-2 Proline. These outcomes collectively suggest that the addition of L-Proline and the conditions created on Rocker-2 elicited a distinctive form of stress that triggered a unique cellular response.

4. Materials and Methods

4.1. Leishmania Tarentolae

ATCC 30143 promastigote cells were grown in a Brain–Heart Infusion (BHI) medium in 50 mL CellTreat culture flasks following the methods of Morgenthaler et al. (2008) [12]. Sterile BHI was supplemented with streptomycin, penicillin, and hemin. *Leishmania tarentolae* has been reported to be a good model system for testing despite not being a human parasite [4]. The axenic *Leishmania* cultures were grown in the dark, at room temperature, in one of three conditions: No rocking (referred to as Static), Rocker-1, and Rocker-2. Both rockers were set to the same speed and percent tilt. All three conditions were set up in the same room at the same time. For each experiment, the same number of cells were inoculated into each flask (using a stock culture). Time was considered as day 0 on the day of inoculation. All work was performed using sterile techniques.

4.2. Microscopy

A Jenco Light Inverted Microscope was used to view the culture while still in the suspension flask. This allows for the cells resting on the bottom of the flask to be viewed and the number of clumps in each culture to be photographed and counted. Magnifications of 250× were used.

4.3. Labnet Rocker 35 and Labnet Rocker 35EZ

Two types of gel rockers were used for the rocked cells. Gel rockers were set to the minimum speed (~10 rocks/min) and an 8% tilt.

4.4. Additions

L-Proline, purchased from Sigma Aldrich (Saint Louis, MO, USA), was added to one flask of the Static, Rocker-1, and Rocker-2 flasks to give a final concentration of 0.5 M.

4.5. MTT Cell Viability Assay

Cell viability was determined by using the MTT assay following the method of Mendez et al., 2014 [13]. From each culture flask, following gentle mixing of the culture, four replicate volumes (100 µL) of the *L. tarentolae* culture were obtained and loaded into separate wells of a Sigma Aldrich Flatbottom 96-well microplate. Ten µL of the MTT reagent (5 mg/mL water) were added and then incubated at room temperature (in the dark). To stop the reaction, 100 µL of a mix of Triton X-100, HCl, and isopropanol (10% Triton X-100, 0.014 M of HCl in isopropanol) was added, mixed well, and then the sample absorbance was evaluated at 595 nm using the Bio-Rad iMark Microplate Reader. Values were normalized by subtracting the A595 nm reading of the cell-free BHI from the values obtained from the test cultures. Values are reported as A595 nm per 1hr of incubation with the MTT reagent following the method of Turner et al., 2012 [14]. Values are reported as mean ± SD for

n = 4 replicates. Significant differences were evaluated using ANOVA and pairwise T-Test Post-test and considered significantly different when $p < 0.05$.

4.6. Clump Counting

Four random areas of each culture flask were photographed using a Canon EOS Rebel T3i camera attached to the Jenco Inverted Microscope. Images were imported into Adobe Photoshop 2021. The Counting Tool in Photoshop was used to mark and count clumps. A clump was defined as a large rosette of *Leishmania* cells. Total Clumps produced is the accumulated sum of the daily count of clumps from the four random areas of the flask over the culture's 13-day growth period.

4.7. SAP Enzyme Assay

Using the method of Dorsey et al. (2018) [15], 1.5 mL volumes were removed from the 13-day old culture flasks. The cells were centrifuged for 1 min at 10,000× *g* using an Eppendorf tabletop model 5415 centrifuge. Cell-free supernatants were removed from these samples and tested for the secreted acid phosphatase activity [15]. BHI without additions or cells was used as blanks for this spectrophotometric assay. Equal volumes of cell free supernatants were mixed with 0.5 M of a sodium acetate buffer (pH 4.5), and then 100 µL of a freshly prepared solution of para-nitrophenyl phosphate (pNPP, 0.5 mg/mL buffer; Sigma Aldrich), as the artificial substrate for the phosphatase enzyme, was added (the final volume of reactions was 1.0 mL) and incubated for 22 h in the dark at room temperature. Following the addition of 10 M of NaOH (100 µL), the absorbance at 405 nm was evaluated. Results (means ± SD) are reported as corrected A405 nm values per 24 h of incubation.

5. Conclusions

Little research has been published regarding the effects of motion on *Leishmania* cell growth and its impact on cell viability and/or metabolic behavior. This could, in turn, influence the infectivity of cells. We report here this new insight into the surprising differences between cultures experiencing motion verses static growth conditions. We speculate that motion will influence drug sensitivity.

It is also of interest to note that the addition to L-proline, an important amino acid especially in collagens, in general had a negative effect on our cells under the conditions of these experiments. More work is needed to understand the molecular mechanisms of these observations.

This simple experimental design resulted in surprising outcomes that clearly indicate that growth conditions affect cell behavior and viability. This experiment started with the same stock cells, medium, and all other environmental conditions (temperature, relative humidity, and light conditions); only the location of the cells in a static or a rocked environment differed. It was surprising to see substantial differences in cell responses. It was particularly surprising to see the difference between Rocker-1 and Rocker-2, which were set on the same benchtop, at the same rocking rate, and had the same degree of tilt.

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