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ORIGINAL ARTICLE

Impact of Low pH on Microbial Growth Rate, ATP Production, and NADH to NAD$^+$ ratio

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Key words:
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ABSTRACT

Background: *Bacterial metabolism is the tendency of bacteria to live, function, and replicate fittingly under their current culture and varied environment conditions. Microorganisms have intricated metabolic regulatory mechanisms to ameliorate environmental stresses.* ***Objectives:*** *We examined the effect of acidic pH, as one of stresses, on growth rate and metabolism of five different microorganisms.* ***Methodology:*** *ATP level, as an indicator for microbial viability, and alterations in NADH/NAD$^+$ ratio, which plays a critical role in microbial metabolism, were assessed.* ***Results:*** *Our results showed that alterations in pH influence metabolism of different bacterial species with different extent. The growth rate of Pseudomonas aeruginosa, Escherichia coli and Bacillus Subtilis were diminished with an elevation in ATP and NADH/NAD$^+$ ratio at low pH. Contrary, MRSA and MSSA showed trivial alterations for ATP and NADH/NAD$^+$ ratio.* ***Conclusion:*** *Ultimately, this study affirmed differences in metabolism between different species and confirmed that alterations in pH influenced the metabolism and hence the pathogenesis.*

INTRODUCTION

Microbial metabolism is known as all the biochemical reactions occurring inside the microorganism to obtain the energy and nutrients it requires to establish and replicate. It includes chemical varieties of substrate oxidation and dissimilation reactions that usually occurred to generate energy [1]. The tendency of bacterial cells to live, function, and replicate fittingly under their current culture conditions and under the varied environment conditions comprise the domain of the bacterial metabolism [2]. Not merely that for the bacterial metabolism, but also it has been proved in numerous studies the involvement of metabolism in virulence regulation and pathogenesis [3-7]. It is unambiguous that all bacteria have optimum growth conditions for their cellular missions and any alterations in the outer milieu such as pH or the limitations of nutrients could presumably command to metabolic disorders and even to cell death [8]. To conquer the changes in the external environment and to establish the infection, numerous physiological and genetic mechanisms have evolved such as pH homeostasis and regulation of metabolism. Each microorganism species uses a diversity of different mechanisms like a reaction to acid tension [9].

From these mechanisms, the regulation of the pH by the bacterial cell as an essential indicator of the physiological state of bacteria in an acidic environment that is referred as pH homeostasis [10] which is essential for bacterial growth and metabolism, inspiring the absorption of nutrients and the assembly of proteins and nucleic acids [11]. Microorganisms have evolved intricate metabolic regulatory mechanisms to ameliorate their acid tolerance in acid milieus. They amended bacterial cofactors and redox factors for persistence, replication, and metabolism in acidic milieu via intensification of the glycolytic process [11]. In an earlier report, the glycolytic rate increased by 70% in acidic condition by virtue of shifting enzyme levels and the metabolic control of the enzyme effectiveness [12]. The raise in enzyme performance recompenses for the repression caused by lowered pH, and saves the metabolism.

Some bacteria remolded their metabolism in response to acid with production of sufficient adenosine triphosphate (ATP) to support bacterial growth and survival[6,13,14]. Microorganisms release ATP via respiration and thereafter harness ATP to implement cellular functions which are important for their existence, growth, and reproduction. This implies that cellular ATP possess vocations in saving and outfitting energy in metabolism, [15]. Furthermore, extracellular ATP has been shown to serve a role in bacterial physiology [16,17]. Extra-cellular ATP and the dynamical alterations in its intensity confirmed that ATP could have essential roles outside the cell beside to its known functions inside the cell [15]. ATP release is not limited to selected bacterial species [15], it has been detected in log phase cultures of *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Staphylococcus aureus* (*S. aureus*) but not the stationary cultures [17]. Although it is established that the ATP level in most types of bacteria was growth phase dependent

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showing the extreme intensity at log phase, its level varied as one of the reaction mechanisms to acid stress[15].

Another metabolic regulatory mechanism that has been promoted by bacteria in regards to acid tension is the modulation of the nicotinamide adenine dinucleotides ($NADH/NAD^+$) ratio. This ratio is considered a key metabolic marker of the bacterial cellular state[18], since NADH and NAD^+ cofactor implements in plentiful redox reactions and controls a lot of enzyme activities [19-22]. Thus, the $NADH/NAD^+$ ratio has a decisive impact on the reservation of the bacterial redox balance, that consider a fundamental for bacterial growth and metabolism [23]. Previous study correlated the alterations in the $NADH/NAD^+$ ratio with the induction of virulence in *P. aeruginosa*[24].

Notwithstanding that cognizing the influence of low pH, as one of the environmental stress aspects affecting bacterial growth, could aid in solving clues about the pathogenesis of the microorganisms inside the host environments and in answering questions about microbial food spoilage, little information is available about the bacterial metabolic responses under acidic milieus. To recognize and to estimate the bacterial responses under low pH, we examined the effect of acidic pH on five different microorganisms on their growth rate and metabolism. We assessed the level of cellular and extra-cellular ATP as an indicator for the microbial viability. By the same token, seeing that fundamental role of the $NADH/NAD^+$ ratio in the microbial metabolism, we additionally, elected to measure the alterations in the $NADH/NAD^+$ ratio

METHODOLOGY

Bacterial strains and growth conditions:

Five microorganisms were involved including both Gram-negative and Gram-positive; *Escherichia coli* (*E. coli*) strain *E. coli* K-12 (ATCC29181), *Pseudomonas aeruginosa* (*P. aeruginosa*) strain PAO1, *Bacillus Subtilis* (*B. Subtilis*) strain (ATCC23059), methicillin resistant *Staphylococcus aureus* (MRSA) strain ATCC 43300, and methicillin susceptible *Staphylococcus aureus* (MSSA) strain ATCC 25923. Bacterial culturing was grown for each microorganism in nutrient broth at 37 °C with stirring at 225 rpm. Subcultures were prepared in two sets in fifteen ml nutrient broth for each microorganism. One of these sets was adjusted to pH 3 using 10 mM HCL and the other one was used with its current pH value.

From each culture, 1ml for OD measurement, 1ml for NADH and NAD^+ values determination, and 100 µl for ATP measurement were taken at different time points; 2, 4, 6, 8, and 24 hours of incubation.

Measurement of growth rate:

Subcultures for each microorganism from the two sets were incubated at 37°C with shaking for 24 hours. The absorbance at 600 nm was measured at 2, 4, 6, 8, and 24 hours of incubation. The growth curve of the optical density (OD_{600}) of each culture was plotted. The experiment was performed in triplicate.

Measurement of cellular ATP and extra-cellular ATP levels:

Cellular ATP and extra-cellular ATP levels were measured in the five tested microorganisms at different time points using the luciferase bio-luminescence assay for the two used sets. Briefly, in a 96-well plate, 100 µl of each bacterial whole culture was combined with same volume of Promega BacTiter-Glo. The samples incubated for maximum 5 minutes and the luminescence was determined utilizing the luminometer (Filter Max F5 Multi-Mode Microplate reader) with SoftMax Pro Easy software to detect the cellular ATP. For Measuring the extra-cellular ATP, bacterial cultures were pelleted and 100 µl of each supernatant was blended with same volume of Promega BacTiter-Glo reagent and analyzed as before. The experiment was performed in triplicate and samples were measured in technical triplicates.

Detection of NADH to NAD^+ ratio:

Bacterial pellets collected from 1 ml of each culture from the two used sets at different time points were dissolved in a basic solution consisting of 0.2 M NaOH with 1% dodecyl-trimethyl ammonium bromide. From each sample, 100 µl were used for NADH determination as a base-treated part. Another 100 µl were combined with 0.4 M HCl for detection of NAD^+ as acid-treated part. The two parts were heated at 60 °C for 15 min. Neutralization using 0.4 M HCl/0.5 M Trizma and 0.5 M Trizma was performed for both the base and the acid-treated parts, respectively. The test was performed using Promega NAD/NADH-Glo™ following manufacturer instructions. Luminescence was measured using the Filter Max F5 Multi-Mode Microplate reader luminometer and SoftMax Pro Easy software. The ratio of $NADH/NAD^+$ was figured by collating the relative light units. The experiment was performed in triplicate and samples were measured in technical triplicates.

Results and Discussion:

Microbial metabolism is the tactics by which the bacteria gets the energy and nutrients it requires to survive and replicate under various environmental conditions. The acidic environment either in food or in the human gastrointestinal tract is considered a critical challenge for many microorganisms. Knowing the bacterial responsiveness to acidic environment enable us to predict bacterial features under this condition such as their growth and metabolism which in turn help in understanding their pathogenesis and in their treatment. It has long been known that the generation time of bacteria is greatly influenced by the changes in pH [25]. Here, we monitored the bacterial growth for five different microorganisms under their current culture pH and under low pH. Their growth curves were plotted as appeared in figure 1; *E. coli*, *P. aeruginosa*, and *B.*

subtilus grew more slowly at low pH than at their culture pH by approximately 1.5~3-fold. While MRSA and MSSA showed reduction in their growth rate by almost 1 or less than 1-fold under acidic pH (Fig. 1). This is in accordance with a previous study which revealed that increasing acidity decreased the growth rate significantly ($P<0.05$) for *E. coli* and *B. cereus* [26]. It was also proved the high correlation between lower growth rates for several bacterial species and the acidic environments. Inasmuch, high relevance between the alterations in pH with the lag times and the growth rates of different microorganisms[26]

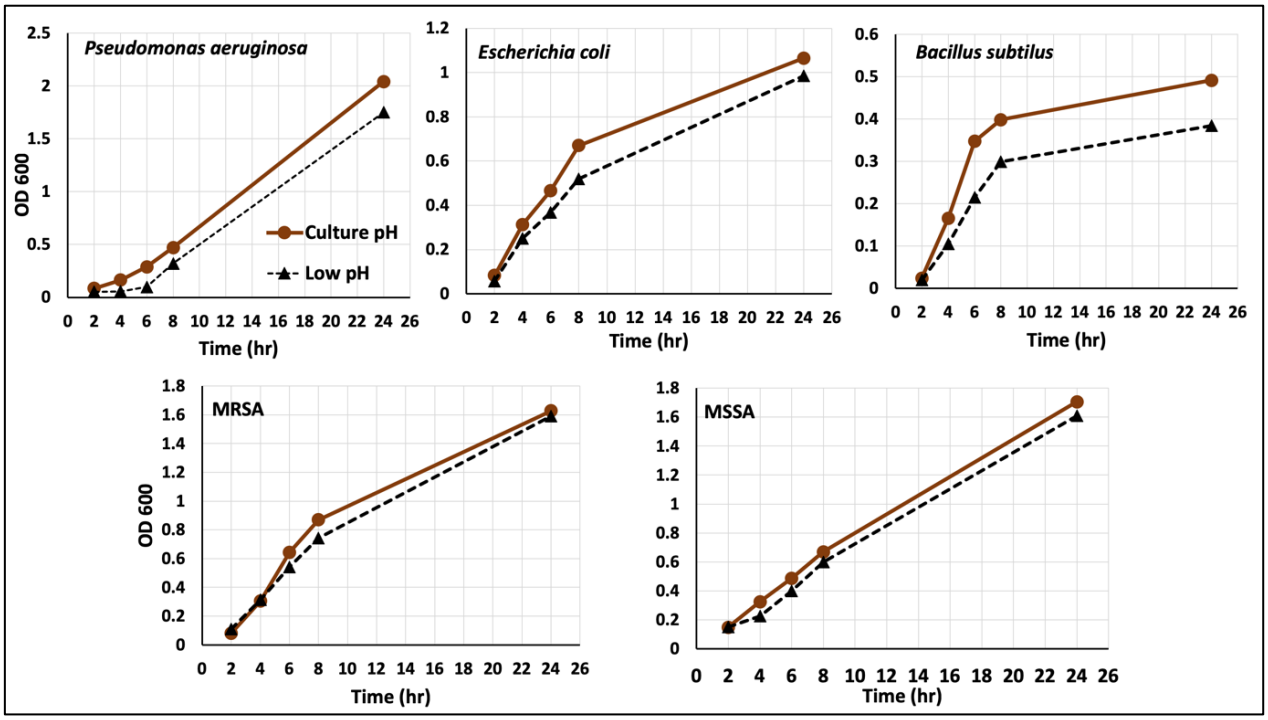

Fig. 1: The growth rate for the five microorganisms used in this study under their current culture pH and under low pH. Data is shown as a representative of triplicate experiments.

Since ATP is considered the energy carrier of most living microorganisms and has been used as an indicator for the microbial viability, we harnessed ATP bioluminescence assay to detect the viability of the microorganisms. Cellular ATP of the whole culture and extra-cellular ATP of the supernatant levels were parsed in the five tested microorganisms at different time under their current culture pH and under low pH. *E. coli* and *P. aeruginosa* cultures showed elevation in the level of both cellular and extra-cellular ATP at low pH than at their culture pH by nearly 2-fold (Figures 2, 3). A striking raising in both ATP levels were observed in *B. subtilus* culture with approximately 4-fold increase at low pH as shown in figures 2 and 3. Previously, typical results were shown for *E. coli*, *P. aeruginosa*, and *B. subtilis* by responding similarly to the alterations in the pH [27]. In a more robust fashion, an eminent relation among bacterial ATP level and pH has been disclosed with the ATP level inversely related to the pH [27]. Several lines of evidence incriminate the alterations in pH in ATP overproduction where the bacteria remolded the metabolic pathways producing sufficient ATP to support growth and survival [6,13,14].

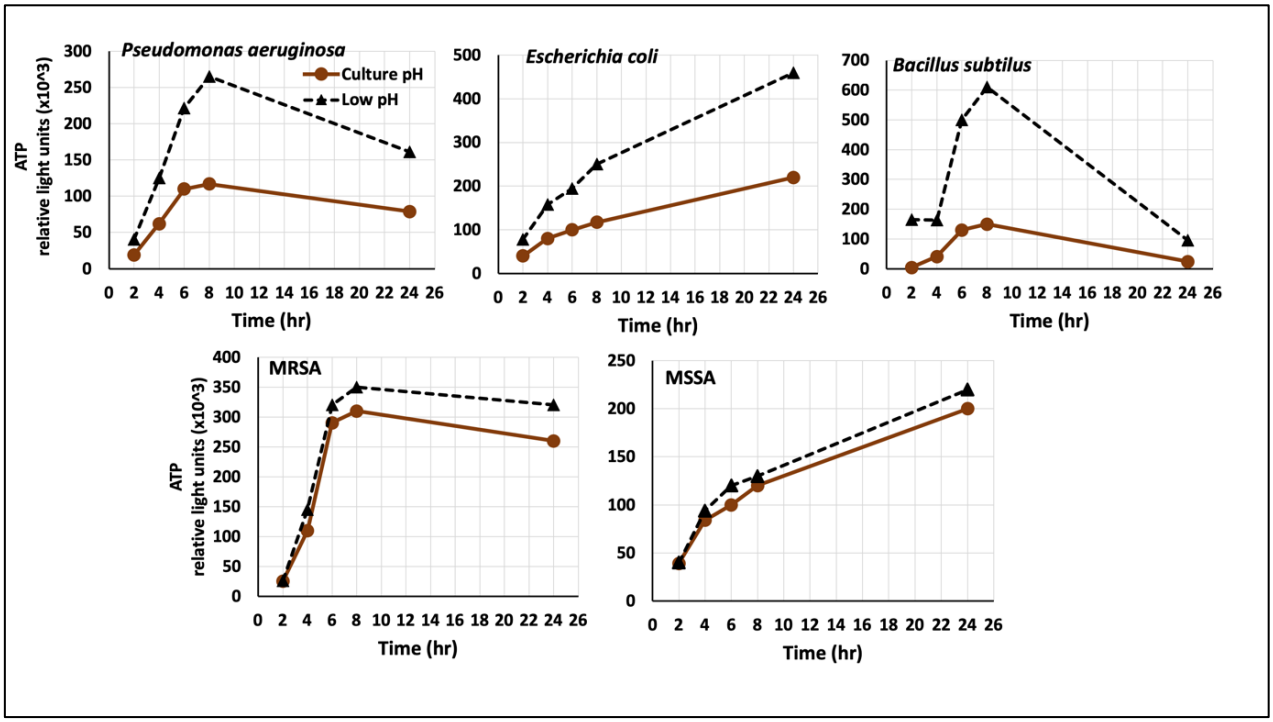

Fig. (2): Comparison of cellular ATP levels in the whole culture of the five microorganisms employed in this study under culture pH and low pH conditions. ATP levels determined by bioluminescence assay. Each measurement was detected from three biological and three technical replicates.

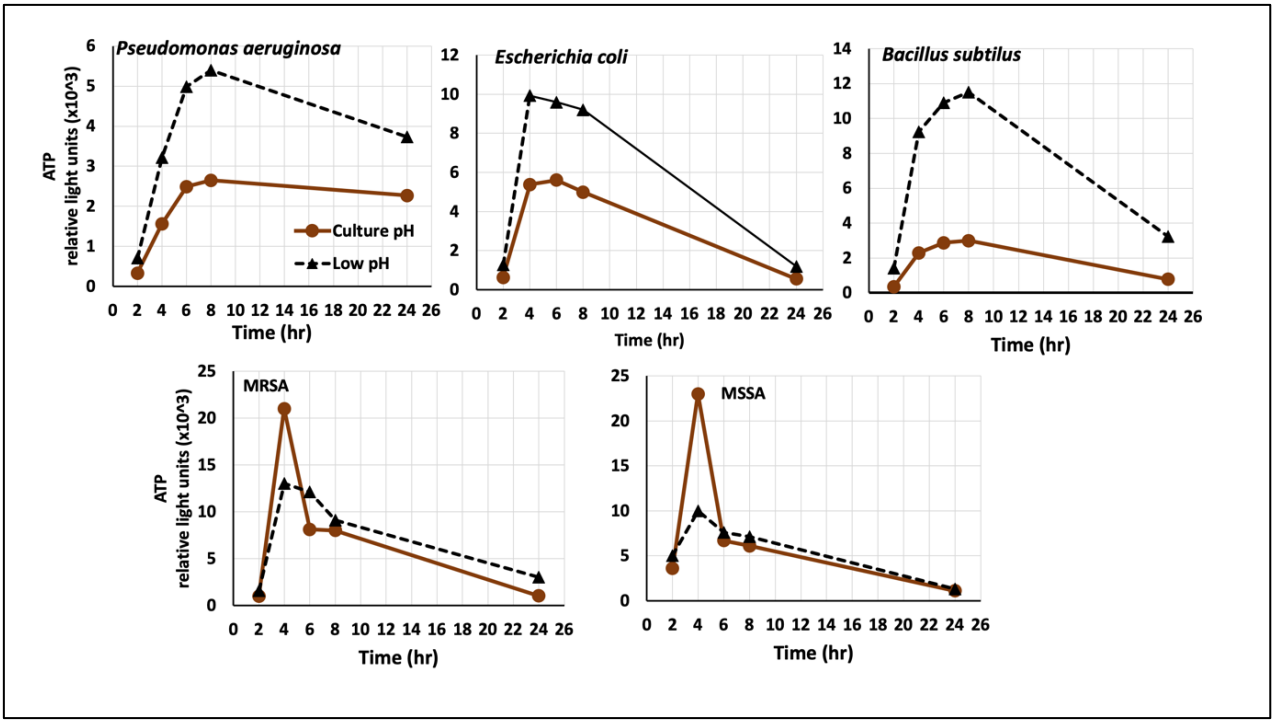

Fig. (3): Comparison of extra-cellular ATP levels in culture supernatant of the five microorganisms employed in this study under culture pH and low pH conditions. ATP levels determined by bioluminescence assay. Each measurement was detected from three biological and three technical replicates.

Unsurprisingly, cultures of *Staphylococcus aureus*; MRSA and MSSA showed trivial ATP levels alterations in acidic pH for both cellular and extra-cellular ATP as shown in figures 2 and 3; respectively. Similar to what has been reported early for *S. epidermidis* which showed no changes in ATP level under acidic pH. The modest disparity associated with Staphylococcus species could be attributed to its nature as an inhabitant of the human skin, that has a pH around 4.7 [28-31]. This corporate the ability of this species to adapt and survive to grow under low pH [32]. Likewise, *S. aureus* is able to utilize different metabolic tactics to get their energy either aerobic respiration, anaerobic respiration, fermentation, or a mix of them [33].

Another coenzyme that have an important major role in bacterial metabolism is the NAD that subsists in two forms either as an oxidized form (NAD^+) or as a reduced form (NADH). It involved in over 300 redox reactions and as a substrate for bacterial DNA ligases [34]. The equilibrium among the both forms of NAD; oxidized and reduced forms, is known as NAD^+/NADH ratio. This ratio is a substantial motif of the microbial redox state [18]. We investigated the NAD^+/NADH ratio as a measurement of the metabolic activities inside the microbial cells of the five microorganisms used in this study at different time under their culture pH and under acidic pH as shown in Fig. 4. In accordance with the ATP levels results, the highest divergence NADH/NAD^+ ratio among the actual culture and the acidic culture was detected for *B. subtilis* with a difference reaching to nearly 25-fold after 4 hr incubation. Likewise, *E. coli* and *P. aeruginosa* cultures showed NADH/NAD^+ ratio disparity under the acidic culture with a variance extending to approximately 10-fold. Our results confirming the elevation in the metabolic activity under the low pH concordant with the ATP levels results. Coherently, Vemuri, *et al*. alluded that the NADH/NAD^+ ratio cog the metabolic fluxes of numerous pathways and the elevated NADH/NAD^+ ratio ameliorates surplus metabolism [35], while the low NADH/NAD^+ ratio promotes the glycolytic flux [36]. Furthermore, the adequate balance of the NADH/NAD^+ ratio is significant for the metabolic pathways that are tangled in virulence [37,38]. Several lines of evidence incriminated the NADH/NAD^+ ratio in the bacterial pathogenesis. Former studies assigned the critical role of the NADH in the induction of *P. aeruginosa* virulence [24,39].

The striking result was the unambiguous change in the metabolic activities for the Staphylococcus; MSSA and MRSA employed in our study. In agreement with the ATP level results for these two microorganisms, the NADH/NAD^+ ratio was slightly elevated under the acidic environment with inconsiderable changes from their actual culture conditions (Figure 4).

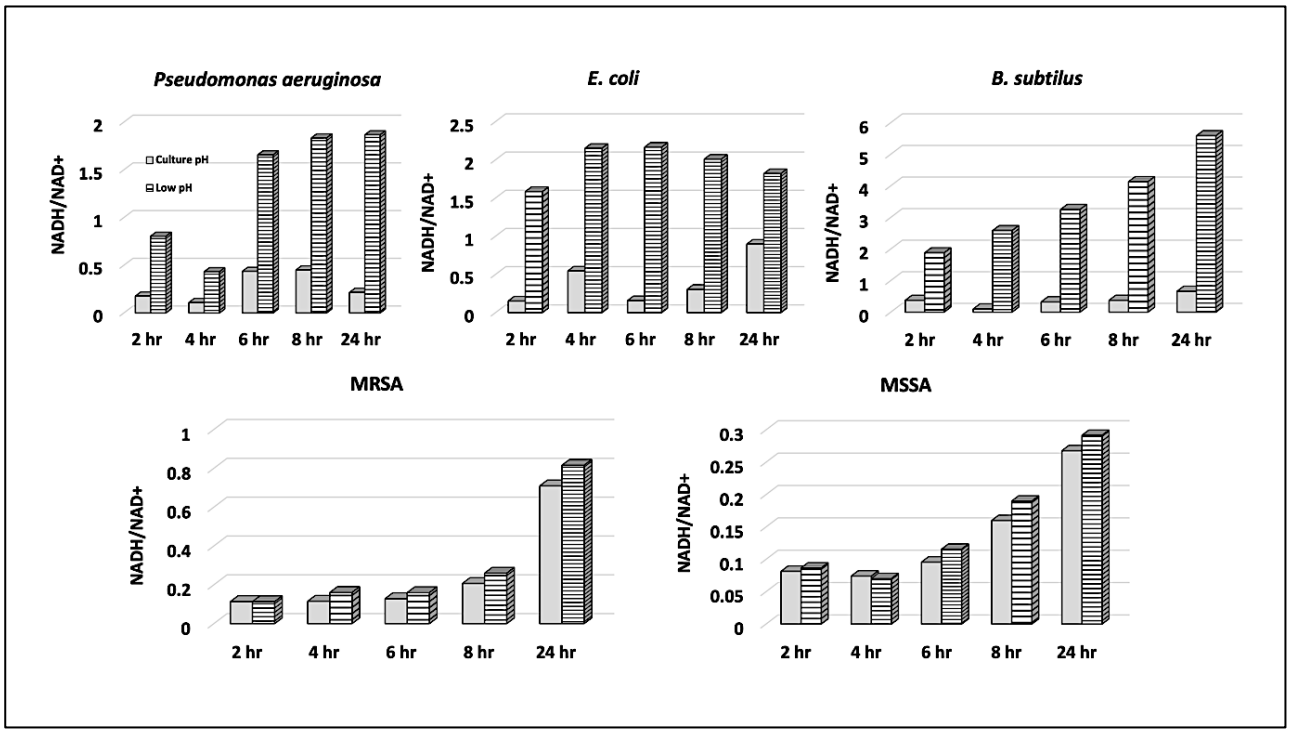

Fig. 4: NADH/NAD+ ratio determined by bioluminescence assay in the culture of the five microorganisms harnessed under culture pH and low pH conditions. Each measurement was detected from three biological and three technical replicates.

The peculiar ability of MSSA and MRSA to adapt in the low pH, most probably, is related to the fact that *S. aureus* is quite acid tolerant [40]. It seems to us noteworthy to remind here that the prosperity of S. *aureus* as a pathogen is attributed to its mastery of precise setting its cellular physiology to overcome the provocations exhibited by various milieus, which warrants it to settle various recess in the host [33] and increases its ability to adjust the metabolism and the bioenergetics to infect any area of the host.

CONCLUSION

This study raised an evidence about the differences in the metabolism between various types of bacteria and confirmed that the alterations in the pH could influence the metabolism and hence the pathogenesis of these microorganisms.

Compliance with Ethical Standards
This study was approved by Suez Canal University ethical board.

Conflicts of interest:
The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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