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Understanding the Tolerance of Different Strains of Human Pathogenic Bacteria to Acidic Environments

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Abstract: It is known that many bacteria are susceptible to low pH, but it is less clear how the acid nature influences this susceptibility. The objective of this work was to evaluate the susceptibility of selected bacteria to different low pH conditions and to understand if their tolerance is affected by the type of acid (organic vs inorganic) in the media acidification. Strains of different species of human pathogenic bacteria were cultivated at different acidic conditions, using organic or inorganic acids. Overall, we verified that tolerance to acidic conditions is dependent on the type of acid used to acidify the growing medium, organic or inorganic. The strains analysed, although having different tolerance to low pH, were shown to be more susceptible to organic than inorganic acids. This result shows that beyond the pH of the media, the nature of the acid affects bacterial growth. Such a result has consequences in the evaluation of the efficiency of an antibacterial extract, which should take into consideration the differential susceptibility of bacteria to low pHs obtained using organic or inorganic acids.

Keywords: pH tolerance; acidic stress; organic/inorganic acids

Citation: Porto-Figueira, P.; Câmara, J.S.; Vigário, A.M.; Pereira, J.A.M. Understanding the Tolerance of Different Strains of Human Pathogenic Bacteria to Acidic Environments. *Appl. Sci.* **2023**, *13*, 305. https://doi.org/10.3390/app13010305

Academic Editors: Ana Carolina Gonçalves and Andreia Filipa Silvestre Duarte

Received: 9 November 2022
Revised: 19 December 2022
Accepted: 20 December 2022
Published: 27 December 2022

1. Introduction

Each bacteria species or strain has its optimal environmental condition for growth. However, as they are constantly challenged by their environment, bacteria develop stress responses to deal with environmental changes, which protect them from adverse and variable conditions.

Therefore, strategies to control the growth and survival of pathogenic bacteria, particularly if they are implicated in healthcare-associated transmission, are crucial for human health. This requires deeper knowledge about the effects different aspects of bacterial biology, including the influence that some environmental factors, particularly acidity (pH), temperature, water activity, oxygen levels, and presence of macro and micronutrients, have on bacterial growth and toxins production [1]. Typically, when bacteria are challenged to grow under suboptimal conditions, they become stressed and adopt different strategies to survive. According to the type of bacteria, these challenges elicit mechanisms that may dictate a growth reduction or they even stop growing, dormancy, or eventually, if all mechanisms fail, die [2–4]. Variations in temperature, salinity, dryness, and pH are common stress conditions sensed by bacteria. Concerning pH, bacteria develop different strategies to cope with the changes in the environmental pH, being differentially tolerant to acidification. Acid stress tolerance developed by pathogenic, or spoilage bacteria, may make them difficult to control or eliminate. On another end, acid tolerance mechanisms developed by bacteria used as probiotics are desirable.

Under acidic environments, the destabilization of the proton motive force leads to the leakage of ions, including protons, which eventually reduces the cytoplasmic pH [3,5]. This event triggers different harmful effects in bacteria [6], and ultimately, the failure to restore physiological pH will damage cellular structures, leading, for instance, to the disintegration of the cytoplasmic membrane [2,3,5,6]. This has led Gram-positive and Gram-negative neutralophilic bacteria to develop different mechanisms to mitigate acid-induced damage [2,3,5–8]. In Gram-negative bacteria, the outer membrane is not a very efficient barrier to the movement of protons caused by acidic conditions because porins are large enough to allow ion passages [5]. In contrast, the cytoplasmic membrane is a better barrier to proton influx and cyclopropane fatty acids (CFAs) and seems to be very relevant in this protection. This is supported by the evidence of *CFA* gene upregulation and augmented CFAs levels correlating with acidic resistance [9,10]. Nevertheless, the ability of different bacteria to survive in acidic environments usually involves a synergistic combination of different regulatory circuits of acid resistance/tolerance, as suggested by studies on the activation and/or mutation of different genes [8,11,12] (Supplementary Figure S1). In *Escherichia coli*, the transcriptomic analysis of two strains exposed to organic and inorganic acids revealed acidulant- and strain-specific acid tolerance responses [13]. In turn, citric acid treatment of burn infections was found to be more effective in the control of *Staphylococcus aureus* than conventional antibiotic therapy [14]. Overall, knowledge about the mechanisms that bacteria use to adapt to surrounding acidic environments is very relevant for the evaluation of antibacterial activity under different conditions and may have important applications, namely in clinical and food safety fields. In food research, for instance, substitution of artificial conservants by natural compounds able to mitigate food spoilage is highly desirable and requested by the consumers. On the other hand, interest and industrial applications for the use of probiotic organisms with health-protective effects in human and animal feeding is increasing exponentially. In this context, the objective of this work was to evaluate the susceptibility of different bacteria, *S. aureus*, *E. coli*, *Klebsiella pneumoniae*, and *Acinetobacter* sp., to different acidic conditions and verify if the bacterial viability was dependent on the type of acid used to obtain such conditions.

2. Materials and Methods

2.1. Microorganisms

The Gram-positive *S. aureus* and Gram-negative *E. coli*, *K. pneumoniae*, and *Acinetobacter* sp. bacteria strains used in this study were isolated from samples at Hospital Dr Nélio Mendonça, Funchal, Portugal, where they were identified by API (Biomeriuex, Lyon, France) and then kept frozen (−20 °C) in the appropriate medium (LB medium 30 % glycerol). Before each experimental procedure, an aliquot of each bacterium was grown in Mueller Hinton agar (MHA, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) medium (pH 7.3) and incubated for 24 h at 36 °C. Then, a well-isolated colony was picked from each plate to a new Mueller-Hinton plate and incubated for an additional 18 h. This procedure was rigorously the same for all studied bacteria, avoiding any exposure to acid stress or differences in phase growth, that could interfere with the results.

2.2. Media Preparation and pH Determination

The organics, acetic acid (AA–CH_3COOH) and formic acid (FA–HCOOH), and the inorganics, hydrochloric acid (HA–HCl) and sulfuric acid (SA–H_2SO_4), were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used to obtain a set of Mueller Hinton Broth (MHB, Fluka, Merck KGaA, Darmstadt, Germany) mediums with pH ranging from 3.0 to 7.0 (36 different conditions). pH was measured using a pH electrode (Mettler Toledo, Schwerzenback, Switzerland) and adjusted dropwise. The final broth media was then sterilized by filtration using 0.22 µm PTFE syringe filters (BGB, Rheinfelden, Germany). pH adjustment of the MHB mediums was made just before the incubation with the bacterial suspension in the 96-well plates (Greiner, Merck KGaA, Darmstadt, Germany, see below). The stability of the pH all over the experiments was observed by measuring, at specific

time points over a 24 h period, the pH of an aliquot of each condition, kept at 36 °C. No significant variations were observed during the 24 h period.

2.3. pH Assays

For each bacterium strain tested, a suspension in a saline solution (0.9 % NaCl) was prepared from an isolated colony of the 18 h incubated agar plate and diluted to achieve turbidity equivalent to a 0.5 McFarland standard. The suspension was further diluted at 1:100 (equivalent to 0.025 McFarland or 1–2 $\times$ 10^5 CFU/mL); 10 µL of this inoculum was then added to each well of a 96-round well-plate containing 100 µL per well of MHB medium, adjusted at different pH using different acids (see above) and incubated at 36 °C during 18 h. As a control for bacterial concentration and viability, 20 µL of the inoculum was plated in MHA (at neutral pH). As a control for bacteriostatic effect, two successive dilutions of the inoculum were done: 10 µL into 140 µL followed by 2 µL into 1 mL and then 50 µL of this suspension were plated on Muller-Hinton agar and incubated for 24 h at 36 °C. Bacterial growth in the liquid medium was then assessed by adding 40 µL of iodonitrotetrazolium chloride (INT, Sigma), 0.2 mg/mL per well, and incubating for an additional 30 min at 36 °C. Bacterial viability was determined by subculturing for 18 h in agar plates, with each condition showing no bacterial growth (no reduction of the dye to a pink colour), plus the first condition in which there was significant growth. Briefly, 2 µL of the selected wells were diluted into 1 mL saline solution and then 50 µL plated on Muller-Hinton agar and incubated for 24 h at 36 °C. It is expected that the number of colonies formed, on plates prepared from wells where a bacteriostatic effect happened, would be much lower than on the plates prepared from wells showing bacterial growth (INT positive ones) and equivalents to the ones obtained on bacteriostatic control plates. Each pH condition was tested in triplicate for each bacteria strain and each experimental set was carried out twice on different days.

3. Results and Discussion

The susceptibility to acidic environments was assessed in four human pathogenic bacteria, *E. coli*, *K. pneumoniae*, *Acinetobacter* sp., and *S. aureus* [15,16]. The experimental conditions assayed were 0.5-pH gradients between 3.0 and 7.0, obtained using either organic (acetic acid–AA, and formic acid–FA) or inorganic (hydrochloric acid–HA, and sulphuric acid-SA) acids. The selected bacteria were cultured in the pH-adjusted MHB mediums, in 96-well microplates, and their ability to grow was verified by adding INT [17]. Reduction of INT to a red-coloured product indicates bacterial growth. Figure 1 summarizes the results obtained, showing the ability of the selected bacteria to grow under the different pH conditions assayed.

A subculture of each well where bacterial growth did not occur and the first condition in which there was significant growth (indicated by an intense pink colour) was then made on MHA plates, with neutral pH, to determine the bactericidal versus the bacteriostatic effect of the different pH media (Figure 2). Under bacteriostatic conditions, cell metabolism is largely affected, but cells survive, although are not able to divide. In this case, no reduction of the INT is observed in the 96-well plates, but isolated colonies will be formed in the subcultured agar plates, although in a small number since no bacterial growth occurred during the incubation time in the well. Table 1 consolidates the results from Figures 1 and 2 and highlights the bactericidal pH conditions (blue boxes) and bacteriostatic (white boxes) for each bacterium. Remain pH conditions do not affect bacterial growth (pink boxes).

Overall, the results obtained reveal that bacteria's ability to cope with pH can vary significantly depending on the species, being *Acinetobacter* sp. the most susceptible, only surviving at pH 5.0 or above, while *K. pneumoniae* was the most resistant bacteria, tolerating pH 3.5, although under a bacteriostatic effect (Figure 2 and Table 1). More importantly, the type of acid (organic or inorganic) used to obtain the acidic environments in which bacterial cells were challenged, also dictates bacterial behaviour. As can be easily verified in Figures 1 and 2, although the selected bacteria can grow at least above pH 5.5, irrespectively

of the acids involved in the media acidification, they are much more tolerant to inorganic (strong) acids (HA and SA) than organic (weak) acids (AA and FA, although to a lesser extension in to *Acinetobacter* sp.). This observation is certainly related to the nature of the acids used. Strong acids, such as the HA and SA, completely dissociate in solution into Cl^- or SO_4^{2-} and H^+, respectively, which cannot diffuse inside the cell due to the low ion permeability of the membrane [2,18,19]. On the other hand, weak acids like AA and FA, when in solution, exist substantially in the undissociated state, CH_3COOH or CHOOH, respectively, which are relatively small and uncharged molecules that can easily diffuse through the cell membrane (using mechanisms like permeases or porins) [7,19].

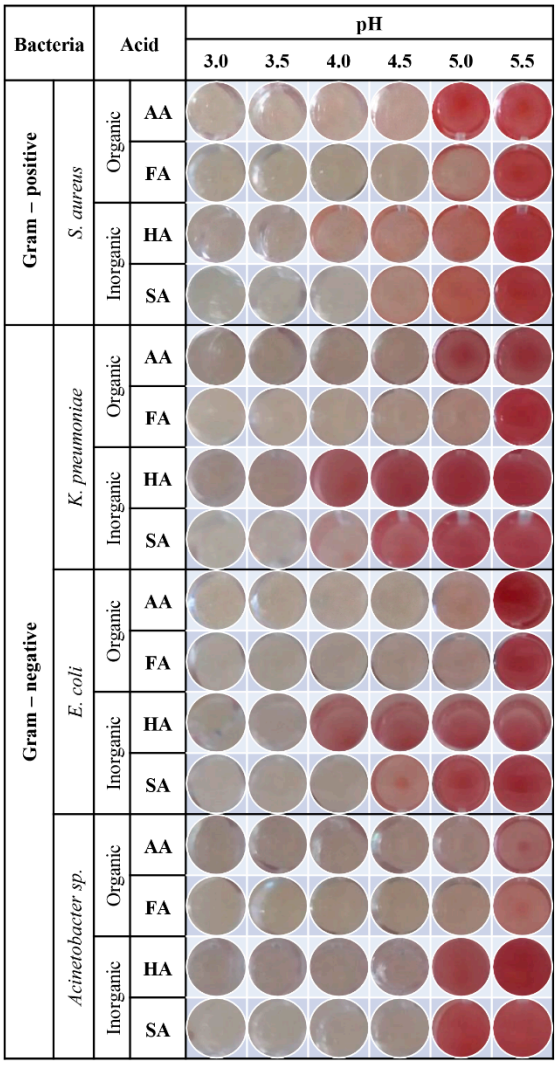

Figure 1. Differences between Gram-positive and Gram-negative bacterial growth in pH-adjusted MHB medium, assessed with the iodonitrotetrazolium chloride (INT) assay) [17]. Wells where bacterial growth occurred show a pink colour. The pH gradients shown were obtained with the addition of organic (AA and FA) and inorganic (HA and SA) acids. For simplification, a representative well for each bacteria species is shown out of the triplicates done in the same assay. Each assay was repeated twice on different days. The conditions above pH 5.5 did not affect bacterial growth and for simplification, they are not shown. Legend: AA–acetic acid, FA–formic acid, HA–hydrochloric acid, SA–sulphuric acid.

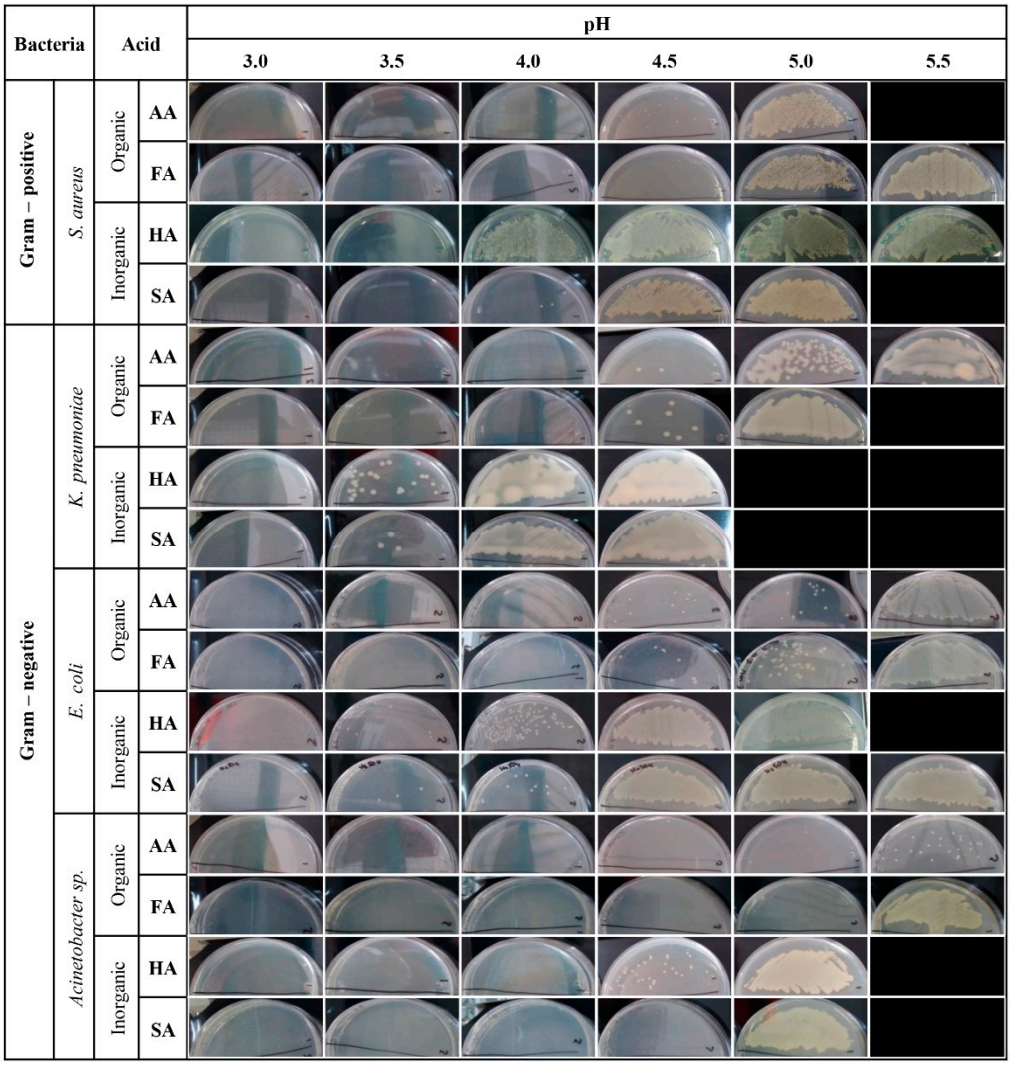

Figure 2. Effect of different acidic environments on bacterial viability, evaluated by subculturing in neutral pH MHA medium after growth in pH adjusted MHB medium. For simplification, only a representative plate for each combination of bacteria/condition out of the triplicate assays performed on two different days is presented. Legend: AA–acetic acid, FA–formic acid, HA–hydrochloric acid, SA–sulphuric acid.

However, once in the cytosol, and due to its higher pH, the undissociated acids easily dissociate, producing CH_3COO^-, $CHOO^-$, and H^+. These forms, being charged, cannot easily diffuse out from the cell, and consequently accumulate intracellularly [7,19]. This proton accumulation reduces the cytosolic pH to dramatic values that deregulate the metabolic machinery of the cell [7]. Therefore, the bactericidal or bacteriostatic ability of an acid (organic or inorganic) will depend primarily on the concentration of its undissociated form, which is determined by the dissociation constant, pKa. This indicates the pH at which the ratio of dissociated to undissociated forms is 50:50. For that reason, AA and FA, with a pKa of 4.8 and 3.8, respectively, are more efficient in inhibiting bacterial growth than HA and SA, whose pKa are −6.3 and −3.0, respectively. Remarkably, the results obtained not only show differences between organic and inorganic acids but also slight variations inside each group (AA/FA or HA/SA). In a simplistic approach, this would be a predictable result for the weak acids used, which originate relatively similar molecules, CH_3COO^- or $CHOO^-$ (AA and FA respectively), and eventually to a larger extent for the strong acids used. In this case, the pKa (−6.3 and −3.0) stoichiometry of the dissociation reactions (one proton versus two protons) and size of the anions generated (Cl^- and $SO_4{}^{2-}$), respectively,

are significantly different. This would be additional evidence supporting the mechanisms of diffusion of weak acids into the bacterial cytoplasm followed by dissociation and internal medium acidification. There are, however, additional considerations that certainly play a role in the results obtained, as the structure of the cell wall of the bacteria studied. Gram positive (*S. aureus*) and Gram negative (*E. coli*, *K. pneumoniae*, and *Acinetobacter* sp.) present significant differences in what concerns to the complexity and composition of their cell walls, for instance in terms of polysaccharides and peptidoglycans proportions and transporters. Furthermore, other metabolic features must be considered in the explanation of the higher toxicity of weak acids, at least in certain bacteria. Acetic acid, for instance, has an important role in *E. coli* metabolism and can be used both as a substrate or product (acetate switch), while formate is only a product [20]. A deeper discussion of these and other mechanisms affecting the response of different bacteria to acidic conditions can be found elsewhere [11,13,14,20–22]. A simplified diagram representing the differences in the mechanism of cytosolic acidification of bacteria is presented in Figure 3.

Table 1. Bacterial tolerance to pH.

Bacteria		Nature of the Acid	Acid	pH								
				3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0
Gram-positive	*S. aureus*	Organic	AA	–	–	–	+					
			FA	–	–	–	–	*				
		Inorganic	HA	–	–	*	*	*				
			SA	–	–	–	*					
Gram-negative	*K. pneumoniae*	Organic	AA	–	–	–	–					
			FA	–	–	–	+	*				
		Inorganic	HA	–	+							
			SA	–	–	*						
	E. coli	Organic	AA	–	–	–	+	+				
			FA	–	–	–	+	+				
		Inorganic	HA	–	–							
			SA	–	–	+	*					
	Acinetobacter sp.	Organic	AA	–	–	–	–	–	*			
			FA	–	–	–	–	–	*			
		Inorganic	HA	–	–	–	+					
			SA	–	–	–	–					

Legend: AA–acetic acid, FA–formic acid, HA–hydrochloric acid, SA–sulphuric acid, "–" blue boxes–the absence of bacterial growth in the 96 well and agar plates (bactericidal conditions), "+" white boxes–bacterial growth only in agar plates (bacteriostatic conditions); pink boxes–bacterial growth in the 96 well, "*" pink boxes–bacterial growth in the 96 well at a reduced rate.

As referred to above, the bacteria studied in this work have been selected by their involvement in human pathogenic situations [15,16], but these are different bacteria in phylogenetic terms. *S. aureus* is the only Gram-positive bacteria assayed in this work and the most divergent of all, while *K. pneumoniae* and *E. coli* are the two bacteria that are phylogenetically more similar. Moreover, all these bacteria grow normally in different niches and pH environments. This has consequences in the mechanisms each specie developed to cope with acidic environments and the organization of the cell membrane is certainly the main factor contributing to this differentiation. *Acinetobacter* sp. is the least tolerant bacteria to acidic environments. Moreover, it is the species that exhibits fewer variations to the acidic conditions according to the type of acid (organic or inorganic) used

to obtain the pH-adjusted media (Table 1). Although it is a Gram-negative bacterium, such as *E. coli* and *K. pneumoniae*, *Acinetobacter* sp. belongs to a different order and family [23]. Moreover, *E. coli* and *K. pneumoniae* are enteric bacteria and as such, they must survive the passage through the stomach [11], where HA, an inorganic/strong acid, is present. It was therefore expected that *E. coli* and *K. pneumoniae* would be more tolerant to acidic challenges than *Acinetobacter* sp. Furthermore, *E. coli* and *K. pneumoniae* were found the most resistant to pH, being able to tolerate pH 3.5 obtained using inorganic acids. Moreover, the differential sensitivity to organic and inorganic acids is again observed in both species, being more pronounced in *K. pneumoniae*, which does not tolerate organic acids below pH 5.0. As already referred, the outer membrane of Gram-negative bacteria is relatively permeable to ions [5] and thus the membrane remodelling, particularly in its composition in CFAs, is an important mechanism of protection against acidic stress and preservation of membrane integrity [9,10]. This was demonstrated in the correlation between the levels of CFAs and resistance to acidic environments observed in *E. coli*, as well as in the increased sensitivity to low pH values exhibited by *cfa* mutants [9,10]. *S. aureus* can grow in an optimum pH range of 6–7 but is killed rapidly at pH 2 [24]. Between these limits, *S. aureus* growth is dependent on the combination of optimum levels of different parameters, such as nutrient availability and temperature [24]. At the molecular levels, the resistance of *S. aureus* to pH relies on the maintenance of the intracellular pH (pHi) by proton pumps, the production of intracellular chaperones, and regulators. In fact, Cotter and Hill have sown that σ^B is directly involved in the survival of *S. aureus* in acidic environments [7,25]. This mechanism seems to be conserved in other Gram-positive bacteria. *L. monocytogenes*, for instance, was reported to be more tolerant to acidic conditions caused by hydrochloric and citric acids than acetic and lactic acids. As in *S. aureus*, this differential resistance has been associated with the ability of *L. monocytogenes* to maintain pH homeostasis [26]. All evidence points that cell membrane plays a role in the protective mechanisms necessary to maintain the cell integrity and reduce proton influx in such hard conditions [5]. Overall, taking into consideration the selected bacteria and acidic environments assayed, the results obtained support previous suggestions that permeant weak organic acids, as the acetic acid here assayed (AA), are more effective against bacteria than inorganic strong acids (e.g., HCl, referred as HA) [27]. This is certainly the main reason why media acidification is used to improve food preservation, particularly fresh and unprocessed foods. However, the results obtained evidence that such acidification should be performed using weak organic acids instead of inorganic strong acids. In this context, it is noteworthy to refer to different reports pointing to the effectiveness of weak acids against bacterial growth and spoilage. Nei et al. [28–30], for instance, proposed gaseous acetic acid treatment to reduce the risk of foodborne illness caused by the consumption of contaminated fresh sprouts and spices. This strategy has a great potential for many other applications, particularly those involving fresh fruits and vegetables [31]. In turn, Sun, et al. [32] has modelled the effect of temperature, pH, and acetic and lactic acid concentrations on the control of the acid-tolerant *Bacillus* spores, showing a synergistic effect of decreased pH and the presence of acetic and lactic acids. This solution offers an alternative for the ambient storage for low-acid pasteurized sauces without involving the use of preservatives such as benzoic and sorbic acids. More recently, de Moraes Motta Machado, et al. [33] reported that the combination of ultrasound and acetic acid presented equal to or greater efficiency than chlorinated compounds in the decontamination of kale. But the potential of acetic acid as a natural preservative is not limited to fresh and unprocessed foods. This was also recently shown by Sheen, et al. [34], who reported the synergetic effect of high hydrostatic pressure and acetic acid in the inactivation of pathogenic *E. coli* in ground chicken.

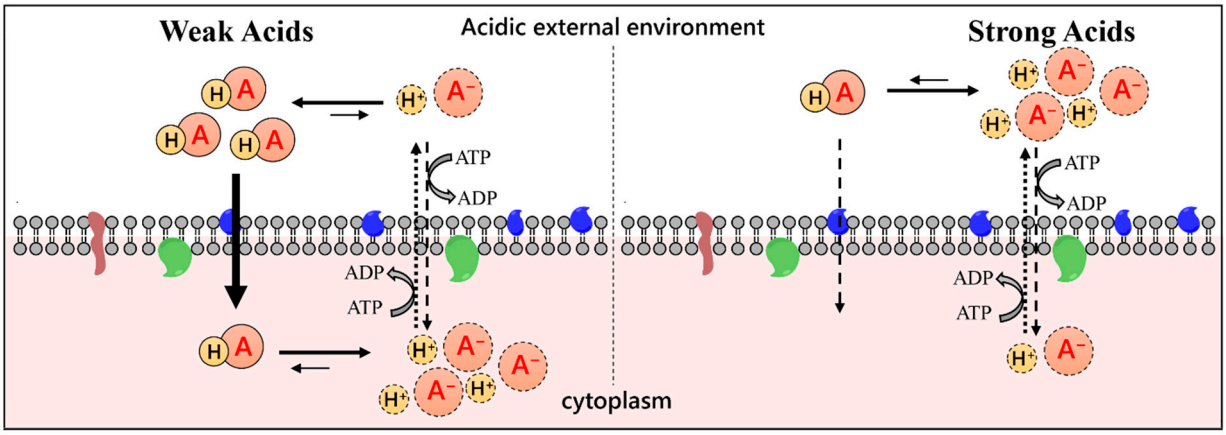

Figure 3. Possible mechanisms of bacterial acidification by weak (organic) and strong (inorganic) acids. Weak acids can easily diffuse into bacteria cytoplasm where they are dissociated and acidify the internal medium. In contrast, strong acids are usually dissociated and will not reach the bacterial cytoplasm as easily as weak acids. A^-–anion, H^+–proton, HA–acid.

4. Conclusions

The present study shows the effect of the media pH in the growth of different bacterial species, putting in evidence for the important role of the type of acid used in media acidification in the survival of the microorganism. We observed that tolerance to the low pH is quite dependent on the type of acid used to acidify the growing medium, being that all studied bacteria were more susceptible to organic than inorganic acids. This suggests that bacteria use different regulatory circuits of acid resistance/tolerance to cope with different types of acidic environments. Moreover, this work also reveals that additional appropriate pH controls are mandatory when evaluating antibacterial activity under acidic conditions to avoid obtaining biased results not considering the effect of the type of acid. Such strategy will be particularly relevant, for instance, in experiments examining the effects of acidic foods and food constituents in the prevention of foodborne bacterial pathogens.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13010305/s1, Figure S1: Different mechanisms used by bacteria to cope with acidic environments.

Author Contributions: Conceptualization, P.P.-F., A.M.V., and J.S.C.; methodology, P.P.-F. and A.M.V.; formal analysis, P.P.-F. and A.M.V.; investigation, P.P.-F. and A.M.V.; resources, P.P.-F. and A.M.V.; data curation, P.P.-F., J.A.M.P., A.M.V., and J.S.C.; writing—original draft preparation, P.P.-F., J.A.M.P., A.M.V., and J.S.C.; writing—review and editing, P.P.-F., J.A.M.P., A.M.V., and J.S.C.; supervision, A.M.V. and J.S.C.; project administration, J.S.C.; funding acquisition, J.S.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by FCT-Fundação para a Ciência e a Tecnologia through the CQM Base Fund-UIDB/00674/2020, and Programmatic Fund-UIDP/00674/2020, and a PhD Grant SFRH/BD/129630/2017 given to Priscilla Porto-Figueira, Portuguese Government funds), and by ARDITI-Agência Regional para o Desenvolvimento da Investigação Tecnologia e Inovação, through the project M1420-01-0145-FEDER-000005-Centro de Química da Madeira-CQM+(Madeira 14-20 Program) and Project M1420-09-5369-FSE-000001 for the Post-Doctoral fellowship granted to Jorge A. M Pereira.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding authors.

Conflicts of Interest: The authors declare no conflict of interest.

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