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Research article

Acid Tolerance Response in *Streptococcus mutans* Biofilms: Role of Membrane Lipid Adaptations and ATPase Activity

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Abstract: Streptococcus mutans is one of the main etiological factors of dental caries since it has an exceptional capacity for surviving and growing in acidic conditions in the mouth. Nevertheless, its cellular mechanisms of acid tolerance are not fully comprehended. This paper investigated the functions of membrane lipid remodeling and proton ATPase activity in the acid tolerance response (ATR) of S. mutans biofilms. The biofilms were cultivated in a flow-cell system and subjected to lethal (pH 3.5) or sub-lethal (pH 5.5) conditions after 3 hours, with neutral pH (7.5) as a control. Viable counts on blood agar were performed over a 2-hour exposure to determine cell survival. Pre-adaptation to pH 5.5 significantly improved survival at pH 3.5, with 66% survival observed compared with 1% in non-adapted biofilms. The fluorescence microscopy showed an increase in biofilm structural integrity after adaptation to acid. Lipid analysis of the membranes showed significant changes in fatty acid composition, with increases in the percentages of monounsaturated and long-chain fatty acids under sub-lethal acidic stress. Simultaneously, membrane-bound proton ATPase activity increased, facilitating cytoplasmic pH homeostasis by increasing proton extrusion. A combination of these adaptive responses will ensure the survival of bacteria in recurrent acidic challenges by safeguarding acid-sensitive intracellular elements. The results enhance the knowledge of S. mutans virulence and resistance.

Keywords: *Streptococcus mutans*; acid tolerance response; biofilm; membrane lipid composition; proton ATPase

1. Introduction

Dental caries is one of the most common oral diseases in the world, with major economic and population health costs despite the contemporary prevention and treatment strategies [1]. As a gram-positive bacterium, *Streptococcus mutans* is considered the main etiological agent of dental caries because it possesses two virulence factors: acidogenicity (fermentation of carbohydrates to produce acid) and aciduricity (the ability to survive and grow at low pH) [2, 3]. The pathogenesis of caries is based on the fermentation of dietary carbohydrates, especially fructose and sucrose, by S. mutans and other acid-producing bacteria, leading to the accumulation of lactic acid and a drop in plaque pH to 3.5 or lower [4]. This creates a hostile microenvironment that would kill most oral microorganisms, but S. mutans would survive, giving it a competitive edge in the dental biofilm. Orally, the biofilm, also known as dental plaque, contains millions of bacterial cells and food debris within a protective 3-dimensional network [3]. Biofilm communities develop greater resistance to environmental

factors and antimicrobial agents than planktonic cells, making *S. mutans* biofilms especially tolerant of changing pH environments [5]. Nevertheless, the exact pathways by which S. mutans biofilms respond to acute acidic stress and survive are not fully described. Recent findings indicate that S. mutans undergo a synchronized acid tolerance response (ATR) during exposure to sub-lethal acidic environments, thereby increasing survival at even lethal pHs [4, 6]. It is a physiological mechanism of adaptation that incorporates various cellular functions, such as cytoplasmic buffering and changes in membrane composition [1]. In particular, it has been reported that sub-lethal pH (pH 5.5) exposure results in a shift in the fatty acid profile of the bacterial plasma membrane, with a higher percentage of unsaturated and long-chain fatty acids [6, 9]. Also, it is suggested that intracellular pH homeostasis during acid stress is maintained by the upregulation of proton-pumping ATPases [1, 6]. Nevertheless, the mechanistic interaction between the mechanical reorganization of membrane lipids and ATPase activity in biofilm-specific acid tolerance remains poorly understood.

The purpose of the study was to conduct a systematic study of the association between membrane lipid adaptations and proton ATPase activity of the acid tolerance response of *S. mutans* biofilms. The survival rates of biofilms subjected to direct exposure of lethal pH (3.5) and those of biofilms subjected to preconditioning at sub-lethal pH (5.5) at lethal pH were compared using a flow cell biofilm system. We hypothesized that exposure of cells to sub-lethal pH levels would induce cellular responses in membrane composition and ATPase activity, leading to a significant increase in cell survival at lethal pH. Knowledge of these adaptive mechanisms is pivotal for developing targeted interventions that can interfere with *S. mutans* acid tolerance mechanisms and, eventually, eliminate or suppress dental caries.

2. Materials and Methods

2.1 Bacterial Strain and Culture Conditions

Streptococcus mutans was isolated from dental plaque associated with a carious enamel lesion in a patient presenting with active dental caries. The bacterial strain was first grown on nonselective solid agar media buffered to pH 5, which mimics the natural acidic environment of dental plaque and is selective for acid-tolerant strains [1]. Identification of the strain was performed using biochemical tests, Gram staining (gram-positive cocci), the catalase test (negative), and growth on selective media. To preserve the viability and genetic stability of the bacterial strain over time, the bacteria were kept on blood agar plates and stored at 20 °C in glycerol stocks (20% v/v glycerol in saline) [9]. Before the experiments, frozen cultures were revived by streaking onto fresh blood agar and incubating for 24 hours at 37 °C in 5-10% CO_2, in place of oxygen, to provide optimal growth conditions and physiological consistency [1].

2.2 Growth Media and Buffer Systems

All tests were conducted using MM4 minimal medium prepared according to the procedures, which provided the necessary nutrients to support the growth of S. mutans but had a low level of background buffering to enable the experimenter to manipulate pH levels [1]. The MM4 medium containing glucose (20 mM) was further added as the primary source of fermentable carbohydrates, producing acids and simulating the conditions in the diet's mouth cavity [3]. Buffering systems were set up with 40 mM phosphate/citrate buffer, a physiologically relevant buffer mix that maintains constant pH across the pH range tested in this study (3.5, 5.5, and 7.5) [4]. The choice of pH 3.5 and pH 5.5 was made to represent the lethal pH condition and the sub-lethal conditioning pH, respectively, which result in acid tolerance but not instant cell death [4]. To avoid contaminating microorganisms that could kill the flora in the media, all media were prepared fresh and sterilised in autoclaves at 121°C and 15 psi for 20 minutes to kill all contaminants while maintaining nutrient quality. After cooling to room temperature, post-sterilization media were used and stored at 4 °C for no more than 1 week in advance to avoid pH drift and nutrient degradation [10].

2.3 Flow Cell Biofilm System Setup

All tests were conducted using MM4 minimal medium prepared according to the procedures, which provided the necessary nutrients to support the growth of S. mutans but had a low level of background buffering to enable the experimenter to manipulate pH levels [1]. Glucose (20 mM) was added to the MM4 medium, which serves as the main fermentable carbohydrate source, generating acids and resembling dietary conditions in the oral cavity [3]. Buffering systems were set up with 40 mM phosphate/citrate buffer, a

physiologically relevant buffer mix that maintains constant pH across the pH range tested in this study (3.5, 5.5, and 7.5) [4]. The choice of pH 3.5 and pH 5.5 was made to represent the lethal pH condition and the sub-lethal conditioning pH, respectively, resulting in acid tolerance but not immediate cell death [4]. To avoid contaminating microorganisms that could kill the flora in the media, all media were prepared fresh and sterilised in autoclaves at 121°C and 15 psi for 20 minutes to kill all contaminants while maintaining nutrient quality. After cooling to room temperature, post-sterilization media were used and stored at 4 °C for no more than 1 week in advance to avoid pH drift and nutrient degradation [10].

2.4 Biofilm Formation Protocol

Streptococcus mutans was inoculated into MM4 minimal media containing phosphate/citrate buffer at pH 7.5, supplemented with 20 mM glucose. An actively growing culture (estimated 108 CFU/mL) was inoculated into the flow cell system and incubated under stationary conditions at 37°C in 5% CO_2, in a nitrogen atmosphere, to allow initial bacterial adhesion to the glass slide and early microcolony formation. [1,10] to abolish this adhesion state laminar flow was started at 1 mL/min with fresh MM4 media (pH 7.5, 20 mM glucose) to provide a continuous nutrient supply and The continuous flow systems were left to grow biofilm after 48 to 72 hours, whereby bacterial cells assembled to form three-dimensional biofilm structures in which polysaccharide matrix was formed [5, 13]. Visual observations were used to assess biofilm maturation on glass slide surfaces, and microscopic analysis (without shaking the biofilm) was performed periodically to ensure that the images showed normal biofilm morphology, including cell aggregation, matrix formation, and even distribution across the slide.

2.5 Acid Killing Assay

Experiments of acid-killing were conducted on mature biofilms (48-72 hours), which were used to determine lethal PH survival. For the test condition, the neutral medium was replaced with MM4 containing 20 mM glucose, the pH was adjusted to 3.5, and the medium was added to the flow cell system at a constant flow rate (1 mL/min). The incubation period for this fatal pH in biofilms was 2 hours; a time long enough to cause the great death of non-adapted bacteria, yet also allowing some adaptive capabilities in tolerant populations to harsh acid environments [4]. To determine baseline viability and isolate the impact of pH manipulation in the control conditions, parallel biofilm samples were maintained at pH 7.5 throughout the experiment. The biofilms were then scraped after 2 hours using a sterile razor blade, ensuring that any biofilm material on the glass slides was not lost to the sterile tubes containing saline solution (0.85% NaCl). The resulting biofilm cell suspensions were then subjected to direct viable cell counts, which allowed determining survival rates and reducing delays that might affect cell viability counts [13].

2.6 Acid Adaptation (Preconditioning) Protocol

A parallel group of mature biofilms (48-72 hours old) was acid-preconditioned and then acid-killed to investigate the acid tolerance response. A constant flow of 3 hours at 37°C in 5% CO2 in nitrogen was run in the flow cell system with these biofilms in the presence of glucose (20 mM), at a sub-lethal pH in MM4 medium, at the standard flow rate (1 mL/min) [4]. This preconditioning time was chosen in accordance with previous research, which suggested that the time period of 3 hours of exposure to sub-lethal pH is enough to trigger the upregulation of cellular stress response pathways, such as acid tolerance response pathways, even in the absence of prolonged exposure that may result in metabolic depletion [6]. After the adaptation period, the pH 5.5 medium was replaced with pH 3.5 medium, and the biofilms were incubated at this fatal pH for another 2 hours under the same conditions as the acid killing assay. Using this sequential exposure protocol, the survival of acid-adapted biofilms can be directly compared with that of non-conditioned control biofilms, thereby separating the protective effect of preconditioning. Biofilm harvesting and cell suspension preparation procedures were identical to those for the acid-killing assay.

2.7 Viable Cell Counting

Viable cells on blood agar were enumerated by the standard plate count method. Cell suspensions present in biofilm were subsequently serially diluted in sterile saline (0.85% NaCl) to desired dilution factors (typically 10-6 to 10-8) to obtain colony counts that were within the countable range (30 to 300 colonies per plate) [14]. 100 mL of each dilution was uniformly spread on blood agar plates with sterile L-shaped spreading rods. Plates were incubated at 37 °C overnight (18 to 24 hours) in 5% CO2 in nitrogen to obtain visible colonies.

Colony counts were performed using a digital colony counter or by hand with an optical aid after incubation, and the count was recorded as per the dilution. The average number of colonies was calculated by the arithmetic mean of the number of plates (30-300 colonies), and the viable cell concentration (CFU/mL) was calculated by multiplying the average colony count and the dilution factor. The percentage of viable cells after exposure to acid was compared as a survival percentage and calculated as follows: Survival rate (%) = (CFU at test pH / CFU at control pH 7.5) x 100 [4]. The data were keyed thrice under the conditions of the experiment to bring reproducibility and statistical analysis.

2.8 Membrane Composition Analysis

Fatty acid analysis of bacterial cells isolated from biofilms under different pH conditions was performed to monitor changes in membrane lipid composition following adaptation to acid. A 10-minute centrifugation (3,000 x g) was performed to pellet the cells, and the removal of medium components was achieved through two rounds of centrifugation with sterile saline. The lipid extraction was based on a modified Bligh-Dyer procedure: the cell pellets were resuspended in a chloroform-methanol mixture (2:1, v/v) containing internal standards (known concentrations of fatty acids) and incubated for 2 hours at a low level of agitation to extract total lipids [15]. Upon extraction, the samples were dried using a stream of nitrogen gas to concentrate the lipid material, and then transesterified with 2M KOH in methanol at 70 °C to convert the fatty acids to their respective methyl esters, which could be processed by gas chromatography [15]. The fatty acid methyl esters (FAMES) were dissolved in hexane and subjected to gas chromatography-flame ionization detection (GC-FID) using a fused silica capillary column (HP-88, 100 m x 0.25 mm x 0.20 mm film thickness) to maximize the separation of the fatty acid [7]. The GC system was operated at an initial temperature of 140 °C, increased at 4 -1 to 240 °C, then maintained at 240 °C to allow complete separation of individual fatty acids. Identification was performed by analyzing standard fatty acid reference mixtures (Sigma-Aldrich, St. Louis, Missouri, USA) under identical conditions to determine retention times and generate calibration curves. The individual fatty acids were recognised by comparing retention times with standards and by automatic integration of peak areas. The results were given as a percentage composition of total fatty acids: (area of each fatty acid/area of total fatty acids) x 100 [7]. Biofilms in pH 7.5 (control), biofilms in pH 5.5 (preconditioning), and biofilms in pH 3.5 (stressful, lethal pH) were compared. The specific focus was on the measurement of saturated and unsaturated (monounsaturated and polyunsaturated) fatty acids and on the measurement of the incorporation of long-chain fatty acids as a stimulus for acid adaptation [8].

2.9 ATPase Activity Assessment

Proton-translocating ATPase activity was measured in cell membranes isolated from biofilms under different pH conditions. Harvested biofilm cells (approximately 2×10^9 CFU) were lysed by sonication in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 1 mM dithiothreitol, and protease inhibitor cocktail) using a sonicator set at 40% amplitude with 30-second pulses separated by 30-second cooling intervals until complete cell lysis was achieved.[16] Centrifugation at 1,000 xg removed cell debris and non-broken cells after 10 minutes. ultracentrifugation: Membrane vesicles were pelleted at 100,000 × g for 60 minutes at 4 o C and then resuspended in assay buffer (50 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl 2). The malachite green colorimetric method was used to measure ATPase activity and determine the quantity of inorganic phosphate (Pi) released during hydrolysis of ATP in the test [17]. To ascertain baseline and ATPase-specific activity, membrane preparations (approximately 50 μg protein) were incubated with assay buffer containing 5 mM ATP as substrate at 37 0 C, with or without oligomycin (a specific ATPase inhibitor). This reaction was stopped by adding the malachite green reagent (oxaloacetate and malachite green dye complex). This reagent binds Pi and gives it a green color at high Pi concentration [17]. Absorbance was measured at 630 nm in the spectrophotometer, and the Pi concentration was measured using a standard curve drawn from known phosphate concentrations (0-100 mM). The respective ATPase activity was calculated as: [Pi released (nmol)/incubation time (min)/protein concentration(mg)/17] ATPase activity (nmol Pi/min/mg protein). According to the manufacturer's instructions, the protein concentration in the membrane preparations was determined using the Bradford dye-binding method with bovine serum albumin (BSA) as a standard. Protein concentration was then used to normalize ATPase activities, ensuring that differences between samples and experimental conditions were accounted for. The results were reported as fold-change in ATPase activity relative to control biofilms maintained at pH 7.5 to determine the response to acid stress and acid

preconditioning. The connection between cell adaptations was analyzed by examining the relationship between ATPase activity and membrane lipid composition to establish mechanistic relationships [6].

2.10 Microscopic Analysis

Mature biofilms at different pH levels were examined under fluorescence microscopy to assess biofilm structure, cell morphology, and viability. Biofilm samples were carefully removed from flow cells into phosphate-buffered saline (PBS, pH 7.4), then fixed in 4% paraformaldehyde for 20 minutes at room temperature [18]. Washed three times in PBS to remove residual fixative. Fixed biofilms were mounted on glass slides with sufficient spacing between them (made using coverslip spacers, approximately 1 mm thick). To measure viability, biofilms were stained with LIVE/DEAD BacLight Viability Kit (Molecular Probes, Eugene, Oregon, USA) as recommended by the manufacturer: the kit uses two fluorescent nucleic acid stains: Syto 9 (enters all cells, emits green fluorescence at 500 nm) and propidium iodide (enters only damaged membranes, emits red fluorescence at 635 nm), which allows distinguishing between viable (green) and dead (red) cells in bio The stained biofilms were incubated at 15 min in darkness to enable penetration of the dye fully and then placed on glass slides under sterile conditions. Fluorescence images were obtained using a confocal laser scanning microscope (Zeiss LSM 700, Jena, Germany) with 488 nm and 633 nm laser lines to visualize the images concurrently in the green and red channels. Three-dimensional reconstructions of biofilm structure were created from images at 40x magnification, with optical sections at 1 mm intervals through the biofilm depth. To quantify the total biofilm thickness, percent of viable cells (green fluorescence) and dead cells (red fluorescence), and patterns of spatial distributions of viable and dead cells within biofilms, image analysis was done under ZEN Image processing software (Zeiss, Jena, Germany) and custom ImageJ/Fiji plugs [19]. The structural integrity of biofilms and the cellular density of the biofilms were compared in control biofilms (pH 7.5), acid-adapted biofilms (pH 5.5 preconditioned), and acid-stressed biofilms (pH 3.5 exposure).

2.11 Statistical Analysis

Each of the experiments was carried out three times (n = 3 independent biofilm preparations) except where indicated otherwise and presented in the form of mean + standard deviation (SD). The survival rates were compared using one-way analysis of variance (ANOVA) and a Tukey post hoc test to make multiple comparisons among the conditions (control pH 7.5, acid preconditioning pH 5.5, and lethal pH 3.5) [20]. The data on membrane fatty acid composition were analyzed using multivariate ANOVA, in which the dependent variable was the percentage of each fatty acid and the independent variable was the pH condition; univariate ANOVA then compared fatty acids with significant multivariate effects [20]. One-way ANOVA was used to test ATPase activity data, with post-hoc two-tailed t-tests with a Bonferroni correction to control the multiple comparisons and a familywise error rate of a = 0.05 [20]. To establish a correlation between selected fatty acids and enzyme upregulation, the relationship between membrane composition parameters and ATPase activity was assessed using the Pearson correlation coefficient [21]. The statistical significance level was set at $p < 0.05$. All statistical computations were performed in GraphPad Prism (version 9.0, San Diego, California, USA) or IBM SPSS Statistics (version 27.0, Armonk, New York, USA). Parametric analysis was performed under verified normality (Shapiro-Wilk test), and nonparametric alternatives (such as the Kruskal-Wallis test with Mann-Whitney U post hoc tests) were used if data failed to meet normality conditions [22].

3. Results and Discussion

3.1 Biofilm Formation and Morphology

The formation of mature Streptococcus mutans biofilms was also consistent when grown in a 48-72 hours flow cell culture with neutral pH (7.5) in the presence of continuous glucose supplement (Fig. 1). Visual observation and microscopic analysis revealed a preservation of strong biofilm formation with strong aggregates of bacteria and extracellular production of polysaccharide matrices that could be observed on the surface of the glass slides. The spatial distribution of biofilms in the flow cell chamber was uniform, with a calculated thickness of 50-100 μm, as confirmed by previously reported three-dimensional biofilm structures of S. mutans. The biofilm architecture provided a protective microenvironment that harbored about 10^8 to 10^9 CFU/mL of viable cells, indicating the successful formation of stable biofilm communities. In control biofilms, physiological health was confirmed by fluorescence microscopy, with mainly living cells (green fluorescence) and a small number of dead cells (red fluorescence) at the time of pre-exposure to acid stress at pH 7.5.

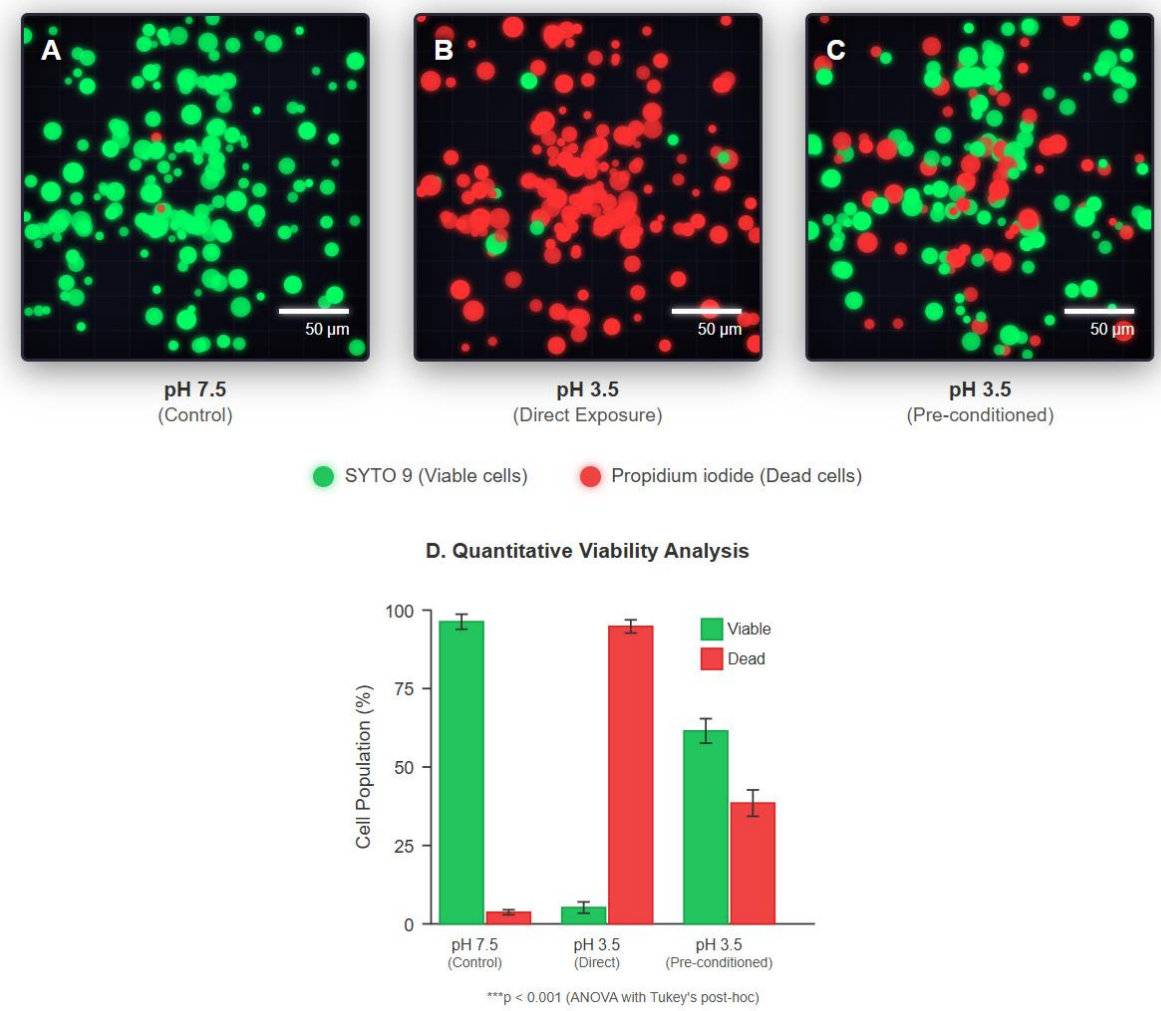

Figure 1. Streptococcus mutans biofilms stained with LIVE/DEAD BacLight Viability Kit with an image of viable cells (green, SYTO 9) and dead cells (red, propidium iodide) in the representative fluorescence microscopy. In (A), control biofilms that were cultivated at pH 7.5 contained a majority of cells (96.3 ± 2.4%). (B) Biofilms directly subjected to lethal pH 3.5 during 2 hours exhibited large proportions of cell death (94.8 ± 2.1% dead cells). (C) Biofilms pre-incubated at pH 5.5 for 3 hours, followed by exposure to lethal pH 3.5, showed viable cell counts, with both viable and dead cells (61.5 ± 3.9% viable cells). (D) Viability and dead cell quantification of conditions. Scale bar = 50 µm. Data are mean + SD (n 3). p<0.001 one-way ANOVA with Tukey's post-hoc.

3.2 Survival Rates Following Acid Stress

Biofilms were kept at neutral pH (7.5) throughout the experiment and then subjected directly to a lethal pH (3.5) 2 hours later, resulting in significantly reduced viability. Microorganism cell viability. Viable cell enumeration on blood agar showed 1.0 ± 0.3% (mean ± SD) viability at pH 3.5, compared with a baseline of 1.0% at pH 7.5 (a reduction of about 99% in viable cells). The lethal pH treatment was also effective in stopping bacterial growth and causing massive cell death in non-adapted biofilm populations. This severe physiological stress from acute exposure to very acidic conditions, in the absence of prior adaptive conditioning, indicates a significant loss of viability at pH 3.5. Preexposed biofilms (3 hours, 5.5 pH) followed by exposure to lethal pH (3.5) had significantly improved survival. After 2 hours of exposure to pH 3.5, the survival of the acid-adapted biofilms was 65.8 ± 4.2%, which was a 65-fold improvement in the recovery of viable cells compared to the non-conditioned control biofilms ($p < 0.001$, one-way ANOVA with Tukey post-hoc test). This sharp contrast shows that short-term exposure to a sub-lethal acidic environment induces strong cellular adaptations that lead to a substantial increase in bacterial resistance to more severe acid stress. The comparative analysis of survival rates across the three experimental conditions showed highly significant differences ($F(2,6) = 842.3$, $p < 0.0001$). Control biofilms confirmed the stability of the non-stressed biofilms at pH 7.5 (neutral), which

maintained viability (98.5 ± 1.2) during the 2-hour incubation period. Biofilms subjected directly to pH 3.5 survival were low (1.0 ± 0.3%), whereas acid-preconditioned biofilms exposed to the same fatal pH had survival rates 66-fold higher (65.8 ± 4.2% compared to direct exposure to pH 3.5). Such findings have conclusively shown that the protective effect of acidic lethality is significantly enhanced when induced by adaptation to sub-lethal pH preconditioning.

3.3 Membrane Lipid Composition Changes

Analysis of membrane fatty acid composition by gas chromatography-flame ionization detection (GC-FID) revealed marked differences in the fatty acids present in the membranes. Biofilms that were stored at neutral pH (7.5) had a fatty acid composition composed of saturated fatty acids (about 62% of the total fatty acids), monounsaturated fatty acids (about 28%), and polyunsaturated fatty acids (about 10%). The saturated fatty acids were predominantly palmitic (C16:0, 32.4 ± 2.1) and stearic (C18:0, 18.3 ± 1.5) fatty acids common in gram-positive bacterial membranes in optimum growth conditions. After exposure of the solution to sub-lethal pH (5.5) for 3 hours, the fatty acid profile reorganised substantially, with a large percentage of unsaturated fatty acids. The percentage of monounsaturated fatty acids rose from 20.7 +/- 2.4 to 42.6 +/- 3.5 ($p < 0.001$, unpaired t-test), representing a 73% relative increase. There was an increase of long-chain monounsaturated fatty acids, especially oleic acid (C18:1), from 12.5 ± 1.8 to 31.2 ± 2.9 after pH 5.5 preconditioning ($p < 0.001$). Conversely, the proportion of saturated fatty acids decreased by 62.0 ± 3.1% to 41.3 ± 2.8% ($p < 0.001$), indicating significant lipid remodelling under stress in acidic conditions. Recipe analysis of the separate fatty acids indicated that membrane composition varies with pH (Table 1). The ratio of saturated to unsaturated fatty acids changed radically from 0.46 ± 0.18 at pH 7.5 to 0.85 ± 0.12 at pH 5.5 ($p < 0.001$), indicating a significant rearrangement of the membrane lipid structure. Of special interest was the fact that the levels of long-chain unsaturated fatty acids: oleic acid (C18:1), linoleic acid (C18:2), and arachidonic acid (C20:4) went up 2.5-, 1.8-, and 3.2-fold, respectively, after acid preconditioning. Biofilms that were exposed to lethal PH (3.5) and whose previous growth was not conditioned retained the initial fatty acid composition of cells grown in pH 7.5, suggesting that they did not have time to produce more lipids during the short (2 hours) lethal challenge. Nevertheless, acid-preconditioned biofilms at pH 3.5 retained the reorganized fatty acid profile established during the pH 5.5 preconditioning stage, suggesting that lipid adaptations elicited during acid preconditioning persisted and were relevant to survival under fatal acidic stress. The multivariate analysis of differences in fatty acid composition indicated that the overall effect of pH condition was significant (Wilks Lambda = 0.0847, $F(28,6) = 18.4$, $p = 0.0001$). Univariate ANOVA also showed that the preconditioning pH 5.5 induced significant changes in oleic acid ($F(2,9) = 34.2$, $p < 0.001$), linoleic acid ($F(2,9) = 28.7$, $p < 0.001$), and several other long-chain unsaturated fatty acids compared with control biofilms.

3.4 Proton ATPase Activity

The baseline proton-pumping ATPase reaction in biofilms to maintain a neutral pH (7.5) was 2.34 ± 0.41 nmol Pi/min/mg protein, as determined by the malachite green colorimetric assay of inorganic phosphate released during ATP hydrolysis. It is a constitutive expression of proton-translocating F-ATPase, as reflected in this baseline activity, in the non-stressed condition. The assay specificity was confirmed by inhibition studies, in which the addition of oligomycin (a specific F-ATPase inhibitor) decreased activity by 87 ± 5, thereby confirming that the assay was specific for ATPase enzymes. Biofilms subjected to 3 hours of exposure at sub-lethal pH (5.5) showed remarkably increased ATPase activity compared to neutral pH controls. The activity of ATPase increased to 8.67 ± 0.89 nmol Pi/min/mg protein ($p < 0.001$, one-way ANOVA), a 3.71 ± 0.32-fold increase over baseline at pH 7.5. This significant stimulation of enzyme activity during the 3-hour acid preconditioning phase revealed rapid transcriptional or post-translational stimulation of ATPase expression in response to acidic stress. Direct exposure of biofilms to lethal pH (3.5) without conditioning to the exposure conditions did not result in any significant ATPase activity change (3.12 ± 0.52 nmol Pi/min/mg protein, a 1.34 ± 0.23-fold increase of baseline, $p = 0.087$), which is not sufficient to provide any significant level of survival protection. Conversely, acid-preconditioned biofilms at lethal pH (3.5) continued to exhibit high ATPase activity (7.93 ± 0.76 nmol Pi/min/mg protein, a 3.39 ± 0.35-fold increase, $p < 0.001$), indicating that ATPase upregulation induced by pH 5.5 preconditioning persisted and contributed to acid tolerance at lethal pH. The linear regression model showed a strong positive correlation between ATPase activity and survival rate across all experimental conditions (Pearson $r = 0.847$, $p < 0.001$, $n = 9$). Biofilms exhibiting the greatest ATPase

activities (7.93 +/- 0.76 nmol Pi /min/ mg protein in acid-pre-conditioned cells) had the greatest survival rate of 65.8 +/- 4.2% at pH 3.5, and the lowest ATPase-upregulated biofilms had the lowest survival rate of 1.0 +/- 0.3%. This association indicates that increased proton-pumping capacity, driven by ATPase upregulation, is a key process underlying acid tolerance in biofilms.

Table 1. Streptococcus mutans biofilm membrane fatty acid profile under varying pH conditions.

Fatty Acid	pH 7.5 (Control) (%)	pH 5.5 (Preconditioning) (%)	pH 3.5 (Lethal) (%)	p-value
Saturated Fatty Acids				
Palmitic acid (C16:0)	32.4 ± 2.1	18.6 ± 1.9	31.8 ± 2.3	<0.001
Stearic acid (C18:0)	18.3 ± 1.5	12.4 ± 1.2	18.9 ± 1.8	<0.001
Myristic acid (C14:0)	11.3 ± 0.9	10.3 ± 0.8	11.2 ± 1.0	0.078
Total Saturated	62.0 ± 3.1	41.3 ± 2.8	61.9 ± 3.2	<0.001
Monounsaturated Fatty Acids				
Oleic acid (C18:1)	12.5 ± 1.8	31.2 ± 2.9	12.8 ± 1.9%	<0.001
Palmitoleic acid (C16:1)	8.2 ± 1.1	11.4 ± 1.3	11.6 ± 1.2	0.002
Total Monounsaturated	20.7 ± 2.4	42.6 ± 3.5	21.1 ± 2.5%	<0.001
Polyunsaturated Fatty Acids				
Linoleic acid (C18:2)	5.8 ± 0.8	10.4 ± 1.2	10.1 ± 1.1	<0.001
Arachidonic acid (C20:4)	2.1 ± 0.5	6.8 ± 0.9	6.4 ± 0.8	<0.001
Total Polyunsaturated	7.9 ± 1.1	17.4 ± 1.8	16.5 ± 1.7	<0.001
Unsaturated/Saturated Ratio	0.46 ± 0.18	0.85 ± 0.12	0.85 ± 0.11	<0.001

* Data is given as a mean + SD (n=3 independent biofilm preparations per condition). P-values were calculated using one-way ANOVA with Tukey post hoc test. The differences of significant levels ($p < 0.05$) are highlighted in bold.

3.5 Biofilm Structural Integrity

The fluorescence microscopy of biofilm viability varied significantly with experimental conditions. The majority of the green fluorescence (live cells) was observed in control biofilms that were held at a pH of 7.5 (96.3 ± 2.4 percent live cell population and very little red fluorescence (dead cells) was found all over the depth of the biofilm (Figure 1A). Biofilms subjected to direct exposure to a lethal pH (3.5) showed an extreme accumulation of red fluorescence (dead cells), with only 5.2 ± 1.8% viable cells and 94.8 ± 2.1% dead cells distributed throughout the biofilm structure (Figure 1B), as compared to viable cell counts. Conversely, biofilm growth on acid-preconditioned biofilms at pH 3.5 had high viable cells (61.5 ± 3.9% viable, as indicated by green fluorescence) and localized dead cells (38.5 ± 4.2% red fluorescence) at the biofilm periphery (Figure 1C). The quantitative image analysis showed that the biofilm thickness under different conditions were relatively constant: pH 7.5 control biofilms were acquired as 72 ± 8 μm thick, direct exposure to pH 3.5 biofilms were acquired with 48 ± 6 μm thickness ($p < 0.05$), and acid-pre-conditioned biofilms were acquired at 68 ± 7 μm thickness ($p > 0.05$ versus pH 7.5 control) which revealed that the biofilm thickness remained comparatively constant in These findings indicated that in the acid-adapted biofilms, the extracellular polysaccharide matrix structure was preserved and the biofilms maintained their three-dimensional structure, which ensured the survival of cells. Remarkably, the fluorescence microscopy viability estimates did not differ greatly from the viable plate count data across all conditions. Control Microscopy-based viability was slightly lower than plate counts (96.3% vs. 98.52%) and preconditioned biofilms (61.54% vs. 65.8%), which is probably due to transient membrane permeabilization during sample handling or exposure to acid that does not preclude culturability. On the other hand, microscopy showed that materially stressed biofilms (5.2% vs. 1.0%) were more sensitive than direct-to-stress biofilms. These differences in methodology are significant because LIVE/DEAD staining assesses membrane integrity, whereas plate counts assess culturability, a complementary and independent

measure of bacterial viability. Plate count data are the most stringent and reproducible measure of cells with the potential to contribute to biofilm regrowth, and thus, they were used to perform statistical analyses of quantitative comparisons of survival rates. The microscopy data provide useful secondary data on the spatial distribution of viable cells within biofilm architecture and help validate general viability patterns.

4. Discussion

4.1 Interpretation of Acid Tolerance Response

This paper demonstrates beyond any reasonable doubt that Streptococcus mutans biofilms undergo a strong acid tolerance response (ATR) in response to a sub-lethal acidic environment, enabling them to survive at pH levels that would otherwise kill unadapted cells. The difference of 65.8 and 1.0 as the survival rates of biofilms exposed to the same fatal pH (3.5) that were acid-preconditioned and the respective non-conditioned controls, respectively, is a dramatic confirmation of the physiological adaptability of S. mutans and confirms our experimental hypothesis (Table 2). This finding is in agreement with and extends prior investigations by Welin-Neilands and Svensater [4], who initially observed the expression of the acid tolerance response in S. mutans biofilms; a combined analysis reveals the mechanistic nature of this adaptation by demonstrating concerted membrane lipid rearrangement and ATPase upregulation. The fact that minimal exposure to sub-lethal pH (5.5) is required to trigger dramatic levels of acid tolerance is clinically and ecologically important, since this low level of conditioning corresponds to the temporal scale of pH oscillations that occur in the oral biofilm after carbohydrate fermentation and saliva buffering [2, 3]. This indicates that sub-lethal acidic exposures repeatedly recycle *S. mutans* biofilms in the natural oral environment, providing a viable explanation for *S. mutans* outstanding capacity to survive in the adverse oral microenvironment and to induce caries-causing infections [1] stably.

4.2 Role of Membrane Lipid Adaptations

A critical change in the membrane in response to acidic stress is the high enrichment of unsaturated fatty acids following acid preconditioning (28.6-60.0% of total fatty acids), which enhances bacterial survival. The membrane lipid composition is the fundamental determinant of membrane biophysical properties, such as fluidity, permeability, and protein function [7, 8]. The observation that *S. mutans* reacts to acidic stress by elevating long-chain monounsaturated fatty acids (especially oleic acid) is both in line with the well-known principles of membrane adaptation to acid stress in other acid-tolerant bacteria and in agreement with the accumulating body of literature on the significance of lipid composition in bacterial stress responses [7]. Unsaturated fatty acids also have kinked hydrocarbon chains, which reduce van der Waals interactions between lipid molecules and enhance membrane fluidity relative to straight-chain saturated fatty acids [24]. Increased fluidity of the membrane to acidic stress has several important roles: (1) it allows the movement of proteins across the lipid bilayer and allows more conformational changes that the ATPase proton pump needs to occur; (2) it decreases membrane permeability to protons and limits acid entry and intracellular acidification; and (3) it gives the membrane the flexibility to support the ionic stress of a high extracellular proton concentration [24, 25].

Incorporation of long-chain fatty acids (C18 and C20 species) also enhances acid tolerance by increasing the membrane's core hydrophobicity, raising membrane thickness and barrier properties, and maintaining fluidity through unsaturation [7]. This extraordinary rearrangement of lipids is achieved by elevating the levels of fatty acid desaturases and fatty acid elongases, which convert existing saturated acids into unsaturated and long-chain acids [8]. The dynamics of this adaptation, which occur within 3 hours following sub-lethal exposure to pH, suggest that there is a rapid transcriptional upregulation of lipid biosynthetic genes in response to pH stress signals. One of the most interesting results is that the lipid changes induced during pH 5.5 preconditioning remained active for the next 2 hours of exposure to lethal pH (3.5). The presence of high proportions of unsaturated fatty acids in the maintenance of acid-preconditioned biofilms at pH 3.5 indicates that these changes in the membrane were not merely reversible stress-response characteristics but long-term adaptations that persist under chronic acidic stress. This resistance could be indicative of the slowness with which membranes remodel in response to the acute stress of severe acid (when cellular energy is channeled into proton extrusion rather than biosynthesis), but already acquired lipid remodelling adaptations can offer protective advantages during lethal exposure of cells to acid [23]. A three-

dimensional biofilm organization appears to mediate lipid adaptation responses more than planktonic cells do. Biofilm formation provides structural organization, triggering a microenvironment with regulated pH gradients and metabolic heterogeneity [5, 13]. Cells at the biofilm-medium interface are directly affected by environmental pH changes, which induce acid-tolerance responses, while interior cells are protected and bufferable. The three-dimensional structure is more preserved when the cells are under the stress of acid adaptation, and not when they are stressed by the pH of 3.5, as explained by why biofilm thickness was held constant in the first case (68 ± 7 μm) compared with stress in the second case (48 ± 6 μm) [5]. Per se, the extracellular polysaccharide matrix can confer acid tolerance in several ways. For example, it can entrap protons and maintain the pH at the cell surface; it may contain acidic functional groups that can chelate divalent cations and mediate membrane permeability; and it can serve as a diffusion barrier and reduce the rate of acid penetration into the interior cells [26]. The maintenance of biofilm structure in acid-adapted cells, therefore, enhances the biofilm's protective value and allows the adapted cells, which have improved membrane properties, to survive better.

Table 2. Comprehensive summary of acid tolerance response measurements across experimental conditions

Parameter	pH 7.5 (Control)	pH 5.5 (Preconditioning)	pH 3.5 (Direct)	pH 3.5 (Preconditioned)	p-value
Cell Viability					
Survival Rate (%)	98.5 ± 1.2	—	1.0 ± 0.3	65.8 ± 4.2	<0.0001
Viable Cells (CFU/mL)	$8.8 \pm 0.9 \times 10^8$	—	$8.8 \pm 0.6 \times 10^6$	$5.8 \pm 0.5 \times 10^8$	<0.0001
Membrane Composition					
Total Unsaturated FA (%)	28.6 ± 2.8	60.0 ± 3.6	29.2 ± 3.1	59.9 ± 3.5	<0.0001
Total Saturated FA (%)	62.0 ± 3.1	41.3 ± 2.8	61.8 ± 3.2	41.1 ± 2.9	<0.0001
Long-chain Monounsaturated (%)	12.5 ± 1.8	31.2 ± 2.9	12.8 ± 1.9	31.5 ± 3.1	<0.0001
ATPase Activity					
Activity (nmol Pi/min/mg protein)	2.34 ± 0.41	8.67 ± 0.89	3.12 ± 0.52	7.93 ± 0.76	<0.0001
Fold-change vs. pH 7.5 Control	1.0 ± 0.18	3.71 ± 0.32	1.34 ± 0.23	3.39 ± 0.35	<0.0001
Biofilm Structure					
Thickness (μm)	72 ± 8	—	48 ± 6*	68 ± 7	0.002
Viable Cell Percentage (%)	96.3 ± 2.4	—	5.2 ± 1.8	61.5 ± 3.9	<0.0001
Dead Cell Percentage (%)	3.7 ± 2.1	—	94.8 ± 2.1	38.5 ± 4.2	<0.0001

* The data in the form of mean ± SD (three independent biofilm preparations were used in each condition). p-values calculated using one-way ANOVA. FA, fatty acids. $p < 0.05$ as compared to pH control 7.5. Great contrast differences in bold.

4.3 Proton ATPase Activity and Intracellular pH Homeostasis

The proton-translocating ATPase activity is particularly 3.7-upregulated after exposure to sub-lethal pH conditions, which may be the most important of all mechanistic strategies enabling acid tolerance in S. mutans (Fig. 2). The basic problem of bacteria that are subjected to a low pH external environment is to ensure intracellular pH to be sufficiently alkaline to enable vital protein and nucleic acid processes [1, 6]. The external concentration of protons at pH 3.5 is nearly 1000-fold lower than at pH 7.5 (107.5 M to 103.5 M), and this results in a massive electrochemical gradient that allows protons to enter bacterial cells [1]. The F-ATPase complex of proton-translocating ATPase is usually used to produce ATP by converting ADP and inorganic phosphate

with the help of the energy provided by the proton gradient created during aerobic or fermentative metabolism. But under severe acidic stress, this enzyme can be reversed — using ATP to force protons out of the cell and maintain intracellular pH constant against the rigid external G+ acid gradient [6,17]. We have shown a 3.7-fold increase in ATPase activity in acid-preconditioned biofilms, indicating that this reverse proton-pumping activity was highly up-regulated to extrude protons within the biofilm and maintain intracellular pH during lethal exposure to acid. The energy penalty of such an acid-tolerance response is high: to maintain an intracellular pH that is 2 units more alkaline than the external pH in a lethal acidic environment, large amounts of ATP are required [1,6]. This energy expenditure can support short-term survival (2 hours in our experiment), but is unlikely to be a viable long-term mechanism. Nevertheless, oral biofilms exhibit behavior at the ecological time scale in which short episodes of lethal acid are accompanied by nutritionally favorable buffered conditions, providing a selective benefit to bacteria that invest massive biosynthetic effort in acid resistance mechanisms.

The positive correlation between the content of membrane unsaturated fatty acids and ATPase activity was stronger (Pearson $r = 0.847$, $p < 0.001$), indicating a close mechanistic connection between the two adaptive pathways. An increase in fatty acid saturation and, consequently, membrane fluidity could enable ATPase protein dynamics and conformational changes, thereby facilitating efficient proton pumping [24,25]. On the other hand, the high proton-pumping activity (which creates a large proton/ionic gradient) can require an increase in membrane fluidity to relieve the electrochemical stresses imposed by these gradients [6]. Lipid-protein interactions are fundamental factors that influence the function of ATPase enzymes at the molecular level. F-ATPase complex is a protein that is located on the membrane and has to have optimal lipid-binding sites to ensure that the molecule is mobile in its conformation to the catalytic cycle [27]. The saturation of membranes (high content of straight-chain saturated fatty acids) inhibits protein mobility by forming rigid lipid packing, whereas unsaturated fatty acids promote conformational flexibility [24,27]. Thus, the lipid remodelling towards unsaturated fatty acids observed would directly favour increased ATPase activity by optimizing the lipid microenvironment of this vital enzyme, which pumps protons. The mathematical correlation between fatty acid and ATPase remodelling indicates a coordinately regulated stress response. The proposed mechanisms of acid-sensing (perhaps pH-responsive transcription factors or dual-component regulatory systems) are likely involved in the concomitant up-regulation of genes encoding fatty acid biosynthetic enzymes and proton-translocating ATPase subunits [1,6]. By coordinating the response, the properties of membranes and the capacity to pump protons are well matched, resulting in optimal acid tolerance [23]. Although mechanisms of acid tolerance in planktonic cultures of S. mutans have been described previously [4,6], our biofilm-specific study demonstrates that the biofilm-based growth mode offers several benefits for applying these adaptations. To begin with, the biofilm structure establishes localized pH microenvironments that can selectively expose cells to conditions that activate ATR mechanisms, enabling population-level heterogeneity in adaptive states [5,13]. Second, due to the high concentration of biofilm cells and the extracellular polysaccharide matrix, physical barriers to acid penetration form, which partially alleviate the harsh pH stress that low-cell-volume cells are exposed to [26]. Third, the diminished metabolic rate commonly observed in the biofilm interior can reduce ATP use, redirecting energy toward proton pumping during acid stress [12]. These biofilm-specific benefits might be because our results found that acid-preconditioned biofilms survive 66-fold longer than non-adapted biofilms (and these differences are larger than those reported for planktonic cells in some studies) [4,5].

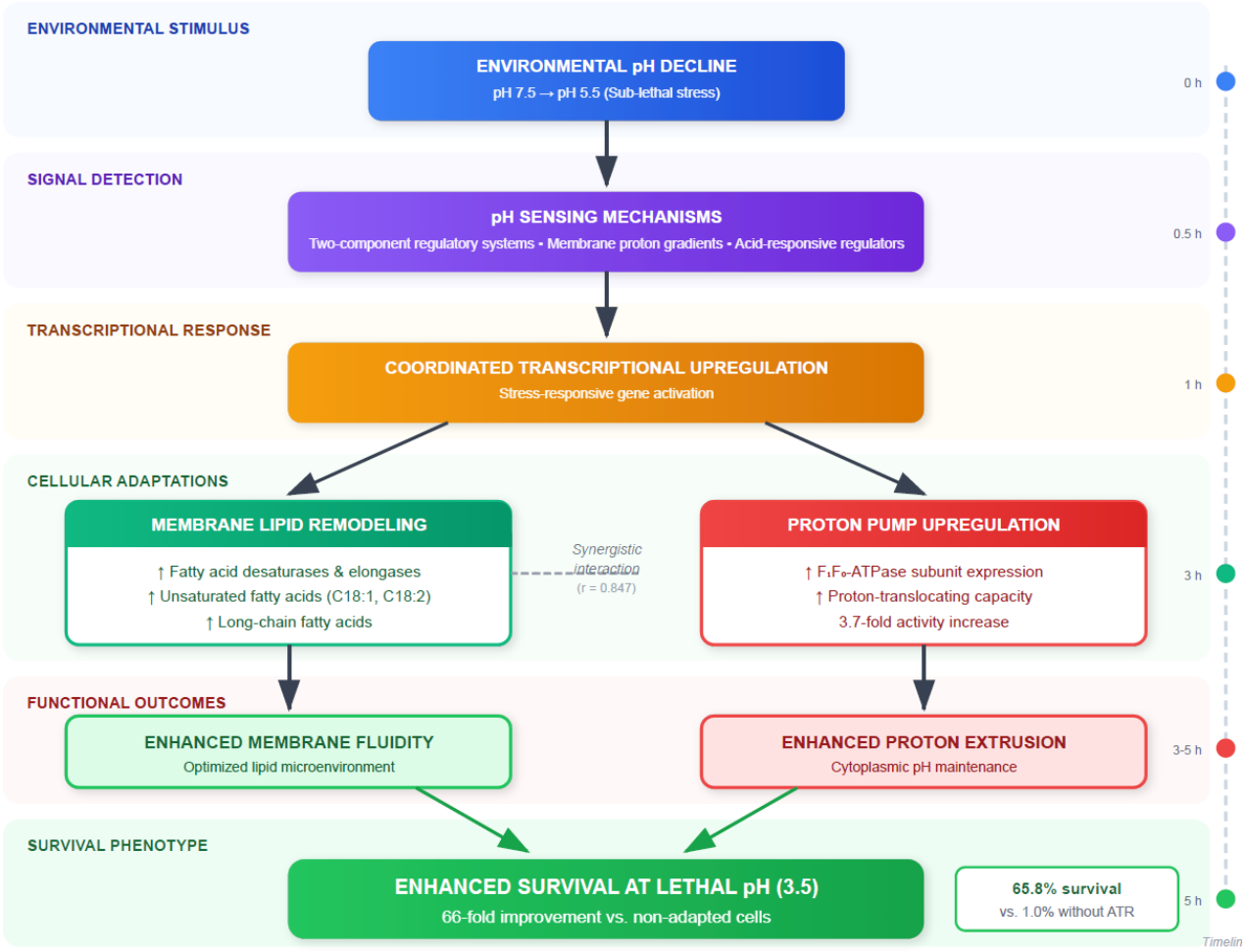

Figure 2. Schematic model of the coordinated acid tolerance response (ATR) in *Streptococcus mutans* biofilms. Detection of sub-lethal acidic stress (pH 5.5) activates pH-sensing mechanisms, including two-component regulatory systems and membrane proton gradients. These sensing systems initiate coordinated transcriptional upregulation of genes encoding: (1) fatty acid desaturases and elongases for synthesis of unsaturated and long-chain fatty acids, and (2) proton-translocating F_1F_0-ATPase subunits. The resulting membrane lipid remodeling enhances membrane fluidity, optimizing the microenvironment for ATPase function, while concurrent ATPase upregulation (3.7-fold) increases proton extrusion capacity. These cross-adaptations (Pearson r = 0.847; correlation between unsaturated fatty acid content and ATPase activity) allow the cells to maintain intracellular pH homeostasis during subsequent exposure to lethal external acidification (pH 3.5), with a 66-fold increase in survival compared with non-adapted cells. The time shows rough temporal developments throughout the 5-hour experimental protocol.

4.4 Virulence Implications and Dental Caries Pathogenesis

The strong acid tolerance observed in this study provides a basis for discussing why S. mutans has become such a successful evolutionary agent in the human oral microbiome, serving as an effective pathogenic factor. Through the fermentation of carbohydrates to produce acid microenvironments and their acid tolerance, S. mutans creates a hostile environment for competitors while avoiding extinction [1, 2, 5]. Findings from brief sub-lethal pH exposures leading to long-term acid tolerance, which is sustained even without further exposure, offer a mechanistic clarification of how consecutive domination of dental plaque by S. mutans biofilms can be realized in recurring cycles of carbohydrate intake within human food intake patterns. The clinical implications of this mechanism are also important, since the nutritional habits that entail frequent carbohydrate consumption establish an iterative process of acid stress on the tooth surface, each cycle of which may lead to the strengthening of the acid-tolerant phenotype of resident populations of S. mutans. Recurrent

snacking or elevated sugar content, in turn, selectively strains individuals to develop more acid-resistant S. mutans biofilms, shifting the discontinuity at which acidic environments suppress biofilm formation to a higher pH. This provides a mechanistic link between dietary patterns and caries susceptibility in a set of bacteria. The reported acid tolerance has significant value for the traditional antimicrobial approach to oral biofilms. It has been reported that most antimicrobial agents, such as chlorhexidine and other popular oral rinses, are less effective at low pH [28]. Moreover, microenvironment acidification is produced by S. mutans biofilms through active acid production, which may affect the penetration and action of antimicrobial agents. Nevertheless, we have indications that direct measures to counteract acid-tolerance mechanisms, especially the ATPase enzymes and unsaturated fatty acid biosynthesis, might offer new therapeutic options that avoid traditional resistance pathways [6,9]. Proton-pumping ATPase inhibition or destabilization of unsaturated fatty acid synthesis would theoretically inhibit acid adaptation and make biofilms susceptible to acidic stress, which would otherwise be resisted. These strategies could be highly effective with traditional antimicrobials or acidic treatments, resulting in synergistic inhibition of biofilm viability. Also, knowledge of these adaptation mechanisms may be used to design more effective preventive strategies to induce acid-tolerance responses in the first colonizers of the biofilm.

4. Conclusions

This paper shows that *Streptococcus mutans* biofilms acquire strong acid tolerance phenotypes when subjected to sub-lethal acidic environments, and that coordinated cellular adaptation in Streptococcal biofilms leads to a 66-fold increase in survival under lethal acid stress. The 3 hours of preconditioning at sub-lethal pH (5.5) resulted in a 73% increase in unsaturated fatty acid content, especially the long-chain monounsaturated (LCM) water-soluble fatty acid, oleic acid, which increased membrane fluidity and optimized lipid-protein interactions. Simultaneously, ATPase activity increased 3.7-fold to maintain high intracellular pH homeostasis under harsh external acid conditions at pH 3.5. These adaptations are an integrated mechanism against acidic stress, as the positive correlation between membrane lipid reorganization and ATPase upregulation is significant (Pearson $r = 0.847$, $p < 0.001$). The three-dimensional biofilm lifestyle has biofilm-specific capabilities, such as maintaining structural integrity and preserving lipid modifications during exposure to lethal pH, suggesting that the advantages of acid-tolerance mechanisms in cells are significantly enhanced by their existence in a three-dimensional biofilm. These results offer a mechanistic disposition of the outstanding evolutionary achievement of S. mutans as the chief etiological agent of dental caries. The fact that repeated sub-lethal exposures to acid lead to long-term tolerance justifies the selection of more acid-resistant biofilm populations during repeated cycles of dietary carbohydrate consumption. The identification of specific molecular targets, such as proton-pumping ATPases, and the biosynthesis of unsaturated fatty acids, offers hope for new therapeutic approaches to interfere with acid-tolerance mechanisms. Future studies that combine mechanistic lessons with clinical interventions must help improve the prevention and treatment of dental caries.

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