Effect of pH on the antimicrobial activity of the macrophage metabolite itaconate

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Abstract

The production of itaconate by macrophages was only discovered in 2011. An increasing number of studies have since revealed essential biological functions for this small molecule, ranging from antimicrobial to immunomodulator. The antibacterial role of itaconate has however been questioned because the estimated concentration of itaconate in macrophages (low-millimolar) is lower than the minimum inhibitory concentration (MIC) of itaconate reported for several bacterial strains (low-to-mid-millimolar). We note that some of these investigations have tended to ignore the high acidity of this small diacid (pKas 3.85 and 5.45), thereby potentially biasing activity measurements. We measured the MIC of itaconate in *Escherichia coli* (not known to metabolize itaconate) and in *Salmonella enterica* serovar Typhimurium (known to metabolize itaconate) at varying pH values to probe the effect that pH has on itaconate toxicity. Herein, we demonstrate that the antimicrobial effect of itaconate is dependent upon the pH of the media and that itaconate does have antimicrobial activity at biologically relevant pH and concentrations. Under nutrient-poor conditions, the antimicrobial activity of itaconate in both *E. coli* and *S.* Typhimurium increased approximately 200-fold when the pH was dropped by one unit, whereas itaconate was not found to be toxic under nutrient rich conditions. Our results also reveal that the activity of itaconate is synergistic with acidity, yet is not a function of increased permeability with protonation. Similar experiments performed with succinate (a pKa-matched diacid) yielded drastically different results, consistent with a target-based mechanism of action for itaconate. Overall, our work shows the importance of controlling the pH when performing experiments with itaconic acid.

Introduction

Itaconate is produced by macrophages upon classical activation by bacterial lipopolysaccharides (LPS).(1) The presence of LPS leads to upregulation of the immune-responsive gene 1 (*irg1*), translated to the protein *cis*-aconitate decarboxylase (Irg1, Acod1, or Cad), which produces itaconate from the citric acid cycle intermediate *cis*-aconitate.(2) Whereas itaconate was recently demonstrated to have a complex immunomodulatory effect on mammalian cells(3-12) in which itaconate inhibits mammalian succinate dehydrogenase (Sdh2)(5) leading to metabolic reprogramming of the macrophage,(3) other reports have suggested that it may also contribute to the antimicrobial activity of the innate immune system.(2, 13) Consistent with the latter role, an itaconate degradation pathway has been identified in some bacterial species known to proliferate in macrophages.(14) Interestingly, Hammerer *et al*. have reported a small molecule able to resensitize bacteria to itaconate, and have proposed that inhibiting itaconate degradation in bacteria might be a new strategy to treat infections.(15)

Itaconate is an inhibitor (K_i of 0.9 – 220 μM) of bacterial isocitrate lyase (Icl),(16, 17) an essential enzyme of the glyoxylate cycle. The two enzymes of this cycle, Icl and malate synthase, enable bacteria to divert citric acid cycle metabolites from decarboxylation to preserve carbon atoms, and are essential for survival within macrophages.(18, 19) Interestingly, itaconyl-CoA was reported to inhibit B_{12}-dependent methylmalonyl-CoA mutase (involved in the degradation of odd-chain fatty acids and some amino acids to replenish the citric acid cycle).(20), (21)

Reports of the concentration of itaconate in macrophages vary significantly. Within activated murine macrophages, the overall concentration of itaconate has been estimated to 3 – 8 mM, (1, 2) whereas this value is only 60 µM for activated human macrophages.(2) In contrast, reported minimum inhibitory concentrations (MICs) for itaconate in bacteria range from 1 – 75 mM.(2, 13, 15, 17, 22-25) From these numbers, whether the itaconate concentration in macrophage is high enough to effectively inhibit microbial growth has been questioned.(26) It has more recently been demonstrated that itaconate is transported to vacuoles containing *Salmonella* and reaching 5 – 6 mM,(27) consistent with itaconate playing an antimicrobial role.(2, 15) Furthermore, the amount of itaconate needed to inhibit bacterial growth may also depend on the environment.

Itaconate is commercially available as itaconic acid, which is acidic enough (pKa of 3.85 and 5.45)(28) to significantly alter the pH of growth media. Authors of previous articles reporting the activity of itaconate against specific bacteria (MICs) have used commercial itaconic acid in their experiments (and not a neutralized form such as disodium itaconate), without mention of adjusting the pH.(2, 13, 15, 22-25) Bacterial growth is well known to be affected by pH,(29-31) and failure to ensure a constant pH may considerably skew MIC measurements. Recent reports have also suggested that there may be synergy between pH and itaconate activity,(32, 33) although a systematic exploration of the phenomenon has yet to be reported. We elected to perform studies with *Escherichia coli* (a species that does not encode itaconate-degrading enzymes) and *Salmonella enterica* ser. Typhimurium (which expresses itaconate-degrading enzymes) to explore the effect of pH on the activity of itaconate and to verify the relevance of the proton shuttle mechanism as a mode of action for itaconate.

Methods

Chemicals, bacterial strains, and media used

Itaconic acid was purchased from Alfa Aesar, sodium succinate hexahydrate was purchased from Chem-Impex, sodium carbonate was purchased from Fisher Scientific, disodium itaconate was prepared by mixing Na_2CO_3 (10.6 g, 100 mmol) with itaconic acid (13.01 g, 100 mmol) in distilled water (100 mL) and stirred for 1 hour before removing the water *in vacuo* to produce a fine white powder. The white powder was incubated at 70°C overnight to remove residual water. Absorbance was measured using Spectramax® i3x microtiter plate reader from Molecular Devices. The bacterial strains used in these studies include: *Escherichia coli* (ATCC® 25922), *Salmonella enterica* spp. Typhimurium (ATCC® 14028), *Acinetobacter baumannii* (ATCC® 19606), *Enterobacter faecium* (ATCC® 19434), *Klebsiella pneumoniae* (ATCC® 13883), *Pseudomonas aeruginosa* (ATCC® 27853), and *Mycobacterium tuberculosis* H37Rv (ATCC® (25618™). Shel Lab® rotary shakers were used for 10 mL incubations, and an Ohaus® benchtop orbital shaker was used for assay incubations. The rich media used for selected experiments were Difco™ Nutrient Broth (NB) except for *M. tuberculosis* where Middlebrook 7H9 media (Difco) supplemented with 0.05% albumin, 0.085% sodium chloride, 0.1% acetate and 0.05% Tyloxaplol (7H9-acetate) was employed. The minimal growth media (M9A) recipe for 1 L is as follows: 6.8 g Na_2HPO_4, 3.0 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl, 4.0 g NaOAc, 2 mL $MgSO_4$ (62.5 mg/mL), 1 mL $CaCl_2$ (11.3 mg/mL), and 150 µL trace elements solution (162 mg $FeCl_3$, 13.6 mg $ZnCl_2$, 24.2 $Na_2MoO_4{\cdot}2H_2O$, 15.9 mg $CaCl_2$, 12.6 $CuCl_2{\cdot}2H_2O$, 6.2 mg H_3BO_3 in 10 mL 1 M HCl). The media were filter sterilized. The pH-specific minimal media (M9A') were prepared as M9A medium, except that a combination of AcOH/NaOAc was used to access the desired pH.

MIC_{90} assays of itaconic acid and disodium itaconate in M9A without controlling the pH

Bacteria were allowed to grow overnight in NB (dipped-loop from frozen stock into 10 mL at 37°C and 200 rpm) before plating onto NB agar and allowing to grow for 10-11 hours at 37°C. Four colonies were picked to inoculate NB liquid media (10 mL) and incubated until the stationary phase was reached, as measured by optical density at 600 nm (ca. 16 hours for *E. coli*; 24 hours for *S.* Typhimurium, *A. baumannii, E. faecium, K. pneumoniae,* and *P. aeruginosa*). An aliquot (100 µL) of the culture was then transferred into M9A media (10 mL). The suspension was incubated at 37°C and 200 rpm until OD_{600} reached 0.6 (ca. 8-10 hours for *E. coli*; ca. 24 hours for *S.* Typhimurium, *A. baumannii, E. faecium, K. pneumoniae,* and *P. aeruginosa*). This bacterial culture was used to inoculate (10 µL) each well of a 96-well microplate (flat clear bottom) containing M9A and itaconic acid or disodium itaconate (0.37, 1.1, 3.3, 10, 20, 40 mM), except for *E. coli* and *S.* Typhimurium in which itaconic acid concentrations were 0.0024, 0.0049, 0.010, 0.020, 0.039, 0.078, 0.16, 0.31, 0.63, 1.3, 2.5, 5.0, 10, 20, and 40 mM. Reference for relative growth was monitored in the absence of itaconic acid. Visible light absorption of the growth medium was subtracted from the readings. All data points are means of 3 independent experiments, each performed in triplicates. The plates were covered with a gas-permeable moisture barrier adhesive seal and incubated at 37°C and 250 rpm for 72 hours.

MIC_{90} assays of itaconic acid in NB with pH control

Bacteria were allowed to grow overnight in NB (loop from frozen stock into 10 mL NB at 37°C and 200 rpm) before plating onto NB agar and incubating for 10-11 hours at 37°C. Four colonies were picked to inoculate liquid NB media (10 mL) before incubation at 37°C and 200 rpm until an

OD600nm of 0.1 at. The bacterial culture was next diluted 10-fold into fresh NB. This bacterial suspension was used to inoculate (10 µL) each well of a 96-well microplate (flat colourless bottom) containing NB (190 µL) and itaconic acid (0.37, 1.1, 3.3, 10, 20, 40 mM). The plates were covered with a gas-permeable moisture barrier adhesive seal and incubated at 37°C and 250 rpm for 18 hours. For reference, the growth was also monitored in the absence of itaconic acid. The absorption of the growth medium alone was subtracted from all readings. All data points are means of 3 separate experiments, each performed in triplicates.

Resazurin microtiter assay (REMA) for *M. tuberculosis*

Itaconic acid and disodium itaconate were tested separately against *M*. *tuberculosis* H37Rv using the resazurin microtiter assay (REMA) in 96-well plates as previously described.(34) Briefly, a mid-logarithmic phase culture of H37Rv (OD_{600nm} approx. 0.5) was diluted in 7H9-acetate media (Middlebrook 7H9 media from Difco, supplemented with 0.05% albumin, 0.085% sodium chloride, 0.1% acetate and 0.05% tyloxaplol) to an OD_{600nm} of 0.001 (approx. 1×10^5 CFU/mL). Bacteria (100 µL) were then dispensed in transparent flat-bottom 96 well plates. On each plate, controls without itaconate or itaconic acid and media alone were included. Plates were incubated for 6 days at 37°C before the addition of resazurin (0.025% wt/vol to 1/10 of the well volume). After overnight incubation, the fluorescence of the resazurin metabolite, resorufin, was determined with excitation at 560 nm and emission at 590 nm, using a TECAN Infinite M200 microplate reader. The minimum inhibitory concentration (MIC_{99}, referred to as MIC) was determined using the Gompertz equation with GraphPad Prism software (version 7). Itaconic acid and disodium itaconate were tested in triplicates.

MIC assay of disodium itaconate and disodium succinate in M9A at controlled pH

Bacteria were allowed to grow overnight in NB (loop from frozen stock into 10 mL NB at 37°C and 200 rpm) before plating onto NB agar and incubating for 10-11 hours at 37°C. Four colonies were picked to inoculate NB liquid media (10 mL) before incubation at 37°C and 200 rpm until an OD60nm 0.1 . The bacterial culture was next diluted 10-fold into fresh NB. This bacterial suspension was used to inoculate (10 µL) each well of a 96-well microplate (flat colourless bottom) containing M9A' (190 µL) adjusted to the desired pH and disodium itaconate or disodium succinate (0.37, 1.1, 3.3, 10, 20, 40 mM for *E. coli*, via addition of disodium itaconate or disodium succinate in a MilliQ water solution, and 3.7, 11, 33, 100, 200, 400 mM for *S.* Typhimurium, via dissolution of disodium itaconate into M9A'). The plates were covered with a gas-permeable moisture barrier adhesive seal and incubated at 37°C and 250 rpm for 72 hours. For reference, the growth was also monitored in the absence of disodium itaconate or disodium succinate. The absorption of the growth medium alone was subtracted from all readings. All data points are means of 3 separate experiments, each performed in triplicates.

MIC assay of disodium itaconate in NB at controlled pH

Bacteria were allowed to grow overnight in NB (loop from frozen stock into 10 mL NB at 37°C and 200 rpm) before plating onto NB agar and incubating for 10-11 hours at 37°C. Four colonies were picked to inoculate liquid NB media (10 mL) before incubation at 37°C and 200 rpm until an absorbance of 0.1 at 600 nm was reached. The bacterial culture was next diluted 10-fold into fresh NB. This bacterial suspension was used to inoculate (10 µL) each well of a 96-well microplate (flat colourless bottom) containing M9A' (190 µL) adjusted to the desired pH, and disodium

itaconate (0.37, 1.1, 3.3, 10, 20, 40 mM for *E. coli*, via addition of disodium itaconate in a MilliQ water solution, and 3.7, 11, 33, 100, 200, 400 mM for *S.* Typhimurium, via dissolution of disodium itaconate into pH-adjusted NB). The plates were covered with a gas-permeable moisture barrier adhesive seal and incubated at 37°C and 250 rpm for 18 hours. For reference, the growth was also monitored in the absence of disodium itaconate. The absorption of the growth medium alone was subtracted from all readings. All data points are means of 3 separate experiments, each performed in triplicates.

Relative growth, statistics, and FICI calculations

Growth was quantified based on the OD_{600} of the bacterial culture. For the variable pH experiments using M9A, relative growth in the presence of itaconate was determined by comparison with the growth observed at pH 7.2 in the absence of itaconate . For the experiments using rich medium or succinate in M9A, relative growth was determined by comparison with the growth observed at the same pH (without itaconate). Statistical significance was determined using two-tailed Students t-test at $p<0.05$. FICI values were determined as previously described.(35, 36)

Results and Discussion

Effect of itaconic acid on the pH of media and bacterial growth

Based on the published MIC values of itaconate against bacteria (1 – 50 mM) and the reported cellular concentrations of itaconate (3 – 8 mM in murine macrophages, and ca. 60 µM in activated human macrophages),(2, 27) it is tempting to conclude that itaconate is unlikely to be produced by macrophages for its antimicrobial activity.(2) These reported MIC values however

may not have been measured at constant pH, and therefore may be biased. We observed that the addition of itaconic acid to growth media (pH 7.2 before itaconic acid addition), such as the M9 minimal medium supplemented with acetate (M9A) or the nutrient broth (NB) has a considerable effect on the final pH of the medium, with an approximate decrease of 2 pH units for M9A (Figure S1a) and 3.5 pH units for NB (Figure S1b) at 40 mM itaconic acid. As expected, the acidifying effect of itaconic acid was greater on NB, which does not have buffering capacity.

It is known that some bacterial species are capable of utilizing itaconate as a carbon source (*e.g. S.* Typhimurium, *P. aeruginosa*, *M. tuberculosis*),(14, 38) which may change their susceptibility to itaconate depending on the growth conditions. We therefore compared the antibacterial activity of itaconic acid and disodium itaconate in M9A media (without adjusting the pH after addition). This growth medium forces bacteria to utilize the glyoxylate cycle, which itaconate inhibits.(17, 37) All bacterial species tested were more susceptible to the former, implying that pH affects the antimicrobial activity of itaconate (Table 1).

Table 1. MIC_{90} of itaconic acid and disodium itaconate for different pathogens in M9A (unadjusted pH)

Species	**MIC_{90} of itaconic acid (mM)**	**MIC_{90} of disodium itaconate (mM)**
Escherichia coli	5	> 40
***Salmonella enterica* spp. Typhimurium**	20	> 40

Pseudomonas aeruginosa	20	> 40
Klebsiella pneumoniae	10	> 40
Acinetobacter baumannii	20	> 40
Enterobacter faecium	20	> 40
Mycobacterium tuberculosis	1*	4*

*These values are MIC_{99}. They were measured in the presence of 0.05% Tyloxaplol. Experiments with *M. tuberculosis* utilized the REMA assay.

Antimicrobial activity of itaconate at controlled pH in M9A media and synergy with acidity

We next aimed to tease apart the effect of acidity on bacterial growth from the intrinsic antimicrobial effect of itaconate. Both *E. coli* and *S.* Typhimurium were found to proliferate equally well in M9A medium (16, 17) from pH 7.2 to pH 6.3, while growth was sharply hampered at pH values below 6.3 (Figure S2).

The effect of itaconate varied significantly with pH, revealing an increasing antimicrobial activity as the pH is lowered. As expected from its ability to metabolize itaconate to acetyl coenzyme A and pyruvate,(14) *S.* Typhimurium was in general more resistant to itaconate than *E. coli* (about 5-fold at pH 7.2 and >500-fold at pH 6.4) (Figure 1). Interestingly, we noticed a small increase in bacterial growth at the lowest itaconate concentrations tested (ca. 0.37 mM) for pH 7.0 and above. This was expected for *S.* Typhimurium, but not for *E. coli* which does not express itaconate-metabolizing enzymes and hence should not be able to use itaconate as a carbon source. However, *E. coli* succinyl coenzyme A synthetase (sucCD) has weak affinity for

itaconate.(39) Itaconate may therefore compete with the formation of succinyl-CoA from succinate (K_m succinate = 0.141 mM, K_m itaconate = 0.475 mM) in *E. coli*, which may enhance growth by slightly increasing the concentration of succinate feeding into the citric acid cycle.

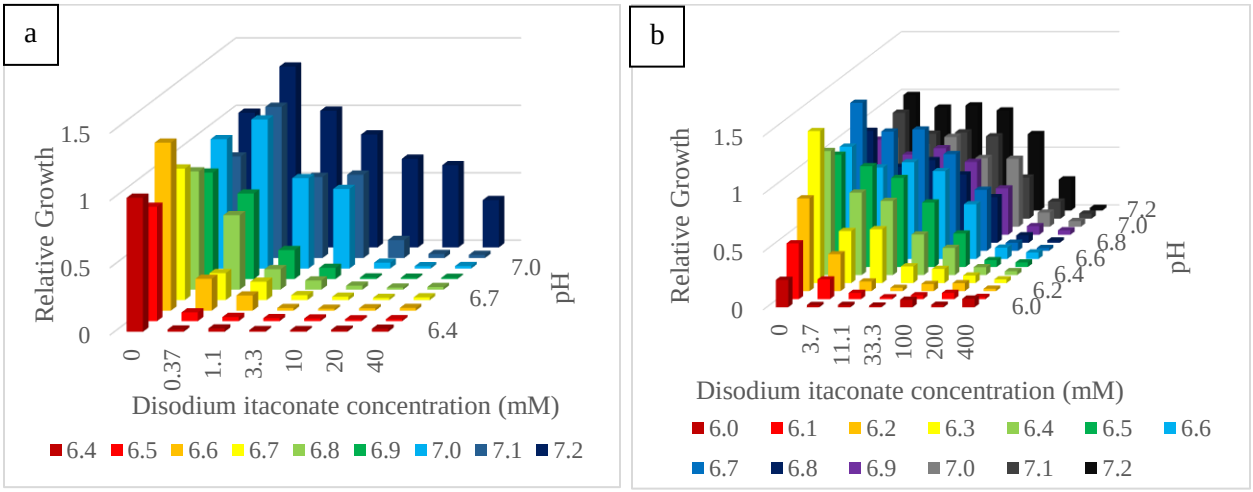

Figure 1. Relative growth measured after 72 hours at 37°C for (a) *E. coli* and (b) *S.* Typhimurium in M9A medium with added itaconate (0 – 40 mM for *E. coli* and 0 – 400 mM for *S.* Typhimurium) at controlled pH values (6.4 – 7.2). The data is presented relative to the bacterial growth observed at 0 mM itaconate and pH 7.2. See Figures S3 and S4 for individual pH graphs with errors (SEM, n = 3) of all conditions tested with statistical analysis.

From these data, the MIC_{90} of itaconate was calculated at different pH values, and these numbers were used to evaluate whether the antibacterial activity of itaconate and acidity are additive or synergistic. Synergy was quantified using the fractional inhibitory concentration index (FICI)(35),(36) (for details, see supplemental data). With *E. coli*, the calculated MIC_{90} of itaconate

at pH 7.2 was 80 mM (Figure S5) and 1×10^{-3} mM for H^+. The FICI were next computed for all $[H^+]$/[itaconate] combinations tested, and the values were less than 0.5 in all cases (Table 2), consistent with synergy between itaconate and acidity for their ability to inhibit the growth of *E. coli*.

Table 2. Calculated FICI values for growth inhibition of *E. coli* at different concentrations of itaconate and pH, where + denotes growth, - denotes no growth.

pH	[ITA] (mM)						
	0	**0.37**	**1.1**	**3.3**	**10**	**20**	**40**
6.4	+	0.4	-	-	-	-	-
6.5	+	0.3	-	-	-	-	-
6.6	+	+	0.3	-	-	-	-
6.7	+	+	+	0.2	-	-	-
6.8	+	+	+	0.2	-	-	-
6.9	+	+	+	0.2	-	-	-
7.0	+	+	+	+	0.2	-	-
7.1	+	+	+	+	+	0.3	-
7.2	+	+	+	+	+	+	+

With *S.* Typhimurium the calculated MIC_{90} were 400 mM for itaconate at pH 7.2 and 3×10^{-3} mM for H^+. The computed FICI values (Table 3) revealed a synergistic relationship between itaconate and acidity at pH values below 6.3, otherwise the effect was found to be additive.

The physiological rationale for the observed synergy between itaconate and acidity is still unclear, yet current knowledge allows us to postulate some contributing factors. An enhanced activity of dicarboxylate and/or mono-basic dicarboxylate transporters at lower pH may partly explain the observed synergy. Succinate transporters are especially relevant given that itaconate

appears to be a ligand for many enzymes that use or generate succinate,(5, 39, 40) suggesting that these transporters may be involved in cell permeation of itaconate. There is evidence that diacid discrimination by transporters may vary with pH.(41-43) For example, the transporter YaaH functions to shuttle both acetate and succinate (with similar affinity) in *E. coli*, and is most effective at pH 6.0.(44) From a quick computational search, we found an analogue of YaaH in *S. enterica,* referred to as SatP (Figure S12). Itaconate may also be transported into *S.* Typhimurium by the dicarboxylate transporter DcuB, which is know to transport succinate.(45)

Table 3. Calculated FICI values for growth inhibition of *S.* Typhimurium at different concentrations of itaconate and pH, where + denotes growth, - denotes no growth.

pH	**[ITA] (mM)**						
	0	**3.7**	**11**	**33**	**100**	**200**	**400**
6.0	+	0.3	-	-	-	-	-
6.1	+	+	0.3	-	-	-	-
6.2	+	+	0.2	-	-	-	-
6.3	+	+	+	+	+	0.7	-
6.4	+	+	+	+	+	0.6	-
6.5	+	+	+	+	+	0.6	-
6.6	+	+	+	+	+	0.6	-
6.7	+	+	+	+	+	0.6	-
6.8	+	+	+	+	+	0.6	-

6.9	+	+	+	+	+	0.5	-
7.0	+	+	+	+	+	+	1
7.1	+	+	+	+	+	+	1
7.2	+	+	+	+	+	+	1

Intracellular *Salmonella* are typically located in acidic vacuoles (pH of ca. 5.0),(46) where the concentration of itaconate is 5 – 6 mM, according to a recent report.(27) Consistent with an antimicrobial role for itaconate in mammals, the MIC of itaconate is much lower at acidic pH and within concentrations that are found in specific macrophage organelles.(27) Other examples of acidic organelles include phagosomes 10 minutes after phagocytosis (pH of 4.5 – 5.0),(47) early phagosomes (pH of 6.1 – 6.5), and late phagosomes (pH of 5.5 – 6.0).(48) In particular, *Mycobacterium*-containing phagosomes have a pH ranging from 5.2 to 6.3, depending on the maturation phase.(49-51) Regardless of their relatively poor growth in our media at lower pH, it is well established that both *E. coli* (pH ≥2.5)(52) and *S.* Typhimurium (pH ≥4.5)(53) tolerate acidic conditions.

Despite the absence of an itaconate-degradation pathway in *E. coli*, the MIC_{90} for itaconic acid is only 4-fold lower in *E. coli* than in *S.* Typhimurium and *P. aeruginosa* (Table 1), both known to produce itaconate-degrading enzymes. Our results show that once the effect of itaconic acid on pH is considered, the different susceptibility of *E. coli* and *S.* Typhimurium to itaconate is more apparent. The MIC_{90} of itaconate was found to differ by 5-fold between these two species at pH 7.2, and by over 500-fold at pH 6.4 (Figure 3 and Tables 2 and 3). Our results also reveal that the

antimicrobial activity of itaconate increases about 200-fold in *E. coli* when the pH of the growth medium is lowered from 7.2 to 6.4 (Figure 1a, Figure S3), and >100-fold in *S.* Typhimurium when the pH is dropped from 7.2 to 6.0 (Figure 1b, Figure S4). Interestingly, at pH 6.0 the MIC_{90} of itaconate in *S.* Typhimurium was found to be 3.7 mM, which is within the concentration range of itaconate found within *Salmonella*-containing vacuoles (5 – 6 mM),(27) implying a potential antimicrobial effect of itaconate in this organelle.

Antimicrobial activity of itaconate at controlled pH in Nutrient Broth excludes a proton shuttle mechanism

Although a number of scientific publications have reported the inhibition of Icl by itaconate, the mechanism of action of this metabolite *in cellulo* remains undetermined. One suggestion is a proton shuttle effect, as proposed by the "weak acid preservation theory", used to describe the antimicrobial activity of organic acids.(29-31) This implies that the organic acid enters the cell in a neutral, protonated form before releasing protons into the more alkaline cytosol, leading to acidification and disruption of the proton gradient.(54, 55) This effect has been observed for acetic acid, sorbic acid and propionic acid among others,(56-58) although these observations may conflate multiple effects.(59)

Itaconic acid inhibited the growth of both *E. coli* and *S.* Typhimurium similarly in NB growth medium (uncontrolled pH) (Figure 2a, 2b). The proton-shuttle effect may be the mode of action for itaconate when the pH of NB is not controlled, leading to similar MIC_{90} values for itaconate against both *E. coli* and *S.* Typhimurium (Figure 2a, 2b). In this case, growth inhibition

is likely non-specific and attributed to the change in pH incurred upon the addition of itaconic acid to the medium (Figure S1).

In contrast, when the pH was controlled (to pH values at which *E. coli* and *S.* Typhimurium are most affected by itaconate; see Figure 1), the inhibitory effect of itaconate was lost (Figure 2c, 2d). This lack of growth inhibition by itaconate when the pH of NB is controlled (Figure 2c, 2d, Figure S6, S7) precludes the sole involvement of a proton shuttle mechanism and is more consistent with a mode of action involving Icl inhibition by itaconate (Icl is not required for growth in NB).

Importantly, the absence of a linear correlation between the growth of *E. coli* or *S.* Typhimurium and the concentration of monoanionic itaconate (ITA^{1-}, Figures S10 – S11, Table S1) implies that increased cell permeability with protonation cannot explain the synergy observed between itaconate and acidity.

These data clearly demonstrate that the activity of itaconate is linked to the carbon source available. This has been previously demonstrated,(15) and is expected to be an important consideration when designing therapeutic strategies for pathogens that are capable of metabolising itaconate (*e.g. S.* Typhimurium, *P. aeruginosa*, *M. tuberculosis*).(14, 38)

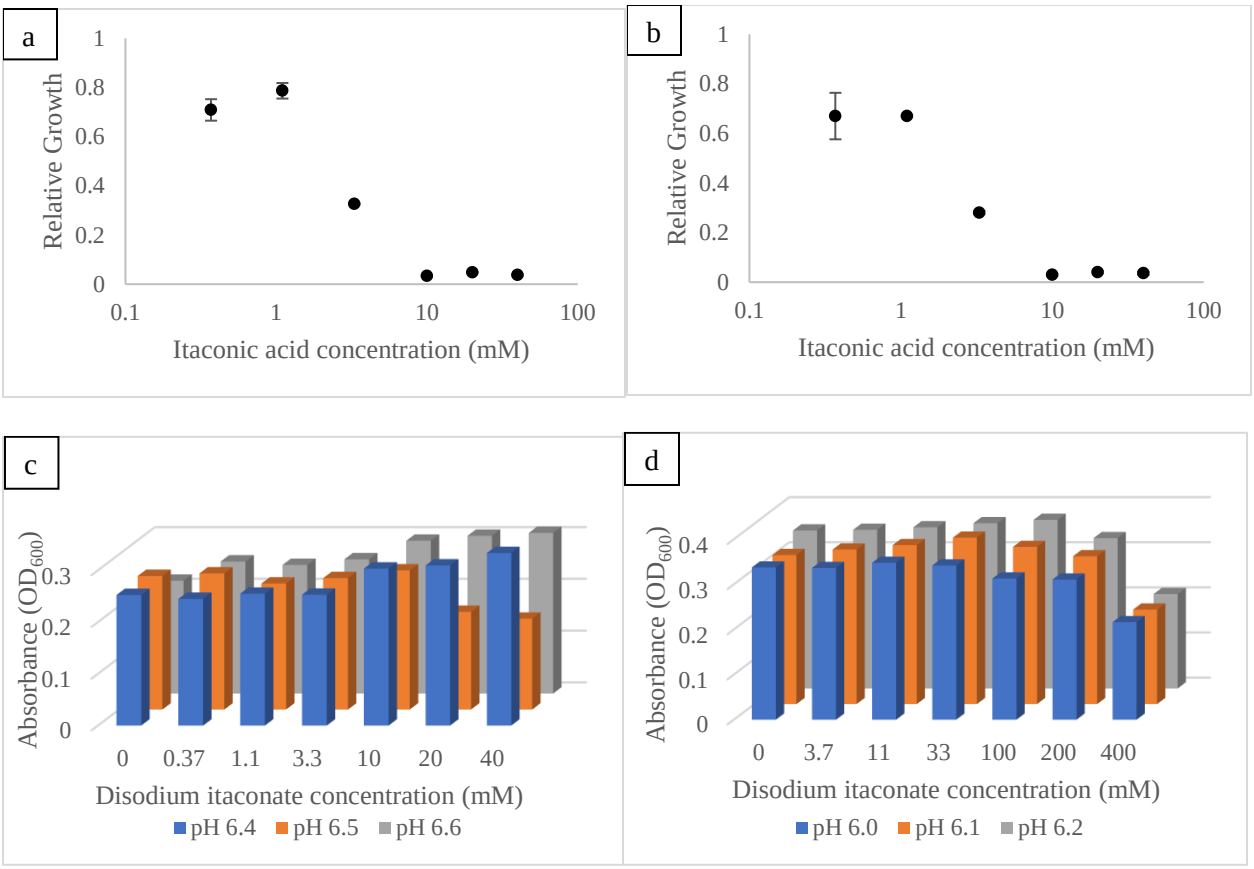

Figure 2. Relative growth in NB medium measured after 18 hours at 37°C for (a) *E. coli* in NB medium supplemented with itaconic acid (0 – 100 mM) without pH control, (b) *S*. Typhimurium in NB medium supplemented with itaconic acid (0 – 100 mM) without pH control, (c) absorbance at 600 nm for *E. coli* in NB medium supplemented with disodium itaconate (0 – 40 mM) at set pH values (6.4 – 6.6), and (d) *S.* Typhimurium in the presence of disodium itaconate (0 – 400 mM) at set pH values (6.0 – 6.2). See Figures S6 and S7 for individual pH graphs with errors (SEM, n = 3) of all conditions tested with statistical analysis.

The antimicrobial activity of succinate at controlled pH in M9A media differs from that of itaconate

To confirm that the antibacterial activity of itaconate observed in M9A media is structure-based (not pH-based) and target-specific, we next evaluated the activity of succinate at controlled pH values. The pKa_2 of succinate (5.48) is similar to that of itaconate (5.45),(28) entailing a similar proton shuttling propensity. These diacids differ in structure only by a methylene group branching off of an α-carbon. Despite this structural difference, many enzymes are known to bind both molecules.(5, 39, 40) In contrast to the effect of itaconate, which displays an MIC_{90} of 0.37 mM at pH 6.4 (Figure 1a), the growth of *E. coli* was not significantly impaired by added succinate (up to 40 mM) within pH 6.4 – 6.7 (Figure 3a, Figure S8). Succinate (up to 400 mM) did not suppress the growth of *S.* Typhimurium either, and even enhanced it at pH 6.1 and 6.2 (Figure 3b, Figure S9). This is clearly different from the effect of itaconate, consistent with a targeted mechanism of action for the latter.

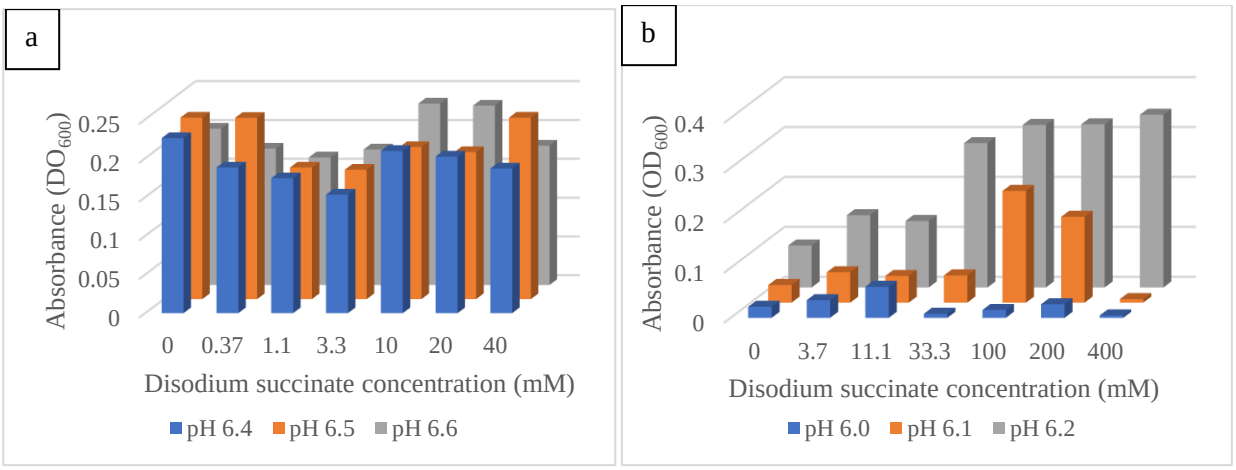

Figure 3. Absorbance at 600 nm measured after 72 hours at 37°C for (a) *E. coli* in M9A medium supplemented with disodium succinate (0 – 40 mM) at set pH values (6.4 – 6.6), and (b) *S.* Typhimurium in M9A medium supplemented with disodium succinate (0 – 400 mM) at set pH

values (6.0 – 6.2). See Figures S8 and S9 for individual pH graphs with errors (SEM, n = 3) of all conditions tested with statistical analysis.

The relationship between macrophages and pathogens is complex, and the exact role played by itaconate remains to be fully clarified. Our results support an inhibitory activity on bacterial growth (possibly via Icl inhibition), in synergy with acidity. They also warrant a more systematic, documented control of the pH in research experiments involving itaconate. The antimicrobial role of itaconate produced by phagocytic cells is also consistent with the fact that several pathogens have evolved mechanisms to limit the harmful effect of itaconate, such as the itaconate degradation pathway encoded by *S.* Typhimurium, *Y. pestis,* and *P. aeruginosa*.(14) Further supporting this hypothesis is the demonstration that *S*. Typhimurium can be resensitized to the antibacterial action of itaconate by small molecule inhibitors of the itaconate degradation pathway,(15) raising the interesting possibility that the antimicrobial activity of itaconate could be exploited to treat infections. This warrants additional investigations to elucidate the pH-dependent response of other pathogens to itaconate, improve our understanding of the expression of dicarboxylate transporters at different pH values, and seek other potential mechanisms of resistance to itaconate.

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Author Contributions

KA and DD made major contributions to the conception of the study. DD acquired and analyzed all data with *Acinetobacter baumannii*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Salmonella enterica* serovar Typhimurium. AL acquired and analyzed all data with *Mycobacterium tuberculosis*. All authors contributed to interpretation of the data, as well as writing and editing of the manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest.

References

1. Strelko CL, Lu W, Dufort FJ, Seyfried TN, Chiles TC, Rabinowitz JD, et al. Itaconic acid is a mammalian metabolite induced during macrophage activation. J Am Chem Soc. 2011;133(41):16386-9.
2. Michelucci A, Cordes T, Ghelfi J, Pailot A, Reiling N, Goldmann O, et al. Immune-responsive gene 1 protein links metabolism to immunity by catalyzing itaconic acid production. Proc Natl Acad Sci U S A. 2013;110(19):7820-5.
3. O'Neill LAJ, Artyomov MN. Itaconate: the poster child of metabolic reprogramming in macrophage function. Nature reviews Immunology. 2019.
4. Cordes T, Michelucci A, Hiller K. Itaconic Acid: The Surprising Role of an Industrial Compound as a Mammalian Antimicrobial Metabolite. Annu Rev Nutr. 2015;35:451-73.
5. Lampropoulou V, Sergushichev A, Bambouskova M, Nair S, Vincent EE, Loginicheva E, et al. Itaconate Links Inhibition of Succinate Dehydrogenase with Macrophage Metabolic Remodeling and Regulation of Inflammation. Cell metabolism. 2016;24(1):158-66.
6. Qin W, Qin K, Zhang Y, Jia W, Chen Y, Cheng B, et al. S-glycosylation-based cysteine profiling reveals regulation of glycolysis by itaconate. Nat Chem Biol. 2019;15(10):983-91.
7. Nair S, Huynh JP, Lampropoulou V, Loginicheva E, Esaulova E, Gounder AP, et al. Irg1 expression in myeloid cells prevents immunopathology during M. tuberculosis infection. J Exp Med. 2018;215(4):1035-45.

8. Bambouskova M, Gorvel L, Lampropoulou V, Sergushichev A, Loginicheva E, Johnson K, et al. Electrophilic properties of itaconate and derivatives regulate the IκBζ–ATF3 inflammatory axis. Nature. 2018;556(7702):501-4.
9. Mills EL, Ryan DG, Prag HA, Dikovskaya D, Menon D, Zaslona Z, et al. Itaconate is an anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1. Nature. 2018;556(7699):113-7.
10. Seim GL, Britt EC, John SV, Yeo FJ, Johnson AR, Eisenstein RS, et al. Two-stage metabolic remodelling in macrophages in response to lipopolysaccharide and interferon-gamma stimulation. Nat Metab. 2019;1(7):731-42.
11. Cordes T, Lucas A, Divakaruni AS, Murphy AN, Cabrales P, Metallo CM. Itaconate modulates tricarboxylic acid and redox metabolism to mitigate reperfusion injury. Mol Metab. 2020;32:122-35.
12. Daniels BP, Kofman SB, Smith JR, Norris GT, Snyder AG, Kolb JP, et al. The Nucleotide Sensor ZBP1 and Kinase RIPK3 Induce the Enzyme IRG1 to Promote an Antiviral Metabolic State in Neurons. Immunity. 2019;50(1):64-76.e4.
13. Naujoks J, Tabeling C, Dill BD, Hoffmann C, Brown AS, Kunze M, et al. IFNs Modify the Proteome of Legionella-Containing Vacuoles and Restrict Infection Via IRG1-Derived Itaconic Acid. PLoS Pathog. 2016;12(2):e1005408.
14. Sasikaran J, Ziemski M, Zadora PK, Fleig A, Berg IA. Bacterial itaconate degradation promotes pathogenicity. Nat Chem Biol. 2014;10(5):371-7.
15. Hammerer F, Chang JH, Duncan D, Castaneda Ruiz A, Auclair K. Small Molecule Restores Itaconate Sensitivity in Salmonella enterica: A Potential New Approach to Treating Bacterial Infections. Chembiochem : a European journal of chemical biology. 2016;17(16):1513-7.
16. Honer Zu Bentrup K, Miczak A, Swenson DL, Russell DG. Characterization of activity and expression of isocitrate lyase in Mycobacterium avium and Mycobacterium tuberculosis. J Bacteriol. 1999;181(23):7161-7.
17. McFadden BA, Purohit S. Itaconate, an isocitrate lyase-directed inhibitor in Pseudomonas indigofera. J Bacteriol. 1977;131(1):136-44.
18. McKinney JD, Honer zu Bentrup K, Munoz-Elias EJ, Miczak A, Chen B, Chan WT, et al. Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. Nature. 2000;406(6797):735-8.
19. Fang FC, Libby SJ, Castor ME, Fung AM. Isocitrate lyase (AceA) is required for Salmonella persistence but not for acute lethal infection in mice. Infection and immunity. 2005;73(4):2547-9.
20. Ruetz M, Campanello GC, Purchal M, Shen H, McDevitt L, Gouda H, et al. Itaconyl-CoA forms a stable biradical in methylmalonyl-CoA mutase and derails its activity and repair. Science. 2019;366(6465):589-93.
21. Shen H, Campanello GC, Flicker D, Grabarek Z, Hu J, Luo C, et al. The Human Knockout Gene CLYBL Connects Itaconate to Vitamin B12. Cell. 2017;171(4):771-82.e11.
22. Hillier S, Charnetzky WT. Glyoxylate bypass enzymes in Yersinia species and multiple forms of isocitrate lyase in Yersinia pestis. J Bacteriol. 1981;145(1):452-8.
23. Nguyen TV, Alfaro AC, Young T, Green S, Zarate E, Merien F. Itaconic acid inhibits growth of a pathogenic marine Vibrio strain: A metabolomics approach. Sci Rep. 2019;9(1):5937.
24. Bellion E, Kelley RL. Inhibition by itaconate of growth of methylotrophic bacteria. J Bacteriol. 1979;138(2):519-22.
25. Shimamoto G, Berk RS. Taurine catabolism. III. Evidence for the participation of the glyoxylate cycle. Biochim Biophys Acta. 1980;632(3):399-407.
26. Luan HH, Medzhitov R. Food Fight: Role of Itaconate and Other Metabolites in Antimicrobial Defense. Cell metabolism. 2016;24(3):379-87.
27. Chen M, Sun H, Boot M, Shao L, Chang SJ, Wang W, et al. Itaconate is an effector of a Rab GTPase cell-autonomous host defense pathway against Salmonella. Science. 2020;369(6502):450-5.

28. Braude EA, Nachod, F. C., editor. Determination of Organic Structures by Physical Methods. 1st ed. New York: Academic Press; 1955.
29. Coban HB. Organic acids as antimicrobial food agents: applications and microbial productions. Bioprocess Biosyst Eng. 2020;43(4):569-91.
30. In Y-W, Kim J-J, Kim H-J, Oh S-W. Antimicrobial Activities of Acetic Acid, Citric Acid and Lactic Acid against Shigella Species. Journal of Food Safety. 2013;33(1):79-85.
31. Theron MM, Lues JFR. Organic Acids and Meat Preservation: A Review. Food Reviews International. 2007;23(2):141-58.
32. Zhu X, Lei H, Wu J, Li JV, Tang H, Wang Y. Systemic responses of BALB/c mice to Salmonella typhimurium infection. J Proteome Res. 2014;13(10):4436-45.
33. Hersch SJ, Navarre WW. The Salmonella LysR family regulator, RipR, activates the SPI-13 encoded itaconate degradation cluster. Infect Immun. 2020.
34. Palomino JC, Martin A, Camacho M, Guerra H, Swings J, Portaels F. Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis. Antimicrob Agents Chemother. 2002;46(8):2720-2.
35. Doern CD. When does 2 plus 2 equal 5? A review of antimicrobial synergy testing. J Clin Microbiol. 2014;52(12):4124-8.
36. Odds FC. Synergy, antagonism, and what the chequerboard puts between them. J Antimicrob Chemother. 2003;52(1):1.
37. Rittenhouse JW, McFadden BA. Inhibition of isocitrate lyase from Pseudomonas indigofera by itaconate. Arch Biochem Biophys. 1974;163(1):79-86.
38. Wang H, Fedorov AA, Fedorov EV, Hunt DM, Rodgers A, Douglas HL, et al. An essential bifunctional enzyme in Mycobacterium tuberculosis for itaconate dissimilation and leucine catabolism. Proc Natl Acad Sci U S A. 2019;116(32):15907-13.
39. Nolte JC, Schurmann M, Schepers CL, Vogel E, Wubbeler JH, Steinbuchel A. Novel characteristics of succinate coenzyme A (Succinate-CoA) ligases: conversion of malate to malyl-CoA and CoA-thioester formation of succinate analogues in vitro. Appl Environ Microbiol. 2014;80(1):166-76.
40. Williams JO, Roche TE, McFadden BA. Mechanism of action of isocitrate lyase from Pseudomonas indigofera. Biochemistry. 1971;10(8):1384-90.
41. Dawson RMC, Elliott, D.C., Elliott, W.H., Jones, K.M. Data for Biochemical Research. 1st ed. Oxford: Clarendon Press; 1959.
42. Karinou E, Compton EL, Morel M, Javelle A. The Escherichia coli SLC26 homologue YchM (DauA) is a C(4)-dicarboxylic acid transporter. Mol Microbiol. 2013;87(3):623-40.
43. Srinivasan L, Baars TL, Fendler K, Michel H. Functional characterization of solute carrier (SLC) 26/sulfate permease (SulP) proteins in membrane mimetic systems. Biochim Biophys Acta. 2016;1858(4):698-705.
44. Sa-Pessoa J, Paiva S, Ribas D, Silva IJ, Viegas SC, Arraiano CM, et al. SATP (YaaH), a succinate-acetate transporter protein in Escherichia coli. Biochem J. 2013;454(3):585-95.
45. Rosenberg G, Yehezkel D, Hoffman D, Mattioli CC, Fremder M, Ben-Arosh H, et al. Host succinate is an activation signal for Salmonella virulence during intracellular infection. Science. 2021;371(6527):400-5.
46. Alpuche Aranda CM, Swanson JA, Loomis WP, Miller SI. Salmonella typhimurium activates virulence gene transcription within acidified macrophage phagosomes. Proc Natl Acad Sci U S A. 1992;89(21):10079-83.
47. Geisow MJ, D'Arcy Hart P, Young MR. Temporal changes of lysosome and phagosome pH during phagolysosome formation in macrophages: studies by fluorescence spectroscopy. J Cell Biol. 1981;89(3):645-52.

48. Flannagan RS, Cosio G, Grinstein S. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. Nat Rev Microbiol. 2009;7(5):355-66.
49. MacMicking JD, Taylor GA, McKinney JD. Immune control of tuberculosis by IFN-gamma-inducible LRG-47. Science. 2003;302(5645):654-9.
50. Schaible UE, Sturgill-Koszycki S, Schlesinger PH, Russell DG. Cytokine activation leads to acidification and increases maturation of Mycobacterium avium-containing phagosomes in murine macrophages. J Immunol. 1998;160(3):1290-6.
51. Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, Haddix PL, Collins HL, Fok AK, et al. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science. 1994;263(5147):678-81.
52. Castanie-Cornet MP, Penfound TA, Smith D, Elliott JF, Foster JW. Control of acid resistance in Escherichia coli. J Bacteriol. 1999;181(11):3525-35.
53. Wilmes-Riesenberg MR, Bearson B, Foster JW, Curtis R, 3rd. Role of the acid tolerance response in virulence of Salmonella typhimurium. Infect Immun. 1996;64(4):1085-92.
54. Stratford M, Anslow PA. Evidence that sorbic acid does not inhibit yeast as a classic 'weak acid preservative'. Lett Appl Microbiol. 1998;27(4):203-6.
55. Lee IY, Gruber TD, Samuels A, Yun M, Nam B, Kang M, et al. Structure-activity relationships of antitubercular salicylanilides consistent with disruption of the proton gradient via proton shuttling. Bioorg Med Chem. 2013;21(1):114-26.
56. van Beilen JW, Teixeira de Mattos MJ, Hellingwerf KJ, Brul S. Distinct effects of sorbic acid and acetic acid on the electrophysiology and metabolism of Bacillus subtilis. Appl Environ Microbiol. 2014;80(19):5918-26.
57. Krebs HA, Wiggins D, Stubbs M, Sols A, Bedoya F. Studies on the mechanism of the antifungal action of benzoate. Biochem J. 1983;214(3):657-63.
58. Piper PW. Yeast superoxide dismutase mutants reveal a pro-oxidant action of weak organic acid food preservatives. Free Radic Biol Med. 1999;27(11-12):1219-27.
59. Russell JB, Diez-Gonzalez F. The effects of fermentation acids on bacterial growth. Adv Microb Physiol. 1998;39:205-34.