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Pre-Exposure to α-Linolenic Acid Reduces Virulence of *Cronobacter sakazakii* in *Galleria mellonella* Infection Model

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Keywords: *Cronobacter sakazakii* | fatty acids | infection model | virulence

ABSTRACT

Cronobacter sakazakii is a food-borne pathogen with infections notifiable in those under 12 months old. Current inhibition methods rely on manufacture and reconstitution guidelines. Fatty acids (FA) are a possible method of control, with reports showing FAs can be bactericidal and regulate virulence mechanisms such as biofilm formation. This research explores the efficacy of the long-chain FA α-linolenic (ALA) to reduce *C. sakazakii* virulence in vivo. *Galleria mellonella* larvae were injected with FA pretreated *C. sakazakii* and viability monitored every 24 h over a total of 96 h. To show the interaction between the host immune system and *C. sakazakii,* haemocytes and bacterial cells were mixed and enumerated following incubation. To investigate the impact on antimicrobial resistance (AMR), 24 antibiotics were tested against *C. sakazakii* pretreated with ALA. Kaplan–Meier survival curves generated showed a dose-dependent increase in larval survival upon increased FA concentration. Regarding the immune evasion assay, data generated show increased *C. sakazakii* and decreased haemocyte counts in the same sample. From the AMR results, there was no statistically significant difference in inhibition zones when compared to the control, indicating no contraindications for current treatment options. α-linolenic acid is a common component of the human diet and is shown here to directly reduce virulence mechanisms including immune system evasion. Pre-treatment with ALA reduces the virulence of *C. sakazakii*, resulting in increased survival of *G. mellonella* larvae. This suggests that ALA may serve as a candidate for further evaluation as a potential agent against *C. sakazakii.*

1 | Introduction

Cronobacter sakazakii infections are rare yet serious and occur in susceptible populations such as neonates and infants. With fatality rates as high as 80% and 20% of survivors developing neurological conditions (Joseph and Forsythe 2012), further insights into the virulence of this pathogen are required.

Insect models are reported to be useful in expanding knowledge of the virulence mechanisms of human bacterial pathogens (Tsai et al. 2016). *Galleria mellonella* (greater wax moth) larvae are a popular alternative to the traditional murine model (Pereira et al. 2018). Use of *G. mellonella* as an infection model does not require ethical approval, nor specialist lab equipment. *G. mellonella*, from the order Lepidoptera

and family Pyralidae, is a common pest of bee hives (Wojda et al. 2020). Having minimal growth and nutritional requirements, these insects' larvae can be incubated at 37°C, reflective of the human body. Although *G. mellonella* has been utilized for study of several bacterial pathogens (Admella and Torrents 2023), to the best of our knowledge, this is the first investigation in this insect host examining *C. sakazakii* virulence following incubation with a fatty acid.

G. mellonella has an innate immune system, including cellular and humoral immune responses, but no adaptive immune system (Curtis et al. 2022). The innate immune system is comparable to that of humans, with haemolymph functioning analogously to mammalian blood (Ménard et al. 2021; Tojo et al. 2000). Circulating in the haemolymph is the cellular immune system, which consists of phagocytic cells, the haemocytes. Haemocytes of *G. mellonella* can be divided into six different types: prohemocytes, plasmatocytes, coagulocytes, granular cells, oenocytoids, and spherulocytes. Functions of these cellular immune cells include phagocytosis, nodulation, and encapsulation (Pereira et al. 2018). The humoral response of *G. mellonella* includes opsonins, antimicrobial peptides, the phenol oxidase pathway, and nucleic acid traps (Tsai et al. 2016).

The infection strategy of *C. sakazakii* is composed of five stages: entry, attachment, colonization, invasion into host cell, and translocation through the cell tissues (Phair et al. 2022). Interaction between *C. sakazakii* and haemocytes provides insight into its ability to evade elements of the immune system. *Cronobacter* species are reported to have several immune system evasion techniques and can survive and replicate within macrophages, both human cell lines (Ji et al. 2021) and murine macrophages (Liu et al. 2024).

Antimicrobial resistance (AMR) is another key virulence trait of *Cronobacter* species, with resistance to commonly used antimicrobials on the rise. Carvalho et al. (2020) reported the AMR of *Cronobacter* species isolated from infant foods. A total of 94.4% were found to be resistant to cefazolin, followed by 9.45% to amoxicillin and 1.35% to trimethoprim/sulfamethoxazole (Carvalho et al. 2020). Another study reported 100% resistance to erythromycin, 45.71% to trimethoprim/sulfamethoxazole, followed by resistance to another 17 antimicrobials ranging from 37.17% to 2.86% (Li et al. 2023). Currently, antimicrobials are the treatment choice for *Cronobacter* infections (Feeney and Sleator 2015). However, rising AMR rates indicate alternative options need to be explored.

In contrast to the broad-spectrum activity of antimicrobials, fatty acids (FA) are specific to bacterial species (Casillas-Vargas et al. 2021) and long-chain fatty acids are reported to impact virulence mechanisms (Borreby et al. 2023). α-linolenic (ALA), a long-chain FA, has been shown to reduce biofilm formation, a key virulence mechanism of *C. sakazakii*, by up to 90% (Phair et al. 2024). Although in vitro studies show promise, it remains to be seen if this FA has any impact on infection in vivo. Reducing *C. sakazakii* virulence through the addition of a food-safe molecule could be exploited for use in the food industry.

This study aimed to highlight *G. mellonella* larvae as a suitable model for *C. sakazakii* infections and demonstrate the impact of ALA on virulence mechanisms. Survival probability and haemocyte evasion are examined in vivo, whereas antimicrobial susceptibility is assessed using the Kirby-Bauer method. Targeting specific pathogenic traits or essential regulatory pathways may add to the arsenal against life-threatening bacterial infections.

2 | Methods

2.1 | Bacterial Culture and Fatty Acid Preparation

Cronobacter sakazakii ATCC 29544 was used for all experiments. Overnight cultures were grown in tryptic soy broth (TSB) (Oxoid, United Kingdom) at 37°C. α-linolenic acid (18:3 α) (ALA) (Cambridge Bioscience, United Kingdom; Cayman Chemical product with purity > 98%) was first dissolved in 99.8% ethanol (Fisher Scientific, United Kingdom), before further dilutions in TSB to obtain final concentrations of 0, 250, 500, and 1000 μM. The highest concentration of ethanol delivered to the bacterium was 1% (v/v); all vehicle controls contained this concentration of ethanol.

2.2 | Pre-Treatment With α-Linolenic Acid

From a fresh overnight culture, 1×10^6 CFU of *C. sakazakii* was transferred to TSB-ALA mixtures (0, 250, 500 and 1000 μM) and incubated at 37°C for 6 h. Growth was measured at 595 nm using a FilterMax f5 (Molecular Devices, United States). Optical density (OD) values are reflective of an OD standard curve generated using the plate count method. Additionally, time zero values for each sample were used to blank all timepoint values. Following this, the bacterial culture was pelleted by centrifugation at $1107 \times g$ for 5 min, washed, and resuspended in phosphate buffered saline (PBS) (Sigma Aldrich, United States).

2.3 | Infection Assay

Larvae of *Galleria mellonella* (Monkfield Reptile Ltd., Mepal, England) at the developmental stage of sixth instar were selected based on the following criteria: weight (200–300 mg), coloration (no signs of melanisation), and viability (successful roll test). Larvae prior to inoculation were stored in the dark at 15°C. Larvae ($n = 10$) were injected with 20 μL of PBS with or without 5×10^4 CFU *C. sakazakii* using a 26G 1 mL syringe (Terumo Europe) for intra-haemocoel injection into the last left proleg. CFU was determined using an OD standard curve generated from serial dilution and plating. Infected larvae were incubated at 37°C in 6-well plates. Viability of the larvae was recorded every 24 h for a total of 96 h. Control groups included an injection control group injected with just PBS and a toxicity control group injected with 1000 μM ALA in PBS. Ten larvae were injected per dose, and each dose was performed three times.

2.4 | Haemocyte Evasion Assay

G. mellonella larvae were injected as described above with heat-killed *C. sakazakii* (5×10^4 CFU in 20 μL). After 24 h of

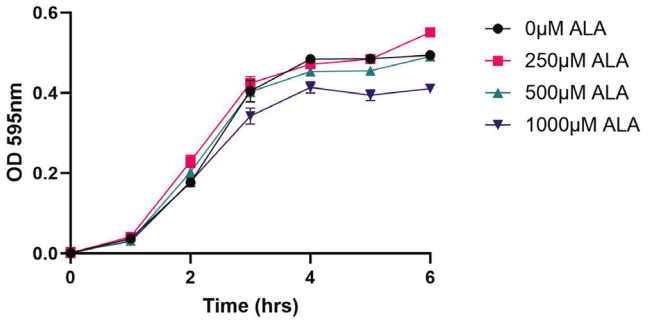

FIGURE 1 | Growth of *C. sakazakii* 29,544 in TSB supplemented with various concentrations of ALA: 0, 250, 500, and 1000 μM. Growth was measured over 6 h, with optical density measured every hour at 595 nm. The mean of three replicates from three independent experiments is plotted. An independent samples *t*-test comparing growth at hour 6 showed no significant difference at the 95% confidence level.

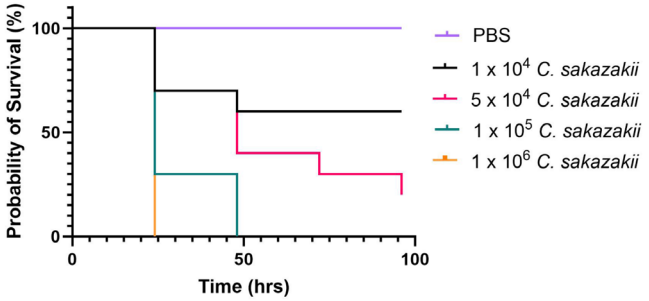

FIGURE 2 | Probability of *G. mellonella* survival when injected with different CFU of *C. sakazakii*. Larvae were monitored over 96 h with results recorded every 24 h. Mean of 10 larvae from three independent experiments is plotted.

incubation, the following method was performed as described by Curtis et al. (2023) with some adjustments. In brief, 500 μL of haemolymph was extracted from the 16 to 18 larvae using a 26G microlance needle (BD, United Kingdom) and kept on ice. Extracted haemolymph was assessed for integrity under a light microscope. Following this control step, the haemolymph was centrifuged at 3075 × *g* for 8 min at 4°C to isolate the haemocytes. Haemocytes were resuspended in 500 μL PBS, and cell-free haemolymph was retained on ice. Enumeration of viable haemocytes was performed using a light microscope and hemocytometer. For opsonisation, ALA pre-treated and control *C. sakazakii* were centrifuged (1107 × *g*, 5 min) and resuspended in the cell-free haemolymph for 45 min at 37°C, before harvesting and resuspension in 500 μL PBS. Bacterial and haemocyte suspensions were mixed in a 1:1 ratio (approximately 1×10^6 haemocyte and bacterial CFU) to a total volume of 1 mL in a 50 mL Falcon (Corning, United States) tube and incubated at 37°C, 150 rpm. At 20-min intervals over 2 h, 20 μL aliquots were collected and serially diluted. This suspension was plated in triplicate on Cronobacter Isolation Agar (Neogen, United Kingdom) and incubated for 15 h at 37°C. To enumerate the haemocytes, 10 μL of this same suspension was transferred to a hemocytometer and visualized under a light microscope.

2.5 | Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was assessed using the Kirby-Bauer method (Hudzicki 2009). ALA pre-treated *C. sakazakii* cultures were transferred to 5 mL of sterile water to match a MacFarland standard of 0.5. This suspension was streaked onto a Mueller-Hinton agar (Sigma-Aldrich, United States), country plate using a sterile cotton swab. Antimicrobial disks (Liofilchem, Italy) were placed onto the agar plate, with four disks evenly spaced out per plate. A total of 24 antimicrobials across nine different classes were tested at the recommended concentrations: ampicillin (10 μg), amoxicillin (25 μg), amoxicillin-clavulanate (20/10 μg), piperacillin-tazobactam (36 μg), cefiderocol (30 μg), cefotaxime (5 μg), cefoxitin (30 μg), ceftazidime (10 μg), ceftriaxone (30 μg), cefepime (30 μg), cefazolin (30 μg), imipenem (10 μg), meropenem (10 μg), aztreonam (30 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), amikacin (30 μg), gentamycin (30 μg), erythromycin (15 μg), tetracycline (30 μg), tigecycline (15 μg), nitrofurantoin (100 μg), chloramphenicol (30 μg) and trimethoprim-sulfamethoxazole (25 μg). Following 18 h incubation at 37°C, inhibition zones were measured and recorded. The susceptibility profile was determined using the breakpoints provided by the EU-CAST guidelines (accessed February 2025).

2.6 | Statistical Analysis

All statistical analysis was performed using GraphPad Prism (v10.1.2) and interpreted considering a 95% confidence level ($p < 0.05$) unless otherwise stated.

3 | Results

3.1 | Growth Over 6 h With Exogenous ALA

Exogenous ALA was introduced in TSB to *C. sakazakii* 29,544 at 250, 500, and 1000 μM. Although a slight difference in *C. sakazakii* growth at 6 h can be observed in Figure 1, statistical analysis showed there was no significant difference between the different growth conditions (ALA concentration of 250, 500, 1000 μM) and the control (ALA at 0 μm).

3.2 | Determination of Optimal *C. sakazakii* CFU Dose in *G. mellonella*

G. mellonella were injected with a series of *C. sakazakii* CFU per 20 μL injection, ranging from 1×10^4 to 1×10^6 (Curtis et al. 2022). Results were recorded as the number of live larvae and the number of dead larvae (Figure S1). The lowest dose, 1×10^4 (Casillas-Vargas et al. 2021), resulted in over 50% viability at 96 h, whereas the highest dose, 1×10^6 CFU/20 μL resulted in 0% viability at just 24 h post-injection (Figure 2). At 48 h, all larvae injected with 1×10^5 CFU/injection were dead (Figure 2).

Based on these results, CFU of 5×10^4 per 20 μL was selected; this dose resulted in 20% survival at 96 h (Figure 2). All further studies used this dose of 5 × 10 (Casillas-Vargas et al. 2021) CFU/20 μL so the efficacy of ALA could be shown even with high pathogen numbers, allowing for greater statistical analysis.

3.3 | Survival of *G. mellonella* Injected With *C. sakazakii* Pre-Treated With Different ALA Concentrations

The probability of survival for *G. mellonella* larvae injected with pre-treated *C. sakazakii* was analyzed and compared to the untreated control. Larvae were also injected only with ALA at the highest tested concentration, with no impact on their survival, proving ALA harmlessness (Figure 3). At the lowest tested ALA concentration, 250 μM (Figure 3, Panel A), the larvae had a much greater probability of survival when compared to the negative control (Figure 3, Panel D). Similarly, pre-treatment with 500 μM (Figure 3, Panel B) and 1000 μM (Figure 3, Panel C) also significantly improved the probability of survival as also shown in Panel D. The control with larvae injected with ALA at 1000 μM in PBS showed 100% probability of survival, showing the FA at this concentration was not detrimental to the viability of the larvae. Results presented here show that ALA pre-treated *C. sakazakii* presents with reduced virulence.

3.4 | Haemocyte Evasion Assay

Interactions between the host immune system and *C. sakazakii* were examined in the haemocyte evasion assay (Figure 4). Without ALA pre-treatment, *C. sakazakii* cell density was initially reduced, followed by a rapid increase in CFU/mL above the initial inoculum of 1×10^6 CFU/mL. The three treated *C. sakazakii* cultures followed a similar trend; however, CFU/mL did not exceed the initial inoculum in the same timeframe. In tandem with this increase in *C. sakazakii* numbers, a decrease in haemocyte numbers was recorded for each tested ALA condition (0, 250, 500 and 1000 μM) (Figure 4). Overall, pretreated cells displayed a slower rate of recovery, that is, when bacterial numbers reach the initial inoculation, when compared to the control, which reached an inoculation number of 1×10^6 CFU/mL at around 60 min.

3.5 | Antimicrobial Susceptibility of ALA Pretreated *C. sakazakii*

C. sakazakii 29,544 was classified as susceptible to 22 antimicrobials and resistant to 2 antimicrobials (Table 1).

Pretreatment with ALA did not change the susceptibility profile (susceptible/intermediate/resistant) nor the inhibition halo of *C. sakazakii* (Figure 5), as determined by two-way ANOVA at the 95% confidence level (Table S1).

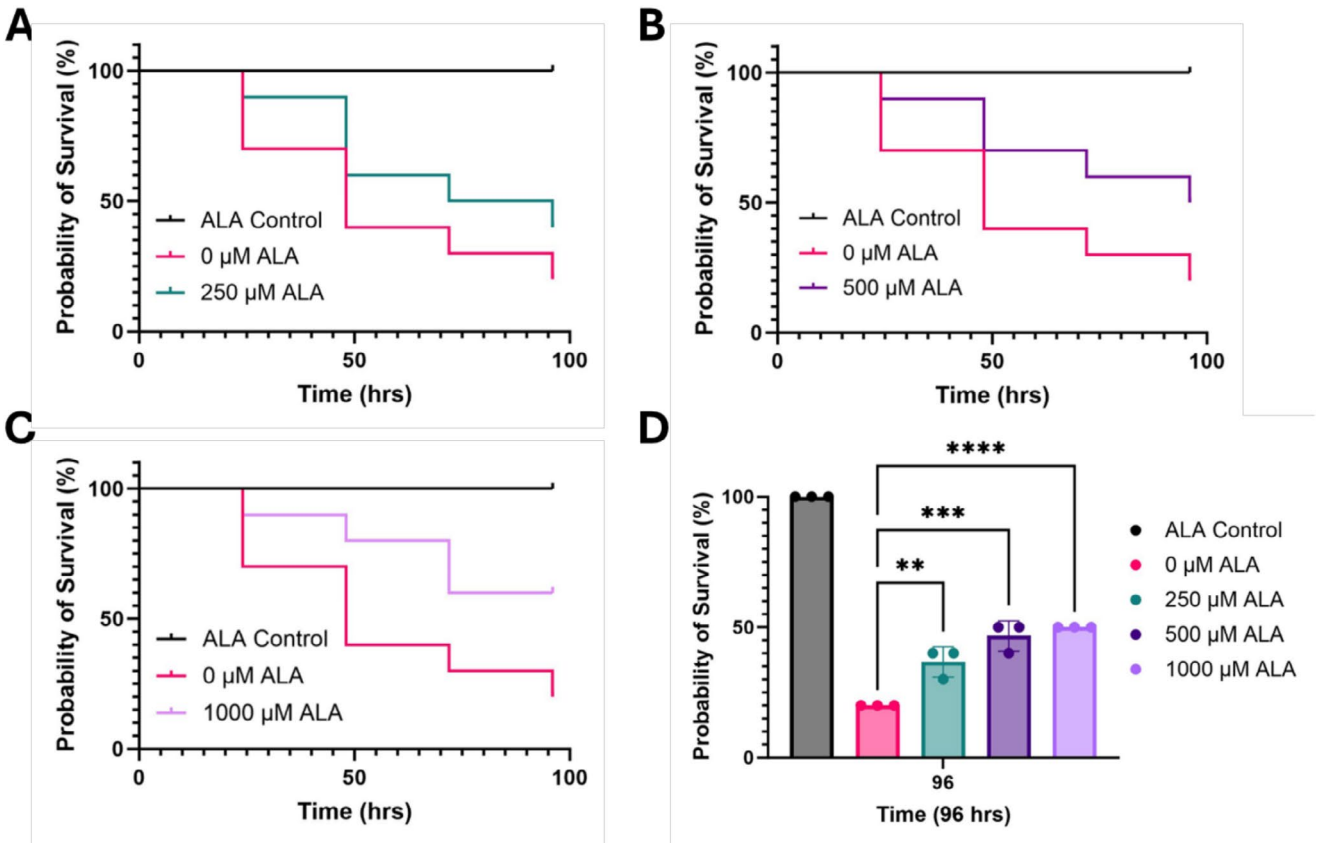

FIGURE 3 | Survival of *G. mellonella* larvae injected with ALA pre-treated *C. sakazakii* at 5×10^4 CFU/20 μL injection. Each graph shows larval survival when challenged with *C. sakazakii* exposed to different concentrations of ALA. (Panel A) ALA 250 μM, (Panel B) ALA 500 μM, (Panel C) ALA 1000 μM. (Panel D) shows the percentage survival at 96 h for each concentration compared to the control with 0 μM ALA. ALA control shows 100% survival of larvae injected with PBS with added 250 μM ALA (Panel A), 500 μM ALA (Panel B) and 1000 μM ALA (Panel C). For each ALA concentration, *C. sakazakii* was exposed for 6 h before each experiment. $^{**}p < 0.005$, $^{***}p < 0.001$, $^{****}p < 0.0001$ calculated using one-way ANOVA comparing to control of ALA 0 μM at 96 h.

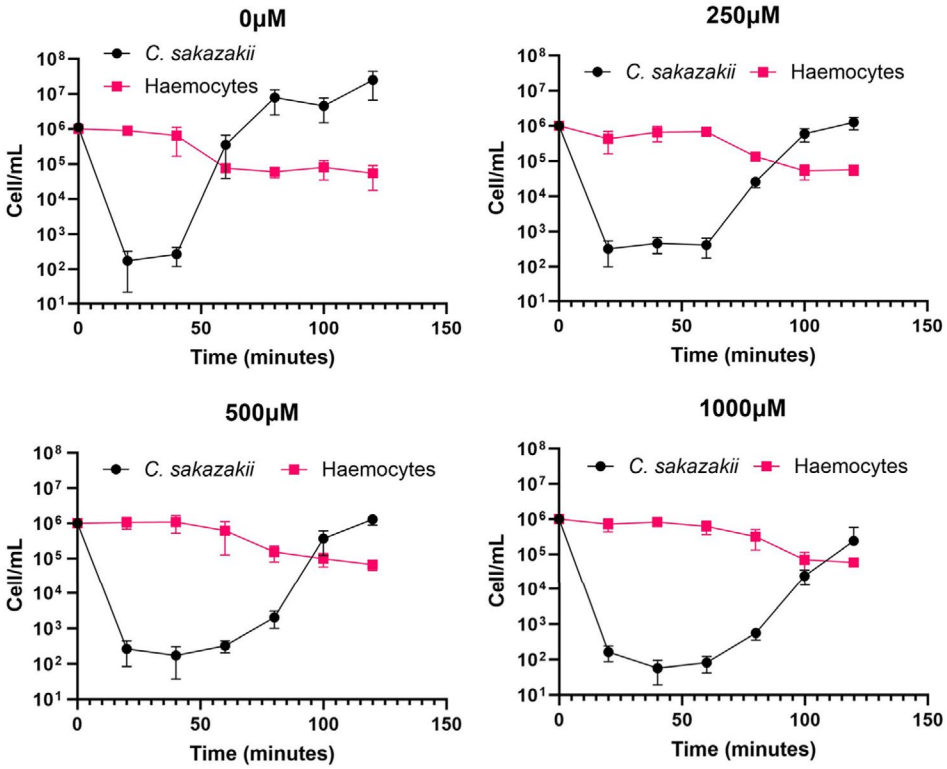

FIGURE 4 | Enumeration of *G. mellonella* haemocytes and *C. sakazakii* at an initial 1:1 ratio of 1 × 10 (Curtis et al. 2022) cell/mL. Results graphed are representative of three independent experiments with triplicate replicates for each concentration of ALA.

4 | Discussion

C. sakazakii infections are serious and life-threatening in susceptible populations. Recently, this has been acknowledged by the Food and Drug Administration (FDA), with *C. sakazakii* infections in infants now notifiable (FDA 2023). Currently, control methods are non-specific, with the prevalence of *C. sakazakii* ranging from as low as 3.9% in aquatic-derived food products to as high as 44.4% in factories producing infant milk formula (Phair et al. 2022). FAs, which are ubiquitous in foods such as fish and avocadoes, are a potential method of *C. sakazakii* control in the food industry, and they pose little risk of toxicity for consumers (Casillas-Vargas et al. 2021). The effect of FAs is dependent on FA choice and concentration, and the pathogen of interest, with this work exploring the use of ALA against *C. sakazakii* and specifically how it impacts its virulence in vivo using the *G. mellonella* infection model. The innate immune system of *G. mellonella* is functionally comparable to that of humans, with haemocytes functioning analogously to white blood cells (Ménard et al. 2021; Tojo et al. 2000). ALA was already shown to impact virulence mechanisms of *C. sakazakii*, including biofilm formation in vitro (Phair et al. 2024).

The infection model *G. mellonella* has many advantages compared to other traditional in vivo models as they require no ethical approval, no specialist equipment, and can be incubated at 37°C to study human pathogens (Pereira et al. 2018). Also, although a non-vertebrate, the innate immune system of *G. mellonella* is comparable to the human (Tojo et al. 2000). The current study demonstrated this infection model to be adequate to show how pretreatment with ALA affects the virulence of *C. sakazakii* in a living entity. Reducing the capability of this pathogen to infect hosts may also enable the host immune system to effectively combat this pathogen.

G. mellonella infection model has been employed to study potential therapeutics as regards enteric bacterial infections. A study by Grounta et al. (2016) showed that treatment with heat-inactivated lactic acid bacteria increased survival of *G. mellonella* injected with *Listeria monocytogenes*. Similarly, Kelly and Jameson (2024) show that a phage cocktail improves survival of *G. mellonella* challenged with the Gram-negative pathogen *Klebsiella pneumoniae*. In both studies, the treatment, injected directly into the larvae either post-infection or in tandem, and similarly to current results, increased survival at a rate dependent on the concentration of the treatment.

Incubation of *C. sakazakii* with ALA at the tested concentrations (250, 500 and 1000 μM) did not statistically impact its growth over 6 h (Figure 1). However, *C. sakazakii*, following this exposure, displayed vastly different infection abilities as regards host mortality and immune system evasion (Figures 2 and 3). *G. mellonella* infected with *C. sakazakii* were monitored for viability, with binary outcome choices, alive or dead (Figure S1), and the optimal dose of *C. sakazakii* to introduce to the larval model was determined (Figure 2). The 5×10^4 CFU/20 μL injection was selected as the best option for further experiments in the current study, considering the 96 h 20% survival rate of the larvae, a value significantly lethal to demonstrate any increase in survival as a result of ALA pre-treatment. Pre-treatment of *C. sakazakii* with 250 μM ALA increased the probability of *G. mellonella* larvae survival from 20% to 40% (Figure 3A). Similarly, the higher ALA concentrations of 500 and 1000 μM (Figure 3B,C) showed increased survival of up to 50%. This overall increase of 20%–30% in *G. mellonella* survival

TABLE 1 | S/I/R profiles of *C. sakazakii* 29,544 treated with ALA as determined by the EU-CAST guidelines (accessed February 2025) S, susceptible; I, intermediate resistance; R, resistant.

Antimicrobial class	Antimicrobial	Conc. (μg)	S/I/R 0 uM	S/I/R 250 uM	S/I/R 500 uM	S/I/R 1000 uM
Penicillins	Ampicillin	10	S	S	S	S
	Amoxicillin	25	S	S	S	S
	Amoxicillin-Clavulanate	20/10	S	S	S	S
	Piperacillin-tazobactam	36	S	S	S	S
Cephalosporins	Cefiderocol	30	S	S	S	S
	Cefotaxime	5	S	S	S	S
	Cefoxitin	30	S	S	S	S
	Ceftazidime	10	S	S	S	S
	Ceftriaxone	30	S	S	S	S
	Cefepime	30	S	S	S	S
	Cefazolin	30	**R**	**R**	**R**	**R**
Carbapenems	Imipenem	10	S	S	S	S
	Meropenem	10	S	S	S	S
Monobactams	Aztreonam	30	S	S	S	S
Fluoroquinolones	Ciprofloxacin	5	S	S	S	S
	Norfloxacin	10	S	S	S	S
Aminoglycosides	Amikacin	30	S	S	S	S
	Gentamycin	30	S	S	S	S
Macrolides	Erythromycin	15	**R**	**R**	**R**	**R**
Tetracyclines	Tetracycline	30	S	S	S	S
	Tigecycline	15	S	S	S	S
Others	Nitrofurantoin	100	S	S	S	S
	Chloramphenicol	30	S	S	S	S
	Trimethoprim-sulfamethoxazole	25	S	S	S	S

rate was statistically significant ($p < 0.005$ *calculated using one-way ANOVA*) and provided incentive to further study both the host immune response to *C. sakazakii* and how ALA impacts the infection strategy of this pathogen.

Following this recorded increased larval survival, the interactions between *C. sakazakii* and *G. mellonella* immune system were further investigated. Adapting the haemocyte kill assay described by Curtis et al. (2023), an immune evasion assay was designed using equal cell numbers of *C. sakazakii* and haemocytes isolated from *G. mellonella*. Haemocytes are an integral part of the innate immune system and are a non-specific line of defense, engulfing foreign entities such as bacterial cells through a mechanism known as phagocytosis (Ménard et al. 2021; Tojo et al. 2000). Other models that have assessed the evasion of phagocytosis by *C. sakazakii* include the use of human macrophage cell lines (Ji et al. 2021) and murine macrophages (Liu et al. 2024).

Results observed in Figure 4 suggested that pre-exposure to ALA impacted the interaction between *C. sakazakii* and haemocytes over the tested 2-h period, highlighting the potential decrease in the ability of *C. sakazakii* to evade phagocytosis. Further analysis is recommended to identify the mechanisms and specific interactions involved.

C. sakazakii displays immune system evasion mechanisms and is reported to survive and replicate in macrophages (Eshwar et al. 2015). Literature suggests several genes and systems that support these evasion techniques. Regarding macrophage evasion, Ji et al. (2021) prepared a *C. sakazakii nlpD* mutant that displayed reduced survival in a human macrophage cell line when compared to the wildtype. This suggests *nlpD*, which encodes an acid resistance factor, supports resistance to macrophage-mediated killing. Additionally, *fkpA* has been identified as instrumental in *Cronobacter* species survival and replication in a human macrophage cell line, with

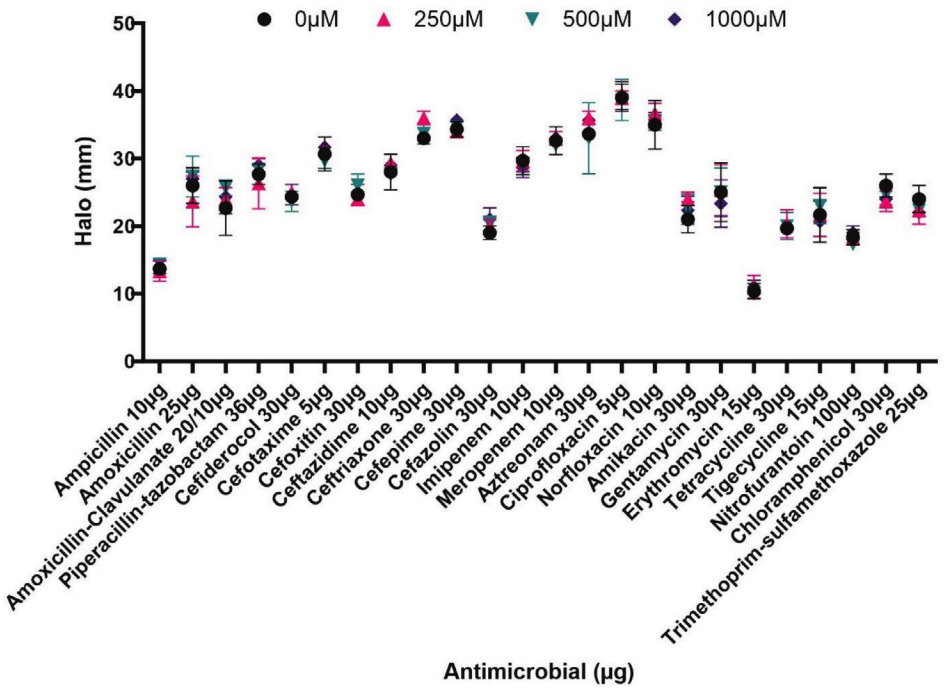

FIGURE 5 | Antimicrobial susceptibility of *C. sakazakii* 29,544 pre-treated with various concentrations of ALA. Each of the 24 antimicrobials was used at the recommended concentration as per EU-CAST guidelines. Results graphed are the average of three independent experiments, each with three technical replicates. Statistical analysis was performed, and no significant difference was identified between the control (0 μM) and each of the ALA concentrations (250, 500 and 1000 μM).

variances observed between the seven *Cronobacter* species (Eshwar et al. 2015).

Similarly, viable but non-culturable (VBNC) *C. sakazakii* has reported resistance to macrophage-mediated killing through recognition evasion and oxidative stress resistance (Liu et al. 2024). It is theorized that the VBNC state suppresses the LPS biosynthesis and regulates genes relating to antioxidation (Liu et al. 2024). Perhaps, the observed decrease in the number of *C. sakazakii* in Figure 4 was due to a short VBNC state, which could be induced upon exposure to the haemocytes, but this hypothesis was not possible to test in the current study.

Pathogenic bacteria have developed mechanisms to survive within macrophages, from evasion of macrophage recognition and resistance to bactericidal agents produced by macrophages to manipulation of macrophage maturation and vacuole escape. Evasion of macrophage recognition is utilized by *Helicobacter pylori and Salmonella typhimurium*, whereby the bacteria modify the lipopolysaccharides on their outer membrane, thereby reducing the Toll-like receptor four recognition (Gaddy et al. 2015; Kong et al. 2012). Resistance to bactericidal methods used by macrophages, including oxidative burst, is reported to be due to bacterial antioxidant defense mechanisms as displayed by *S. aureus* in murine macrophages (Das and Bishayi 2009). Manipulation of macrophages, including maturation regulation, is used by *Mycobacterium tuberculosis* to inhibit phagosome acidification (Zhai et al. 2019). Lastly, vacuole escape involves escaping from the harsh environment of the lysosomal compartment and into the cytosol, which is nutrient rich. Pathogens capable of vacuole escape include *Listeria monocytogenes*, which produces the toxin listeriolysin, causing pore-formation in the vacuoles (Cossart and Lebreton 2014).

Regarding the ability of other pathogens to evade phagocytosis, Admella and Torrents (2023) show that bacterial survival in the haemolymph of *G. mellonella* is species dependent. In larvae infected with *Staphylococcus aureus*, no bacterial cells were recovered; however, both *Pseudomonas aeruginosa* and *Mycobacteroides abscessus* could be isolated, albeit in reduced numbers (Admella and Torrents 2023).

It is clear that macrophage evasion is a key virulence trait, and in the case of *C. sakazakii*, there are several systems that contribute to its immune evasion, such as the genes *nlpD* and *fkpA*. ALA has an impact on the evasion mechanisms employed by *C. sakazakii*; however, the exact mode of action remains to be elucidated.

Reduced susceptibility to antimicrobials is a global concern with an estimated 1.14 million deaths every year attributed to AMR pathogens, and a projected 10 million deaths per year by 2050 (Naghavi et al. 2024). Although *Cronobacter* species are broadly susceptible to antimicrobials, recent studies have reported increasing resistance rates to several commonly used antimicrobials (Carvalho et al. 2020; Li et al. 2023). As antimicrobials remain the current therapy to treat *C. sakazakii* infections, control methods used in the food manufacture environment should be analyzed for potential impacts on its antimicrobial susceptibility.

In the current study, a total of 24 antimicrobials were tested against *C. sakazakii* 29,544 pre-treated with differing concentrations of ALA. Although results indicated no statistically significant

difference between treated and untreated samples, this study was limited to one strain that was susceptible to all tested antimicrobials, except two. Future studies should examine if clinical isolates, already presenting with full or intermediate resistance to more antimicrobials, can become antimicrobial susceptible following ALA treatment.

Regardless, the observed decreased virulence when treated with ALA (Figures 3 and 4) is of relevance, since it can support the human immune system to fight *C. sakazakii* infection. Thus, even without changing its antimicrobial susceptibility, ALA appeared to indirectly support the activity of antimicrobials. Experiments to test this can be run in the future to help clarify this hypothesis, with the *G. mellonella* infection model as a valuable tool, as demonstrated by the current study.

Identification of novel antimicrobial agents is crucial to reduce reliance on currently available treatment options such as broad-spectrum antimicrobials. Results here promote ALA as a potential anti-virulence agent and show that in an in vitro lab model, pretreatment with ALA does not impact current therapeutic options for *C. sakazakii* infections.

C. sakazakii is an opportunistic foodborne pathogen which predominately affects neonatal and elderly populations. To combat this pathogen, fatty acids (FAs) are a promising option, with ALA capable of reducing biofilm formation and altering growth (Phair et al. 2024). Reducing the virulence of this pathogen can lead to better clinical outcomes, as shown here in a larval infection model (Figure 3). Further investigation into the impact of haemocytes on *C. sakazakii* viability showed that, like studies in macrophages, *C. sakazakii* is capable of evading phagocytosis. Pre-treatment with ALA slowed the recovery rate of *C. sakazakii* when incubated with phagocytic haemocytes. This could be contributing to the increased survival of the *G. mellonella* challenged with the pre-treated *C. sakazakii*. This novel work presented here gives insight into the in vivo effect of ALA on *C. sakazakii* virulence. In a broader context, these results give insights into the role of antimicrobial lipids, which although not bactericidal, have significant impact on bacterial virulence. Further studies are needed to elucidate the mechanism of action by which ALA reduces the virulence of *C. sakazakii* as shown in this *G. mellonella* infection model.

5 | Conclusion

G. mellonella is a suitable in vivo model to study *C. sakazakii* infections and immune system evasion abilities. Pre-treatment of *C. sakazakii* with α-linolenic acid improved larval survival by up to 50%, whereas the same pre-treatment reduced the recovery rate of *C. sakazakii* when incubated with *G. mellonella* haemocytes. Regarding antimicrobial susceptibility, α-linolenic acid did not impact *C. sakazakii* susceptibility to the 24 antimicrobials tested. Reducing the virulence of *C. sakazakii* may enable the host immune system to more effectively eliminate this pathogen.

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Graphical abstract was created in BioRender. Katie Phair (2025) https://BioRender.com/seb8gv3.

Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** (Panel A) *Galleria mellonella*, (Panel B) injection technique, (Panel C) living *G. mellonella*, (Panel D) dead *G. mellonella* (melanized). **Table S1:** Full breakdown of inhibition zones of antimicrobials against *C. sakazakii* treated with ALA at different concentrations.