

## Antibiotic susceptibility and Biofilm formation in *Cronobacter* spp.

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**Abstract:** Bacteria displaying antimicrobial resistance pose a significant global healthcare predicament. This study focused on identifying the antimicrobial susceptibility characteristics of an emerging foodborne pathogen, *Cronobacter sakazakii*, and comparing these characteristics to other strains within the *Cronobacter* genus. The British Society for Antimicrobial Chemotherapy (BSAC) method of disc diffusion was used throughout this study. Five antibiotics were used, gentamicin, ampicillin, chloramphenicol, cefpodoxime and nalidixic acid. The ability of this microorganism to form biofilms in various media was also examined in comparison to common pathogenic biofilm-forming bacteria. Biofilm formation was evaluated using a crystal violet based microtiter plate assay. It was found that all strains of *C.sakazakii*, excluding NCTC 8155, were susceptible to the five antibiotics tested, as were all other species of *Cronobacter* used in the study. Three strains of *Cronobacter* formed more biofilm than *E.coli* at 37°C in powdered infant formula, and two strains formed more at 25°C. All strains of *Cronobacter* formed more biofilm than *Pseudomonas aeruginosa* at both temperatures. It is clear that this group of bacteria possess traits which may increase its ability to cause disease in the future.

**Key words:** *Cronobacter sakazakii*, antibiotic susceptibility, biofilm

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### INTRODUCTION

*Cronobacter* is a recently defined genus previously known as *Enterobacter sakazakii*. The genus currently comprises seven species, *C.sakazakii*, *C.malonaticus*, *C.turicensis*, *C.muytjensii*, *C.dublinensis*, *C.condimenti* and *C.universalis* (Iversen *et al.*, 2008; Joseph *et al.*, 2011). *Cronobacter* species are considered as emerging foodborne pathogens and have been identified as the causative agent of several outbreaks or sporadic cases of very serious neonatal infections causing meningitis, septicaemia or necrotising enterocolitis in infants (Miled-Bennour *et al.*, 2010). Surviving patients often develop acute neurological complications such as hydrocephalus, quadriplegia, brain abscess and retarded neural development (Mullane *et al.*, 2007). In most cases the source of the bacterium has not been confirmed but studies corroborated by epidemiologic investigations have implicated rehydrated powdered infant formula as well as equipment and utensils used to prepare this non-sterile product in hospital settings (Gurtler *et al.*, 2005). Many studies to date have focused on the identification and classification of this genus, however, very little is known concerning its virulence and pathogenicity (Townsend *et al.*, 2007). Although *Cronobacter* spp. appear to be a group of widely distributed microorganisms found in various foods, their natural environmental niche still remains to be elucidated. Their presence in a great variety of environments suggests that this opportunistic organism is ubiquitous, making control more difficult (Terragno *et al.*, 2009).

*Cronobacter* spp. possess several characteristics which promote their environmental persistence and protect them from environmental stresses such as heat, dry conditions, antibiotics, osmotic stress and UV light, making them harder to inactivate. These characteristics include biofilm formation, adherence to hydrophilic and hydrophobic surfaces, production of extracellular polysaccharides, and signalling mechanisms with other bacteria (Kim *et al.*, 2010). Microbial biofilms are of great concern in the food industry due to possible product contamination with spoilage or pathogenic bacteria (Hartmann *et al.*, 2010). The formation of biofilms is a critical pathogenic mechanism enabling pathogens, but also opportunistic bacteria, to survive the host immune defences and systemic antibiotic therapies (Campoccia *et al.*, 2010). There are a number of hypotheses concerning the mechanisms of resistance in microbial biofilms including; slow or incomplete penetration of the antibiotic into the biofilm, the deactivation of the antibiotic in the surface layers at a higher rate than it can diffuse, effects of the altered chemical microenvironment within the biofilm such as the presence of anaerobic niches and local accumulation of acidic waste products, reductions in cell activity and permeability and the presence of cells in a spore like state (Stewart and Costerton, 2001). Although the rate of resistance to most

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antibiotics is not yet high in this organism, the potential for acquiring antibiotic resistance due to the misuse of antibiotics in the environment including farms and aquaculture is a very real risk (Kim *et al.*, 2010).

Much of the research on *Cronobacter* spp. has been conducted before its taxonomic reclassification (Iversen *et al.*, 2008). Therefore, these studies require repetition on a species specific level in order to elucidate any differences between the newly classified species and subspecies. The present study aims to evaluate the antibiotic susceptibility and biofilm formation capabilities of several *Cronobacter* strains in comparison to common biofilm forming pathogens.

## METHODS AND MATERIALS

### **Source Of Bacterial Strains And Growth Conditions:**

Type strains of *Cronobacter sakazakii* (ATCC 29544<sup>TM</sup>) and *Cronobacter muytjensii* (ATCC 51329<sup>TM</sup>) were obtained from the American Type Culture Collection (ATCC). Type strains of *Cronobacter turicensis* (DSM 18703) and *Cronobacter malonaticus* (DSM 18702) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). Additional strains of *C.sakazakii* (ATCC 29004, ATCC 12868) were also obtained from the ATCC. *C.sakazakii* (NCTC 8155) was obtained from the National Collection of Type Cultures (NCTC). It should be noted that this strain is currently named *Enterobacter sakazakii* in the collection but was reclassified as *C.sakazakii* after the recent taxonomic change (Iversen *et al.*, 2008). All non *Cronobacter* species used were obtained from recognised cell banks and catalogue numbers will be indicated where relevant. Unless otherwise stated, fresh cultures were obtained by direct inoculation of Nutrient Agar (LabM) formulated according to ISO 6579:2002, ISO 10273:2003 and ISO 16654:2001 and tested in accordance with ISO/TS 11133-2:2003. The inoculated media were then incubated overnight at 37°C.

### **Antibiotic Susceptibility Testing:**

The disc diffusion method for antimicrobial susceptibility testing as described by The British Society for Antimicrobial Chemotherapy (BSAC) in Version 10.2 of the BSAC Methods for Antimicrobial Susceptibility Testing (BSAC, 2011) was used throughout this study. This method uses the minimum inhibitory concentrations (MICs) of drugs in order to define bacterial strains as one of three groups. These groups are: clinically resistant; level of antimicrobial susceptibility which results in a high likelihood of therapeutic failure; clinically susceptible; level of antimicrobial susceptibility associated with a high level of therapeutic success and; clinically intermediate; a level of antimicrobial susceptibility associated with uncertain therapeutic effect. Mueller Hinton agar (LabM) was used as an appropriate medium for these tests and was prepared and sterilised according to the manufacturer's instructions. 25 ml aliquots of this agar were aseptically pipetted into 90 mm Petri dishes (Sarstedt Ltd.) in order to give a depth of 4 mm  $\pm$  0.5 mm. The surface of the agar was allowed to dry before use. The control organisms, *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 29523) and *Pseudomonas aeruginosa* (NCTC 10662) were chosen from the list provided in the BSAC protocol. 10 ml aliquots of sterile Mueller Hinton broth (LabM) were prepared in sterile, glass screw-capped, containers. These were labelled and inoculated with an isolated colony from the strains of interest and the control strains. Each inoculum was grown to the density of a 0.5 McFarland Standard. A spectrophotometer (Jenway 6300) was used to measure the absorbance of the suspensions at a wavelength of 625 nm. The acceptable absorbance range for the standard is 0.08-0.13. The suspensions were then diluted according to the BSAC guidelines. For *Enterobacteriaceae* this meant a 1:100 dilution of the suspension (density adjusted to that of a 0.5 McFarland standard) in distilled water. This level of density and dilution is used in order to produce semi-confluent growth on the agar. Within 15 minutes of dilution the suspension was used to inoculate the prepared plates. A sterile-cotton swab was dipped into each suspension and excess liquid removed by turning the swab against the side of the container. The inoculum was spread evenly over the surface of the agar by swabbing in three directions. The plate was allowed to dry (no longer than 15 minutes) before application of the chosen antibiotic discs. Appropriate disc contents for the organisms tested were chosen by referring to the interpretation tables provided in the BSAC guidelines. The antibiotics used in this study were: gentamicin 10  $\mu$ g; ampicillin 10  $\mu$ g; cefpodoxime 10  $\mu$ g; nalidixic acid 30  $\mu$ g and; chloramphenicol 30  $\mu$ g (All Oxoid). After application of the antimicrobial discs, the plates were incubated at 37°C for 18-20 hours. Each species was tested in this manner in triplicate at the least.

### **Measuring Zones Of Inhibition And Interpretation Of Susceptibility:**

The plates were assessed upon removal from the incubator to ensure that semi-confluent growth had been achieved. The methods described in the previous section give reliably semi-confluent growth with most isolates. A denser inoculum will give reduced zones of inhibition while a lighter one will have the opposite effect (BSAC, 2011). Acceptable inoculum density range for a Gram-negative rod is shown in Figure 1.

The diameters of zones of inhibition were measured to the nearest millimetre using callipers. The zone edge was taken as the point of inhibition as judged by the naked eye and tiny colonies at the edge of the zone were excluded. Using the tables provided in the BSAC guidelines it was confirmed that the zones of inhibition for the control strains were within the acceptable range for each of the antibiotics used. The zones for all plates were recorded and the average of the replicates for each strain calculated before interpreting the results using the tables also provided in the BSAC guidelines.

#### **Crystal Violet Microtiter Plate Assay For Assessment Of Biofilm Formation:**

For the purposes of this study, this assay served to assess the level of biofilm formation in powdered infant formula (PIF) by members of the *Cronobacter* genus in comparison with other common biofilm forming organisms. LB broth (LabM) was used as a comparison medium. This experiment was carried out at 25 and 37°C respectively. The seven strains of *Cronobacter* mentioned previously were used as well as *Pseudomonas aeruginosa* (NCTC 10662), *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922). Each bacterium of interest was grown overnight (i.e. to stationary phase) in 5ml of LB (LabM) broth. The cultures were diluted 1:100 in the chosen medium (reconstituted PIF made to manufacturers guidelines or LB broth) and 100µl of each was pipetted in triplicate into individual wells in a non-tissue culture treated flat bottom 96 well plate (Sarstedt). The plates were covered and incubated at the chosen temperatures for 48 hours. After this time, planktonic bacteria were removed by a wash step. 125µl of 0.1% crystal violet solution was then added to each well and allowed to stain for 10 minutes at room temperature. The crystal violet which had not specifically stained adhered cells was then removed by means of another wash step. The plates were then allowed to air dry. 200µl of an appropriate solvent (see Table 1) was then added to each well and the dye allowed to solubise for 10-15 minutes. The solvent recommended for *E.coli* was also used for *Cronobacter* strains as this was the most similar species in the guidelines for this method. The contents of each well were briefly mixed by gentle pipetting and removed to an optically clear sterile flat bottomed 96 well plate. The optical density of each well was measured at a wavelength of 490nm using a spectrophotometer (Wallac Victor) (Method adapted from Merritt *et al.* (2005)).

#### **Statistical Analysis Of Data:**

Results for all tests described were recorded and analysed using Microsoft Excel Software. Methods of presenting the data obtained were chosen based on a literature review of similar studies in the area of biofilm formation and antibiotic susceptibility testing. The t-Test (Paired Two Sample for Means) was carried out where appropriate. This test was also conducted using Microsoft® Excel Software with the Data Analysis “Toolpak” add-in.

## **RESULTS AND DISCUSSION**

#### **Antimicrobial Susceptibility Testing:**

All testing was conducted according to the BSAC Methods for Antimicrobial Susceptibility Testing, Version 10.2 (BSAC, 2011). This method is used to classify bacterial strains as; clinically resistant, clinically susceptible or clinically intermediate to a chosen antibiotic. The characteristics of these categories are outlined in Table 2.

This method is based on the 1966 publication by Bauer *et al.* which described a disc diffusion method which aimed to consolidate and update previous descriptions of the method and provide a concise outline for its performance and interpretation (Bauer *et al.*, 1966). In this particular study, five common antibiotics were tested against ten strains of bacteria. The concentrations used were based on the guidelines for members of the Enterobacteriaceae family. Seven strains of *Cronobacter* were included along with *E.coli* (ATCC 25922), *S.aureus* (ATCC 25923) and *P.aeruginosa* (NCTC 10662) which served as controls. These controls were chosen from a list of recommended controls (BSAC, 2011) based on availability and suitability for the antimicrobials being tested. Examples of the plates after completion of the recommended incubation period are shown in Figure 2.

The diameters of each zone of inhibition were measured and an average zone diameter calculated for each organism and for each antibiotic used. Using the tables provided by the BSAC guidelines, the bacterial strains were then classified as being in one of the categories outlined in Table 2. The zone diameters of the three control strains were compared to the values provided in the BSAC guidelines and were found to be within an acceptable range. The results obtained are therefore presumed to be valid. It is important to note that the interpretive zone diameters differ between particular antibiotics not only because of variances in disc potency, but also because the antibiotics possess different diffusion and solubility properties in the chosen medium. For this reason also, the largest zone of inhibition may not be the antibiotic of choice in a given case, it only indicates whether an organism is resistant/susceptible to that particular drug (Bauer *et al.*, 1966). It is therefore imperative that a

standard method is followed as closely as possible, when testing antimicrobial susceptibility, in order for the results to be valid.

All strains of *Cronobacter* tested were susceptible to ampicillin, chloramphenicol, nalidixic acid and gentamicin. This correlates with previous studies (Terragno *et al.*, 2009; Kim *et al.*, 2010; Binsztein, 2009) which found that all strains of *Cronobacter* tested were susceptible to these antibiotics. Six of the *Cronobacter* strains were susceptible to cefpodoxime, a third generation cephalosporin broad spectrum antibiotic (Kakumanu *et al.*, 2006). *C.sakazakii* (NCTC 8155) was shown to be resistant to this antibiotic. While susceptibility to this particular drug has not been tested previously, other third and second generation (Terragno *et al.*, 2009; Binsztein, 2009) and even first generation cephalosporins (Kim *et al.*, 2010) have been found to be effective against *Cronobacter* species. Kim *et al.* (2010) did find resistance to cephalothin, a first generation cephalosporin, in 5.3% of food isolates tested. Nonetheless this does not explain the observed resistance of *C.sakazakii* (NCTC 8155) to cefpodoxime. This finding may require further investigation in the future but was unfortunately outside the scope of the current study. It should also be noted that *C.malonaticus* (DSM 18702) demonstrated a significantly lower level of susceptibility to ampicillin in comparison to the other strains of *Cronobacter* tested. The average zone of inhibition for this antibiotic produced by *C.malonaticus* (DSM 18702) was 15 mm, only just qualifying the bacteria as susceptible to this drug.

#### **Assessment Of Biofilm Formation In PIF:**

This experiment was conducted in order to assess the level of biofilm formation of seven strains of *Cronobacter* in comparison with three strains of common biofilm forming bacteria namely, *E.coli* (ATCC 25922), *S.aureus* (ATCC 25923) and *P.aeruginosa* (NCTC 10662). The level of biofilm formation was assessed at 25°C and 37°C in reconstituted PIF and LB broth (LabM). Both media were prepared according to the manufacturers' instructions. It should be noted that after the 30 minute cooling period, recommended by the PIF manufacturer, the boiled water had cooled to below 50°C which is significantly lower than the 70°C recommended by the World Health Organisation (WHO) for the safe preparation of PIF (FAO and WHO, 2004). The method used to assess the level of biofilm formation in these media and at the chosen temperatures was a variation of the crystal violet staining method which utilised microtiter (96 well) plates.

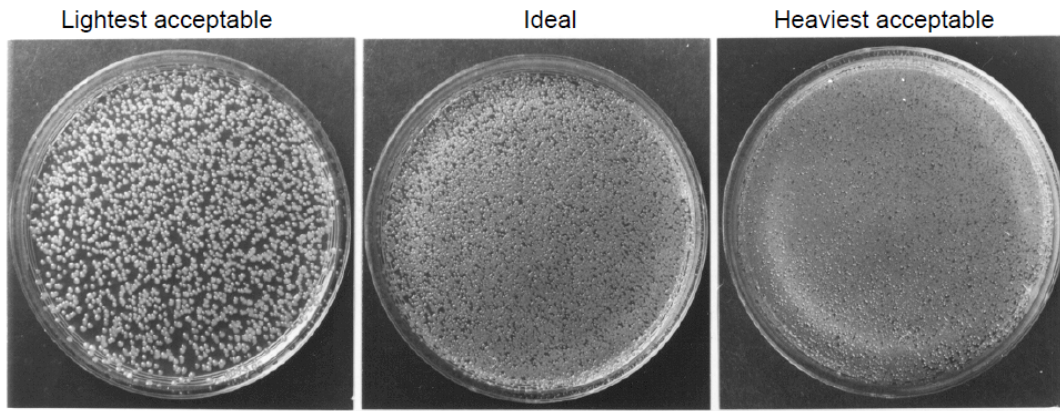
Crystal violet (CV) is a basic dye which stains both living and dead cells as well as the surrounding matrix. It works by binding to the negatively charged surface molecules and polysaccharides in the extracellular matrix (Peeters *et al.*, 2008). Advantages of the CV assay are its cost-effectiveness, straightforward protocol and its common usage for the quantification of biofilms in a wide range of species. The main disadvantage of this assay is that it does not differentiate between living and dead cells, therefore reducing its suitability for biofilm susceptibility testing (Peeters *et al.*, 2008). For the purposes of this study, however, the CV assay was deemed to be suitable and produced consistent results.

Before conducting more in-depth comparisons between bacterial strains, a number of general observations can be made from the findings presented in Figure 3. Firstly, all bacteria formed more biofilm in PIF than in LB broth. Overall, at both temperatures there is a highly significant difference between the average amount of biofilm formed in LB broth and the amount of biofilm formed in PIF ( $P < 0.001$ ). A more in depth analysis of the data shows that the only strain tested for which this is not true is *P.aeruginosa* (NCTC 10662). At 25°C this particular strain did not form significantly more biofilm in PIF ( $P > 0.05$ ), in fact it did not form a detectable biofilm in either media at this temperature. Secondly, it is clear that there is a discernible difference between biofilm formation at 25°C and biofilm formation at 37°C. For both media tested this difference proved to be statistically significant ( $P < 0.05$ ). For the majority of strains tested an improvement in biofilm formation was observed at 37°C, however for *C.sakazakii* (NCTC 8155) this is not the case. While Figure 3 shows a higher average level of biofilm formation for this strain at 25°C, statistically this bacterium showed no significant preference for either temperature ( $P > 0.05$ ).

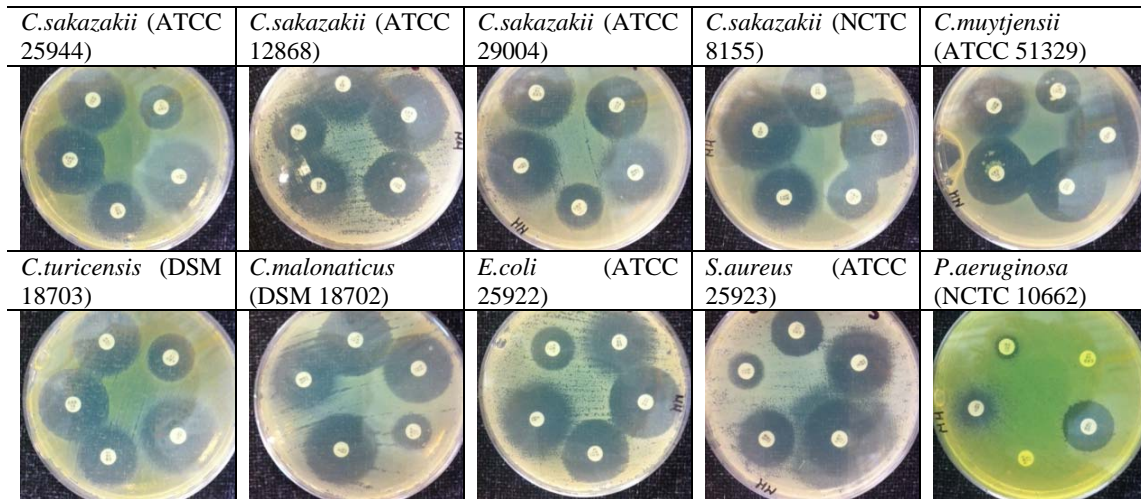
After analysing the overall differences between temperatures and media, a more in depth analysis was conducted, comparing the type strain, *C.sakazakii* (ATCC 29544) to the other strains of bacteria used in this experiment. At 25°C, in LB broth, there was no significant difference between the biofilm formation of this bacterium and any of the other strains ( $P > 0.05$ ) (see Figure 3). At 37°C in LB broth there were again no significant differences between *C.sakazakii* (ATCC 29544) and the other tested strains of bacteria. The average amount of biofilm formed by the strains of *S.aureus* and *P.aeruginosa* were higher, as shown in Figure 3, but were not statistically significant ( $P > 0.05$ ).

At 25°C in PIF, significant differences in the ability of strains to form biofilm are apparent. Of the seven strains of *Cronobacter* used, *C.sakazakii* (ATCC 29544) produced on average the lowest amount of biofilm with only *P.aeruginosa* producing less biofilm overall. As mentioned before, *P.aeruginosa* (ATCC 10662) did not produce detectable biofilms at this temperature in either LB or PIF. The level of biofilm produced by *C.sakazakii* (ATCC 29544) was not significantly different than *C.turicensis* (DSM 18703) and *C.muytjensii* (ATCC 51329) under these conditions ( $p > 0.05$ ) but was significantly different to all other bacterial strains used ( $p < 0.05$ ). *S.aureus* (ATCC 25923) was the most prolific biofilm former at this temperature in PIF (see Figure 3).

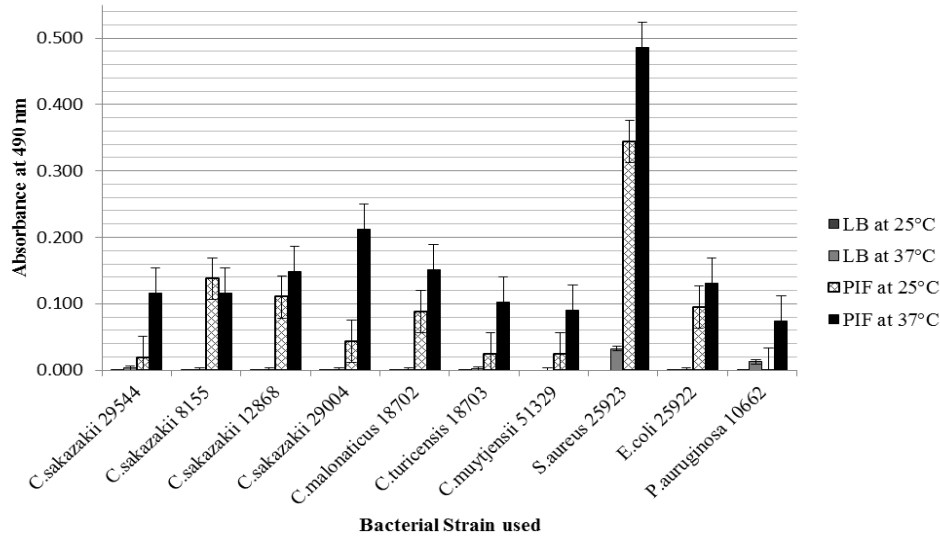
At 37°C in PIF, *S.aureus* (ATCC 29523) was by far the most prolific biofilm former (see Figure 3). However, due to inconsistencies in the absorbance readings for *S.aureus* (ATCC 29523) the difference between these means was not found to be statistically significant. There was no significant difference between the ability of *C.sakazakii* (ATCC 29544) to form biofilm at this temperature in PIF and the abilities of the additional six strains of *Cronobacter* and *E.coli* (ATCC 25922) to form biofilm under the same conditions ( $p>0.05$ ). However there was a significant difference in the amount of biofilm detected for *P.aeruginosa* (NCTC 10662) versus the amount detected for the *C.sakazakii* ( $p<0.05$ ). *P.aeruginosa* is a well-known biofilm forming bacterium due to its association with ventilator associated pneumonia (VAP) and cystic fibrosis (CF) (Ficks-Lima *et al.*, 2008). The significantly lower amount of biofilm that was apparently produced by this bacterium in this experiment may be misleading as the intrinsic qualities of *P.aeruginosa* biofilms may call into question the suitability of the chosen assay for this particular bacterium. While generally the repeatability of the CV assay is high, it appears to be less suitable for the quantification of *P.aeruginosa* biofilm biomass. A higher CV concentration of 0.5% is suggested by some sources to counteract the inadequate fixation caused by the high levels of water found in the slimy matrix of *P.aeruginosa* biofilms (Peeters *et al.*, 2008). This is a factor that will be taken into consideration for future studies.



**Fig. 1:** Acceptable inoculum density range for a Gram negative rod (BSAC, 2011)



**Fig. 2:** Photographic representations of the plates after the recommended 18-20 hour incubation period at 37°C



**Fig. 3:** Biofilm formation in PIF versus LB broth at 25°C and 37°C. These values were obtained by taking absorbance values at 490 nm and subtracting the blank/control which was reconstituted PIF/LB broth that had not been inoculated with bacteria

**Table 1:** Solvents for solubisation of stained biofilms [12]

Organism	Incubation temperature (°C)	Solvent
<i>Escherichia coli</i>	25	ethanol : acetone (4:1)
<i>Pseudomonas aeruginosa</i>	25-37	ethanol : water (19:1)
<i>Staphylococcus aureus</i>	37	glacial acetic acid : water (1:2)

**Table 2:** Antimicrobial Susceptibility Testing category definitions [11]

Category	Definition
Clinically resistant	Level of antimicrobial susceptibility which results in a high likelihood of therapeutic failure
Clinically susceptible	Level of antimicrobial susceptibility associated with a high likelihood of therapeutic success
Clinically intermediate	A level of antimicrobial susceptibility associated with uncertain therapeutic effect. It implies that an infection due to the isolate may be appropriately treated in body sites where the drugs are physically concentrated or when a high dosage of drug can be used; it also indicates a buffer zone that should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretation

The main finding of this experiment is that all seven strains of *Cronobacter*, under the chosen conditions, produced similar amounts of biofilm when compared with each other. The amount of biofilm produced by these strains was also very similar to that produced by *E.coli* (ATCC 25922). This is not surprising as both *E.coli* and *Cronobacter* share many characteristics, being both of the family *Enterobacteriaceae* (Iversen *et al.*, 2008). A more interesting finding was the ability of all strains of *Cronobacter* to form biofilm in PIF at 25°C. This is especially apparent for *C.sakazakii* (NCTC 8155), *C.sakazakii* (ATCC 12868) and *C.malonaticus* (DSM 18702), all three of which produced similar, and in some cases higher, mean absorbancy readings than *E.coli* (ATCC 25922) at this temperature. This finding demonstrates the importance of following stringent guidelines for the safe preparation and storage of PIF, as bottles left to cool and stored at room temperature could provide suitable conditions for biofilm formation.

**Conclusions:**

*C.sakazakii* and the other strains of *Cronobacter* spp. used in this study largely displayed similar susceptibility patterns to that of *E.coli*. There is a possibility that antimicrobial resistance in this genus may emerge in the future due to horizontal transfer with closely related species. Worryingly, all *Cronobacter* species displayed an impressive ability to form biofilm in PIF, performing better than other pathogenic bacteria such as *P.aeruginosa* and *E.coli* under certain test conditions.

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