Growth of *Cronobacter* spp. under Dynamic Temperature Conditions Occurring during Cooling of Reconstituted Powdered Infant Formula

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ABSTRACT

Reconstituted infant formulae are excellent growth media for *Cronobacter* spp. (formerly *Enterobacter sakazakii*) and other microorganisms that may be present in such products. Immediate consumption or rapid cooling and storage at a low temperature are therefore recommended as control measures to prevent microbial growth. Placing a container filled with reconstituted liquid formula in the refrigerator, however, does not mean that the temperature of the liquid is directly the same as the set-point of the refrigerator. This study describes the temperature profiles and methods to predict lag time and possible growth of *Cronobacter* spp. during the cooling process in three types of containers. The overall heat transfer coefficients ($\alpha$) were determined and were shown to have a very large variability in both household refrigerators and an air-ventilated refrigerator equipped with a fan. A mathematical model was built to predict the growth of *Cronobacter* spp. under dynamic temperature conditions using three models for the lag time. The various estimations for the lag time had a remarkably strong impact on the predicted growth. The assumption of a constant $k$-value ($k$ = lag time $\times$ specific growth rate $= \lambda \times \mu = 2.88$) fitted the experimental data best. Predictions taking into account the large variability in heat transfer showed that proliferation of *Cronobacter* spp. during cooling may be prevented by limiting the volume to be cooled to portion size only, or by reconstituting at temperatures of 25°C or lower. The model may also be used to predict growth in other situations where dynamic temperature conditions exist.

*Cronobacter* spp., until recently known as the species *Enterobacter sakazakii*, is a group of opportunistic pathogenic microorganisms belonging to the family of Enterobacteriaceae (8, 13). Although only recently brought to wider attention of infant formulae manufacturers and the public through several outbreaks and public recalls, *Cronobacter* spp. have been associated with neonatal deaths as early as 1958 (32). Cases of neonatal meningitis or necrotizing enterocolitis due to *Cronobacter* infection have in certain cases been associated with powdered infant formulae (4, 6, 29). Several investigations showed that the microorganism can be found in a variety of environments and that contamination may occur during manufacturing and during preparation of the bottles (12, 16, 19, 20, 22). Concentrations in dry powdered formulae ranged from 0.002 to 0.92 CFU g$^{-1}$ (7).

In dry powdered infant formula *Cronobacter* spp. are not able to grow; but, after the addition of water, reconstituted infant formula is a good medium for growth, with only the barriers of short storage and low temperature to prevent bacterial growth. After reconstitution, *Cronobacter* spp. can multiply at temperatures between 3.6 and 47.6°C (12, 17, 23). At room temperature (25°C) the generation time was determined to be 0.59 h (17). A study by Pagotto et al. (24) suggests that $10^5$ CFU ml$^{-1}$ of certain *Cronobacter* spp. strains could cause illness in suckling mice after ingestion.

To prevent multiplication of microorganisms, most manufacturers prescribe consumption of infant formula directly after preparation. For practical reasons, however, caregivers sometimes prefer to prepare all the bottles needed for one day in advance (26). Specifically in hospitals it is not uncommon that infant formulae are prepared once per day and placed in a refrigerator to be used within 24 h (27) or within 30 h with the refrigerator set at 4°C (1). However, even if the temperature of the refrigerator is controlled, the temperature of the reconstituted formula itself is not yet controlled. Many manufacturers instruct that their powdered infant formulae should be reconstituted with moderately warm (lukewarm) water to assure that the powder dissolves well. The liquid formula then needs time to cool down in the refrigerator. The cooling rate of the liquid depends on the temperature, the filling rate, and the air velocities in the refrigerator and the geometry of the food container, the thermal properties of food and bottle, and the volume of the container to be cooled.

In household refrigerators, also referred to as static refrigerators, heat transfer at the container surface is principally due to natural convection by a very limited airflow caused by variations in air density. These variations are mainly related to differences in temperature, filling of...
TABLE 1. Parameters used in equations 2 and 3

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Dimension</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific heat capacity of infant formula ( (c_p) )</td>
<td>3.93</td>
<td>kJ kg^{-1} \cdot °C^{-1}</td>
<td>3</td>
</tr>
<tr>
<td>Density of infant formula ( (\rho) )</td>
<td>1.032</td>
<td>kg m^{-3}</td>
<td>30</td>
</tr>
<tr>
<td>Radius of 1-liter measuring beaker ( (r) )</td>
<td>0.05</td>
<td>m</td>
<td>—</td>
</tr>
<tr>
<td>Radius of bottle type 1 ( (r) )</td>
<td>0.025</td>
<td>m</td>
<td>—</td>
</tr>
<tr>
<td>Radius of bottle type 2 ( (r) )</td>
<td>0.02</td>
<td>m</td>
<td>—</td>
</tr>
<tr>
<td>Height of 1-liter measuring beaker ( (h_t) )</td>
<td>0.14</td>
<td>m</td>
<td>—</td>
</tr>
<tr>
<td>Height of bottle type 1 ( (h_t) )</td>
<td>0.115</td>
<td>m</td>
<td>—</td>
</tr>
<tr>
<td>Height of bottle type 2 ( (h_t) )</td>
<td>0.95</td>
<td>m</td>
<td>—</td>
</tr>
<tr>
<td>Thermal conductivity of infant formula ( (k_{liquid}) )</td>
<td>0.52</td>
<td>W m^{-1} °C^{-1}</td>
<td>30</td>
</tr>
<tr>
<td>Thermal conductivity of bottle wall, polycarbonate ( (k_{container \ wall}) )</td>
<td>0.21</td>
<td>W m^{-1} °C^{-1}</td>
<td>2</td>
</tr>
</tbody>
</table>

the fridge, and humidity gradients. In air-ventilated refrigerators, mechanic ventilation forces air convection, which improves heat transfer (18). Air-ventilated refrigerators are often, but not always, used in hospitals and nurseries.

To our knowledge, investigations into the cooling process of baby bottles are scarce. Rosset et al. (27) studied temperature profiles in 25 neonatal care units and concluded that cold storage was the operation with the largest impact on potential growth of Cronobacter spp. In the report of the technical meeting on Enterobacter sakazakii and Salmonella in powdered infant formula (9), a risk assessment model was discussed that estimates the fate of Cronobacter spp. during various scenarios of preparation and storage. The aim of our study was to make a detailed investigation, both experimentally and by modeling, of the growth opportunities of Cronobacter spp. during cooling of reconstituted infant formula in refrigerators and to use the findings to propose control measures that may reduce and prevent the growth of Cronobacter spp. during the cooling process.

MATERIALS AND METHODS

Preparation of reconstituted infant formula for cooling experiments. Powdered infant formula suitable for infants younger than 6 months of age was bought locally and had a bacterial count of less than 10^2 CFU g^{-1}, which did not influence the experiments (data not shown). In accordance with the instructions on the label, tap water was brought to a full boil in a covered water boiler, cooled down on the bench to approximately 45°C, and mixed with the powdered infant formula to obtain reconstituted formula with a temperature of 40 ± 3°C.

Cooling experiments. A total of 25 cooling experiments were performed using an experimental setup whereby three different container types with reconstituted infant formulae were placed in the refrigerator and the temperature during cooling was recorded. The following three polycarbonate containers were used: a measuring beaker equipped with a lid with a volume of 1,000 ml and a diameter of 0.10 m, (baby) bottle type 1 (maximum 260 ml) with a diameter of 0.05 m, and (baby) bottle type 2 (maximum 120 ml) with a diameter of 0.04 m (Table 1). Reconstituted infant formula was divided among the measuring beaker and the two types of baby bottles. While the bottles had different geometries, both were filled with 120 ml of formula. The measuring beaker held 1,000 ml. The three containers were placed in the center of either an incubator ventilated with air or in one of the nine different static air refrigerators (further referred to as household refrigerators). Eight of nine household refrigerators were located in households and contained different amounts of other food materials. The ninth household-type refrigerator was located in the laboratory and was empty except for the three containers. The temperature of the household refrigerators varied, which was recorded as a given parameter and not set as a controlled experimental parameter. During cooling the temperature was recorded in the center and near the wall of each container every 5 min for 24 h using thermistor metal oxide sensors (CM-UU) and a squirrel data logger (both: Eltek Ltd., Cambridge, UK). The temperature measured in the center of the bottles was slightly higher than the temperature measured near the wall, and the difference was 0.5°C at most. The temperature measured in the center of the bottle was chosen for use in the model predictions, for a more fail-safe approach. The internal air temperature in the refrigerator was measured with a thermistor as well.

Cooling model for reconstituted infant formula. Equation 1 describes the change in temperature of a bottle or beaker \( (T_C) \) assuming that the temperature in the liquid is homogeneous and that heat is transferred from the liquid via the wall of the bottle to the air.

\[
V \cdot \rho \cdot c_p \frac{dT_C}{dt} = -\pi A (T_C - T_R)
\]

in which \( V \) is the volume of the reconstituted infant formula in the measuring beaker or bottle \( (m^3) \), \( \rho \) is the density of the reconstituted infant formula \( (kg \ m^{-3}) \), \( c_p \) is the specific heat capacity of the reconstituted infant formula \( (J \ kg^{-1} \cdot °C^{-1}) \), \( t \) is the time \( (s) \), \( \pi \) is the overall heat transfer coefficient \( (W \ m^{-2} \cdot °C^{-1}) \), \( A \) is the surface area of the object \( (m^2) \), \( T_C \) is the temperature \( °C \) of the prepared liquid formula, and \( T_R \) is the air temperature in the refrigerator \( °C \). Values of the parameters are shown in Table 1. After integration and rewriting, this equation transforms into:

\[
T_{CI} = T_R + (T_{C0} - T_R) \times e^{-r_{s}/r_{p} \cdot c_p} \]

where \( T_{C0} \) is the temperature in the center of the formula in the measuring beaker or bottle at the beginning of the cooling process \( °C \) and \( r \) is the radius of the measuring beaker or bottle \( (m) \). We hereby assume that both the bottles and the measuring beaker can be described as a cylinder and that only the side surface area of the liquid \( (A = 2 \pi r \cdot h) \) contributes to heat transfer, not the top or the bottom of the container. The ratio of surface area and volume \( (A/V) \) can then be described as \( 2r^{-1} \).
As shown in equation 3, the overall heat transfer coefficient \( \alpha \) consists of the external heat transfer coefficient in air, \( \alpha_{\text{external}} \), the coefficient of the wall, \( \alpha_{\text{container wall}} \) the ratio of thermal conductivity of the container material \( (k_{\text{container wall}}) \) and the thickness of the wall], and the internal heat transfer coefficient, \( \alpha_{\text{product}} \):

\[
\frac{1}{\alpha} = \frac{1}{\alpha_{\text{external}}} + \frac{1}{\alpha_{\text{container wall}}} + \frac{1}{\alpha_{\text{product}}} \quad (3)
\]

The external heat transfer coefficient largely depends on the movement of air in the refrigerator. When a fan is present, this coefficient can be expected to be higher than in household refrigerators that are not equipped with a fan. The internal heat transfer coefficient depends on the movement of liquid in the bottle. During cooling of the bottles the liquid may circulate slowly due to free convection. If free convection does not occur, the internal heat transfer coefficient can be estimated by the ratio of the thermal conductivity of the reconstituted infant formula \( (k_{\text{liquid}}) \) and the radius of the container (\( r \)).

**Test organism.** Stock cultures of *Cronobacter sakazakii* (formerly named *Enterobacter sakazakii*) ATCC 29544 were maintained at \(-80^\circ C\) in cryo vials (Greiner Bio-one GmbH, Frickenhausen, Germany), containing a stationary-phase culture suspension in brain heart infusion (Difco, Becton Dickinson, Maryland) broth with 30% (vol/vol) glycerol (Fluka-chemica, Buchs, Switzerland).

**Growth medium, inoculation, and sampling.** The inoculum of strain ATCC 29544 was prepared by transferring 100 µl of the stock culture into 100 ml of brain heart infusion broth, followed by incubation for 18 to 24 h at \(37^\circ C\). Cells were harvested, washed, sprayed onto dry infant formula to obtain a final concentration of \(10^3\) to \(10^6\) CFU g\(^{-1}\), and stored for 3 to 10 days as described elsewhere (17). The powder containing *Cronobacter* spp. was then mixed with water, similar to the procedure described at “Preparation of reconstituted infant formula for cooling experiments,” to obtain reconstituted formula with \(10^2\) to \(10^5\) CFU ml\(^{-1}\) at a temperature of \(37\pm3^\circ C\). This formula was distributed between the type 1 bottle and the measuring beaker and placed in the laboratory refrigerators immediately. No other products were present in the refrigerators. Samples were taken every 30 min during the first 4 h and subsequently every 60 min, by inserting a 1-ml disposable pipette with balloon, leaving the bottles in the refrigerator. If appropriate, samples were first diluted in peptone saline solution (NaCl 8.5 g liter\(^{-1}\) supplemented with 1 g liter\(^{-1}\) neutralized bacteriological peptone from Oxoid, Basingstoke, England) and then plated in duplicate onto tryptone soy agar (Oxoid Ltd., Basingstoke, Hampshire, England) using a spiral platter device (Eddy Jet, Leerdam, The Netherlands). Plates were incubated for 20 to 24 h at \(37^\circ C\) and counted manually. The inoculation levels chosen are higher than the very low numbers found in infant formulae (7, 11, 21, 23). A minimum level required to accurately count the organisms plated by the spiral plater, however, is \(6 \times 10^2\) CFU ml\(^{-1}\), and inoculum levels were chosen to be slightly higher than this minimum.

**Predicting growth of *Cronobacter* spp. under dynamic temperature conditions.** A growth model was built that uses the lag time and the specific growth rate of *Cronobacter* spp. during the constantly changing temperature conditions that occur during cooling. The specific growth rate \( (\mu_{\text{opt}}) \) for *Cronobacter* spp. at each temperature \( (T_i) \) was described with the secondary growth model (equation 4) of Rosso et al. (28) using cardinal parameters previously established (Table 2) (17).

If \( T_{\text{min}} < T_i < T_{\text{max}} \) then

\[
\mu_{\text{opt}}(T_i) = \left( \frac{(T_i - T_{\text{max}})(T_i - T_{\text{min}})}{b} \right) - \left( \frac{(T_{\text{opt}} - T_{\text{min}})}{T_{\text{opt}} + T_{\text{min}} - 2T_i} \right)
\]

\[
\times \left( T_{\text{opt}} - T_i \right) \mu_{\text{opt}} \quad (4)
\]

and

\[
\mu_{\text{opt}}(T_i) = 0 \quad \text{if} \quad \left\{ \begin{array}{l} T_i \leq T_{\text{min}} \quad \text{or} \quad T_i \geq T_{\text{max}} \end{array} \right. \]

where \( T_i \) is the temperature (°C) at each moment in the center of the volume of the infant formula in the measuring beaker or the bottle; \( T_{\text{min}} \) is the extrapolated minimum temperature (°C), \( T_{\text{max}} \) is the extrapolated maximum temperature (°C), \( T_{\text{opt}} \) is the temperature (°C) at which the specific growth rate is maximal, and \( \mu_{\text{opt}} \) (h\(^{-1}\)) is the \( \mu_{\text{opt}} \) at this optimal temperature (28).

Three models were used to estimate the lag time. First, lag times were estimated using the inverse of the secondary Ratkowsky model (equation 5) with parameters previously determined as shown in Table 2 (17).

\[
\ln[\lambda(T_i)] = \ln \left( \frac{b(T_i - T_{\text{min}})[1 - e^{(T_i - T_{\text{max}})}]}{c} \right)^{\frac{1}{2}} \quad (5)
\]

where \( b \) (°C\(^{-1}\) h\(^{-0.5}\)) and \( c \) (°C\(^{-1}\)) are the so-called Ratkowsky parameters (34). \( T_i \) (°C) is the average temperature of the simulation interval. The \( T_{\text{min}} \) and \( T_{\text{max}} \) parameters were determined from previous growth experiments at temperatures ranging from 8 to 47° C (17).
Second, the lag times were estimated using a constant $k$-value, which is the product of the specific growth rate and the lag time (equation 6). It is known that the $k$-value has a large variability, but it can be considered constant over a wide range of temperatures (34).

$$k = \lambda(T_i) \cdot \mu_m(T_i)$$  

(6)

Third, the logarithmic model was used to estimate lag times (equation 7) (25).

$$\log[\lambda(T_i)] = c_1 \cdot \text{ln}(T_i) + b_1$$  

(7)

where $\lambda$ (h) is the lag time, and $b_1$ and $c_1$ are parameters as used in the microbial risk assessment model (25).

It should be noted that all specific growth rate and the lag time models (equations 4, 5, 7) are used with earlier estimated parameters (17) and are therefore predictions. To specifically estimate lag time effects in this study, the $k$-value (equation 6) was determined from experiments taking into account the effect of a dynamically changing temperature on a lag time value.

To determine the length of the lag time at constantly changing temperature, the fraction of the lag time elapsed ($\lambda f_i$) in every time step was calculated numerically according to equation 8.

$$\lambda f_i = \left[ \frac{\Delta t}{60 + \lambda(T_{t-1} - 1)} \right] + \lambda f_{i-1}$$  

(8)

where $\Delta t$ is the time step of the simulation (5 min) and $\lambda f_i$ is the fraction of the lag time elapsed at step $i$ and $\lambda(T_{t-1})$ (h) is the average of the lag time of the current and the previous time step. Each fraction $\lambda f_i$ was added to the value of the previous time step. Once $\lambda f_i \geq 1$, the lag time was assumed to be completed, and growth was assumed to be starting with a specific growth rate corresponding to the temperature prevailing in the bottle at that moment.

To include changing temperature also during the growth stage, the dynamic first-order model for bacterial growth was used and was solved numerically. In each time step the average temperature (and consequently the specific growth rate) was taken as constant, providing the exponential function as outcome (equations 9A to C). As a conservative estimate, growth was predicted assuming $\lambda = 0$, using equation 9A only, thus assuming that Cronobacter spp. started growing instantaneously after the addition of water. So, any further steps can be described by equation 9A, assuming that during the full time step ($\Delta t$) there is exponential growth. When there was a lag time, the fraction of the lag time elapsed was calculated using equation 8. When the fraction of the lag time elapsed was less than 1, the number of microorganisms was stable (equation 9B). To exactly determine the commencement of growth after the lag phase, in the step when $\lambda f_i$ has become greater than 1, equation 9C describes growth in the remaining fraction ($\lambda f_i - 1$) of this time step. Equation 9C is valid only in the first iteration after the lag phase has been completed.

After the first $\lambda f_i > 1$:

$$N_i = N_{i-1} \cdot e^{\mu_m(T_i) \cdot \Delta t}$$  

(9A)

For $\lambda f_i < 1$:

$$N_i = N_0$$  

(9B)

For the first value of $\lambda f_i \geq 1$:

$$N_i = N_0 \cdot e^{\mu_m(T_i) \cdot (\lambda f_i - 1) \cdot \Delta t}$$  

(9C)

where $N_i$ (CFU ml$^{-1}$) is the number of microorganisms present at time step $i$; $\mu_m(T_i)$ (h$^{-1}$) is the specific growth rate at the temperature prevailing in the current time step $i$, and $\lambda f_i$ is the fraction of the lag time elapsed at step $i$.

**Data analysis.** Temperature profiles during cooling were fitted into equation 2 using the solver function in Microsoft Office Excel 2003 by minimizing the residual sum of squares to estimate the overall heat transfer coefficient ($\alpha$) and the air temperature ($T_R$). Experimental growth curves were fitted to the dynamic growth model to estimate the $k$-value using the same software. Standard deviations are reported as ±. Confidence intervals were calculated in Excel. The increase in numbers of Cronobacter spp. cells assessed in the duplicate growth experiments and the $z$ estimated for the different type of refrigerators were analyzed by analysis of variance (ANOVA), univariate analyses using SPSS (SPSS release 12.01 for Microsoft Windows, SPSS Inc., Chicago, IL). Significant differences are presented at a 95% confidence level ($P \leq 0.05$).

RESULTS AND DISCUSSION

**Temperature variability within refrigerator.** In our experiments large variations in air temperatures were observed both over time and between different locations in the same empty household refrigerator (Fig. 1A). Starting 1 h after closing the door with the temperature set at 6.3°C, the temperature near the cooling element in the back of the upper department varied between −8.4°C and +10.4°C. This variation is due to the refrigerator’s engine switching on and off. The lower, middle, and upper compartments of the refrigerator showed more constant temperatures and were on average 5.5 ± 0.8, 6.5 ± 1.3, and 9.9 ± 0.7°C.
respectively. These results are in line with data reported elsewhere. James and Evans (14) surveyed 252 household refrigerators without a fan and found that the coldest spot was on average 2.9°C colder than the warmest spot within the same refrigerator, whereas the greatest range of average temperatures observed in a refrigerator was 12°C. Individual (not averaged) temperature differences ranged from 4.5 to 30.5°C in the same refrigerator. Door openings resulted in an increase in average temperature and in an even larger temporal variability (15). A variation in local air temperature does not immediately change the temperature of a bottle placed in that refrigerator, but varying temperature profiles may change the air flow and thus affect the heat transfer coefficient (\(a\)). Additionally, the speed of cooling, as described in equation 1, will be affected by variations in air temperature.

In air-ventilated refrigerators equipped with a fan, the air is continuously in motion and the variation in air temperature is limited (Fig. 1B). Near the air inlet the temperature varied from 5.4 to 7.3 (average 6.3°C; standard deviation 0.5°C), near the door the temperature was 7.7 ± 0.1°C, and all other locations had temperatures of 6.7 ± 0.1°C.

As a result of the variations in air temperature it was not possible to establish one value for the air temperature required to fit the data to equation 2. Therefore, both the heat transfer coefficient (\(a\)) and the air temperature (\(T_R\)) were fitted simultaneously to the experimental data according to equation 2. The differences between the measured \(T_R\) and the fitted \(T_R\) were on average less than 1°C (data not shown).

**Overall heat transfer coefficient (\(a\)).** Figure 2 shows typical cooling profiles of the 1-liter beaker and both bottles placed in either a household refrigerator (2A) or an air-ventilated refrigerator (2B). During the cooling process the temperature fluctuated slightly due to variations in the air temperature. In a household refrigerator set at 6.3°C, the time required for cooling the prepared formula to 10°C was typically 7.5 h for 1,000 ml of formula in the measuring beaker and 3.5 h for the 120-ml volume in the two types of bottles. In an air-ventilated refrigerator, cooling times typically were 2 h less.

With the three containers, 25 cooling experiments were performed in nine different household refrigerators, and 9 experiments were performed in an air-ventilated refrigerator. All cooling profiles \((3 \times 25 + 3 \times 9)\) were fitted to equation 1 to obtain the overall heat transfer coefficient \([\sigma(W m^{-2} °C^{-1})]\), a parameter independent of the reconstitution temperature, the air temperature, and within a limited range independent of the size of the object. No consistent differences were found between the \(\sigma\)-values determined for the refrigerators that were empty and those that also contained other materials; therefore, the data were pooled and used as a single data set. Figure 3A shows the \(\sigma\)-values with their standard deviation as a function of air temperature in household refrigerators. An effect of air temperature on the heat transfer coefficient \(\sigma\) was not observed. The \(\sigma\)-values ranged from 5.4 to 12.9 (W m\(^{-2}\) °C\(^{-1}\)) in this series of experiments in which temperature settings and filling rates of the nine household refrigerators were variable.

Figure 3B shows a selection of the \(\sigma\)-values that were measured in one single household refrigerator and one air-ventilated refrigerator, both empty except for the experimental setup consisting of the three containers. In this well-defined series of experiments, the range of \(\sigma\)-values [6.1 to
Estimated overall heat transfer coefficient of type 1 and type 2 bottles, each containing 120 ml, and a measuring beaker containing 1,000 ml, of reconstituted infant formula as measured in one household-type refrigerator and one air-ventilated refrigerator.

<table>
<thead>
<tr>
<th>Type of refrigerator</th>
<th>Avg overall $\alpha$ (W m$^{-2}$ °C$^{-1}$)$^a$</th>
<th>No. of experiments</th>
<th>SD of avg</th>
<th>95% confidence interval (W m$^{-2}$ °C$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measuring beaker</td>
<td>Household 9.9</td>
<td>11</td>
<td>1.4</td>
<td>6.8–13.0</td>
</tr>
<tr>
<td></td>
<td>Air-ventilated 14.0</td>
<td>9</td>
<td>2.4</td>
<td>8.4–19.7</td>
</tr>
<tr>
<td>Bottle type 1</td>
<td>Household 10.0</td>
<td>11</td>
<td>1.5</td>
<td>6.7–13.3</td>
</tr>
<tr>
<td></td>
<td>Air-ventilated 15.7</td>
<td>9</td>
<td>2.5</td>
<td>9.8–21.5</td>
</tr>
<tr>
<td>Bottle type 2</td>
<td>Household 7.7</td>
<td>11</td>
<td>1.5</td>
<td>4.4–11.1</td>
</tr>
<tr>
<td></td>
<td>Air-ventilated 13.9</td>
<td>9</td>
<td>2.8</td>
<td>7.4–20.4</td>
</tr>
</tbody>
</table>

$^a$ $\alpha$, heat transfer coefficient.

12.9 (W m$^{-2}$ °C$^{-1}$)] in the single household refrigerator was almost as wide as in the nine different household refrigerators shown in Figure 3A. The fitted heat transfer coefficients as shown in Figure 3B are significantly higher ($P = 0.01$) than in the household-type refrigerators. The coefficients in this well-controlled refrigerator varied over the range of 9.9 to 19.0 W m$^{-2}$ °C$^{-1}$. Average values and confidence intervals are shown in Table 3.

The average values for the beaker and the type 1 bottle corresponded to cooling rates of $1.0 \times 10^{-4}$ and $2.0 \times 10^{-4}$ s$^{-1}$, identical to the cooling rates assumed in the Food and Agriculture Organization of the United Nations and the World Health Organization microbial risk assessment model (25). For bottle type 2, $\alpha$ was significantly lower in household refrigerators (ANOVA univariate analysis, $P = 0.001$) than the corresponding values of the measuring beaker and bottle type 1. The type 2 bottle, which is the smallest baby bottle available on the Dutch market, was filled to its maximum volume. It might be that the area available for heat transfer was slightly less than assumed in equation 2, since the bottle is rounded at the bottom and not a perfect cylinder. Another explanation is that the lower $\alpha$ might be due to the fact that in this narrow bottle, with a radius of 0.02 m, internal heat transfer resistance can not be overlooked. When a circular flow does develop in a container during cooling, the internal heat transfer coefficient $\alpha$ can be estimated to be in the range of 150 to 250 W m$^{-2}$ °C$^{-1}$ using the theory of free convection from a vertical plate (5) and assuming a temperature difference of 0.5°C between core and the wall. If in a narrow bottle such a circular flow does not develop, the internal heat transfer coefficient depends solely on the ratio of the thermal conductivity of the reconstituted infant formula ($k_{\text{liquid}}$, 0.52 W m$^{-1}$ °C$^{-1}$) and the radius of the container (0.02 m).

The resulting internal heat transfer coefficient is 26 W m$^{-2}$ °C$^{-1}$, and thus is in the same order of magnitude as the resulting overall coefficient (see Fig. 3). It may therefore have contributed significantly to heat transfer resistance. To rule out the potential effect of insufficient liquid mixing, the experiments testing the growth of Cronobacter spp. during cooling were continued using bottle type 1. Furthermore this bottle size and type is used by the majority of the caregivers in The Netherlands.

**Growth during cooling.** On the basis of the estimated overall heat transfer coefficient, the temperature in a bottle

![FIGURE 4. Prediction and experimental values of growth during cooling of infant formula in a 1-liter measuring beaker in a household refrigerator set at 7.1°C. The reconstituted formula was prepared from artificially contaminated powder and had a temperature of 38°C at the start of the experiment. (A) (×) measured and (solid line) predicted temperature with 95% confidence interval (dashed lines). (B) Predictions of (●) specific growth rate and (lines) lag times at the prevailing temperature. (C) Fraction of lag time elapsed and (D) number of C. sakazakii (×) measured and (lines) predicted. Various lines represent the following: (gray dashed line) lag = 0, (black dashed line) lag time according to Ratkowsky, and (gray line) lag time predicted with the logarithmic model.](https://example.com/figure4)
or measuring beaker could be predicted during the entire cooling process. Figure 4A shows the temperature of a 1-liter beaker placed in a household refrigerator at 7.1°C. With the temperature at each time point known, both the specific growth rate and the lag time of *Cronobacter* spp. were calculated using equations 4 through 7. Figure 4B shows that the specific growth rate dropped sharply during the first 5 h of the cooling process. In the same 5-h period, the lag time increased to values of 20 h and more. The construction of the lag time under dynamic temperature conditions is shown in Figure 4C. According to the inverse of the Ratkowsky model (equation 5) the lag time was 3.2 h. An initial estimate for the k-value of 4.05 (−) taken from previous research (17) led to a predicted lag time of 3.3 h (data not shown), while the logarithmic model (equation 7) predicted a lag time of 4.8 h. The resulting predicted and experimental numbers of *Cronobacter* spp. under dynamic temperature conditions are shown in Figure 4D. Growth of the organism was apparent in the 1-liter measuring beaker that was cooling from 38°C in the household-type refrigerator set at 7°C. The increase in numbers during the entire 24 h was 0.7 log CFU ml⁻¹. Figure 5 shows graphs similar to Figure 4D for both the 1-liter measuring beaker and the type 1 bottle at 5, 7, 10, and 16°C. This range of temperatures was chosen to mimic the range of temperatures that can be found in household refrigerators, including the 30% of household refrigerators that exceed standard temperatures (10, 31).

**Impact of lag time.** In the predictions a small difference in lag time resulted in considerable differences in the estimated growth. With a longer lag time (Fig. 4C), the temperature of the formula (Fig. 4A) will be lower at the moment that growth commences, whereas also the specific growth rate (Fig. 4B) will be lower, together resulting in a considerably smaller increase in cell count.

When the lag time was assumed to be zero (gray dashed lines), the exponential growth function overestimated the experimental values considerably in all cases shown in Figure 5. Although *Cronobacter* spp. cells are known to commence their growth quickly in infant formula, they do seem to require some time to adjust to their new environment and start multiplying. The logarithmic model overestimated the lag time and thus underestimated the growth in all cases (25). The results of the use of a k-value were often comparable to the Ratkowsky model. The Ratkowsky model (black dashed lines) predicted the growth during cooling at 7 and 10°C quite well, but at other temperatures this approach underestimated growth. Optimal fits to the experimental data were obtained with $k = 1.75$ at 4°C, $k = 3.49$ at 7°C, $k = 4.07$ at 10°C, and $k = 2.06$ at 16°C (gray solid lines). The best fit over all eight experiments was obtained with $k = 2.88$ (−) (black solid line). These values are all within the range of lag values previously reported for individual experiments at a constant temperature (17, 34).

It should be noted that the experiments shown in Figures 4 and 5 were performed with *Cronobacter* spp. cells that were present in the dry powder; these can be expected to be injured and therefore show relatively long lag phases. It could be that even longer lag times might have been observed when lower levels of inocula were used than in the current study and when naturally contaminated powered infant formula was used. On the other hand, in an additional series of experiments using an inoculum of cells grown overnight in brain heart infusion, lag times were shorter and could be predicted best by assuming k-values ranging from 1.2 to 1.9 (−) (results not shown).

**Prediction of growth during cooling.** Using the parameters for heat transfer and lag time derived from the experiments, a series of simulations were performed to evaluate the effect of reconstitution temperature and refrigerator temperature. Regarding the lag time, the value $k = 2.88$ (−) as found in this study was assumed in all predictions.

**Cooling time and growth during 24 h.** Table 4 presents an overview of the estimated time required to cool infant formula from 40°C to 10°C in refrigerators set at 7°C
TABLE 4. Prediction of the time required to cool from 40 to 10°C and the increase in cell counts after 24 h in type 1 and type 2 bottles, each containing 120 ml, and a measuring beaker containing 1,000 ml, of infant formula reconstituted at 40°C and placed in refrigerators with an air temperature of 7°Ca

<table>
<thead>
<tr>
<th>Type of refrigerator</th>
<th>Predicted time to reach 10°C (h)</th>
<th>Predicted increase in cell counts in 24 h (log CFU ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measuring beaker</td>
<td>Household</td>
<td>6.8 (5.3–10.0)b</td>
</tr>
<tr>
<td></td>
<td>Air-ventilated</td>
<td>4.8 (3.5–8.1)</td>
</tr>
<tr>
<td>Bottle type 1</td>
<td>Household</td>
<td>3.4 (2.6–5.1)</td>
</tr>
<tr>
<td></td>
<td>Air-ventilated</td>
<td>2.2 (1.6–3.5)</td>
</tr>
<tr>
<td>Bottle type 2</td>
<td>Household</td>
<td>3.6 (2.5–6.2)</td>
</tr>
<tr>
<td></td>
<td>Air-ventilated</td>
<td>2.0 (1.3–3.7)</td>
</tr>
</tbody>
</table>

a Overall heat transfer coefficients with their confidence intervals were taken from Table 3, and for lag time the optimal value \( k = 2.88 (-) \) was assumed.

b Values in parentheses are 95% confidence intervals.

and the increase in *Cronobacter* spp. cells in bottles placed in such refrigerators for 24 h. In a household refrigerator, a 1-liter portion of formula reconstituted at 40°C needed at least 5.3 and up to 10 h to reach a temperature of 10°C; in an air-ventilated refrigerator, at least 3.5 h were required. Bottles needed on average 3.5 h in the household-type and 2.1 h in the air-ventilated type to cool down to 10°C.

In 1-liter portions, growth of *Cronobacter* spp. was predicted to vary in both types of refrigerators from 0.2 to 2.3 log. In bottles placed in household refrigerators, the increase in numbers was on average limited to 0.2 or 0.3 log. The maximum increase in bottles placed in household refrigerators was 1.1 log for refrigerators with the poorest heat transfer properties. In most air-ventilated refrigerators set at 7°C, no increase (0.0 log on average) was predicted in bottles during 24 h, while the upper limit of the confidence interval was 0.3 log. The variability of both the time to reach 10°C and the increase in cells over 24 h mainly originated from the considerable variability of the overall heat transfer coefficient (Table 3).

**Varying refrigerator temperature.** Figure 6 shows the effect of air temperature in the refrigerator on the predicted growth of *Cronobacter* spp. during cooling from 40°C in the 1-liter measuring beaker and in the type 1 bottle filled with 120 ml. Assuming an average heat transfer coefficient of 10 W m⁻² °C⁻¹, no growth was predicted to occur in the bottle if the air temperature was 6°C or lower. Over 6°C, the numbers were predicted to increase slightly within 4 h, whereas up to 24 h more significant multiplication was predicted, depending on the temperature.

In contrast to the bottle, the 1-liter container was predicted to support growth at all refrigerator temperatures. This can be explained by the fact that the resulting temperature of the formula \( T_s \) during cooling in this container is higher than in the bottles, due to a higher \( V/A \) ratio (equation 1) in the 1-liter container. At the common range of refrigerator temperatures (below 7°C), growth was predominantly in the first 4 h of cooling, while growth was limited in the period between 4 and 24 h. At higher temperatures, however, *Cronobacter* spp. was able to continue multiplying after the first 4 h. With the refrigerator set at 16°C, a 4.2-log increase in the bottle and an almost 5.5-log increase in the beaker were predicted.

**Effect of reconstitution temperature.** Figure 7A shows the impact of reconstitution temperature on the predicted growth of *Cronobacter* spp. in formula placed for 24 h in a household refrigerator at 4°C with a heat transfer coefficient that is either average or at the upper or lower

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**FIGURE 6.** Increase in cell counts as a function of refrigerator temperature at (diamonds) 4 h and (triangles) 24 h after reconstitution at 40°C, assuming an average (10 W m⁻² °C⁻¹) heat transfer coefficient. Open symbols with solid lines represent the 1-liter measuring beaker, and closed symbols with dotted lines represent the type 1 baby bottle.

**FIGURE 7.** The effect of reconstitution temperature on (A) increase in log count (this study) and (B) increase in relative risk (25) during cooling for 24 h in a refrigerator with an air temperature of 4°C. Heat transfer coefficients are assumed to be (triangles) average (10 W m⁻² °C⁻¹), and (crosses) at lower (6.7 W m⁻² °C⁻¹) and (squares) at upper (13 W m⁻² °C⁻¹) limit of confidence interval. Open symbols represent a 1-liter beaker and closed symbols the type 1 bottle filled with 120 ml of infant formula.
limits of its 95% confidence interval. In the 1-liter beaker, growth was predicted to occur at reconstitution temperatures over 25, 32, or 37°C, depending on the assumption for the heat transfer coefficient. Below a reconstitution temperature of 25°C, no growth was predicted under any circumstances. For the 120-ml bottle our model did not predict growth, unless the reconstitution was over 35°C and the heat transfer coefficient was at its lower confidence limit. Reconstitution at more than 50°C was not included, since our experiments and simulations were not suitable to measure and to predict cell numbers after thermal inactivation, which likely plays a role at these temperatures. For comparison with our study, results obtained using the microbial risk assessment model (25) are shown in Figure 7B as an increase in relative risk compared to a base-line scenario, which is reconstitution at 25°C. Simulations were limited to 24 h cooling. Although the end points were not identical, the microbial risk assessment model and our study concluded that, in bottles, growth will either not occur (both studies), or will be limited to 0.45 log units in case of poor heat transfer (this study).

When cooling 1-liter containers, however, the reconstitution temperature was found to affect the proliferation of Cronobacter spp.; it may well affect growth of other microorganisms that could possibly be present in the reconstituted infant formula. Our study predicted growth of Cronobacter spp. already present in 1 liter of formula after reconstitution at 26°C and placement in a household refrigerator with the poorest heat transfer properties.

It should be noted that the latter situation is not a worst case scenario. The heat transfer coefficient assumed in our study was measured in an empty refrigerator. Refrigerators in home situations may have even poorer heat transfer properties, because the refrigerator may be packed with other food products and the door may be opened frequently or bottles may be placed in the refrigerator door, where heat transfer is at its minimum (15). Moreover, the Cronobacter spp. cells used in this study were present in the powder and can be expected to have a longer lag phase than actively growing cells. When formula is contaminated during reconstitution with cells that are actively growing, a shorter lag phase and quicker multiplication may be expected.

Possible control measures. Focusing on the cooling phase only, there are several control measures that may help to prevent and minimize growth of Cronobacter spp. in situations where bottles and/or syringes of infant formula have to be prepared in advance from powdered formula for use within 24 h. For example:

(i) Cooling reconstituted infant formula in portion-size containers only, such as bottles, may prevent multiplication quite drastically as compared with cooling in 1-liter portions.

(ii) Replacing household-type refrigerators with air-ventilated refrigerators that have better heat transfer properties is effective in reducing growth during cooling. However, air-ventilated refrigerators cannot totally prevent growth in 1-liter portions of formula with reconstitution temperatures around 40°C.

(iii) Our study predicts no growth during cooling after formula reconstitution at or below 25°C. This may suggest that limiting the reconstitution temperature to 25°C might be an effective control measure, provided that reconstitution is followed by immediate cooling of bottle-sized portions in a refrigerator. The formulae used in this study dissolved well at lower temperatures; for those powders that may not dissolve at room temperature and need to be reconstituted at 40°C or more, quick cooling of bottle-sized portions (for instance using running water or ice) may be an alternative way to prevent growth during cooling. Reformulation to allow powders to dissolve at lower temperature would, however, be preferred.

(iv) Although lowering the set points of refrigerators in neonatal care units and/or households to 2°C or less might seem a likely control measure, our results suggest that this may have only a very limited effect on multiplication during cooling. Only when the current set point is above 7°C might such a measure have a significant effect.

This study provides measurements of both heat transfer characteristics and microbiological growth of Cronobacter spp. in containers of reconstituted infant formula, along with a model that is able to estimate the variability of these processes taking place simultaneously under dynamic temperature conditions. Powdered infant formula manufacturers, neonatal health care professionals, and (inter)governmental organizations may use the specific findings to further improve guidelines and best handling practices (33).

The lag time, which is known to be highly variable, has a remarkable impact on the overall growth opportunities of Cronobacter spp., as it affects both the moment in time and the temperature at which the organism starts to grow. The predictive model proposed can be used to estimate the proliferation of Cronobacter spp. and potentially of other microorganisms that might be present in powdered infant formula. Using the predictive model, exposure of consumers can be simulated for different scenarios of preparation and consumption of infant formulae, thereby aiding governments and industries to identify effective control measures to protect the vulnerable consumers of this product.

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REFERENCES


