Treatment of *Escherichia coli* O157:H7 with lactic acid, neutralized electrolyzed oxidizing water and chlorine dioxide followed by growth under sub-optimal conditions of temperature, pH and modified atmosphere

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**Abstract**

The utilization of sub-lethal decontamination treatments gains more and more interest due to the increased consumers' demand for fresh, minimally processed and convenient food products. These products rely on cold chain and hurdle (combination) technology to provide microbiological safety and quality during their shelf life. To investigate the ability of surviving cells to resuscitate and grow in a food simulating environment, sub-lethal decontamination treatments were coupled with subsequent storage under sub-optimal growth conditions. For this purpose chlorine dioxide (ClO2) and neutralized electrolyzed oxidizing water (NEW)-treated cultures of *Escherichia coli* O157:H7 were inoculated in TSB-YE of pH 5.8 and aw 0.99, and stored at 10°C–14°C, 12.5°C–14°C and 15°C, under four different atmospheres (0%, 30% and 60% CO2 balanced with N2, and air). Due to the severity of injury, lactic acid-treated cells were inoculated in TSB-YE pH 7.0. Data obtained reveal that the fraction of sub-lethally injured *E. coli* O157:H7 undergoes an additional inhibitory effect during the storage period under of sub-optimal conditions. Observed extension in the lag growth phase was a direct consequence prior sub-lethal injury. The effects of liquid ClO2 and NEW were less pronounced in comparison to lactic acid. The current study signifies the potential utilization of appropriate combination of different extrinsic and intrinsic factors in the elimination or growth inhibition of food-borne pathogens.

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1. Introduction

Since the recognition of its pathogenic character in 1982 (Riley et al., 1983) *Escherichia coli* O157:H7 became known as a notorious major cause of bloody diarrhea and hemorrhagic colitis (HC) that can progress to potentially fatal hemolytic uremic syndrome (HUS) (Ahn et al., 2008; Amirlak and Amirlak, 2006; Karmali, 2004). Outbreaks of *E. coli* O157:H7 are most commonly associated with the consumption of undercooked beef products and raw milk (Denny et al., 2008; Hussein and Bollinger, 2005; Sartz et al., 2008). However, the recent internationally spread outbreaks indicated that other foods, including fresh produce and minimally processed foods, can be a transmission vector of this pathogen (Abdulraouf et al., 1993; Anon, 2006a; Hsin-Yi and Chou, 2001; Vojdani et al., 2008). Moreover, the consumers' demand for more convenient, minimally processed and fresh-like food is increasing nowadays. Therefore a great need exists for the optimization of decontamination treatments in order to ensure the microbiological safety of different foods (Manas and Pagan, 2005). Those treatments should induce sufficient inactivation of inherent pathogenic and spoilage microorganisms, and, at the same time, should not alternate native sensorial properties, leave no residues, be acceptable for the consumer and the legislator, and should be environmentally friendly (Corry et al., 1995). Although sub-lethal treatments have been shown to be effective in inactivating vegetative cells of bacteria, yeast and moulds, they are usually not able to provide complete inactivation of food-borne pathogens in foods, with the surviving population being, most likely, sub-lethally injured (Jasson et al., 2007; Manas and Pagan, 2005). Some of the most studied decontamination treatments are treatments with organic acids, chlorine dioxide, electrolyzed oxidizing water, high hydrostatic pressure, pulsed electric fields and light pulses (Aymerich et al., 2008; Dickson and Anderson, 1992; Gomez-Lopez et al., 2007, 2009; Hugas et al., 2002; van Netten et al., 1995).
Modified atmosphere packaging (MAP) has been effectively used to prolong the shelf life of fresh and minimally processed food, by replacing the air that surrounds the food in the package by specific gas mixture (Phillips, 1996). The gases most commonly used in MAP are carbon dioxide (to inhibit bacterial growth), oxygen (to prevent anaerobic bacterial growth and maintain the color of the meat products) and nitrogen (to prevent package collapse and avoid oxidation of fats) (Eberhart-Phillips et al., 1997; Rao and Sachindra, 2002). The major antimicrobial agent in MAP is carbon dioxide and its effectiveness depends on the used gas concentration, storage temperature, food product and the type of present microflora (Dixon and Bell, 1989).

To extend the use of novel decontamination treatments in the food industry, the combination of these treatments with traditional or emerging preservation technologies during storage is needed (hurdle technology) (Gould, 1996; Leistner, 2000; Leistner and Gorris, 1995). Few studies have reported the great synergistic effect of decontamination treatments such as irradiation, high hydrostatic pressure and ultrasound coupled with subsequent MAP (Chouliara et al., 2008; Raso and Barbosa-Canovas, 2003). The aim of this study was to investigate the effect of sub-lethal decontamination treatments with lactic acid, chlorine dioxide and neutralized electrolyzed oxidizing water on post-treatment resuscitation and growth dynamics of E. coli O157:H7 under sub-optimal conditions of pH and modified atmosphere. Such information should help to elucidate the effect of injury on the behavior of food-borne pathogens under food and storage relevant conditions.

2. Material and methods

2.1. Bacterial strain

E. coli O157:H7 wild type 476 (Laboratory of Food Microbiology and Food Preservation (LFMP), University of Gent, Belgium), which was used in this study, was the fecal isolate (Verhoey-Rozand et al., 2000). Among four attenuated strains present in the culture collection of LFMP, this strain showed moderate resistance against all three bactericidal treatments with approx. 2 log CFU/ml reduction, and therefore was the stain of choice. The reference E. coli O157:H7 cells (100-fold dilution of working culture in Physiological Salt Solution (PSS, 0.85% w/v NaCl)), in order to obtain the effective concentration of 5 ppm ClO2. Test tubes for ClO2 treatment was covered with aluminum foil to avoid degrading effect of light on ClO2. After 2 min of exposure to ClO2 (Zhang and Farber, 1996) the reaction was terminated by transferring ten ml suspension into empty sterile tube eliminating the residual activity of ClO2 (Van Houteghem et al., 2008).

2.2. Decontamination treatments

Three sub-lethal decontamination treatments with lactic acid, chlorine dioxide (ClO2) and neutralized electrolyzed oxidizing water (NEW) were applied into E. coli O157:H7 broth cultures. The preliminary screening experiments were performed to obtain suitable parameters for each decontamination treatments leading to a 1–2 log CFU/ml reduction in cell counts. The surviving cells were exposed to suboptimal conditions mimicking pH of different foods (Rahman, 2009) and were monitored under sub-optimal growth conditions.

2.2.1. Lactic acid treatment

Three millilitres of E. coli O157:H7 culture, containing 108 CFU/ml (10-fold dilution of working culture in TSB-YE) were transferred into a sterile falcon tube containing 27 ml of lactic acid solution (8% v/v lactic acid (Fluka, Germany), pH 3.0). The static exposure time was set to 4 min followed by centrifugation of 2 min at 10 400 × g (Sigma, Laboratory Centrifuges, Göttingen, Germany). The supernatant was decanted and cells were resuspended in 15 ml of TSB-YE to serve as inoculum for the storage under sub-optimal conditions, as described later in this paper.

2.2.2. Chlorine dioxide treatment

2.2.2.1. Determination of ClO2 concentration. Due to volatile character of ClO2, the concentration of the solution was determined before each experiment using an iodometric method (APHA, 1995). Briefly, 5 ml of ClO2 stock solution (Vernagene, Bolton, UK) was mixed with five ml of KI (Sigma–Aldrich, Steinheim, Germany) buffer (pH 7.0) in an Erlenmeyer flask. The mixture was titrated with 0.01 N sodium thiosulfate solution (Na2S2O3, Aldrich, WI, USA) until the colorless end-point, using soluble starch (Difco, Becton Dickinson, Maylan, France) as an indicator. The concentration of ClO2 was calculated according to the volume of utilized Na2S2O3 for the titration.

2.2.2.2. ClO2 treatment. The volume of 1 ml 50 ppm ClO2 solution was transferred into the tube containing 9 ml of TSB-YE/m of E. coli O157:H7 cells (100-fold dilution of working culture in Physiological Salt Solution (PSS, 0.85% w/v NaCl)), in order to obtain the effective concentration of 5 ppm ClO2. Test tubes for ClO2 treatment was covered with aluminum foil to avoid degradation effect of light on ClO2. After 2 min of exposure to ClO2 (Zhang and Farber, 1996) the reaction was terminated by transferring ten ml suspension into empty sterile tube eliminating the residual activity of ClO2 (Van Houteghem et al., 2008). The cell suspension served as inoculum for the storage under sub-optimal conditions, as described later in this paper.

2.2.3. Neutralized electrolyzed oxidizing water treatment

2.2.3.1. NEW generation. Neutral electrolyzed oxidising water (NEW) was generated using an ecosid® 0.02 (Ecosid NV, Schoten, Belgium). The NaCl solution (0.1% w/v) was pumped into the NEW generator, with current and flow rate set up at 0.6 A and 10 l/h, respectively. The concentration of free chlorine was determined using the DPD method (HANNA instruments HI19311, Hungary) (APHA, 1995).

2.2.3.2. NEW treatment. Four ml of NEW (80 ppm free chlorine) was transferred into the tube containing 6 ml 107 CFU/ml E. coli O157:H7 cells (100-fold dilution of working culture in PSS (0.85% w/v NaCl)). After 1 min the reaction was terminated by adding 2 ml of TSB-YE in test suspension to eliminate the residual activity of NEW (Oomori et al., 2000). Surviving cells were used as an inoculum for storage under sub-optimal conditions, as described later in this paper.

2.3. Modified atmosphere packaging

The growth medium used was TSB-YE (Oxoid) modified to pH 5.8 (adjusted with 5 M HCl) and a 0.099 (0.125% w/v NaCl), while for lactic acid-treated cells TSB-YE pH 7.0 was used as TSB-YE 5.8 did not allow survival of the treated cultures.

Experiments were performed in 600 ml glass side arm flasks, where top bottle neck was closed with the cap, and the side arm was secured with silicon septa surrounded by an open cap. The glass flasks were filled with 120 ml of prepared broth in order to obtain ratio liquid/gas 1:4, and autoclaved at 121 °C for 15 min. The flasks were flushed using gas packaging-flush machine (KG DS8454, Witt-Gasetechnik, Witten, Germany) to remove air and obtain desired gas concentrations (0% CO2/100% N2; 30% CO2/70% N2 or 60% CO2/40% N2). Flushed and closed glass flasks were stored at 10 °C for 24 h to reach gas equilibrium between headspace and broth. The gas concentration was checked after 24 h using an O2–CO2 analyzer (Checkout 9900-PBI Dansensor, Denmark). Control flasks contained air instead of modified atmosphere.
Growth under sub-optimal conditions was monitored for sub-lethally treated (lactic acid, ClO2 or NEW-treated) and non-treated E. coli O157:H7 cells. A 24 h culture diluted 1000-fold in TSB-YE served as a non-treated control suspension. The volume of 1 ml of either treated or non-treated E. coli O157:H7 cells was inoculated in the flask to obtain initial inoculum of 10–10⁴ CFU/ml. Inoculated flasks were incubated at 10, 12.5 and 15 °C for at least 30 days.

Samples were analyzed prior to and during (every second day) incubation. Samples were plated out on non-selective (TSA-YE, Oxoid) and selective agar plates (MacConkey Sorbitol Agar supplemented with Cefixime Tellurite supplement, CT-SMAC, Oxoid). The growth curves were constructed by plotting the log CFU/ml versus time.

The percentage of sub-lethally injured E. coli O157:H7 cells (Jasson et al., 2007; Ray and Speck, 1973) was calculated using the following formula:

\[
\% \text{ sub-lethally injured cells} = \left( \frac{\left( \text{ CFU/ml on non-selective media} \right) - \left( \text{ CFU/ml on selective media} \right)}{\text{ CFU/ml on non-selective media}} \right) \times 100
\]

Duplicate experiments were performed for each of the combinations of decontamination treatments and atmosphere/temperature conditions.

2.4. Statistical analysis

The significance of differences between treated and non-treated cultures was analyzed using one-way ANOVA (Microsoft Excel). Statistical significance was set at a P-value of <0.05.

3. Results

3.1. Lactic acid treatment

No surviving cells were detected upon inoculation of lactic acid-treated E. coli O157:H7 into TSB-YE pH 5.8 under any of conditions tested. E. coli O157:H7 remained below the detection limit of 1 log CFU/ml at all times in all three repetitions performed (data not shown). However the growth of non-treated cells in TSB-YE pH 5.8 was not hampered. To evaluate the extent of injury caused by lactic acid in terms of pH sensitivity of remaining cells under modified atmosphere conditions, further experiments were performed at pH 7.0. Growth data obtained at 10, 12.5 and 15 °C under different atmospheres is presented in Fig. 1 indicating an important additional bactericidal effect of CO2 from modified atmospheres (30% and 60% CO2) and airborne O2 (20.95% O2) at 10 °C (Fig. 1a). The number of surviving cells dropped below the detection limit of 1 log CFU/ml after 14 and 27 days when air and 60% CO2/40% N2 were used at 10 °C, respectively. Only in one repetition was severely retarded growth observed after 27 days under 100% N2 (Fig. 1a). The additional reduction of approx. 1.5 log CFU/ml was seen for lactic acid-treated cultures upon incubation at 12.5 °C (Fig. 1b).

Although the temperature of 12.5 °C was more permissible for the resuscitation and subsequent growth of sub-lethally injured E. coli O157:H7 cells it still provided an important extension in the lag phase of stressed cells defined as a sum of resuscitation time (Jasson et al., 2009) and the lag phase of healthy cells. This extension was shorter than at 10 °C, but longer than at 15 °C. Lactic acid-treated cells needed 12, 14, 16 and 20 days for the resuscitation at 12.5 °C, when gas mixtures of 100% N2, air, 30% CO2/70% N2 and 60% CO2/40% N2 were used, respectively (Fig. 1b). The temperature of 15 °C overrode the effect of atmosphere allowing faster resuscitation of sub-lethally injured cells. The growth was detected after 6 days when air and 100% N2 atmosphere were used, while at the gas mixtures of 30% CO2/70% N2 and 60% CO2/40% N2 the growth was initiated after 8 days (Fig. 1c).

Non-treated cells grew significantly faster than lactic acid-treated cells under all atmospheres at 10 °C and at 12.5 °C (p<0.05), with the latter having an exception under 100% N2. No significant difference between lactic acid-treated and non-treated cells was observed when the storage temperature of 15 °C was used (p>0.05).

3.2. Chlorine dioxide treatment

Growth of E. coli O157:H7 initially treated with liquid ClO2, inoculated in TSB-YE (pH 5.8) and stored under different atmospheres at 10, 12.5 and 15 °C is presented in Fig. 2. ClO2 treatment induced resuscitation and lag phase of 8 days prior the growth commenced under 100% N2 and air, whereas under 30% CO2/70% N2 this was 12 days, all at 10 °C. High CO2 concentration in the gas mixture of 60% CO2/40% N2 at 10 °C did not permit growth of ClO2-treated cultures in one repetition while in another repetition growth was noticed only after 20 days of storage. To avoid biased data interpretation based on the mean of two extreme values (no-growth vs. growth), both repetitions are presented in Fig. 2a.

When the storage temperature of 12.5 °C was used, ClO2-treated cells showed lag phase extension which was in the range from 4 to 8 days for all different atmospheres (Fig. 2b), being doubly longer than at 15 °C (Fig. 2c).

The difference between the ClO2-treated and non-treated cells was found to be statistically significant (p<0.05) only when the storage under atmosphere of 60% CO2/40% N2 and temperature of 10 °C was combined.

3.3. Neutralized electrolyzed oxidizing water treatment

Fig. 3 shows the growth of NEW-treated and non-treated E. coli O157:H7 cultures in different atmospheres at three storage temperatures (10, 12.5 and 15 °C). At 10 °C, air and 100% N2 atmosphere allowed growth of NEW-treated cultures after 12 and 16 days, respectively, while no growth occurred for other tested atmospheres. It is noticeable that CO2-enriched atmospheres did not reduce the number of E. coli O157:H7 present, with initial inoculation level of approx. 10⁵ CFU/ml being stable during 35 days of storage (Fig. 3a).

Treatment with NEW resulted in a delayed growth of 4, 6 and 10 days at 12.5 °C when treated cells were stored under the atmosphere of 100% N2, air and 30% CO2/70% N2, respectively. In the case of 60% CO2/40% N2, the growth commenced only after 14 and 20 days for two repetitions (both growth curves are shown in Fig. 3b).

The results obtained at 15 °C indicated considerably shorter lag phases than ones obtained at lower temperatures. A delay of 4 and 6 days in growth onset was observed under atmospheres of 30% CO2/70% N2 and 60% CO2/40%, respectively, while under 100% N2 and in air, the observed delay was 2 days (Fig. 3c).

The significant difference between NEW-treated and non-treated E. coli O157:H7 cells was determined for the atmospheres of 60% CO2/40% N2, 30% CO2/70% N2 and air at 10 °C (p<0.05), and for 60% CO2/40% N2 at 12.5 °C (p<0.05). Analyses have shown that all
Fig. 1. Growth of lactic acid-treated (open symbols) and untreated (closed symbols) E. coli O157:H7 in TSB-YE (pH 7.0) at 10 °C (a), 12.5 °C (b) and 15 °C (c). The results present the colony count on non-selective media (TSA-YE). Error bars represent standard error of the mean (n = 2). ○ – 0% CO₂/100% N₂; ▽ – 30% CO₂/70% N₂; □ – 60% CO₂/40% N₂; △ – air.
Fig. 2. Growth of ClO₂-treated (open symbol) and untreated (closed symbols) E. coli O157:H7 in TSB-YE (pH 5.8) at 10 °C (a), 12.5 °C (b) and 15 °C (c). The results present the colony count on non-selective media (TSA-YE). Error bars represent standard error of the mean (n = 2). ○ - 0% CO₂/100% N₂; ▽ - 30% CO₂/70% N₂; □ - 60% CO₂/40% N₂; ○ - air. Exceptionally for 10 °C: □ - 60% CO₂/40% N₂ (repetition 1); Δ - 60% CO₂/40% N₂ (repetition 2).
Fig. 3. Growth of NEW-treated (open symbol) and untreated (closed symbols) E. coli O157:H7 in TSB-YE (pH 5.8) at 10 °C (a), 12.5 °C (b) and 15 °C (c). The results present the colony count on non-selective media (TSA-YE). Error bars represent standard error of the mean (n = 2). ○ – 0% CO2/100% N2; ▽ – 30% CO2/70% N2; □ – 60% CO2/40% N2; ○ – air. Exceptionally for 12.5 °C: □ – 60% CO2/40% N2 (repetition 1); Δ – 60% CO2/40% N2 (repetition 2).
other observed differences were above the threshold of statistical significance ($p > 0.05$).

### 3.4. Sub-lethal injury

The range of percentages of sub-lethally injured cells determined for all three different decontamination treatments and their evolution under different storage conditions is shown in Table 1. Obtained values were compared to a threshold value of 48% (Jasson et al., 2007), compensating for a fraction of healthy population that is not able to grow on CT-SMAC, while growing on TSA-YE. The results for day 0 indicate that lactic acid treatment induced almost 100% sub-lethal injury in *E. coli* O157:H7 cultures, while ClO2 and NEW led to 76.8% and 83.9% of sub-lethally injured cells in the population, respectively. At all times, the percentage of sub-lethal injury for non-treated cultures was below the statistical threshold value of 48% (Jasson et al., 2007) (data not shown).

#### 4. Discussion

Formation of the sub-lethally injured fraction of cells is a recognized downside of sub-lethal decontamination (Wu, 2008). However, the impaired intercellular wellbeing can be utilized to achieve further control over a food-borne pathogen. In the present study, the effect of sub-lethal decontamination on the lag phase duration and subsequent growth of *E. coli* O157:H7 was quantified under different sub-optimal conditions. Data obtained reveal that the effect of decontamination treatment is not terminated with the end of the treatment procedure.

A fraction of sub-lethally injured *E. coli* O157:H7 undergoes an additional inhibitory effect during the storage period under sub-optimal conditions throughout which secondary stress extends the time needed for the recovery and multiplication. Certain stress conditions can even provide a supplementary bactericidal effect. Observed extension in the lag phase, under constraints of lowered atmosphere.

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</table>

+++ > 80% sub-lethally injured; ++++, 60~80% sub-lethally injured; ++, 48~60% sub-lethally injured; +, <48% sub-lethally injured; n.d., not determined.
pH, lowered temperature and enriched CO₂ atmospheres, was a direct consequence of prior sub-lethal injury. For all conditions tested, the direct relationship was established between the extension of lag phase and extent of sub-lethal injury. Moreover, the duration of the lag phase for the injured cells was found to be dependent on the type of decontamination, i.e. type and extent of injury. The effects of liquid ClO₂ and NEW were less pronounced in comparison to lactic acid. Lactic acid treatment induced cells almost 100% sub-lethal injury and has consequently severely delayed growth during sub-optimal storage in comparison to ClO₂ and NEW treatments that caused 76.8% and 83.9% of sub-lethal injury, respectively. Although later percentages are above previously defined threshold values of statistically significant injury of 48% (Jasson et al., 2007), the absolute numbers expressed in CFU/ml behind them diminish biological significance and the resulting impact on the growth of the total population. The differences in resuscitation time could be influenced by the level and the type of the injury (Beales, 2004; Huang et al., 1997; Liao et al., 2007). The type of the injury is not only treatment dependent, but also organism dependent, which may explain why E. coli O157:H7 in this study reacted differently than did Listeria monocytogenes to lactic acid and ClO₂ treatments (Rajkovic et al., 2009). The severity of injury caused by lactic acid was demonstrated by complete inactivation of treated cells upon inoculation in the medium of pH 5.8, whereas non-treated cells could multiply under these conditions. The change of the stressful pH 5.8 to more favorable pH 7.0 allowed a better survival of injured cells. However, the lag phase of lactic acid-treated cells in pH 7.0 medium remained longer when compared to ClO₂- and NEW-treated cells in the medium of pH 5.8. E. coli O157:H7 cells are not able to multiply and grow at temperature lower than 7 °C (Anon, 2006b). However, temperature abuse that may occur at retail and wholesale level can enhance the growth of this food-borne pathogen (Tamplin, 2002). In our study three abusive temperatures were used in order to determine their influence on the growth of E. coli O157:H7 cells which survived sub-lethal decontamination treatments. As expected, the results revealed that the storage temperature of 10 °C applied for all decontamination treatments and all atmospheres provided the longest lag phases, followed by 12.5 °C and 15 °C. The small differences in temperature imposed an important influence on the bacterial ability to grow. Apart from the well-known bacteriostatic effect of temperature on the bacterial growth, the solubility of CO₂ increases as temperature decreases (Genigeorgis, 1985; Phillips, 1996). It is important to note the valuable preservation effect that atmospheric concentrations of O₂ in the air provided by imposing lethal effect on injured cells within 14 days of storage at 10 °C in the case of lactic acid treatment. In the same period MAP only provided delayed growth. The lethal effect of 60% CO₂ appeared much later, indicating that injury caused by lactic acid rendered cells more sensitive to the oxidative stress than to the antimicrobial effects of CO₂ when stored at 10 °C. Oxygen, which might be toxic for the cells at elevated concentration, can induce activation of intercellular protective mechanisms such as enzymatic activity of oxygen dismutase and catalase (Gregory and Firdovic, 1973). It is possible that tested injured E. coli O157:H7 in the present study was not able to activate these systems in the presence of airborne oxygen (20.9%). Golden et al. (1989) and Bromberg et al. (1998) also reported that heat-injured Aeromonas hydrophila and E. coli O157:H7 grew faster when exposed to the atmosphere containing 100% N₂ than air. 

The storage at 12.5 °C, apart from extending the lag phase, also induced an initial reduction (log CFU/ml) of lactic acid injured cells during 10–15 days before the multiplication occurred. However, the same phenomenon was not seen for ClO₂- and NEW-treated cells where the number of cells remained constant throughout the lag phase. As previously mentioned, this may be explained by different level and nature of sub-lethal injury caused by prior decontamination treatments applied (Beales, 2004; Jasson et al., 2007; Manas and Pagan, 2005).

In conclusion, the results obtained in this study indicate that repair of sub-lethally injured E. coli O157:H7 can be significantly delayed with the appropriate combination of the preservation conditions (temperature, pH and appropriate gas mixture in the packaging) and sub-lethal decontamination treatment inducing a significant percentage of sub-lethal injury. The post-treatment behavior of sub-lethally injured cells should be taken as a parameter in the predictive modelling and risk assessment studies as the respective outcome will be different in comparison to the current studies in which healthy cells were used.

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