Studies on Some Microbial Safety Aspects of Elopak Gable Top Cartons Intended to Be Used in Low Acid Aseptic Applications

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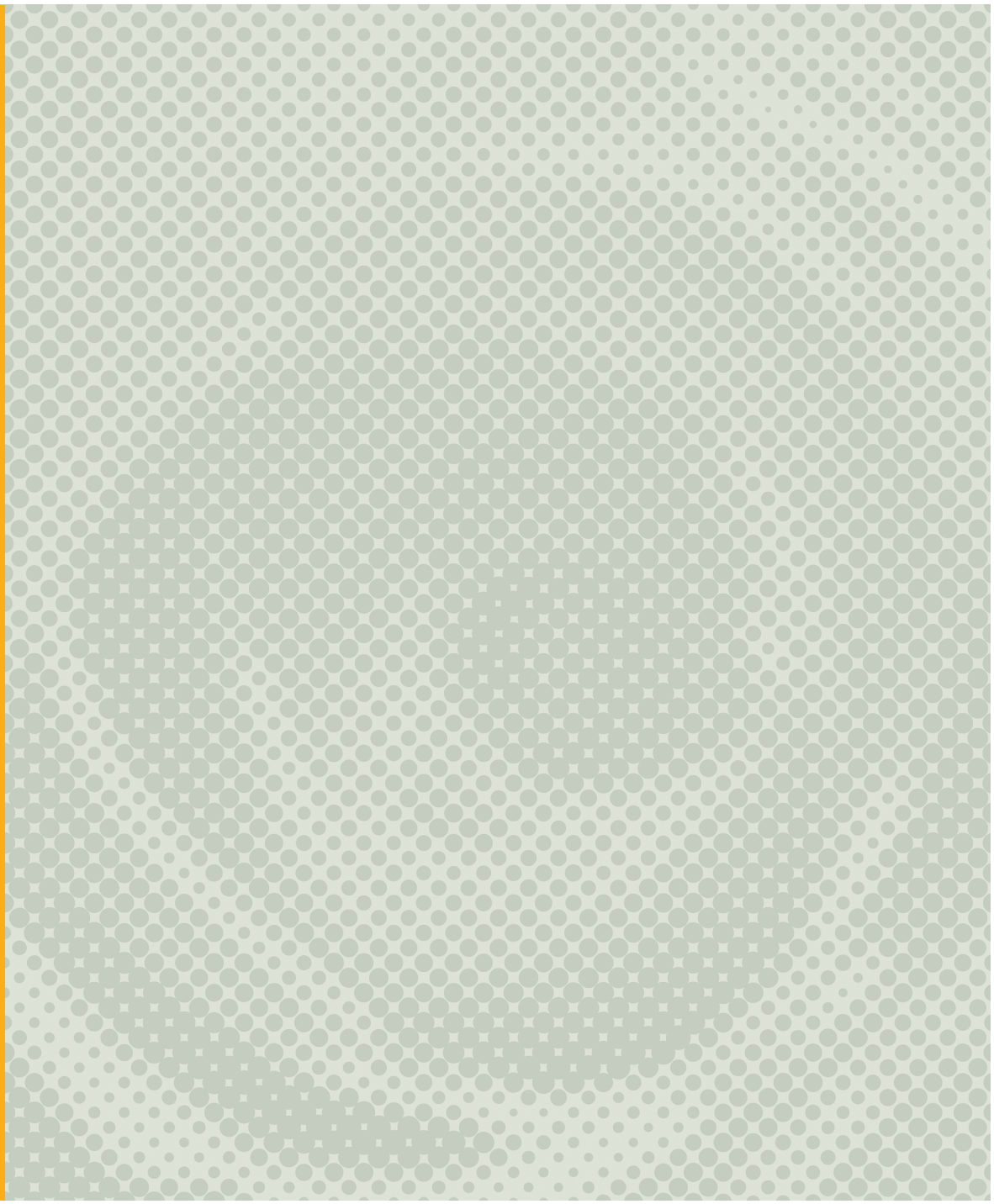

NORWEGIAN UNIVERSITY OF LIFE SCIENCES

Studies on some microbial safety aspects of Elopak gable top cartons intended to be used in low acid aseptic applications.

By

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"Until you try, you don't know what you can't do."

Henry James

BC 52 DIAMOND CURVE

GABLE TOP CARTONS

EXECUTIVE SUMMARY

In developing a low acid aseptic system, much attention is given to the efficiency of the sterilization system and the microbiological quality of the packaging materials. Considering that low acid foods are very vulnerable if contaminated by a variety of microorganisms, the microbiological quality of the packaging material (carton) is considered a critical factor in determining the success of the whole process.

This thesis ascertain critical microbiological aspects of the Elopak gable top carton which will be used for filling low acid aseptic liquid products by the low acid aseptic filling machine currently under development at Elopak AS. Due to the unrelated nature of the different topics studied in this thesis, the thesis is detailed in four different sections;

Section one is based on investigations that were carried out during the optimization phase of the sterilization system. It investigates structures in the carton that contributes in making decontamination in the corners very hard to achieve.

The nature of the corners was determined by electron microscopy. Attention was also paid to how spores were distributed in the corners. A model (sterilization rig) of the newly developed sterilization system was used to evaluate kill rate in three types of cartons with different corner topology. Comparative analysis by Electron microscopy was also done between the corners of the Elopak gable top aseptic carton and corners in cartons from a company with a commercially functional low acid aseptic processing system, carrying out carton sterilization in a similar way to that used by Elopak.

The investigation showed that the Elopak gable top carton had numerous structures of different morphologies in the corners. The cartons from the competitor company had fewer structures which were far wider than those present in the Elopak carton. Kill rates in cartons with no structures in the corners were higher than in cartons with structures in the corner.

This section concluded that the structures in the Elopak gable top carton were partially responsible for the low decontamination observed in the corners, and smoothening of these corners would increase decontamination in them. Comparative analysis revealed that the competitor company avoided low decontamination at the corners by maneuvering corner topology in their cartons.

The next sections pay more attention to microbiology related aspects of the Elopak gable top carton.

In section two, the type of spore formers found in paperboard and those contaminating carton blanks after production were identified. Spore formers in dust samples in the production premises were also of interest in this section.

Paperboard and dust samples were homogenized and plated on agar plates. Flat blanks were exposed in blank storage halls at the convertor plant, and contact plates used to grow the contaminating spores on these flat blanks. Some colonies from the different samples were selected based on unique colony morphology, and their 16s rRNA genes were sequenced to identify them.

In this study, the bacteria isolated from paperboard and those identified as contaminants on flat carton blanks were of *Bacillus spp. Clostridium spp.* were also found to be present in dust samples. 16s rRNA gene sequencing hinted that *Clostridium botulinum* might be present in the dust samples.

The presence of *Clostridium botulinum*, a lethal food pathogen which will readily grow in low acid food foods under favorable conditions was speculated in dust samples within the Elopak converter plant by results from section 2. This necessitated the need for a proper investigation of the incidence of *Clostridium botulinum* in dust samples. Section 3 therefore investigated the presence of botulinum neurotoxin genes in the dust samples from which 16s rRNA gene sequencing gave hints of the presence of *Clostridium botulinum.*

The dust samples were inoculated in nutrient broth to enrich for *Clostridium botulinum*. Primer sets specifically designed to target and amplify botulinum neurotoxin genes where used in a real-time polymerase chain reaction to detect the presence of these genes in the enriched dust samples.

Clostridium botulinum serotype B and F were found to be present in dust samples from the boxing area and rail in front of the entrance hall respectively.

In the fourth section, the risk of potentially harmful bacteria in paperboard crossing through tiny holes in the layers laminating the paperboard to contaminate products stored in the carton was studied.

Holes about four times bigger than the micro holes found in the laminating layers of the gable top carton were artificially made in the layers laminating paperboard in the gable top carton blanks. The perforated carton blanks were then sterilized and filled with aseptic validation medium (AVM) using the new low acid aseptic filling machine. The filled cartons were incubated at 30 ^{0}C for 5 weeks.

No growth in medium was observed after incubation, suggesting that bacteria might not be able to migrate through the micro holes present on the laminating layers, or that more time would be needed for bacteria to pass through them.

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ABREVIATIONS

Alu	Aluminum
AVM	Aseptic Validation Medium
CDC	Center for Disease Control
DRCA	Differential Reinforced *Clostridium* Agar
DRCM	Differential Reinforced *Clostridium* Medium
FDA	Food and Drug Administration
HP	Hydrogen Peroxide
LCR	Log Cycle Reduction
PE	Polyethylene
PCA	Plate Count Agar
PCR	Polymerase Chain reaction
RCA	Reinforced Clostridia Agar
RCM	Reinforced Clostridia Medium
TPGY	Tryptone-peptone-Glucose-Yeast extract
VPHP	Vaporized hydrogen peroxide.

Definitions

Decontamination: This is the process of reducing the microbial load on a surface or in an environment, but does not go as far as eliminating the total microbial load, as is the case with sterilization.

Commercial sterility: This refers to the absence of microorganisms capable of reproducing in the food under normal non-refrigerated conditions of storage and distribution, as well as being free of all viable microorganisms (including spores) of public health significance.

Flat bed blanks: Flat blanks whose scorelines are created by pressing the already cut flat blanks on a flat surface, with a scoring tool. This is unlike the normal way of scoring laminated paperboard rolls by rolling through a scoring machine.

Pinholes: Micro holes in the layers laminating the paperboard

Sealing area at carton bottoms: area below the bottom horizontal scorelines of the carton blanks that is activated and sealed during bottom sealing of carton blanks to form cartons

Scorelines: lines or depressions created on laminated flat blanks that will help the flat blanks fold into cartons.

Skived cartons: Cartons in which the raw paperboard edges will not be in direct contact with the product.

Unskived cartons: Cartons in which the product will be in direct contact with the raw paperboard edges.

1 General introduction

Food is a suitable ecological niche for many microorganisms. Some of the microorganisms which inhabit food cause food deterioration over time, releasing their metabolites in food and or other substances to ease their metabolism of the food. Some of these metabolites and substances are toxic to humans. Some of the microorganisms that inhabit food are themselves pathogenic. Storage of food at ambient temperatures requires that these organisms be eliminated from the food, and the food protected from recontamination by these microorganisms. To a manufacturer of shelf stable foods, success in production is achieved when product sterility is maintained throughout the shelf life of product (Placencia et al, 1986). This is achieved by sterilizing food after packaging or packaging it aseptically (Gidney, 2000).

Aseptic packaging is the process of filling commercially sterile food into decontaminated packages under sterile condition, with proper hermetic sealing of package after filling (Elopak, 2011). The role of the packaging material in aseptic packaging cannot be underestimated. The packaging guarantees food quality by protecting it from microorganisms and physically or chemically induced changes (Rahman, 1999). Aseptic packaging in paperboard based cartons has a proud history of its contribution to food safety due to its compliance to guidelines for food grade paperboard (May 1994).

But what is the borderline between a high quality aseptic package and a low quality aseptic package? This question has been a topic for review over the years, especially in the packaging of foods with low acid content; which are most vulnerable to bacterial contamination (Tanner, 1940, Sanborn 1945; May, 1994).

According to the Food and Drug Administration (FDA), the paperboard in aseptic an carton should contain less than 250 colony forming units (CFU) per gram of the homogenized paperboard, and the food contact surface of such a carton should contain 50 colonies for 1 L cartons, and 10 colonies for cartons carrying less than 100 mL, as determined by the rinse method (FDA, 1993) “But is total count a real measure of sanitary quality?” (May, 1994). Tanner, (1940) suggested that counts, as well as bacteria species should be taken into consideration. He argues that 100000 harmless saprophytes in paperboard present less danger than 10 harmful pathogens. May, (1994) suggested that current microbiological standards for

food grade paperboard packages needed some readjustments. Currently, no universally agreed standards exist on determining the microbiological quality of carton blanks.

At Elopak AS, a company producing carton based package filling systems, and gable top cartons for the filling of non carbonated liquid foods, the carton blank microbiological quality standards have been drafted based on the FDA's recommendations with slight modifications, making Elopak's standards even more austere.

Current research at Elopak is aimed at developing a filling system for low acid liquid food products. To fulfill the conditions required for low aseptic liquid food filling in paperboard cartons, the company faces 2 major challenges with respect to the carton sanitation;

- To develop a package sterilization system capable of a log 5 cycle reduction of spores of *Bacillus atrophaeus* in the aseptic cartons.
- To evaluate the microbiological qualities of the current aseptic cartons and determine the risk it poses in the newly developed low acid aseptic processing chain.

On the model sterilization rig, an optimal sterilization condition with a kill rate of Log 5 for spores of *Bacillus atrophaeus* in cartons is being sought by varying those parameters which affect sterilization conditions in the system, while monitoring the kill rates in cartons artificially inoculated with spores of *Bacillus atrophaeus.* At the end of the optimization process, the setting with the best kill rate, i.e. ≥ LCR of 5 for spores of *Bacillus atrophaeus* will be use to validate the sterilization system of the newly developed machine. Thus far, the required kill rates of Log 5 for spores of *Bacillus atrophaeus* has been achieved in certain areas in the carton, but in certain areas, the kill rate is still at log 3, (Cold spots), notably, the bottom corners in the carton.

The present focus is now on understanding the cold spots to help solve the problem of low decontamination in them, and evaluating the microbiological quality of carton blanks, as well as carrying out an effective risk assessment of the new low acid aseptic processing system.

The aim of this thesis was to

- Provide detail understanding of cold spots

- Determine the bacterial types present in paperboard used for producing Elopak aseptic blanks, and on the blanks sheet surfaces exposed to ambient air during their production
- Investigate the incidence of *Clostridium botulinum* in blank production environment.
- Determine the risk of product exposure to contaminating bacteria in paperboard through micro holes present in the layers laminating the paperboard in aseptic cartons

Due to the disparities in the different topics covered in this thesis, the thesis will be presented in four different sections.

SECTION 1

Structures that Structures that reduce the efficiency of sterilization at the bottom corners in the Elopak gable top cartons.

Introduction.

Aseptic packaging of low acid foods requires a high level of decontamination to be achieved in all parts of the food package. Different systems such as the PET system, web-based systems etc. accomplish this requirement in different ways. In the web-based process of package sterilization, rolls of flat blanks are passed through 35% of hydrogen peroxide at about 80°C before forming them into cartons. In such a process, an even decontamination is achieved on all surfaces in the carton. For systems carrying out decontamination in preformed cartons, attaining even decontamination in all areas of the carton is a greater challenge. Preformed cartons have different geometric structures such as folds and gaps within them. According to Sigwarth and Staek (2003), and Rogers et al (2005), the ability of hydrogen peroxide (HP) to inactivate spores on a surface is partially depended on surface topography, porous (rough) surfaces will reduce decontamination efficiency. Another factor that contributes to this challenge is the hydrophobicity of polyethylene (PE). The wetting ability of hydrogen peroxide on PE is low. When liquid HP is sprayed in a carton, it condenses and forms tiny droplets on the surface with little or no spreading (Knaup, 2011). This, in addition to the complexity of the surface geometry, reduces the evenness of hydrogen peroxide distribution on the surface of the carton even more. This reduces the balance in

decontamination in the carton. As a result, inactivation of spores is greater in certain parts within the carton than others. Regions with the lowest level of decontamination are termed cold spots. For a package to be fit for usage for low aseptic filling, adequate inactivation of spores is recommended in these cold spots.

When developing a new sterilization system, or optimizing an existing one, worst case scenarios of contamination in cold spots are often mimicked in a challenge test to demonstrate the efficiency of the system in decontaminating cartons. During such a test, machine parameters such as temperature, sterilant concentration, airflow rates, etc., which influence decontaminating conditions in a system are varied, and the setting with an optimal inactivation in the cold spots is then adopted to validate the new system.

The newly developed low aseptic sterilization system at Elopak is designed to work with totally vaporized hydrogen peroxide. Gaseous HP is more evenly distributed than fine condensates on a surface. Besides distribution, gaseous hydrogen peroxide is able to access different areas more easily compared to the condensates, due to its higher diffusion coefficient (Mcmurtrie and Keyes, 1948).

Using vaporized hydrogen peroxide (VPHP) has limited the cold spots to the bottom of the Elopak gable top (Knaup, 2011). However, some food researchers recommend that a log 5 cycle reduction (LCR) of resistant spores be achieved in the whole carton before low acid aseptic food filling in them (Moruzzi, 2008).

Three main reasons may account for the corners being cold spots;

- Inappropriate flow of VPHP at the bottom of the cartons.
- Condensation in the corners of the carton.
- Structures in the corners of the carton.

According to Ansari and Datta (2003), increasing heat and sterilant concentration will increase the efficiency of a sterilization system. This means that increment in energy and chemical more than is currently being used might be needed to attain the required inactivation in the cold spots of the gable top cartons. However, the current optimization of the low acid aseptic sterilization system in Elopak seeks to reduce energy and chemical consumption by the system, while maintaining high decontamination efficiency. Much is being done regarding

manipulating machine parameters, in order to find a setting with a high decontamination capability using less energy and chemical than is currently being used. Surprisingly, little attention has been given to the carton itself, even though the cold spots in the carton are directly linked to this challenge. The aim of this study was to observe the nature of cold spots and to investigate their effects on spore inactivation. This will give a better understanding of the cold spots and shed light on what can be done to increase decontamination in them. The hypothesis is that the presence of structures such as holes and cracks in which microorganisms can hide in the corners directly contribute to this problem. Structures in the corners and/or micro condensation are responsible for the low decontamination observed in these areas. Further, a comparison of internal features at the bottom will be made with cartons produced by a competitor company. We will also try to look at the distribution of spores within these structures, which might shed light on how they escape inactivation

2 Background.

2.1 Antimicrobial effects of hydrogen peroxide and Vaporized hydrogen peroxide.

Hydrogen peroxide (HP) is widely used for disinfection, sterilization and antisepsis purposes, and is the only chemical sterilant recognized by the FDA for decontamination of packaging materials (Ansari and Datta, 2003). HP has a broad spectrum efficacy against viruses, bacteria, yeasts and bacteria spores (Block 1991). A number of factors influence the antimicrobial properties of HP, including concentration and temperature (Russell, 1990). The decontamination activity of hydrogen peroxide increases significantly with increasing concentration and temperature (Smith and Brown 1980; Toledo et al., 1973). At a concentration of about 6%, HP shows bactericidal activity, but only slightly sporicidal (Russell, 1990). HP alone as a molecule has no antimicrobial activities, but produces short lived oxidative intermediates such as singlet oxygen, superoxide radicals and hydroxyl radicals during its dissociation to oxygen and water. These intermediate radicals destroy almost all important cellular components (Davidson and Branen, 1993). The hydroxyl radical causes damages to all essential cell components: carbohydrates, nucleic acids (mutations), lipids (lipid peroxidation) and amino acids (e.g. conversion of Phe to m-Tyrosine and o-Tyrosine (Reiter et al., 1995). Davidson and Branen (1993) found that hydrogen peroxide was most biocidal against anaerobic and gram negative bacteria. According to Maillard et al., 2010, vaporized hydrogen peroxide (VPHP) is said to have a greater antimicrobial efficacy than liquid HP. They speculated that this was due to the fact that VPHP could diffuse through

the 3 dimensional structures to oxidize buried residues in the interior of protein molecules. Exactly how HP inactivates spores is poorly understood (Khadre and Yousef, 2001). Low sporicidal activity of HP at low concentrations has been attributed to associated spore catalase which destroys HP in the vicinity of the spores (Cerf and Metro, 1977; Russell, 1990). Setlow and Setlow (1993) found no unusual DNA damage in surviving *Bacillus subtilis* spores that were treated with HP. Shin et al. (1994) noticed that *Bacillus subtilis* spores treated with HP had damaged spore coats and cortex. Despite obvious damages to spore coat and cortex by HP, Setlow and Setlow (1993) believe that spore inactivation was partially caused by DNA damage by oxidative radicals.

2. Technical information.

2.1 Functionality of the sterilization system rig.

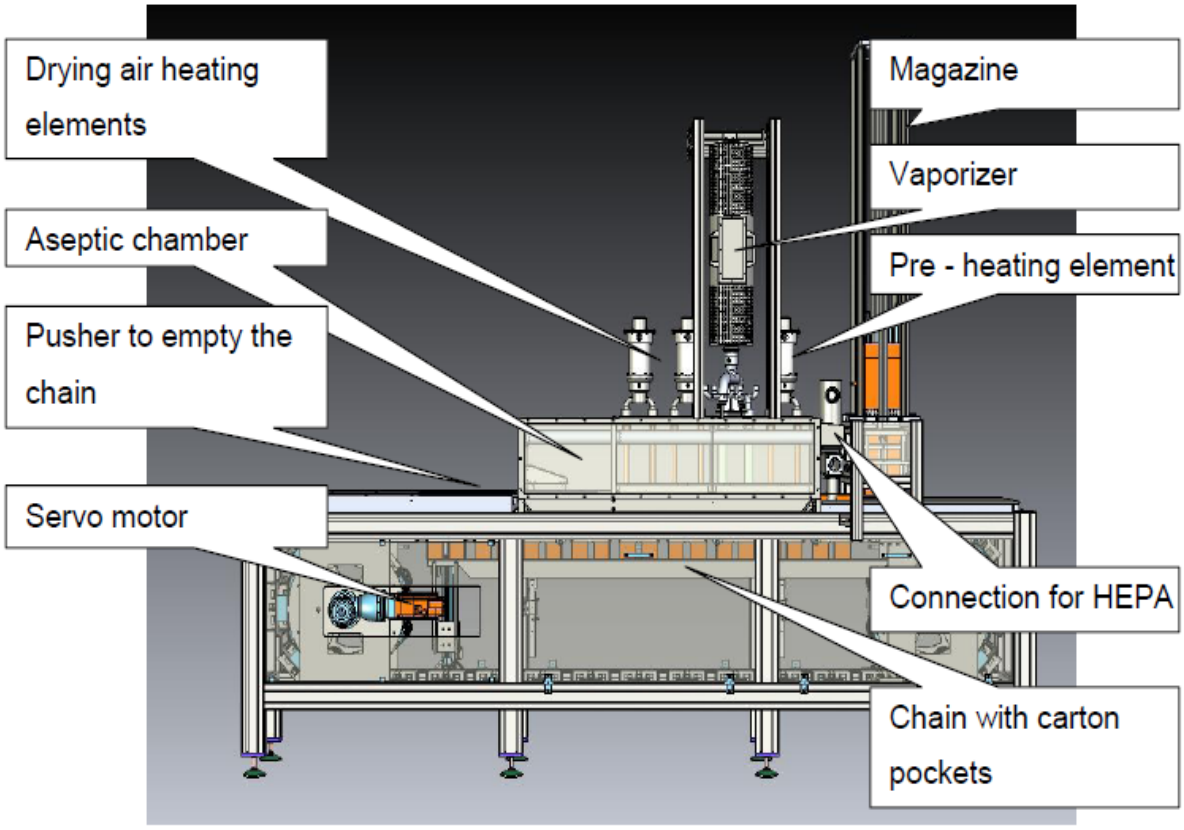


Figure 2.1 Annotated diagram of the sterilization rig A model version of the newly developed sterilization system for low-acid aseptic filling machine (Knaup, 2011)

During carton sterilization, bottom sealed cartons are fed into the aseptic chamber, by placing them in the chain pockets at the end containing the magazine. Upon introduction in the aseptic

chamber, hot air from the pre-heating element is blown into these cartons. The temperature and the rate of flow of the air can be changed by adjusting the heating elements and the nozzle size through which the hot air flows into the carton. After heating, the carton then moves to the peroxide station where it is sprayed with VPHP (35%), and then to the drying station where hot air is again blown into the carton to remove the peroxide residues. At the end of the aseptic chamber, the sterile carton is pushed out of the chain pockets.

2.2. Aseptic blanks.

Aseptic blanks are rectangular sheets paperboard laminated with very thin layers of aluminum and polyethylene which are folded along printed scorelines to form a carton.

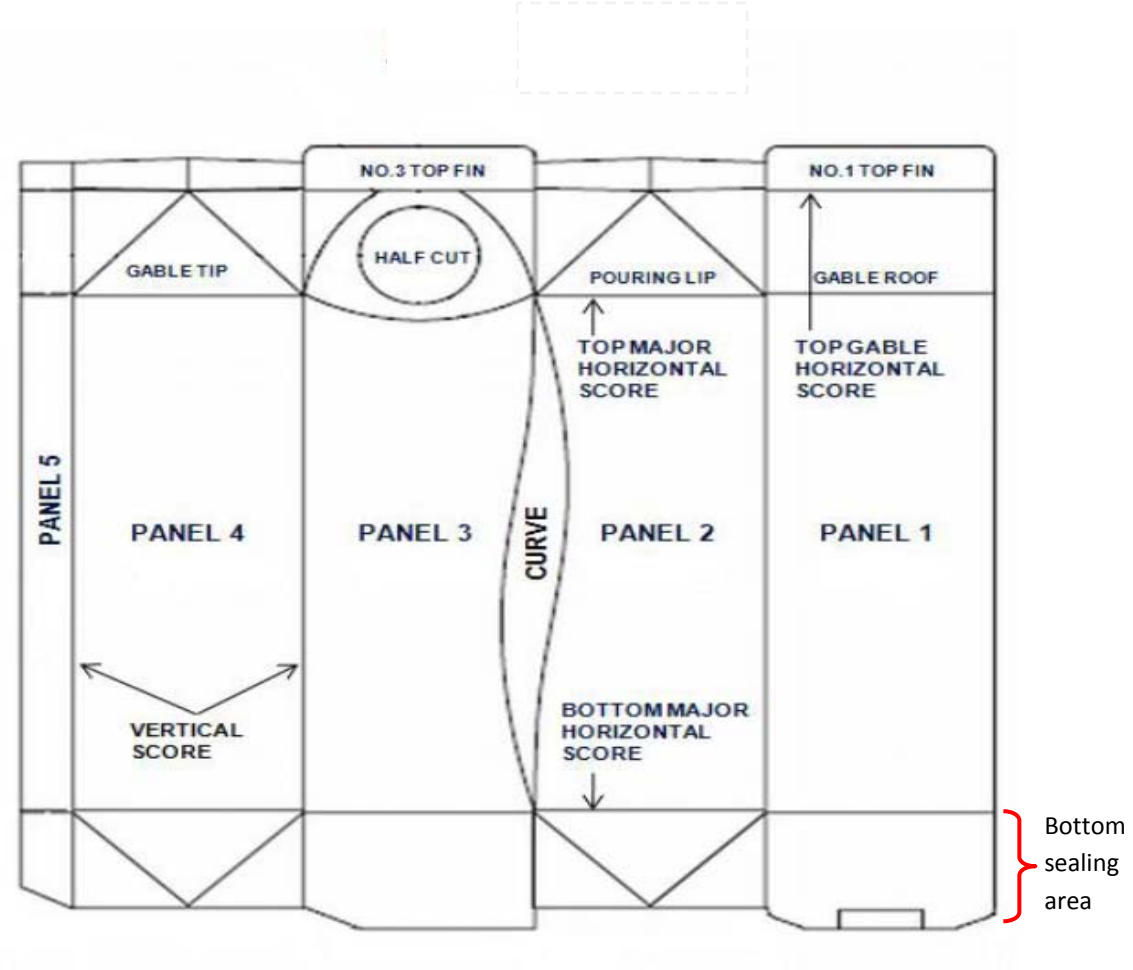


Figure 2.2 Carton blank showing scoring lines. Cartons are formed by folding blanks along the scorelines indicated above (Knaup, 2011)

2.3. Log cycle reduction (LCR).

LCR is a parameter used to evaluate the efficiency of a sterilization system. It can be defined as the log load of spores a sterilization system will reduce to one spore. For example, a system with an LCR of 4 would reduced reduce 10,000 spores (log4 spore load) to 1 spore. This means that in a decontamination process, a system with an LCR of 4 will most likely only spare 1 spore out of 10,000 spores. Assuming that a blank contains one spore, it means such a sterilization system in theory would only fail to sterilize 1 carton out of 10,000 cartons.

3. Materials and methods.

3.1 Materials

Chmical/ material	**Supplier**
Gable-top carton; diamond curved BC 52	Elopak AS, Norway.
Bacillus atrophaeus ATCC 51189/DSM 2277	Fraunhofer- Institut fur Verfahrensechnik und Verpackung , Germany.
Ethanol (96%)	Kemetyl Norge AS, Norway.
Hydrogen peroxide 35% Oxypac S.	Ecolab AS, Norway.
Plate Count Agar	Merck KGaA, Germany.
Temperature logger DQ 1862	Datapaq Ltd, United Kingdom
Sputter coater	Quorum Technologies, UK
Scanning electron microscope	Zeiss EVOI-50-EP SEM (Carl Zeiss SMT Ltd, U.K

3.2. Methods

3.2.1. Electron microscopy.

Serial dilutions of log4, 5 and 6 spores' ml^{-1} in 96% ethanol were made from a stock solution of *Bacillus atrophaeus*.

The nature of 72 corners from 18 cartons was viewed in this experiment; 13 Elopak aseptic gable-top cartons, 3 of which were inoculated with spores of *Bacillus atrophaeus* and 5 cartons from the competitor company. The 3 preformed Elopak aseptic gable-top cartons were inoculated by pipetting 10 µl of spore suspension containing log 4 spore loads per ml in the bottom corners, at the crossing point of the vertical and the horizontal scorelines. Inoculated cartons were then bottom sealed after bottom sealing of the 10 uninoculated cartons. The 5 cartons from the competitor company were purchase filled with product. The product was discarded, and cartons thoroughly cleansed by gentle shaking warm clean water in them. Only those with intact corners were considered in this experiment.

Cutting of corners from the bottom of the gable top cartons was done very gently with a hot blade in order to maintain the original nature of the carton corners.

The cut corners were placed on Scanning Electron Microscope (SEM) sample holders using conductive adhesives. They were then mounted into a Sputter coater, and their surfaces coated with a very thin layer of platinum. This was done in order to conduct electrons away from the surfaces of the cartons when observing in an EM, as accumulation of elections will result in blurring.

After coating, the carton corners were observed under a SEM at different magnifications.

3.2.2: Sealing of structures at the corners

The inert substance- epoxy was used to create thin films on the inner surfaces of the cartons, sealing off the cracks and the micro holes found in the corners.

To examine the effects of structures in the corners on decontamination, three set of cartons with different internal bottom surface geometry were used; 20 Elopak aseptic gable top cartons, 20 "test" aseptic gable top (flat bed blanks) cartons with inverted (inward) scorelines,

produced at the test center in Spikkestad-Norway and 20 Elopak aseptic gable top cartons with the bottom corners and horizontal scorelines sealed with a thin film of epoxy. The corners were sealed by applying 3 ml of epoxy into bottom sealed cartons and swirled to create a thin film in the bottoms of the cartons. The cartons with epoxy were allowed to rest for 10 min at room temperature before heating for 20 min in an oven maintained at 60 ^{0}C.

All sample cartons were then inoculated with *Bacillus atrophaeus* spore suspension. 6 cartons per log load were inoculated in each sample group by pipetting 0.5ml of the spore suspension into the bottom of the cartons while swirling the carton bottom in a circular manner, distributing the spore suspension at the bottom to all four corners. Swirling was carefully done making sure that all corners got sufficient amounts of suspension over them. Then the cartons were allowed to dry over night under a laminar flow cabinet.

3.2.3. Sterilization of carton samples.

Carton decontamination was carried out in the sterilization system rig (Figure 2.1) after drying of spore suspension in the cartons. Cartons with the lowest log load were sterilized first. Table 2.1 below shows the setting that was used in this experiment.

Table 3.1 Parameter settings for the sterilization test rig.

H2O2 flow (l/hr)	*evaporator air flow(l/min)*	*Vaporizer concentration (%)*	*Evaporator temp (^{0}C)*	*H2O2 nozzle size (ø)*	*Preheat temp&flow rate (^{0}C&m^3/hr)*	*Exposure time(sec)*
1.6	*160*	*4.66*	*280*	*6*	*230/80*	*2.4*

3.2.4. Agar overlay

After carton sterilization, the cartons were cut aseptically about 5cm from the bottom. Molten glue was placed round the corners in order to limit the agar to the corners. Upon solidification of the glue, Plate count agar (PCA) was poured into the glue frame, just enough to cover the corners (see figure 3.1a and 3.1b). Samples were left in a laminar flow cabinet for PCA to solidify. Using a sterile loop, small channels to provide oxygen to surviving bacteria were made through the agar into the corners. The cut bottoms, placed on sterile flat blanks were put in racks. Racks were placed in plastic bags and incubated at 30 ^{0}C for 3 d.

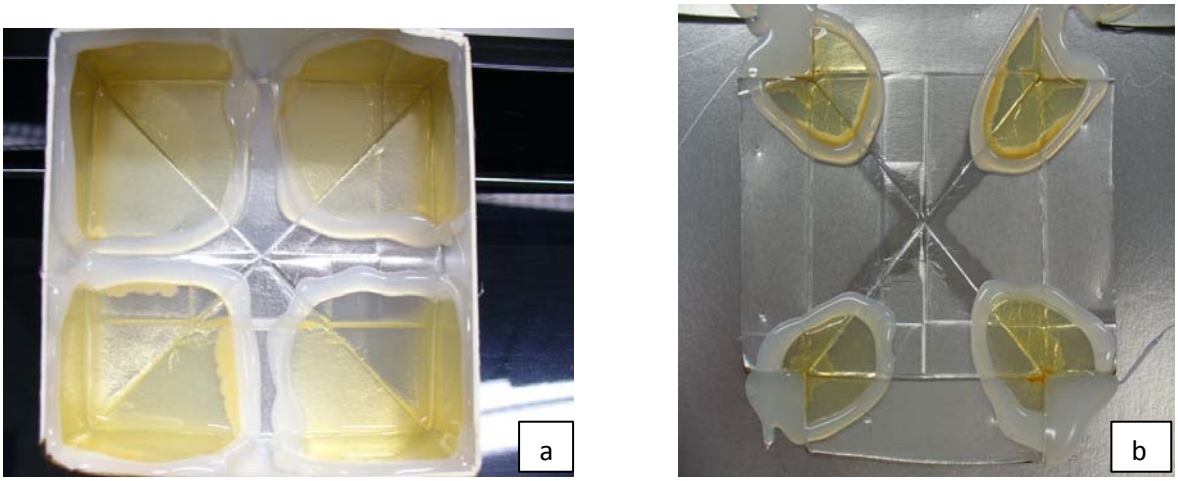


Figure 3.1 Cut carton bottoms with agar on corners. Note the glue frame around the corners restricting agar to the corners. a: Intact bottom (new method). b: Flat bottom (previous method). Growth on agar indicates survival of spores after d the decontamination process.

3.2.5 Heat pattern of Bottom sealer.

Datapaq logger DQ 1862 (Heat sensors), were placed at specific positions at the bottom of a side sealed blank using adhesive tape (Figure 3.2). This side sealed blank containing sensors was placed on mandrel of the bottom sealing rig. The rig was programmed for heat activation of the polyethylene layer only, without application of pressure for sealing. Then after activation, the temperatures at positions where sensors had been place were read off the sensors, and the heat pattern of the bottom sealer was determined.

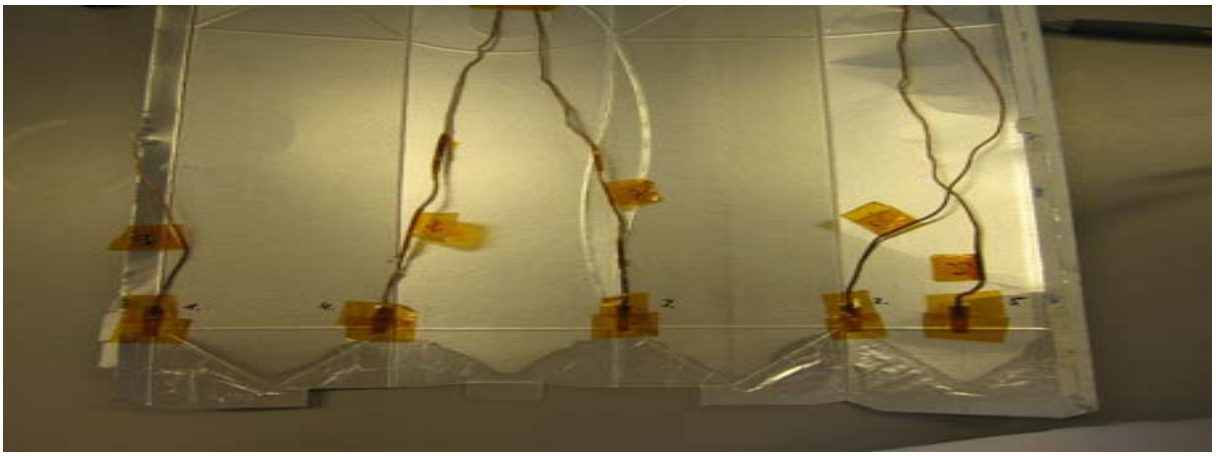


Figure 3.2 Datapaq logger positioned at different positions in the bottom of a carton to determine the heat pattern of the bottom sealer.

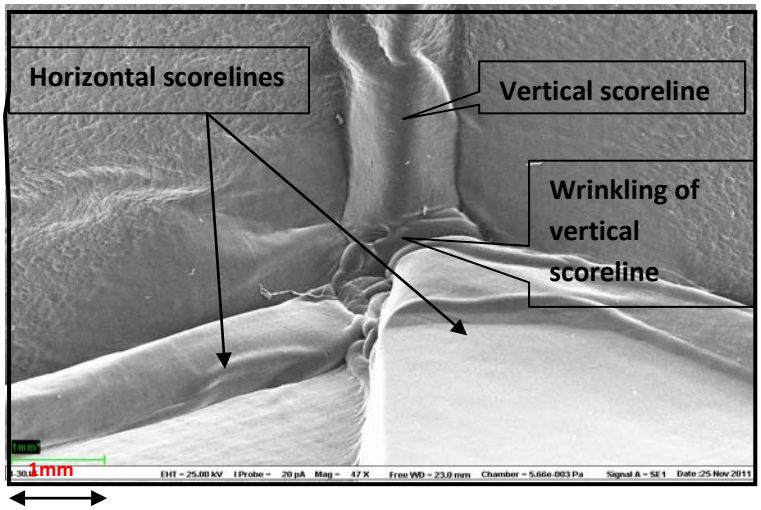
3.2.6. Controls for “Crack sealing” experiment.

For the positive controls, two cartons in each sample type were inoculated as described in section 3.2.2. The cartons were run through the sterilization rig 30 min after Hydrogen peroxide (HP) flow had been turned off. While negative control was achieved by running 6 uninoculated aseptic gable top cartons through the rig with just the air on. This was done after all other cartons had been decontaminated including passing of the positive controls through the rig. PCA was then laid on all control samples as described in 3.2.4

4 Results

4.1 Microscopy

Observation of corners under the SEM revealed unique folding pattern in the corners of all Elopak aseptic gable top cartons. Different features were observed at different magnifications. The most notable observation was the folding of scorelines due to interaction with each other in the bottom corners of sealed Elopak aseptic gable top cartons, seen at very low magnifications (Figure 4.1). At a magnification of 108X, folds and holes were visualized within the area where scoreline interactions had occurred (Figure 4.2a). At 375X magnification, tiny trench-like features were observed (Figure 4.2b). By increasing the magnification to 493, micro cracks and micro-holes (pinholes) were also observed in these same areas (Figure 4.3).



EM lab, UMB

Figure 4.1 General folding patterns of scorelines in the corners of a gable top carton at 47X magnification. Notice the folds due to scoreline interaction at the center of the corner

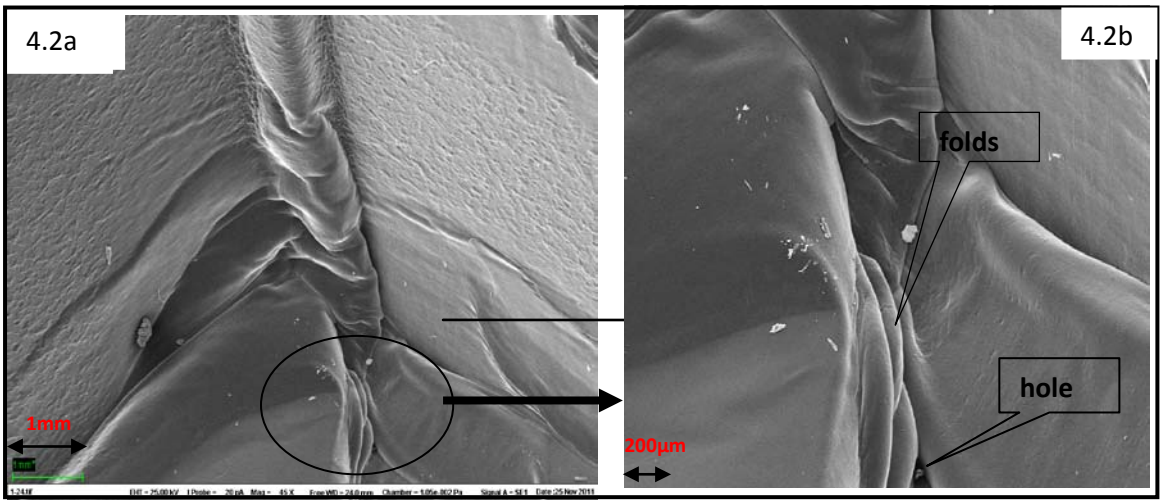


Figure 4.2a Wrinkled horizontal scorelines. Structures such as holes and folds resulting from the wrinkling of horizontal scorelines as they crush against each other in the corners.

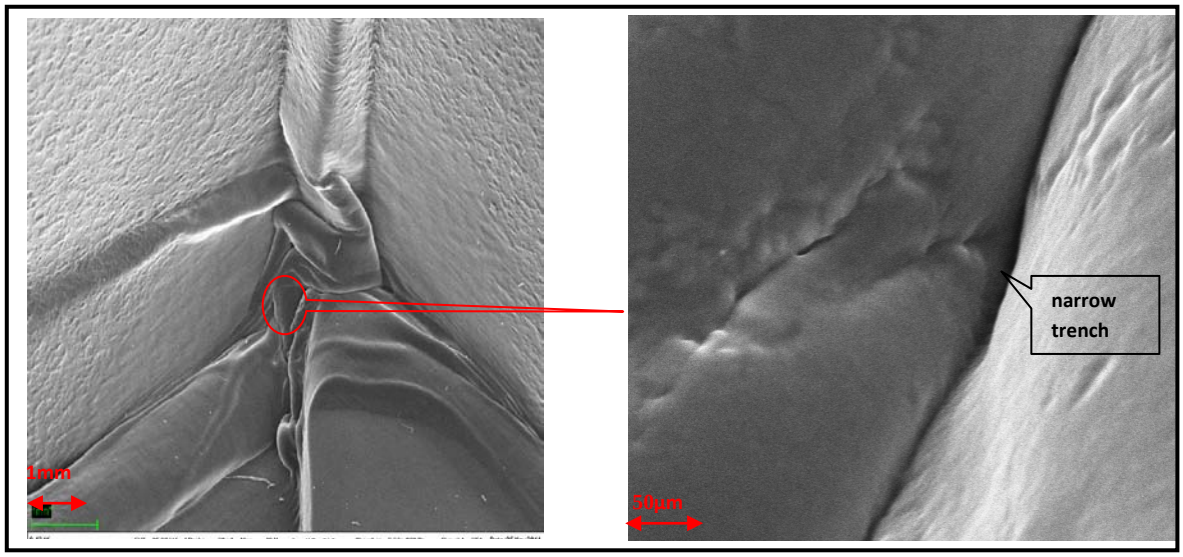
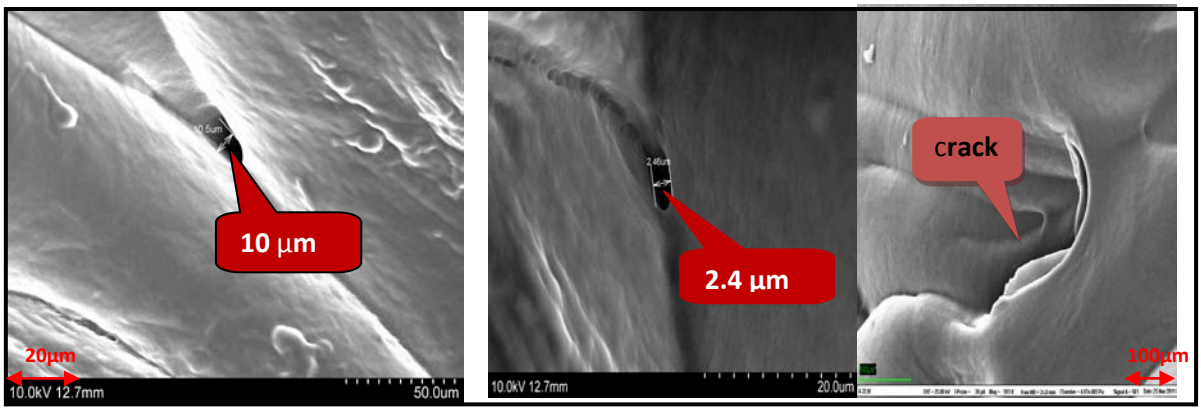


Figure 4.2b wrinkled vertical scoreline. Narrow trenches formed on a vertical scoreline crushed between the horizontal scorelines.



EM lab, UMB

Figure 4.3 Small cracks and micro-holes present on the PE layer in the corners of bottom sealed gable top cartons from Elopak.

Furthermore, numerous tiny micro bubbles were also seen on the surface of the PE in these corners (Figure4.4a). In some cases, structures that appeared to be burst bubbles (Figure 4.4b) were observed.

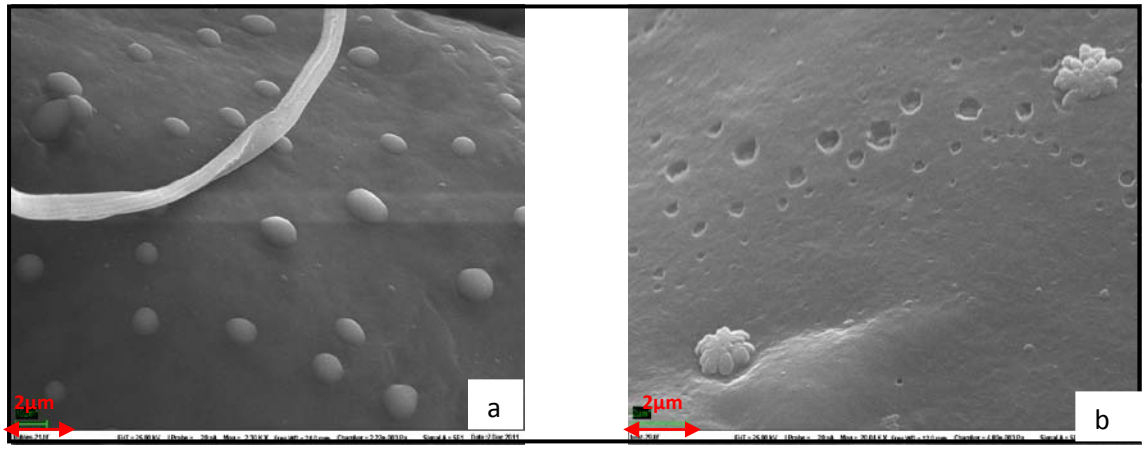


Figure 4.4 Micro bubbles (a) and burst bubbles (b) on the PE layer in the corners.

Micro bubbles in corners where spores were inoculated were had spores in them (Figure 4.5). Some spores where buried beneath the PE layer in and around the corners. (Figure4.6). In some cases, lumps of spores were observed on the surfaces, with some seaming to be buried in the PE (Figure4.7).

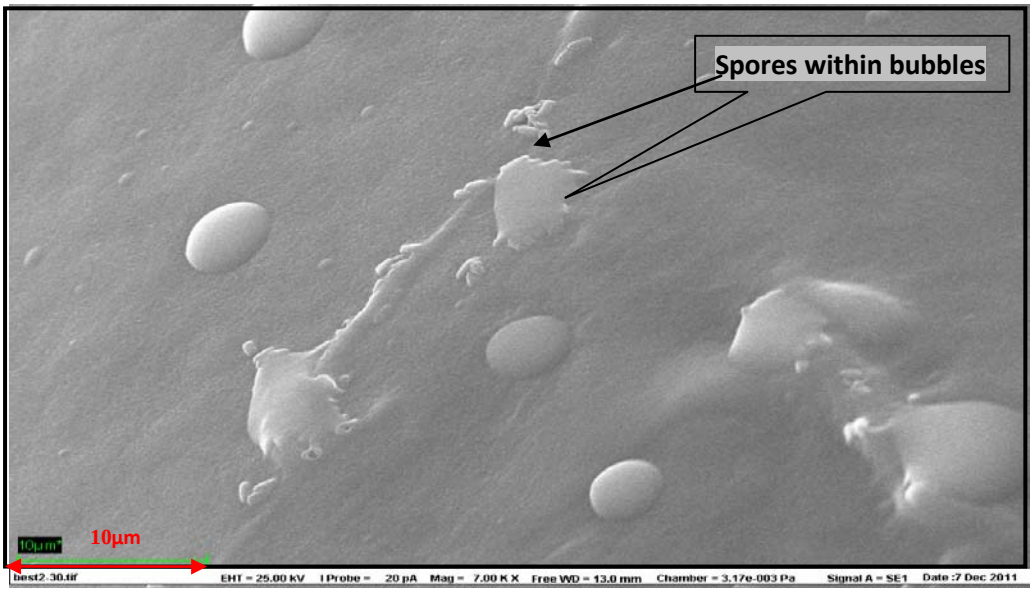


Figure 4.5 Spores trapped inside micro bubbles on the PE surface in the corners.

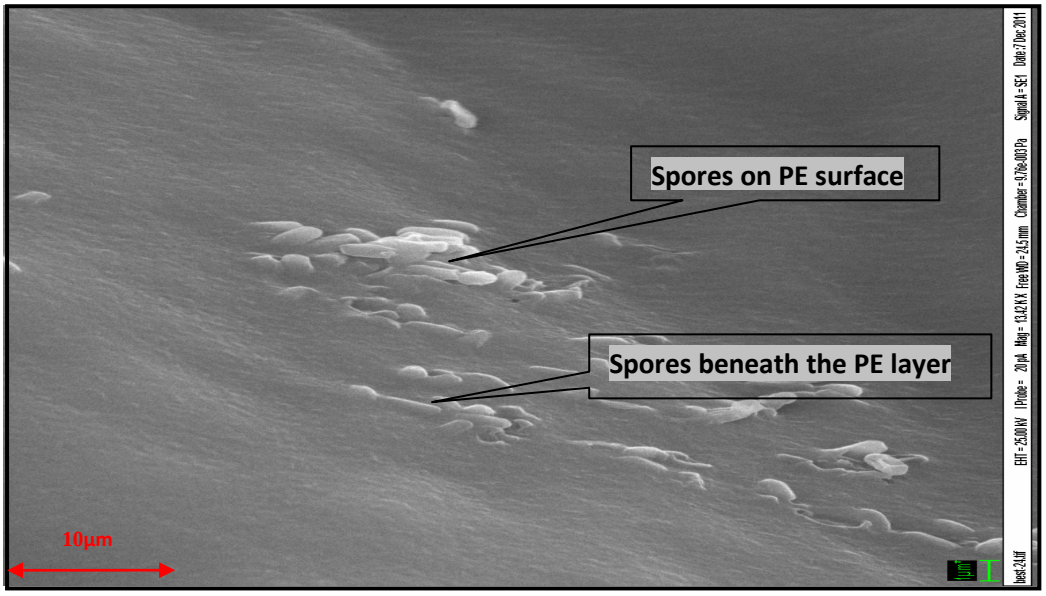


Figure 4.6 lumps of spores on the PE surface. Some appear buried under the PE.

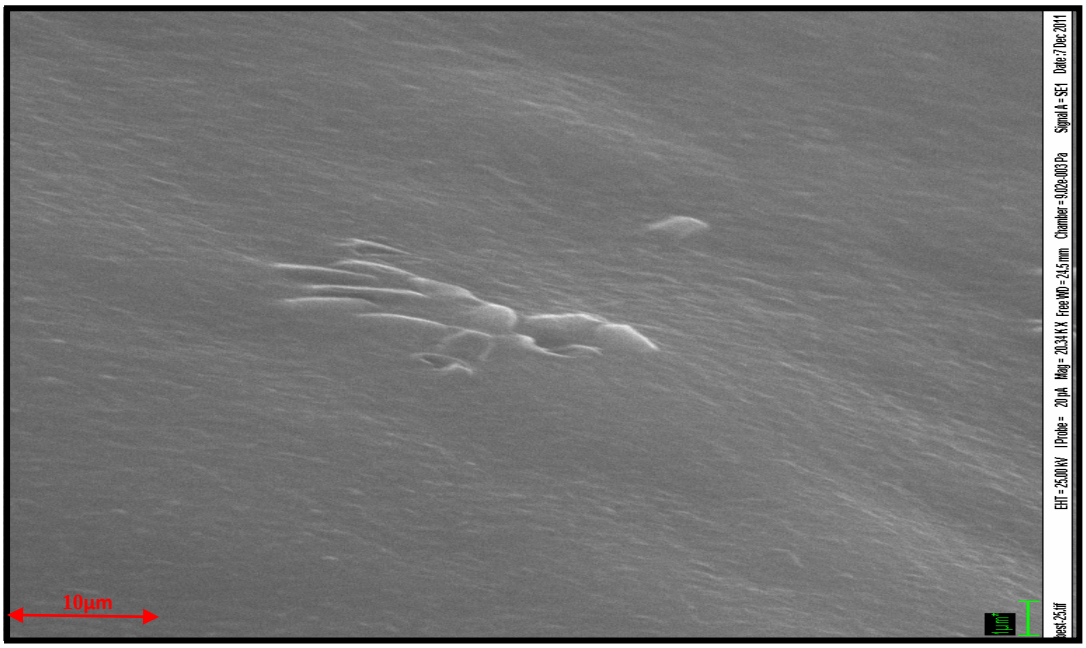


Figure 4.7 Spores embedded deep beneath the PE layer.

4.2. Heat pattern of bottom sealer.

The temperature at different places at the bottom were read off the Datapaq logger 1862.

Temperatures higher than 100 ^{0}C were recorded on food contact surfaces, just above the bottom scorelines (Figure 4.8)

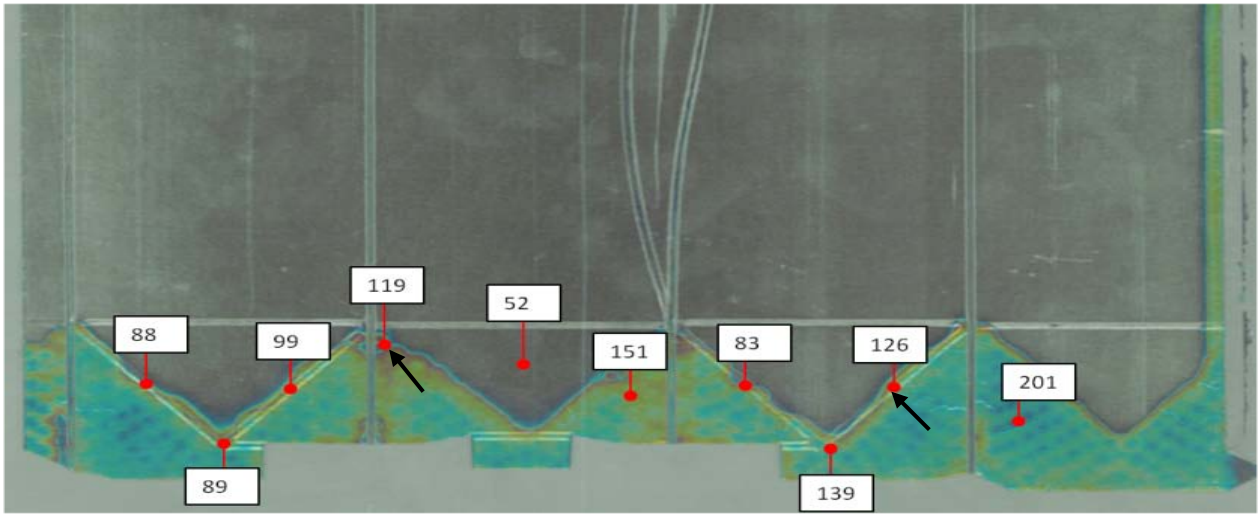


Figure 4.8 Temperatures (0C) recorded at different parts of the bottom by datapaq logger during the process of bottom sealing. Arrows show food contact surfaces activated at temperatures greater than 100 ^{0}C

4.3 Comparative studies of Elopak cartons and cartons from company X

Visual inspection of flat blanks from company X revealed that their scorelines were thinner and lower than the scorelines in Elopak aseptic gable top cartons. The horizontal scorelines on the latter blanks terminated a few millimeters away from the vertical scorelines just before the corners at the bottom. In formed cartons from company X, there were no interactions between the vertical and the horizontal scorelines in the corners. Compared to the corners in formed Elopak gable top cartons, corners from company X had a relatively smoother geometry (Figure 4.9).

The many folds which were seen in the Elopak carton corners at the point of interaction of the scorelines (4igure4.9a) such as small cracks micro holes and narrow trenches were not observed in the corners of aseptic cartons from company X (4.9b). Gaps (indicated by the red arrows red arrow) were observed in the areas between the folded bottom surface and the vertical scoreline in the corners of aseptic cartons from company X. These gaps were considerably wider and shallower than those that were observed in the Elopak cartons (Figure 4.2b). The vertical scorelines of the Elopak aseptic gable top cartons were wrinkled and crushed at the corner due to interaction with the horizontal scorelines (Figure 4.9a), while the vertical scorelines in cartons from company X was bent at the corners when the carton bottoms were sealed (Figure 4.9b)

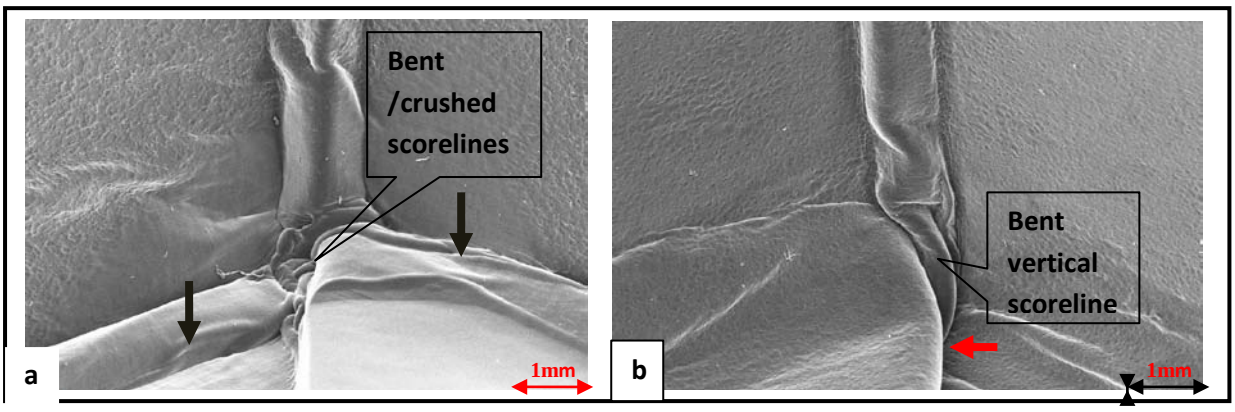


Figure 4.9 Effect of bottom sealing on scorelines in the bottom corners of the Elopak aseptic gable top carton and aseptic cartons from company X. (a) Elopak carton with black arrows indicating the horizontal scorelines which crash against the vertical scorelines.(b) Carton from company X, with red arrow indicating vertical scoreline bending at the bottom.

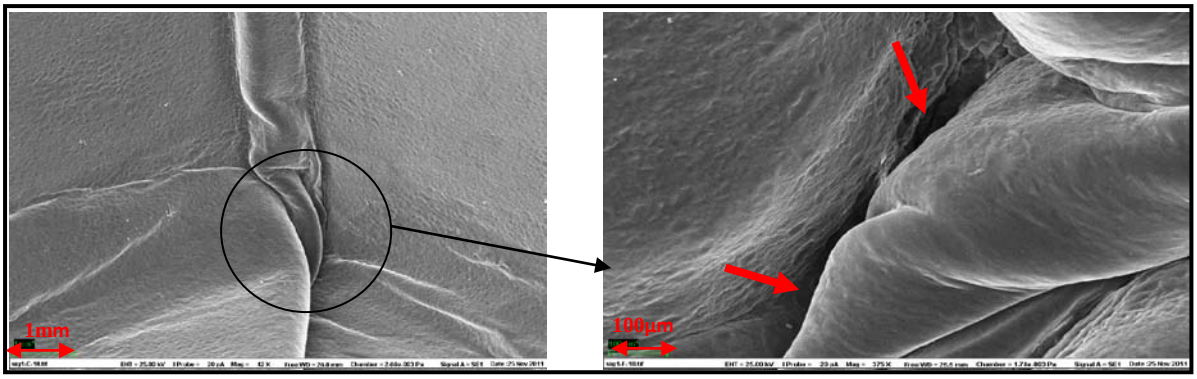


Figure 4.10 structures at the corners in cartons from company X. Wide gaps between folded bottom surface and bended scoreline are prominent. No holes or tight openings. Notice the difference in the size of the gap compared to the size of the gap present in a similar position in the Elopak carton (Figure 4.2a).

4.4 "Crack sealing" experiment.

Upon incubation of inoculated cartons, the number of corners with growth at each log load in the different sample groups was counted. Cartons with inverted scorelines showed the highest number of unsterilized corners, followed by the Elopak aseptic gable top carton. No growth was observed at all the log load of spores in the corners of cartons that were sealed with epoxy (Figure 4.11).

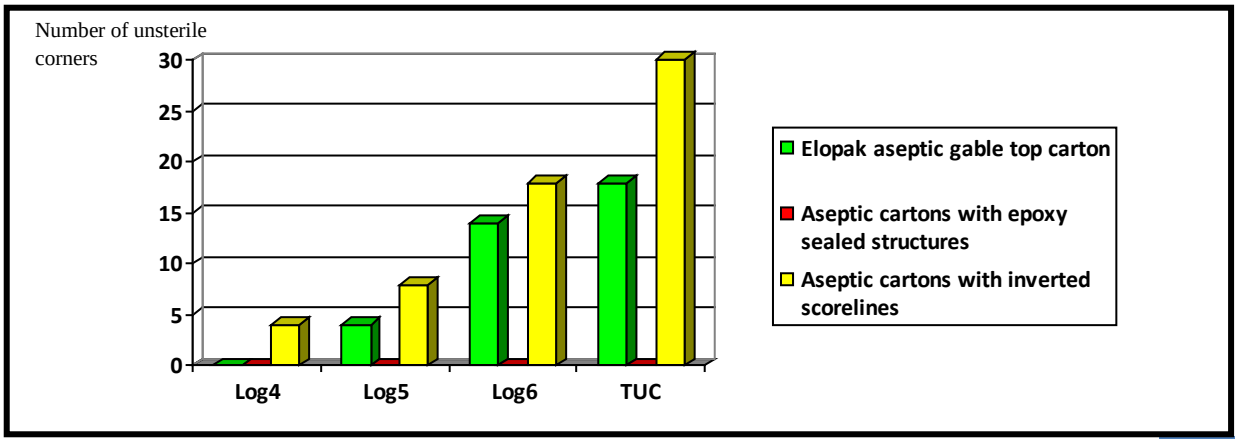


Figure 4.11 Bar chart showing the number of unsterile corners in each carton sample against the corresponding load of *Bacillus atrophaeus*. TUC: Total number of Unsterile Corners.

The log cycle reduction (LCR) by the sterilization system in each carton sample group was calculated using the formula described in Moruzzi et al. (2000), and the resulting LCR compared (Table 4.1). The LCR value for crack sealed cartons could not be determined, but the decontamination of all corners indicated that the LCR in these cartons would be higher than in the other two carton samples.

Table 4.1 LCR delivered the sterilization system at the corners in the different carton samples.

	Crack sealed cartons	Normal Elopak cartons	Cartons with inverted scorelines
LCR	NA	6.48	5.43
95% Confident interval (LL)	NA	6.06	5.04
95% confident interval (UL)	NA	6.89	5.83

LL: lower limit. **UL**: upper limit. **NA**: not available.

5. Discussion

5.1 Microscopy

Direct action of vaporized hydrogen peroxide (VPHP) on a contaminating microorganism is necessary for the VPHP to inactivate the microorganism (Unger et al 2007). Therefore obstruction of direct activity of VPHP on microbes reduces inactivation of the microbes by the sterilizing gas (VPHP). The results in this study indicated that spores trapped in structures at the bottom corners of the aseptic gable top cartons remained active after decontamination of the cartons. This phenomenon was also observed by Knaup (2011). In an investigation to determine the efficiency of VPHP in carton sterilization, Knaup (2011) reported that spores found in cracks could not be reached by VPHP, hence were not inactivated. He suggested that scoreline folding resulted in wrinkles in the corners of the carton. This created structures in which spores could be trapped, protecting them from the action of VPHP. This suggestion was confirmed in our observations. Several structures resulting from the folding of scorelines

were observed in all the corners from the Elopak gable top cartons analyzed in this study. Structures such as tiny holes of a few microns in diameter, wrinkles on the vertical scorelines, folds with tight gaps between them and cave-like openings in overlapping folds were visualized. All these structures were found at the point where the three scorelines, i.e. the two horizontal scorelines and the one vertical scoreline meet at the corner after bottom sealing. Most of these structures had very narrow openings (< 20 μm), and could trap and harbor spores. According to Opfell et al (1964), narrow passages hinder gas diffusion through them. Though gases generally have a high diffusion coefficient (McMurtrie and Keyes, 1948), studies have shown that sterilization by VPHP is not very effective on porous surfaces (Dobbelaere et al., 2001; Rogers et al., 2005; Unger et al., 2011). Porous surfaces i.e. surfaces with crevices, cavities and holes etc. like those found in the corners of the gable top carton provide cover from the effect of a sterilant for spores trapped within them (Tietjen et al., 1992). Therefore, spores trapped in some of the structures found in the gable top carton corners would be protected from VPHP activity. This could be one of the reasons why decontamination was lowest in these corners.

According to Unger Bimczok et al (2011), sterilization affectivity in cavities decreases with reducing gap size and increasing depth. However, the depth of the holes and cavities were not determined in this experiment. Another factor that could have contributed to low decontamination due to the presence of porous structures (whether shallow or deep) is micro-condensation. Although it has been reported that micro-condensation may increase the inactivating potential of HP (Watling et al., 2002), however, on rough hydrophobic surfaces such as those of the Elopak gable top carton corners, micro-condensation will instead have an adverse effect on sterilization. (Knaup, 2011**).** When VPHP condenses on PE surfaces, it forms droplets. This is because of the inability of liquid hydrogen peroxide (HP) to wet the hydrophobic PE surface. Some of these droplets are deposited on the tiny mouths of the cracks and crevices in the corner, and because HP cannot wet the PE surface completely, these droplets remain on the mouth of these crevices, rather than penetrate into them. Hence the droplets act as shields, shielding VPHP from getting inside the cracks, holes and crevices. Consequently, spores inside shielded structures will not be assessable to the sterilizing VPHP, and will remain active.

The heat pattern of the bottom sealer revealed that temperatures greater than 100^0C were reached on some parts of the food contact surfaces in the bottom corners during bottom

sealing (Figure 4.8). At these temperatures PE melted resulting in bubbles on the PE surface after solidification of molten PE. During the process of bubble formation, spores were mixed with molten PE and some of these spores became trapped beneath the re-solidified PE at the corners of bottom sealed cartons. Microscopic bubbles with air were also observed, some of which seemed to contain spores in them. The location of spores beneath the PE layer and in bubbles raised doubts on the method of laying agar on flattened cartons (Figure 4.1b). Cutting the corners through areas where spores where embedded beneath the PE, or in bubbles during bottom sealing released these spores unto the surfaces of the flatten carton bottom (Figure 5.1). Being trapped beneath the PE layer and/or in bubbles during carton sterilization, these spores would have been protected from inactivation by VPHP. Consequently, growth of these spores was observed when agar was laid on the flatten cartons. Prior to this discovery, the growth of these spores was thought to be an inefficiency of the sterilization system, and attempts to optimize the systems let to no significant changes in its efficiency.

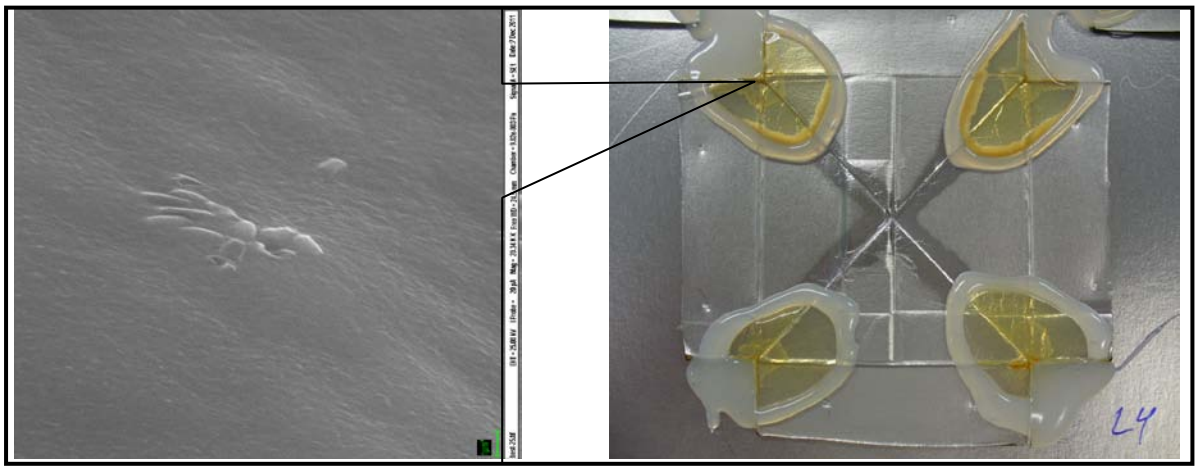


Figure 5.1 Spores embedded at the corner of a carton escape inactivation but are released to grow in agar when carton corners are flatten in the agar overlay method.

This method was changed in favor of laying agar on intact bottoms (Figure 3.1a). In the new method, the carton bottoms were simple cut, but not flattened. Hence, spores that were embedded underneath the PE were not released, avoiding false results that would have occurred due to their release. This change of method had a profound effect on the subsequent LCR results, and the sterilization effect of different settings was clearly visible. By using intact bottoms, only spores which are on the surface of the PE layer, or hidden is the geometric structures found at the corners were exposed to agar. Whatever was buried

underneath the PE layer, remained buried, and did not influence the decomtamination oberved.

However, because of the presence of burst bubbles and some spores which were only partially embedded into the PE layer (Figure 4.4 and Figure 4.6), more testing should be conducted to show that the spores embedded beneath the PE and within the bubbles will not be released during the shelf life of the packaged food.

5.2. "Crack sealing" experiment.

Smoothening the bottom corner surfaces with epoxy blocked all structures such as micro holes and cracks in which spores could hide, and on which microcondensate droplets could form in the corners of the carton. This left spores lying on the epoxy-coated PE surfaces in these corners. Consequently, these spores were directly acted upon by VPHP, and were all inactivated. This result suggested that carton sterilization would be more effective in cartons with smoother surfaces. Unfortunately, it was not possible to directly compare the quality of decontamination in epoxy sealed cartons with that in gable top carton because LCR in cartons with no contamination can not to calculated using the formula of Moruzzi et al., (2000). This formula demands that at least one corner must show growth of bacteria, and therefore could not be applied in epoxy-sealed cartons in which all spores were inactivated (Figure 4.11). However, inactivation of all spores in these cartons implied that LCR would be higher than in the other two carton samples. In gable top cartons which were not sealed with epoxy, some of the inoculated were not inactivated. Cracks and holes in the corners of these cartons could have provided covers for the spores that were traopped in them, protecting them from being inactivated by VPHP. Growth of these spores was observed in PCA after 2 d of incubation at 30 ^{0}C. A similar effect of rough surface topology on the efficiency of VPHP as a sterilant was also reported by Rogers et al., (2005), who examined the sterilizing effect of VPHP on surfaces with different porosity. They found that decomtamination with vapour phase hydrogen peroxide was far more effective on smooth surfaces than on rough surfaces, as as we observed in our results. This results support the claim that structures in the corners reduced the efficiency of decontamination that was observed in them.

The necessity of scorelines in folding flat blanks into cartons is a limiting factor in producing cartons with with very smooth internal surfaces. Folding of these scorelines always results in structures of some type. However, Unger Bimczok et al., (2011), stated that on rough

surfaces, the efficiency of decontamination increases with increasing gap size. Therefore, wide structures should have a lesser effect on the efficiency of decontamination by VPHP compared to tight structures. This was our rational behind making cartons with inverted scorelines. Scoring of flat blanks in an inverted manner create predominantly furrows with wide gaps in the corners when these flat blanks are folded and sealed to form cartons. According to Unger Bimczok et al., (2011), decontamination should be more efficient in such cartons. Unfortunately, this was not the case with cartons having inverted scorelines in this in this experiment. These cartons had the lowest LCR among the three types of carton samples that were analyzed. This was probably due to the fact that these cartons did not fit well on the mandrel of the bottom sealer test rig. Hence sealing was not optimal, and the unsealed areas in the corners presented more hiding places for spores, thereby drastically reducing efficiency.

It is worth noting that the values of LCR calculated in this report are higher than would normally be achieved during the actual sterilization process. This is because spores were inoculated after the bottom of the cartons had been sealed, instead of the usual way of inoculating before bottom sealing. During the process of bottom sealing, spores present on the surface are carried into resulting structures by the folding process, trapping them in these structures. This mimicks real life scenario when blanks are contaminated with spores or other microbial forms of life prior to bottom sealing and carton sterilization. In contrast, bottom sealing of cartons before inoculation leads to the deposition of the spores on the surfaces of already formed structures. Since spores are non motile, only a few are carried along in solution into some of the structures formed in the bottom corners. Most of the remaining spores lie on the surfaces, exposed to VPHP and are inactivated. In such cases, calculated LCRs are very high. Using bottom sealed carton in this experiment was in order to be able to relate and compare the results in all three samples, as epoxy could only be laid in already bottom sealed cartons.

5.3. Comparative analysis

Company X is also a company involved in the production of systems for low acid liquid food filling. This company successfully applies the principle of sterilization by the condensation technique, a technique which has been tested and failed in Elopak low aseptic system (Elopak, 2008). Although condensed hydrogen peroxide on the surface of cartons is less penetrative than VPHP, due to the higher diffusion coefficient of gases over liquids, this method is said to

be efficient in low aseptic systems developed by company X. Microscopic inspection and comparison of cartons used by both companies has enlightened packaging researchers at Elopak on a possible reason why this method failed in Elopak systems, but succeeded in the systems of company X. The different structures in the corners of the Elopak aseptic gable top cartons, especially structures microscopic openings were absent in the corners of cartons from company X. The latter cartons had a few structures which had wider openings as compared to those that were observed in the Elopak cartons (Figure 3.10). According to Unger Bimczok et al., (2011), sterilization is more effective on surfaces with wide structures than on surfaces with fine structures.

A complex geometry at corners of company X was avoided by terminating the horizontal scorelines just before they entered the corners. Only the vertical scorelines passed through the corners in these cartons. This prevented the interactions between scorelines at the corners when the carton bottoms were folded. Interaction of scorelines was observed in the corners of bottom sealed Elopak gable top cartons carton. Scoreline interaction at the corners formed most of the structures that were observed in the Elopak carton corners. Furthermore, the scorelines in cartons from company X were thinner and lower than those in Elopak aseptic gable top cartons, resulting in little or no wrinkling of the vertical scoreline after bottom sealing. The effect of thick and high scorelines was very obvious in the Elopak carton corners, with much wrinkling of the vertical scorelines which created structures in the corners after bottom sealing of the cartons (Figure 4.1 and 4.9a).

The absence of tiny structures on which micro-condensation could deposit condensate droplets means condensed HP in these cartons will be deposited directly on the spores leading to their inactivation.

The results of this study suggests that optimizing the geometry of the bottom sealing area will increase the sterilization efficiency of decontamination system, contrary to the findings of Unger et al. (2007), who stated that surface properties such as wettabilty and roughness do not directly affect the rate at which spores are inactivated.

Unfortunately, this is a very expensive way to approach the problem of low decontamination in the corners, due a huge cost that will be incurred in redesigning of flat blank scoring machine. However, if present optimization of machine parameters fails to give a setting with

the required LCR of 5 in all areas in the carton, this could be the only option for Elopak if a log5 LCR is actually required at the corners of the carton.

With present market demand for systems with very high decontamination efficiencies, more work is need to optimize sealing in cartons with inverted scorelines. This could be the way forward for Elopak in challenging and taking a lead in the low acid aseptic packaging market.

SECTION 2

Spore forming bacteria in paperboard, on flat blank surfaces exposed in storage halls, and in dust samples within the Elopak converter plant at Terneuzen.

1 Introduction.

The Elopak laminated paperboard rolls for the production of aseptic cartons are made up of about 87% paperboard, coated with very thin sheets of aluminum and polyethylene. At the convertor plant in Terneuzen (Netherlands) these rolls are cut depending on the size of the carton to be formed, into flat blanks. These flat blanks are temporarily stored in large storage halls before being side sealed into carton blanks. The side sealed blanks, are then packed into corrugated cardboard boxes or wrapped in paper wrappers and shipped to filling plants around the world.

Paperboard is a network of cellulose fibers from wood, most often mixed with starch and resins. It is known to contain spores of bacteria (mostly *Bacillus* and related genera) and fungi (Väisänen et al, 1991; Pirttijärvi et al, 1996; Delgado et al, 2012). Contamination of paperboard is mostly from the raw materials used in its production (Hallaksella et al, 1991; Raaska et al, 2002), and can also occur during various stages of production from the air, from handling by factory workers or by direct contact with contaminated surfaces of processing machines (Värnamo, 1982), as well as water used in paper pulp suspension. Lamination of paperboard with polyethylene (PE) and aluminum (Alu) sheets prevents these microorganisms from gaining access to the packaged food.

Few microorganisms are present on the PE layer laminating the paperboard. (Von Bockelmann and Von Bockelmann, 1986), and are mostly due to airborne contamination after its production Toledo et al. (1973).

Previous internal studies at Elopak on the hygienic standards of carton blank production at Terneuzen indicated high bacteria counts within the plant. An internal report on flat blank contamination suggested that the bacteria counts on the flat blanks surfaces significantly increased during storage prior to side sealing. An analytic study on dust samples within the production plant indicated high counts of both aerobic and anaerobic spores (Elopak, 2012a). Paperboard from the Stora Enso plant, used in the production of blank rolls was also found to

harbor a large number of bacteria, approximately 1500 colony forming units per gram of paperboard (1500 CFU/g), of mostly of spore-forming bacteria (Elopak, 2012b)

The efficiency of the package sterilization depends on the number and types of microorganism present on the food contact surface (Smith and Brown 1980). Surfaces contaminated with bacteria spores are not easy to sterilize, and bacterial susceptibility to the sterilant varies depending on the bacteria type (Teitjen, 1992). In a sterilization process, the number of spores present on the material being sterilized has a direct impact on the failure rate of the process (Von Bockelmman, 1985). Nakayama et al., (1996) showed that there is a significant degree of variation in resistances between different bacterial spore types. Therefore, numbers as well as bacterial types on the blank surfaces are important elements of blank microbiological quality to be considered in evaluating the efficient of a sterilization process. Another important aspect regarding the microbiological quality of aseptic paperboard cartons is the bacteria count in paperboard. It has been shown that the presence of defects on the layers laminating paperboard could lead to ingression of the bacteria present in paperboard into the stored food (Kamei et al., 1991).

Thus, given that intrinsic conditions in low acid foods will support the growth of a great variety of bacteria, including deadly pathogens such as *Clostridium botulinum*, it is very essential to determine the identity of bacteria in order to be able to evaluate the risk of product exposure to food spoilers and human pathogens in case of a bridge of the integrity of the laminating layers. Severity of human exposure will depend on the pathogenicty of the bacteria found in paperboard and on the blanks surfaces.

Although legislative recommendations for the microbiological quality of paperboard and blanks to be used in low acid aseptic food packaging are based on the number of spores present in and or on paperboard and blanks respectively, quantifying risk based on numbers alone could be misleading. Numbers give no indication of resistance or pathogenicty. Hence to be able to qualify low acid aseptic blanks with respect to microbiological risk, it is essential to identify the type of bacteria present in paperboard, and on the blanks. Such was the intention of this study.

The study aims at establishing the identities of the spore-formers that are present in paperboard from Stora Enso, as well as in dust samples from within the convertor plant at Terneuzen that could serve as reservoir for blank contamination. Spore formers that

contaminated flat blanks exposed in the storage hall before side sealing were also will also be identified. Special interest was paid on anaerobic spore formers especially the Clostridia, which has the deadly *Clostridium botulinum* as a member.

2. Literature Review

2.1 Bacteria in paperboard

Microbiological studies on paperboard production processes indicated that the microbiological quality of the finished paperboard product was determined by both the number, and type of bacteria in the raw materials (Tanner et al, 1940). Previous studies have indicated that bacteria are the most dominant type of microorganisms present in paperboard, far more than yeasts and moulds (Sammons, 1999). The association between plants and bacteria has been documented (Seldin et al 1984: Elo et al 2001, Von der Weid et al, 2002.**).** Hallaksella et al (1991) isolated *Bacillus spp.* from live forest trees;-*Picea abies*, commonly used in wood pulp production. Starch and wood pulp used in paperboard production contain sugars that bacteria can metabolize (Väisänen et al, 1991). Sanborn (1933) stated that the solution resulting from diluted paper pulp during the paperboard production provides a good medium for bacteria growth, with enough sugars from the wood. Starch, according to Raaska et al (2002), is a suitable ecological niche for bacteria, with plenty of available carbohydrates and other nutrients. According to Raaska et al, (2002), paperboard is not a suitable ecological niche for vegetative bacteria cells because of the low water activity in paperboard. In an experiment to prove this concept, Negro artificially inoculated paperboard with vegetative bacteria. Viable bacteria survived for a couple of hours to a few days after the board was dried, depending on the initial inoculation load. He concluded that paperboard was not a transmission medium for bacteria (Tanner 1948). Contaminating bacteria found in paperboard were mostly spores, since the entire process of paperboard production is very microbiocidal to vegetative cells (Tanner 1948). Paperboard production involves harsh processing like bleaching with Chlorine, heating of pulp, and drying of paper rolls, therefore almost all vegetative forms of microbial life are destroyed during paperboard production (Raaska et al, 2002, Tanner 1948). In an effort to demonstrate the harshness of board drying after production, Tanner et al (1940) sprayed paper rolls with inocula of *Escherichia coli, Staphylococcus aureus,* and an aerobic spore former before roll drying. After drying, only the

spore former could be recovered. The presence of predominantly spore formers in board is well documented, with a majority of these organisms being *Bacillus spp* and related genera (Väisänen et al, 1991; Pirttijärvi et al, 1996; Raaska et al, 2002; Delgado et al, 2012). Spores are resistant to chemical, biotic and abiotic stresses (Stieglmeier et al, 2009) enabling them to escape the bactericidal actions of the paperboard production process, and survival in the environments in paperboard.

2.2 Bacteria on blank surfaces stored factory halls.

Paperboard used for the production of food cartons is often coated with Aluminum and water/air tight plastic materials, usually polyethylene in the case of aseptic cartons to form carton blanks. The materials used in coating are usually applied in molten form. Upon production, there are almost no microorganisms on the blank surfaces (Von Bockelmann and Von Bockelmann, 1986). Toledo et al. (1973) suggested that the microorganisms found on PE-laminated packaging materials came from the environment in which there were being stored and were airborne. Simard et al (1983) stated that about 70% of airborne bacterial contamination on material stored indoors was due to gram positive cocci of human origin, while Green et al, 1962 suggested that dust (soil) was the main source of contamination by *Bacillus spp.* in indoor environments. Suprisingly very little documentation is available regarding contamination of packaging material surfaces. In a rare study, Pirttijarvi et al (1996) conducted microbial analysis on food contact surfaces of liquid food packages lined with polyethylene and found the bacteria counts on polyethylene-lined paperboard packages to be very low. Von Bockelmann and Von Bockelmann (1986) differentiated the bacterial flora on food packaging materials lined with a layer of polyethylene as 44.4% micrococci, 3.1% bacterial spores (*Bacillus*), 3.7% streptococci, 16% others.

3. Materials and methods

3.1 Sample collection.

3.1.1. Paperboard

Paperboard from different paperboard rolls were collected at the Stora Enso board production plant. The board samples were wrapped in aluminum foil and sealed in an air-tight bag. The samples were then transported to the microbiology laboratory of Elopak at Spikkestad for analysis.

3.1.2. Dust

Table3.1: Dust samples from within the convertor plant at Terneuzen

Sample ID	Sample Source/Site
1	1 Box Packer (dust)
2	1 Box Packer (dust)
3	1 Box Packer (dust)
4	1 Box Packer (dust)
5	11 Boxing area (dust)
6	11 Boxing area (dust)
7	1 Corrugated box (whole)
8	1 Corrugated box (whole)
9	3 Corrugated box (without outer layer)
10	3 Corrugated box (without outer layer)
11	1 Corrugated box (whole)
12	3 Corrugated box (without outer layer)
13	4 Corrugated box (without outer layer)
14	10 External mud, April 2012

Dust samples were collected from different locations within the converter plant at Terneuzen. The samples were allocated different numerical ID depending on the site of collection, and placed in sterile tubes, corked air-tight, before transporting to the microbiology laboratory of Elopak AS at Spikkestad-Norway for analysis.

3.1.3. Blanks

Flat blanks were collected from the middle of various stacks in the two storage halls (hall 1 and 4) at the Elopak converter plant at Terneuzen (Netherlands), and exposed to ambient air within these halls for 24hrs and 48hrs. Then these blanks were sealed in sterile packages carefully avoiding any contact with their surfaces, and transported to the microbiology laboratory of Elopak at Spikkestad.

Picture 3.1 Exposure of flat blanks to ambient air in the storage halls at Terneuzen

3.2 Microbiological analysis.

3.2.1 Paperboard.

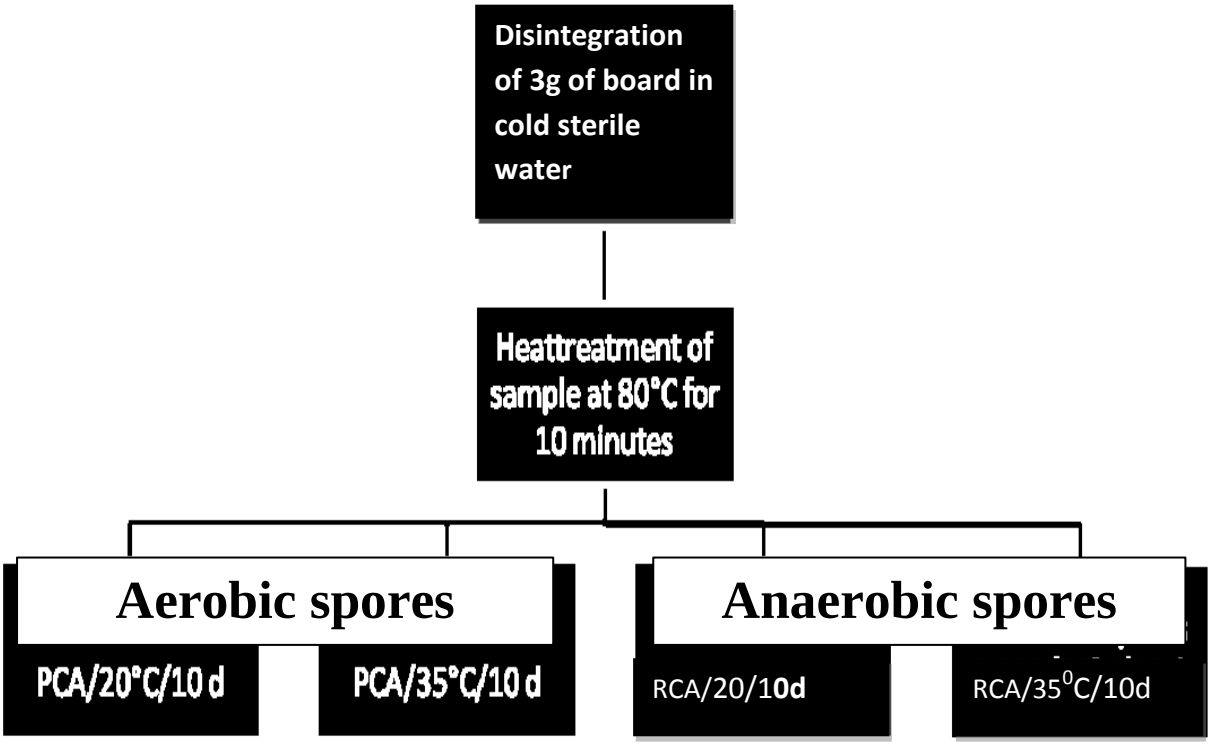


Figure 3.1 Flow diagrams for paperboard analysis.

Paperboard samples were aseptically cut into 20 mm x 20 mm using sterile scissors and forceps. 3g of board pieces were suspended in 300 mL of cold sterile water and homogenized using a Waring blender at high speed for 2 min. The blender jar was disinfected with 95% ethanol and flaming after each disintegration process.

2 x 30mL of the resulting homogenate was placed in 2 sterile tubes with screw caps, corked tight and submerged in a water bath maintained at 82^{0}C. The required heating temperature of 80^{0}C was achieved in the tubes in approximately 10minutes, and then tubes were left submerged in the water bath, maintaining the heating temperature of 80 ^{0}C for 10 min, making a total heating time of 20 min

3.2.2 Dust.

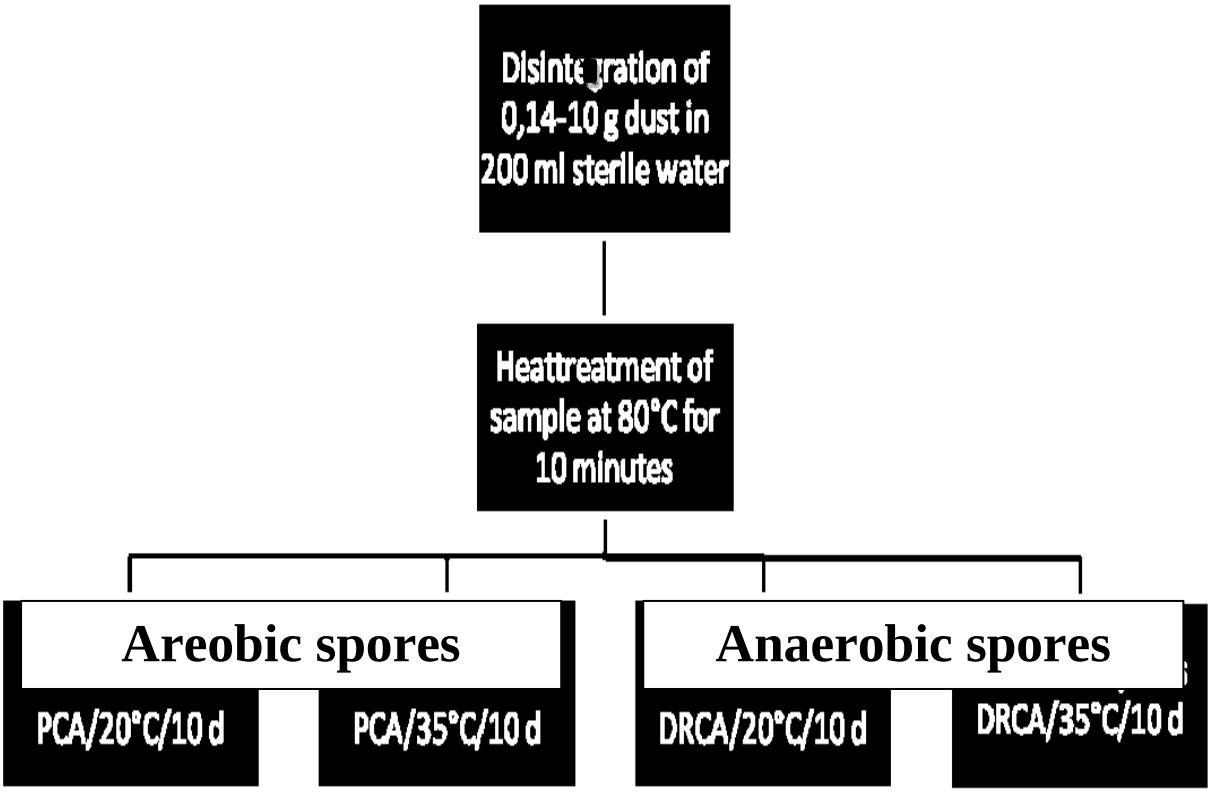


Figure 3.2: Flow diagram for dust analysis.

0.037-0.2g of dust was suspended in 200 ml of cold sterile water, and disintegrated using a warring blender at high speed for 2 min. 2 x 30 mL of the resulting homogenate were transferred into two sterile glass tubes with screw caps, corked tight and submerged in a water bath maintained at 82^0C. A temperature of 80^0C was achieved in the tubes in approximately 5 min and tubes were left submerged in water bath for an additional 10 min. The blender jar was disinfected with 95% ethanol and flaming after each disintegration process.

3.2.3: Blank surfaces.

Contact plates made from different agars were used to analyze different bacteria types on the blank surfaces. Differential reinforced clostridia medium agar (DRCA) (Peptone 5.0 g; peptone 5.0 g; meat extract 8.0 g; yeast extract 1.0 g; starch 1.0 g; D(+)glucose 1.0; L-

cysteinium chloride 0.5 g; sodium acetate 5.0; sodium di-sulfite 0.5; ammonium iron(II) citrate 0.5 g; sodium resazurin 0.002 g; agar 15 g: water 1L) was used for analysis of anaerobic spores, reinforced clostridia medium agar (RCA) (Meat extract 10.0; peptone 10.0; yeast extract 3.0; D(+)glucose 5.0; starch 1.0; sodium chloride 5.0; sodium acetate 3.0; L-cysteinium chloride 0.5; agar 15g; water 1 L) was used for aerobic spore analysis. Both agars were made in the Microbiology laboratory of Elopak at Spikkestad

9 replica contact plates were pressed against the inner surface of each blank by placing a weight of 500 g on the plate for 5 s for analysis of corresponding bacteria type.

3.3. Plating and Incubation

3.3.1. Paperboard and dust.

After heating, tubes were rapidly cooled to room temperature in an ice water bath. 10ml of heat treated homogenate were distributed in 5 (9cm) petri dishes. Plate Count Agar (PCA) was poured into these 5 plates, swirled, and allowed to dry under a laminar flow cabinet. The petri dishes were put in polyethylene bags and incubated under aerobic conditions at 20^0C and 35^0C for 10days. For anaerobic analysis; Reinforced Clostridial agar medium was used for paperboard and Differential Reinforced Clostridial agar Medium, DRCA was used for dust analysis. Anaerobic incubation was carried out in an anaerobic jar with 3 generators (BioMerieux Marcy-L'Etoile) at 20^0C and 35^0C.

3.3.2. Blank surfaces

Contact plates pressed against exposed flat blank surfaces were put in plastic pouches and incubated at 20^0C and 35^0C, under aerobic and anaerobic conditions for 10 days using same incubation parameters shown in figure 1 and 2.

3.4. Pre-enrichment of selected colonies

Some colonies from anaerobically incubated cultures in 3.3.1 and 3.3.2 were picked on the basis of unique colony morphologies and enriched in RCM broth for 2 d and incubated under anaerobic conditions as previously described (Figure 1 and 2). Then 1 ml of enriched culture was collected in 1.5 ml eppendorf tubes for DNA extraction.

3.4.1. Pre-enrichment of “black colonies”

Colonies that appeared black on DRCA were picked with a sterile pipette tip, and the pipette tips containing the colony were dropped into 45ml bottles containing DRCM broth. The bottles were put in anaerobic jars with 3 generators and incubated at 35 ^{0}C for 2 d. Upon incubation, a pipette was used to collect 10 µl of broth close to the bottom of the tubes, and the broth was distributed and streaked on 5 DCRM agar plates, and incubated in an anaerobic jars containing 3 generators for 2 d. After 2 d, black colonies which were not covered by spreading colonies were picked and enriched in DRCM broth under the same incubation conditions as previously described. 1 µl of the enriched broth was again streaked on DRCM agar and again incubated for 2 d. Resulting black colonies were picked and enriched in TPYG broth for 2 d. Then 1.5 ml of the incubation broth was used for DNA extraction.

3.4 Control of Anaeroboisis.

Germination and subsequent growth of *Clostridium sporogenes* spores were used as a test for anaeroboisis in media and anaerobic incubation conditions throughout this experiment.

RCM and DRCM agar plates were made and inoculated with spores of *C. sporogenes*. The plates were placed in an anaerobic jar with 3 generators. DRCM broth was also inoculated with spores of *C. sporogenes* and incubated in an anaerobic jar.

3g of autoclaved paperboard samples were inoculated with 0.5ml of log 3 *C. sporogenes* spore solution, and allowed to dry under a laminar flow cabinet. The inoculated paperboard samples were homogenized, and plated on DRCA plates.

3.5 DNA extraction

DNA was extracted by enzymatic treatment using GenElute Bacterial Genomic DNA kit (Sigma-Aldrich), according to the manufacturer’s instructions; briefly; 1ml broth of enriched “black colonies” was collected in 1.5ml centrifuge tubes (after thorough shaking of incubation tubes). These tubes were centrifuged at 6500 rpm for 2 min, and the resulting supernatant discarded. The pellets were suspended in 200 µl of lysozyme/mutanolysin solution (45mg/ml) and incubated for 1 h at 37 ^{0}C. After incubation, the solutions were added 20µl of RNAse solution and incubated at room temperature for 2 min. Then 20µl of proteinase K and 200µl of

lysis solution C were added, the solution vortexed, and incubated at 55 ^{0}C for 20 min. Lysed cell solutions were added 200 µl of 96% ethanol in each tube and vortexed. Then the lysed solutions were transferred into binding columns and centrifuged at 6500xg, eluate was discarded. 500 µl of wash solution was added to each of the columns and centrifuged again. Then columns were transferred into new 1.5 ml tubes, added 30ul of elution buffer and incubated for 1 min. Tubes with columns were centrifuged and DNA was eluted in eluate.

3.6 Polymerase chain reaction (PCR) and PCR conditions.

PCR was carried out in 0.2 ml PCR tubes in a Peltier Thermal Cycler (PTC) 200 PCR machine. The PCR mixture for each sample contained 5µl of 10x PCR buffer, 1µl of dNTP mix (10mM), 0.5µl of 16s rRNA gene universal primer 1F and 5R(100pmol/µl) each, 0.3µl of Taq polymerase (5U/µl). 3µl of template DNA was used for DNA extracted with the extraction kits and 39.7µl of milliQ water to make up 50µl PCR reaction mix. For DNA extracted using the freeze-boil techniques, template 10µl of template was and 32.7 µl milliQ water were added. The PCR was carried out with an initial denaturation phase at 95 ^{0}C for 3min, followed by 30 cycles of denaturation at 94 ^{0}C for 30 sec, elongation at 55^{0}C for 30 sec, annealing at 72 ^{0}C for 3 min, and a final extension at 72 ^{0}C for 10 min. Amplified 16s rRNA gene products were observed on a 1% ultrapure agarose gel containing Ethidium bromide under UV illumination

3.7 Cleansing of PCR products

PCR products were cleansed using the Qiagen-QIAquick purification kits according to the manufacturer's instructions. Briefly, 250µl of buffer PB was added to the PCR product, and placed in QIAquicks column contained in 2ml tubes. The tubes were centrifuge at 13000rpm for 1minute, and the resulting eluate discarded. The DNA, bound in the columns was washed by centrifuging 0.75ml of buffer PE through the columns for 1minute. DNA was eluted into new sterile 1.5ml tubes by adding 30µl into the columns and centrifuging for 1minute.

3.8 16s rRNA gene sequencing.

The purified DNA was sent to GATC Biotech AG (Jakob-Stadler-Platz 7. 8467 Konstanz, Germany) for 16s rRNA gene sequencing**.**

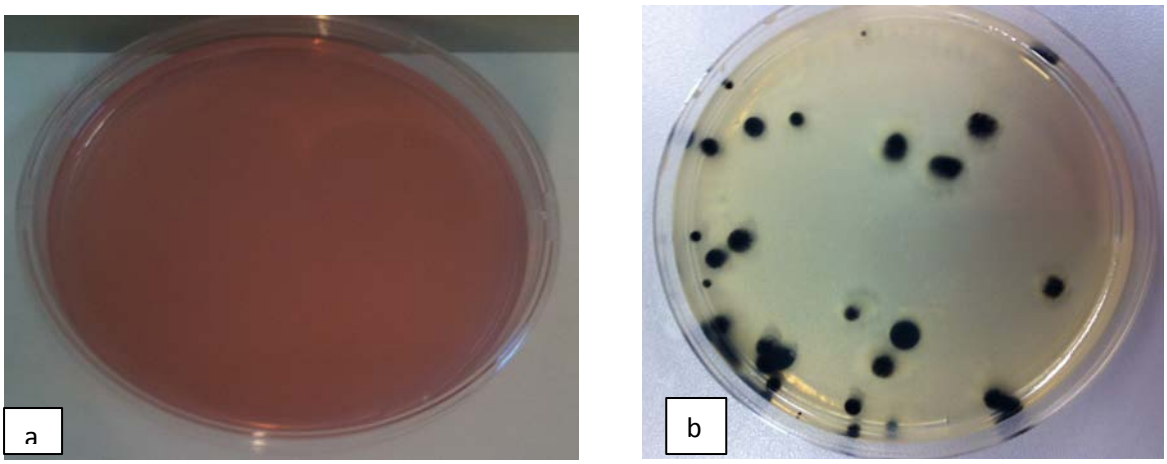
3.9 Specie identification.

Sequences of 16s rRNA genes received from GATC were compared with 16s rRNA gene sequences in the nucleotide database of National center for biotechnology information (NCBI) using blast search tool to determine the closest relatives on the bases of maximum sequence identity.

4 Results

4.1 Control of anaerobiosis.

Inoculated *Clostridium sporogenes* spores in paperboard and on the RCM and DRCM agar and broth all germinated. These spores formed black colonies on the DRCM agar (picture 4.1**)** and turned DRCM broth black after incubation in the anaerobic jars with 3 generators.



Picture 4.1 DRCM plates inoculated with spores of *Clostridium sporogenes*. 1a: Plate before incubation. 1b: Plate after 2 d incubation. Notice the color differences between a and b. Oxygen removal by anaerobic generators in jars; DCRM agar plate showing black colonies of *Clostridium sporogenes* after incubation.

4.2 Identified bacteria isolates from dust, paperboard and surface blanks.

All isolates identified from a selected number of colonies from paperboard cultures and on blank surfaces, including the non-black colony forming bacteria from soil were identified by the sequence of their 16s rRNA genes to *Bacillus spp*. *Bacillus Lichenformis* and *Bacillus cereus* were the common isolates from paperboard while *Sporolactobacillus spp* were dominant dust samples (Table 4.1).

Table 4.1 Bacteria isolates in paperboard from Stora Enso, on blank surfaces exposed at Terneuzen and from dust sample within the convertor plant at Terneuzen.

Sample ID	Growth condition	Closest relative	16S Max. Iden(%)
Paperboard	Anaerobe	*Bacillus licheniformis*	98
Paperboard	Anaerobe	*Bacillus licheniformis*	98
Paperboard	Anaerobe	*Bacillus cereus*	99
Paperboard	Anaerobe	*Bacillus cereus*	99
Paperboard	Anaerobe	*Bacillus licheniformis*	98
Dust	Anaerobe	*Sporolactobacillus terrae*	98
Dust	Anaerobe	*Sporolactobacillus laevolacticus*	97
Dust	Anaerobe	*Bacillus coagulans*	99
Dust	Anaerobe	*Sporolactobacillus laevolacticus*	99
Blank surface	Anaerobe	NE	NE
Blank surface	Anaerobe	*Bacillus Lichenformis*	98
Blank surface	Anaerobe	NE	NE
Blank surface	Anaerobic	*Bacillus lichenformis*	98
Dust	Anaerobe	*Bacillus coagulans*	99
Dust	Anaerobe	*Sporolactobacillus terrae*	97

NE- Note available

4.3 16s sequence identification of black colony forming bacteria.

Blast search of the NCBI database for the closest relatives of the black colony forming bacteria isolated from dust showed that they were *Clostridium spp*. The different serotypes of *Clostridium botulinum* and *Clostridium sporogenes* showed identical sequence similarity in most of the isolates, making identification difficult (Table 4.2)

Table 4.2 Blast search results from the NCBI database for black-colony forming bacteria in dust samples from Terneuzen. Table shows the first two closely related species to the query isolates from dust and their sequence resemblance.

Dust ID	First two identified islolates	Query coverage	Sequence ID (Max ID)
1	*Clostridium sporogenes*	100 %	99 %
	Clostridium botulinum serotype A	98 %	99 %
2	*Clostridium botulinum* serotype A	100%	99%
	Clostridium sporogenes	100%	99%
3	*Clostridium celerecrescens*	100 %	99 %
	Desulfotomaculum guttoideum	100 %	99 %
4	*Clostridium sporogenes*	86 %	98 %
	Clostridium botulinum serotype F	86 %	98 %
5	*Clostridium sporogenes*	99 %	99 %
	Clostridium botulinum serotype A	99 %	99 %
6	*Clostridium sporogenes*	99 %	99 %
	Clostridium botulinum serotype A	99 %	99 %
7	*Desulfotomaculum guttoideum*	99 %	96 %
	Clostridium celerecrescens	97 %	96 %
8	*Clostridium botulinum* serotype F	97 %	97 %
	Clostridium sporogenes	97 %	97 %
9	*Clostridium botulinum* serotype A	98 %	97 %
	Clostridium sporogenes	98 %	97 %

5 Discussion

The aim of this study was to identify the spore-forming bacteria contaminants in the gable top carton and environmental isolates that also posed a contamination threat to the cartons during its production

All the spore formers isolated in paperboard from Stora Enso, both aerobic and anaerobic, were *Bacillus spp*. Most *Bacillus spp*. are facultative anaerobes, with an unibiquitous presence is the environment (Tamara et al., 1995). Wood and starch used in paperboard production are known to harbor *Bacillus spp* (Hallaksella et al., 1991; Pirttijarvi 2000; Raaska et al. 2002). The resistant nature of the endospores produced by these bacteria species helps them to survive the harsh processes involved in paperboard production, ending up in finished paperboard (Tanner, 1948, Raaska et al. 2002). These results are similar to results from past studies of bacteria flora in paperboard (Bendt, 1985; Vaisanen et al, 1989; Pirttijarvi et al, 1996; Pirttijarvi, 2000 and Pirttijarvi et al 1996), which all reported aerobic spore-forming *Bacillus spp* as the main bacterial flora in paperboard. Low acid foods will readily support the growth of bacteria, including food pathogens if they become contaminated with these bacteria. Strains of *Bacillus cereus* have been implicated in human food poisoning (Granum et al., 1995; Ombui et al., 2001, Kim et al., 2010; Bottone, 2012). Also, *Bacillus cereus* is a notorious food spoiler (Turnbull, 1996), From this, it is most likely that the presence of *Bacillus cereus* spores in paperboard from Stora Enso might pose a risk to products filled in the low aseptic gable top cartons made from this paperboard, if product becomes exposed to the paperboard during storage and distribution. Internal studies have shown that the Elopak gable top aseptic cartons have micro holes of approximately 20 μm in the sheets laminating the paperboard. These holes might connect paperboard and stored products, causing bacteria in paperboard to gain access to the products in the cartons during storage, as demonstrated by Kamei et al., (1991).

According to Tanner (1940), the number of bacteria, and the bacteria species in and /or on a packaging material are important in assessing microbiological quality of a package. The FDA recommends that paperboard intended for low acid aseptic use should contain less than 250 CFU/g of homogenized paperboard (FDA, 1993). Results from this study showed that paperboard from Stora Enso contained spores of *Bacillus cereus*; a bacterium often implicated

in human food poisoning (Granum et al., 1995). In addition to previous studies which indicated that this same paperboard has more than 1000 CFU/g (Elopak, 2012b), our results demonstrated that paperboard from Stora Enso does not meet the microbiological quality requirements for low acid aseptic food packaging.

Using such a paperboard would reduce the sanitary quality of the low acid aseptic carton. This would increase the risk of product contamination during storage and distribution in case of mechanical damages in the layers laminating the paperboard, that lead to product exposure to the paperboard

Airborne contaminants of flat blanks in storage halls at the Elopak converter plant in Terneuzen were mostly *Bacillus spp*. as determined by this study. In general, spores are a serious problem to the modern day food industry due to their ability to survive most decontamination processes (Leonardi et al., 1990). The defective rate of a package sterilization system depends on the spore counts on the surfaces to be sterilized (Bockelmann and Von Bockelmann, 1986). Therefore, the presence of *Bacillus* spores on low acid aseptic blanks is especially critical. *Bacillus* spores are the most difficult microorganisms to eliminate (Wayne et al., 2000), and some member of this bacteria group are pathogenic. Contamination of cartons blanks with *Bacillus spp.,* which might include pathogenic strains would most likely increase the package defective rate of the sterilization system, with a possibility of some defective packages containing food pathogens. This would increase the probability of human exposure to food poisoning, thereby compromising the overall performance of the system.

At Terneuzen, newly produced flat blanks are usually stored in stacks exposed to the ambient environment within large storage halls (same halls in which flat blanks were exposed in this experiment) waiting to be side sealed into carton blanks. Although the blanks are sometimes covered with polyethylene sheets, repetitive use of the sheets without decontamination means the sheets themselves could also be contamination vectors. As indicated by the results in this study, the top blanks on the stacks in the storage halls are most likely contaminated with *Bacillus* spores. This would create problems for the low acid aseptic sterilization system of Elopak if these blanks are to be used for low acid aseptic product filling.

Much has to be done to improve the microbiological quality of the air in the storage halls at Terneuzen to prevent airborne contamination of blanks before side sealing. Otherwise the top blanks on the stacks could be discarded since contamination is limited to them.

Dust analysis in this study indicated the presence of *Bacillus* and *Clostridium spp*. in the dust samples. The close proximity of the sites of collection of these dust samples to the carton and/or flat blanks meant that these dust could have been serving as reservoirs for spores of *Bacillus* and *Clostridium* to contaminate the blanks. Dust serves as a reservoir of spores within a processing plant, and itself is a major source of contamination (Long and Tauscher, 2006). Corrugated boxes and paper wrappers are used by Elopak to pack and transport carton blanks from the converter plant to filling plants. The packing process creates dust which would settle on the carton blanks. Also, at filling plants, cutting of the corrugated boxes, and or paper wrappers to remove the blanks releases dust from them unto the carton blanks. Hence blanks transported inside these packages risk being contaminated with spores of *Clostridium* and *Bacillus spp* from the corrugated boxes. External mud just outside the plant would also serves as a contamination reservoir. Movement of trucks and forklifts in and out of the plant carry the external mud into the plant. The mud dries up and aerosolizes, becoming airborne contaminants within the plant. Toledo (1973) stated that blank contamination is mostly airborne. Subsequent settling of dust from the external mud on the flat blanks in the storage halls would lead to contamination of flat blanks by the bacteria present in the dust (mud), in this case *Clostridium* and *Bacillus* spp.

Most of the *Clostridium* species isolated from the different dust samples had the same 16s rRNA gene sequence identity and query coverage with strains of *Clostridium sporogenes* and proteolytic *botulinum* strains (Types, A, B of F) in the NCBI database. Whole genomic comparison of *Clostridium sporogenes* and the proteolytic strains of *Clostridium botulinum* by Bradbury et al. (2012), revealed a very close evolutionary relationship between them. Hutson et al. (1993) reported that strains of proteolytic *Clostridium botulinum* shared a 99.7-100 % 16s rRNA gene sequence identity and were phylogenetically identical to *Clostridium sporogenes*. A limiting factor of 16s gene sequencing analysis is its inability to differentiate between closely related bacteria species (Tang et al., 1998). However, these results hinted on the possibility of the presence of *Clostridium botulinum* in these dust samples

Conclusion and recommendations

The results from previous internal studies at Elopak determined that the spore count in paperboard from Stora Enso was about 3 times high than what is recommended by the FDA. Whether this is of any significance to public health and food safety is still unclear. Relying on spore counts alone does not form strong bases for rejecting and this paperboard. 100000 spores from harmless saprophytes will pose less risk than 10 spores from pathogenic bacteria. However, this study has demonstrated the presence of *Bacillus cereus*; a bacteria which has often been implicated in food poisoning. By combining these two results, it could be argued that paperboard from Stora Enso does not meet the microbiological requirements for paperboard intended for low acid aseptic food packaging.

Showing proof that this paperboard actually contains pathogenic microorganisms is necessary for an objective assessment of the microbiological quality of this paperboard. The pathogenicity of *Bacillus cereus* in this paperboard could be tested using the *Bacillus cereus* Enterotoxin Reverse Passive Latex Agglutination (BCET-RPLA) tests and/or *Bacillus* Diarrhoeal Enterotoxin Visual Immunoassay (BDEVIA). Also, detection of genes encoding cytotoxin K (CytK), haemolysin BL (Hbl A, Hbl C, Hbl D), non-hemolytic enterotoxin (NheA, NheB, NheC) and EM1 specific of emetic toxin in the *Bacillus spp* found in this paperboard would give indications of the pathogenic nature of the *Bacillus cereus* found paperboard from Stora Enso.

How much of a risk this is to products stored in aseptic cartons made from this paperboard would greatly depend on the integrity of the layers laminating the paperboard.

Indications of a possible presence of *Clostridium botulinum* within the premises of carton blanks production and transportation must be taken seriously. Intrinsic conditions in low acid foods will support *Clostridium botulinum* growth if these foods are contaminated with the bacteria. Any effective risk assessment should involve investigating the presence of *botulinum* neurotoxin genes in these dust samples.

These results also show that airborne contamination of flat blanks with spores of *Bacillus spp*. occurs in the storage halls. Although the results did not indicate the presence of pathogens on the blanks, an important point to note is that these samples were collected during the winter months. We recommend that studies be done in all seasons of the year in order to adequately assess exposure. Rintala et al., (2008) noticed a significant variation in both bacteria counts

and species diversity in indoor environment during the different seasons of the year, with the lowest counts and diversity observed in the winter. Therefore any effective exposure assessment must take into account seasonal changes in species diversity and bacteria counts in and around the converter plant.

SECTION 3

Presence of spores of *Clostridium botulinum* in dust found in paperboard laminating plant.

1 Introduction

Clostridium botulinum is spore-forming, obligate anaerobic bacteria commonly found in soils and marine sediment (Cato et al, 1986). This bacterium if famous for it's a deadly neurotoxin; the botulinum toxin it produces during its growth (Hatheway, 1993).

Human outbreaks of food borne botulism are mostly due food contamination by *Clostridium botulinum* types: A, B, E, F and occasionally G. Low acid can foods, and poorly handled protein foods were the most implicated in botulism food poisoning (Elopak, 2011, FDA, 2012).

Implementation of tradition methods of preservation such as adding salt and nitrites to food, and high temperature treatment in canning led to a drastic drop in food borne botulism (Lindström, 2003). However, recent rise in consumer demands for minimally heat treated foods, with little or no additives, and a corresponding emergence of new food processing technologies to meet up with market demands has led to reemerged fears of human outbreaks from botulinum intoxication (Dahlenborg et al, 2001). Evaluation of the incidence of *Clostridium botulinum* is now being considered a necessary step in risk assessment studies in the modern food industry.

At Elopak, particular emphasis has been placed on the *Clostridium botulinum* during the development of the low acid aseptic filling machine. The presence of *Clostridium* spores on flat blanks, and around blank production environment is assumed to be a significant risk element. Finding from previous internal investigations at Elopak indicated very high clostridia spores in the dust samples within Elopak's carton blank production plant. 16s rRNA gene sequencing analysis hinted on the incidence of *Clostridium botulinum* in these dust samples (This thesis).

However, the inability of 16s rRNA gene sequencing identification method to distinguish between two very closely related species (Tang et al., 1998), prevented any conclusion on whether *Clostridium botulinum* was actually present in these dust samples, or if hints of its presence were due to the presence of its non toxigenic surrogate; *Clostridium sporogenes.*

Clostridium sporogenes and proteolytic *Clostridium botulinum* are known to differ only by the presence of the neurotoxin genes carried by *Clostridium botulinum* (Bradbury et al., 2012).

In this study, the presence of human toxin producing *Clostridium botulinum* is investigated in dust samples from within the Elopak paperboard convertor plant at Terneuzen. Spore enrichment followed by real-time polymerase chain reaction based technique was applied to specifically amplify neurotoxin genes carried by *C. botulinum* spp. in these dust samples.

2 Background

2.1 Classification of human pathogenic strains of *Clostridium botulinum*.

Clostridium botulinum is specie defined solely by the production of a lethal neurotoxin, the botulinum toxin (BoTN) (Sugiyama and Smith, 1988; Peck et al, 2011). Genetically, a huge divergence exists among bacteria classified as *Clostridium botulinum*. It is said that *Bacillus subtilis* and *Staphylococcus aureus* have a closer evolutionary relationship than strains that make up the species-*Clostridium botulinum* (East and Collins 1998). Phylogenically, 4 different types of *Clostridium botulinum* exist (Types I-IV) Human pathogenic strains of *Clostridium botulinum* can be divided into two groups proteolytic (group 1) and nonproteolytic (group 2) depending on their ability to digest complex proteins (Sugiyama, 1988). Proteolytic strains (group1) can utilize amino acids as an energy source, and readily ferment glucose and fructose, but fermentation of other sugars is limited (Sugiyama, 1988). The group 2 members do not metabolize amino acids (are nonproteolytic), but are saccharolytic, fermenting a variety of sugars (Sugiyama, 1988). *Clostridium botulinum* is also grouped according to the type of toxins produced. 7 distinct *botulinum* neurotoxin types (types A-G) produced by the different strains (Lindström, 2003). Strains that make up group 1 include the *botulinum* types A, B, and F, while group 2 members include botulinum types B, E and F. *Clostridium baratii* and *Clostridium butyricum* have been reported to produce neurotoxins E and F respectively (Hatheway, 1993)

2.1 Detection methods

With the recognition of the impact of *Clostridium botulinum* on public health safety in 1793 (Geiger, 1941), the incidence and prevalence of these group of bacteria has since become an integral part in risk assessment analysis in the food industry (Yousef et al., 2007). However,

detecting and demonstrating the presence of *Clostridium botulinum* is still a big challenge to food researchers. The absence of a selective media for Clostridium botulinum (Lindström, 2003) and resemblance to other members of other *Clostridium* genera complicates investigations involving *Clostridium botulinum* (Broda et al, 1998). Most documented investigations on the incidence of *Clostridium botulinum* begin with pre-enriching the *C. botulinum* bacteria in an enriched non specific medium (Dahlenborg et al, 2001; Brett et al, 2004, Yousef et al, 2007; Lindström 2003, Sagua et al, 2005). The only recommended method by the FDA for demonstrating the presence of *Clostridium botulinum* is by mouse lethality bioassay test (Lilly et al, 1998), which demonstrates the presence of the toxins by their lethal effects on rats. However, this method is very slow and time consuming (Kirchner et al., 2010) and the excessive and brutal killing of animals makes this method ethically wrong. Detection of the *Clostridium botulinum* antigens by the ELISA technique has been described for investigating the presence of *Clostridium botulinum* in Clinical and food samples (Poli et al., 2002; Ferreira et al., 2001). Detection methods by demonstrating highly specific proteolysis of SNARE- protein the Zinc –endopeptidase in the BoTN toxins have also been described for each toxin producing type; Ekong et al., (1997), Hallis et al., (1996) for type A, Hallis et al., (1996) for type B, and Ekong et al., (1999) for type E. Molecular methods based on demonstrating the presence of the toxin genes through polymerase chain reactions are the most commonly described techniques employed in the detection of *Clostridium botulinum(*Dahlenborg et al, 2001; Yousef et al, 2007; Lindström 2003, Sagua et al, 2005) . A number of reports have published oligonucleotides primers for specific amplification of the different *botulinum* toxin genes using polymerases in thermo-cyclic reactions (Lindström et al. 2001; Fach et al., 2002; Medici et al., 2009 and Kirchner et al., 2010).

2.2 Choosing media for pre-enrichment of *Clostridium botulinum* spores.

Due to these differences in nutritional requirements amongst *Clostridium botulinum* species, a selective enrichment specific for *Clostridium botulinum* is difficult to achieve and poorly understood. However, strict anaerobic methods, including creating anaerobic conditions in the media and incubation in anaerobic conditions are an essential requirement for the cultivation of *Clostridium botulinum* (Lindström, 2003). Successful germination of *Clostridium botulinum* spores in media is influenced by 3 main factors;

1. level of oxygen in the media (oxidation reduction potential),

2. Nutrient (proteins and or sugars) concentration of media,

3. Acidity of media (Perkin et al, 1987).

Table2.1 Chemical/nutrition make-up of TPGY, PGYS and RCM

Nutrients (g)	TPGY	PGYS	RCM
Peptone	5	5	10
Tryptone	50	5	NA
Yeast extract	20	10	3
Meat extract	NA	10	10
Glucose	4	5	5
Starch	NA	1	1
Agar	NA	NA	NA
Cy. hydrochloride	NA	0.5	0.5
Na thioglycolate	1	NA	NA
Na Chloride	NA	2	3
pH		7 +/_ 0.2	6.8+/- 0.2
Distilled water	1L	1L	1L

NA: Sodium. **Cy**: Cysteine.

Anaerobic conditions in media are usually achieved by adding a chemical reducing agent. cysteine hydrochloride and sodium thioglycolate are commonly used (Fukushima et al., 2002). However, Sodium thioglycolate is a better reducing agent, scavenging oxygen constantly and faster than cysteine hydrochloride. This enables media with Sodium thioglycolate to reach and maintain lower redox potential compared to media with cycteine Hydrochloride (Fukushima et al., 2002). Chopped meat glucose starch (CMGS) medium (CDC, 1998), cooked meat (CM) medium (Robertson, 1916; Quadliaro, 1977) Trypticase

peptone glucose yeast extract (TPGY) broth (Medici et al, 2009; Yousef et al, 2007; Lindström et al 2001), sometimes with the addition of trypsin (Lilly et al, 1971), and reinforced clostridium (RCM) medium (Gibbs et al, 1956). Peptone yeast extracts glucose starch (PYGS) (Yousef et al, 2007), have been used to successfully culture *Clostridium botulinum* and other clostridia. TPGY is the media of choice by the Food and Drug Administration (FDA) for the enrichment of *Clostridium botulinum* (Lilly and Solomon, 1998). Table 2.1 above evaluates the makeup of TPGY against some other *Clostridium botulinum* enrichment media

Clostridium spores germinate better in relatively nutrient concentrated medium than a more diluted one (Louis and Smith, 1975). By supplementing beef infusion medium with 1% Trypticase, 0.2% glucose, and o.3% agar, Louis and Smith (1975) obtained higher numbers of Clostridia cell counts from soil samples. Their results were better than those from a similar experiment by Gibbs and Freame (1965), who used Differential Reinforced Clostridial Medium, another known Clostridia enrichment media, as their enrichment medium. Louis and Smith (1975) demonstrated that in excess of proteins in a sterile medium, spores on proteolytic *Clostridium botulinum* were able to germinate at pH 4.1, underlining the importance of nutrient concentration in the germination of *Clostridium botulinum* spores. From table 2.1, TPGY is by far a more nutritionally concentrated media than RCM and PGYS. TPGY also contains Sodium thioglycolate, which according to Fukushima et al., (2002) is a stronger reducing agent than Cysteine hydrochloride in RCM. This means lower redox potential are quickly achieved and maintained in TPGY than RCM. The advantage of using maintaining lowest possible partial oxygen pressures in a medium during enrichment of *Clostridium botulinum* spores was iterated by Wong et al., 1988. At oxygen levels less than 2 µl/L, they noticed spore germination, and toxin production, even under acidic conditions. And slight increments in the oxygen levels led to very minimal growth. They considered low oxygen level, alongside nutrient concentration as the most influential factors in the germination of spores of strict anaerobes in any medium. Yousef et al (2007) recommended TPGY as preferable anaerobic media for experiment with media manipulation and sample inoculation carried out in aerobic conditions. In a media enrichment sensitivity test experiment with spores of all four human strains of *Botulinum*, Yousef et al (2007) had superior recovery from TPGY than PGYS (which somewhat similar in nutrient content and concentration to RCM), at different incubation times and temperatures.

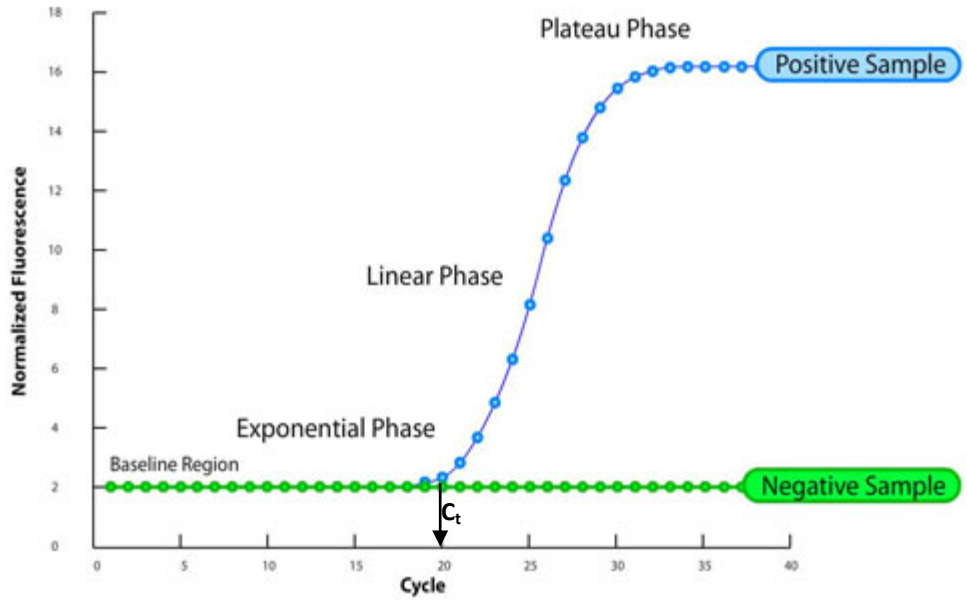
3 Technical information

Real-time or quantitative PCR: Real-time or Quantitative Polymerase Chain Reaction (PCR) is a PCR-based molecular technique used to amplify and quantify targeted DNA simultaneously. In the PCR process, a dye which fluorescents when bound to double stranded DNA is used to tag the amplicon during its amplification. Detected fluorescence indicates the presence of double stranded DNA (dsDNA), and the intensity of the former corresponds to the amount of the latter.

Threshold cycle (C_t) or Crossing point (C_p): During amplification, double stranded amplicons are bounded by a dye which fluorescents after bounding to dsDNA. Fluorescent intensity below the baseline i.e. intensity corresponding with that of the background fluorescence is not detected. When Fluorescent intensity from the sample crosses the baseline, it is detected and its intensity increase with increasing concentration of dsDNA (amplicon). The cycle number at which fluorescence from the sample surpasses the background signal (baseline), indicating the amplification of a targeted sequence is called the threshold cycle (C_t) and correspond to the Crossing point values for use in the LightCycler™ (Roche Applied Sciences)

$C_p \leq$ 29 = Strong signal; $C_p \geq 30 \leq 37$ = moderate signal; $C_p \geq 38 \leq 40$ = weak signal

Melting temperature (T_m): During PCR reaction, DNA passes through stages; denaturation (melting), annealing with primers, and elongation, in a repetitive series of thermo-cycling. The melting temperature of DNA is defined as the temperature at which 50% of the DNA exists as single stranded nucleotide sequences, and is depended on the Guanine: Cytosine content of the DNA, the sequence length of the DNA and the salt concentration in the DNA solution (McClatchey, 2002). This means that under constant reaction conditions, T_m values for DNAs with different sequence length and identities differ and vice versa. In the real-time PCR, the melting point of DNA is marked by a sharp drop in fluorescence, due dissociation of bounded dye from single stranded DNA, causing lose of Fluorescence by the dye.



The baseline region: the initial cycles of PCR during which, despite the theoretical doubling of product, there's little change in fluorescence signal above the background.

The exponential phase: during this phase the theoretical doubling of product at every cycle creates exponential signal growth and the reaction is very specific and precise.

The linear phase: reaction components are starting to become limiting and the reaction efficiency is falling so that the signal no longer grows exponentially.

The plateau phase: reaction components have been exhausted and the reaction can generate no more fluorescence.

Adopted from Abbott Molecular Global

Figure3.1. Real-time PCR data analysis

4 Materials and methods

4.1 Media preparation

TPGY was prepared by mixing the reagents shown on table 2.1 above in 1L of distilled water. The mixture was heated with continuous stirring until all the ingredients were completely dissolved. The broth was then transferred into bottle with air-tight corks. The corks were loosely placed on the bottles and autoclaved at 121^0C for 15 min. Bottles were quickly corked

air-tight after autoclaving and rapidly cooled in ice cold water to room temperature, then stored until use.

4.2 Soil treatment.

Approximately 0.5 g of each dust samples from Terneuzen **(**Table 4.1) were each placed in 5 mL of sterile distilled water in sterile tubes. The tubes were closed air tight and vortexed for 30 s x 30 s to loosen soil aggregates.

Table 4.1 Dust samples collected from within the convertor plant at Terneuzen.

Dust ID	Location of collection	Month of collection.
1	Box packer	June
2	Paper wrapper	June
3	Entrance Floor	June
4	Tooling covers near entrance	April
5	Rail-near Entrance Hall	April
6	Hot air side seal after skive section	April
7	Hot air side seal after skive section	April
8	External mud on surface between real storage and plant entrance	April
9	External mud on surface between real storage and plant entrance	April
10	External mud on surface between real storage and plant entrance	April
11	Boxing area	June
12	Soil sample near car park	April
13	Vacuum dust	April
14	Vacuum desk	April

The resulting homogenates were submerged in a water bath maintained at 60 ^{0}C for 10 min. Heat treatment was followed by quickly adding 40 mL of sterile TPGY into each tube. Then the bottles without corks were quickly placed in an anaerobic jar with 4 generators and an indicator and incubated for 2 weeks at 30^{0}C

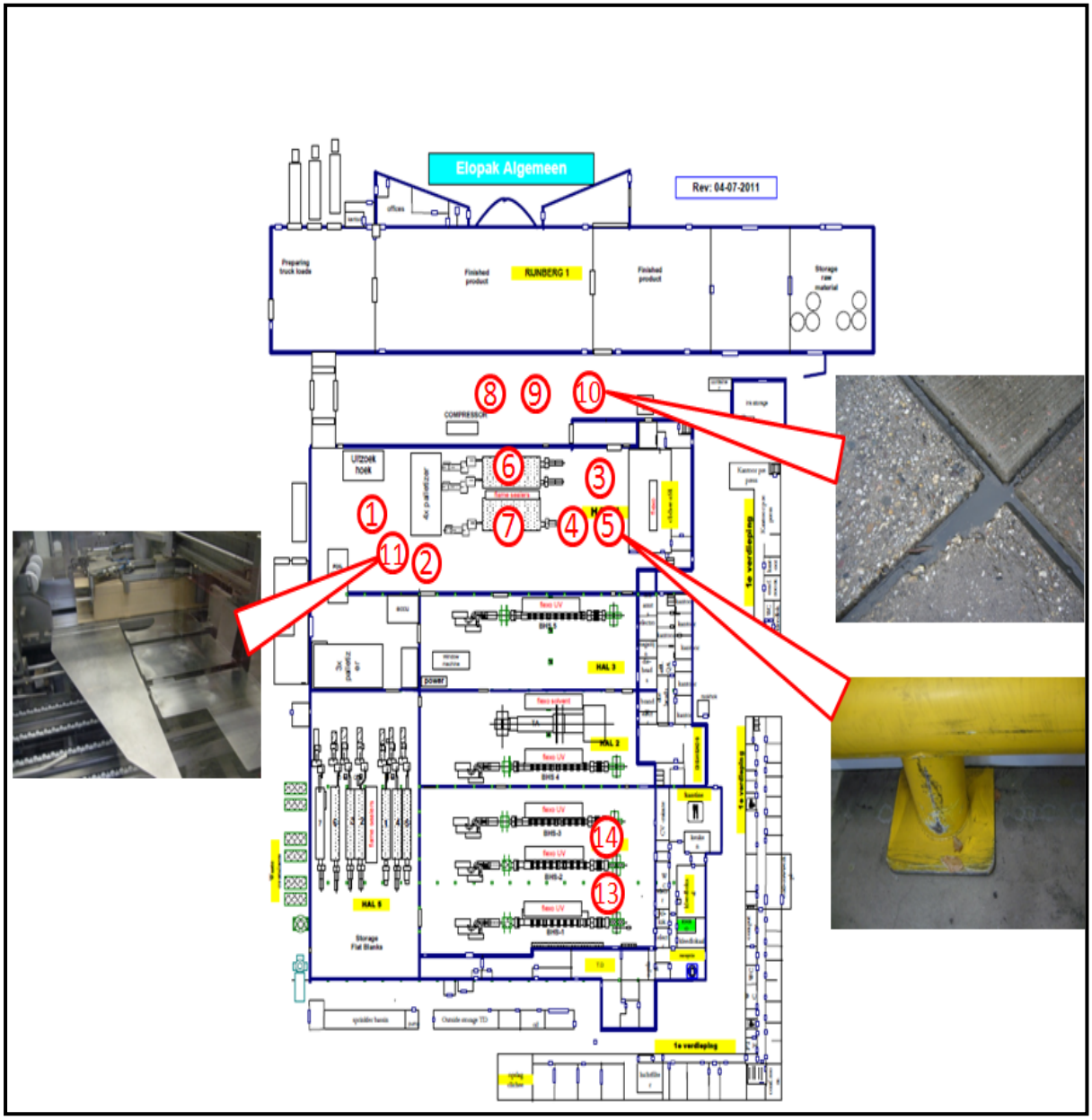


Figure 3.2 Dust sampling sites at the paperboard converter plant of Elopak at Terneuzen

4.3 DNA Extraction.

4ml of enriched TPGY broth for each sample was pelleted by serial centrifugation at of 1ml at 13 000rpm for 10 min. Pellets were then resuspended in 100 µl of STAR buffer. 100 µl of the suspended pellets were then transferred into FastPrep tubes containing 0.3 g of acid-washed glass beads of < 108 µm in diameter (Sigma-Aldrich). The tubes were then agitated twice in a MagNaLyser (Roche Diagnostic GmbH) at 6000 rpm for 40 sec, with 40sec rest between runs, keeping the samples cold during rest. After the last run, samples were then centrifuged at 3500 rpm for 5 min. 70 µl of the supernatant was transferred from the tubes into the KingFisher (KF) 96 plate. 50 µl of lysis buffer and 5 µl of proteinase was added to the wells, and mixed properly by pipetting. Then the plates were placed into the KingFisher robot (Thermo Scientific), and the ProteinaseMagMiniLGC procedure was used to incubate the samples at 55 ^{0}C for 10 min. In the next step, the KingFisher robot was programmed to add reagents only to wells containing samples.

MagMidi plates were prepared according to the table below.

Plate	Plate type	Content	Volume
1	KF well 96	Magic particles, Ethanol Samples	16 µl 105 µl 50 µl
2	KF well 96	Wash buffer 1	170 µl
3	KF well 96	Wash buffer 2	175 µl
4	KF well 96	Wash buffer 3	175 µl
5	KF well 96	Elution buffer	50 µl
6	KF well 96	Tip comb	

The different plates were placed in the KingFisher robot compartment (in the order above), and start the MagMiniLGC procedure. After the run was ended, pure DNA was then used in PCR.

4.4 PCR and PCR conditions.

Singleplex real-time was PCR performed on the LightCycler 480 II system in a total volume of 20 µl using a white LightCycler 480 Multiwell Plate 96 covered with adhesive sealing foil (Roche, Mannheim, Germany).

The PCR set up was as described by Kirchner et al.,(2010) with slight modifications. Briefly; thermal cycling followed a two-step PCR protocol: activation of the *Taq* DNA polymerase at 95°C for 15 min, followed by 45 cycles of 95°C for 15 s and 60°C for 40 s. The fluorescence data were collected at the end of every 60°C step, and runs were analyzed using LightCycler 480 software (Roche). Primers singleplex PCR are listed in table 4.1.

The singleplex reactions contained 2 µl of template, 4 µl of 5× EVAgreen QPCR mix, 0.6 µl primer (10pmol/ µl) for *Clostridium botulinum* serotypes A, B, E and F (Table), and H_2O added to a final volume of 20 µl

Table 4.2 Primer sets for botulinum neurotoxin genes (Kirchner et al., 2010)

Target	Primer	Sequence (5'-3')
bont/A	BoNT_A_F	ATTAGAGGTTATATGTATCTTAAAGGGC
	BoNT_A_R	CTAACAATATTATCTTYATTTCCAGAAGC
bont/B	BoNT_B_F	TTGCATCAAGGGAAGGCT
	BoNT_B_R	ATCCACGTCTATTAAATATACTTGCG
bont/E	BoNT_E_F	TGAAAATAATGTCAATCTCACCTCTTCA
	BoNT_E_R	AAATAATGCTGCTTGCACAGGTT
bont/F	BoNT_F_F	CCGGMTTCATTARAGAACGGAAG
	BoNT_F_R	TGATATTTCTTSTAACAAAACTTYCCCTG

4.5 Melting temperature (T_m)

The LightCycler 480 software version 1.5 was programmed to collect data during the PCR process for melting peak analysis.

4.6 Positive controls/PCR standard curves.

10000 folds serial dilutions were made from each of the *Clostridium botulinum* serotype A, B, E and F DNA, starting from an initial concentration of 100000 copies (0.043µg/µl). These dilutions were amplified as described in (4.4)

4.7 Negative controls

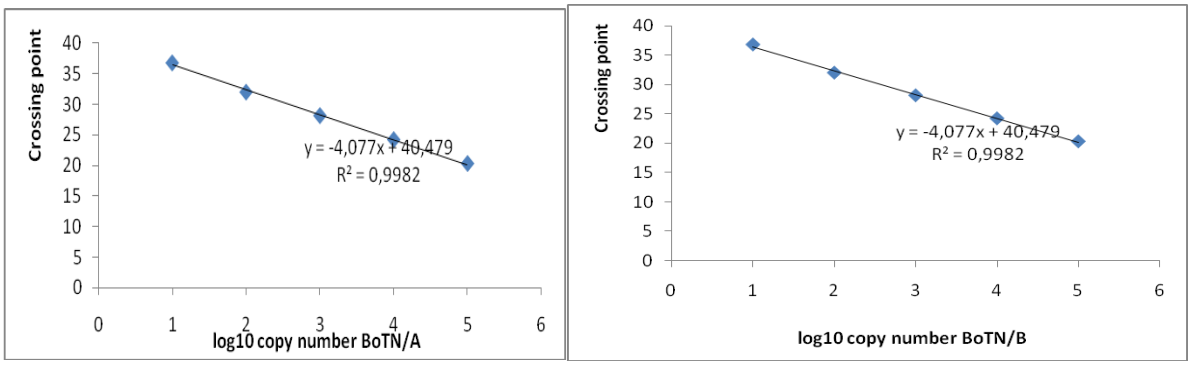
4- 18 µl PCR mixes corresponding to *Clostridium botulinum* serotype A, B, E and F were prepared as in 4.4, but without the template. 2 µl of sterile distilled water was then added to the PCR mix in place of the template and amplified as previously described in 4.4.

5 Results

5.1 Real time PCR performance (Control)

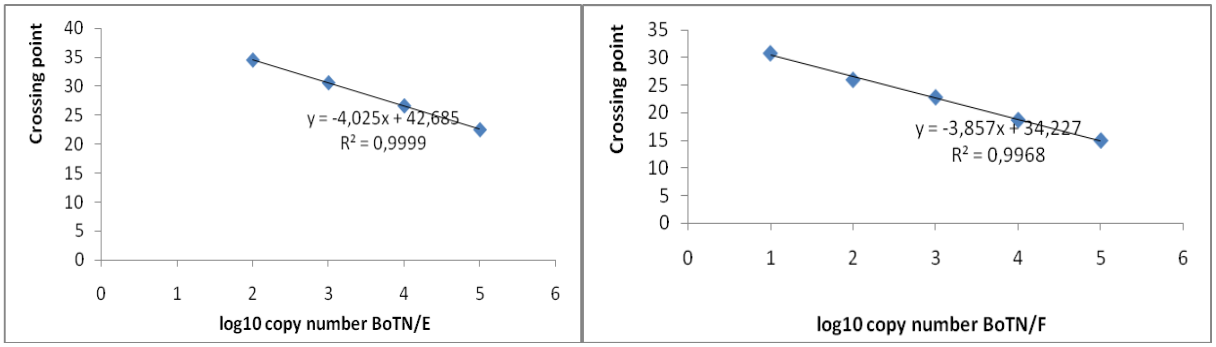
Fluorescent signals and melting curve temperatures were obtained from the quantification software of the LightCycler 480 software version 1.5 (Roche). The crossing point (C_P) values of each dilution, of each serotype, plotted against log10 of its corresponding copy number giving the PCR standards curves.

Real-time PCR assays for the control serotype DNAs showed a strong linear correlation ($R^2 >$ 0.99) between the C_p value and the template concentration over a range 10-100000 copy numbers (Figure 5.1a and 5.1b)



A B

Figure 5.1a Singleplex real-time PCR standard curves for *Clostridium botulinum* serotype A and B. Log10 of the copy numbers are plotted against their corresponding Crossing point (C_p) values.



E F

Figure 5.1b Singleplex real-time PCR standard curves for *Clostridium botulinum* serotype E and F. Log10 of the copy numbers are plotted against their corresponding Crossing point (C_p) values.

No fluorescent signals were detected from the negative control wells.

5.2 Sample analysis.

Clostridium botulinum serotype F neurotoxin genes were detected in dust samples 5 (rail in front of the main hall entrance) after about 39 PCR cycles (Figure 5.4), and serotype B neurotoxin genes in dust sample 11 (dust from boxing area) after about 40 PCR cycles (Figure 5.2).

Melting curve analysis was carried out for all the samples using the LightCycler 480 software version 1.5 to check that the right genes were being amplified in the dust samples. The melting peak temperatures were approximately 77.77 ^{0}C, 80.50 ^{0}C, 76.72 ^{0}C and 77.78 ^{0}C for the control serotype A, B, E and F genes respectively. Neurotoxin gene B detected in dust sample 11 had a melting peak temperature of 81.60 ^{0}C, 1.10 ^{0}C higher than the melting peak temperature for the control DNA of serotype B neurotoxin gene (Figure 5.3), while the neurotoxin gene F detected in dust sample 5 had a melting peak temperature of 80 ^{0}C, 2.32 ^{0}C higher than the melting peak temperature for the control DNA for the serotype F neurotoxin gene (Figure 5.5)

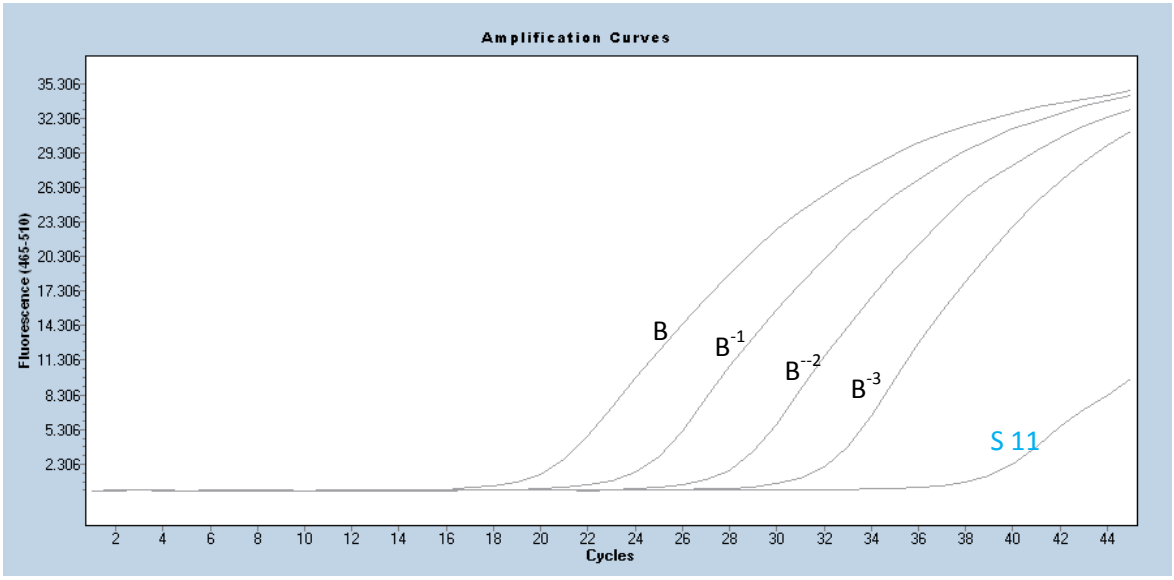


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Figure 5.2 Amplification curves for *Clostridium botulinum* serotype B neurotoxin gene. B, B-1B-2 and B-3 are serially diluted control DNA for serotype B while **S11** is the detected serotype B neurotoxin gene in dust sample 11.

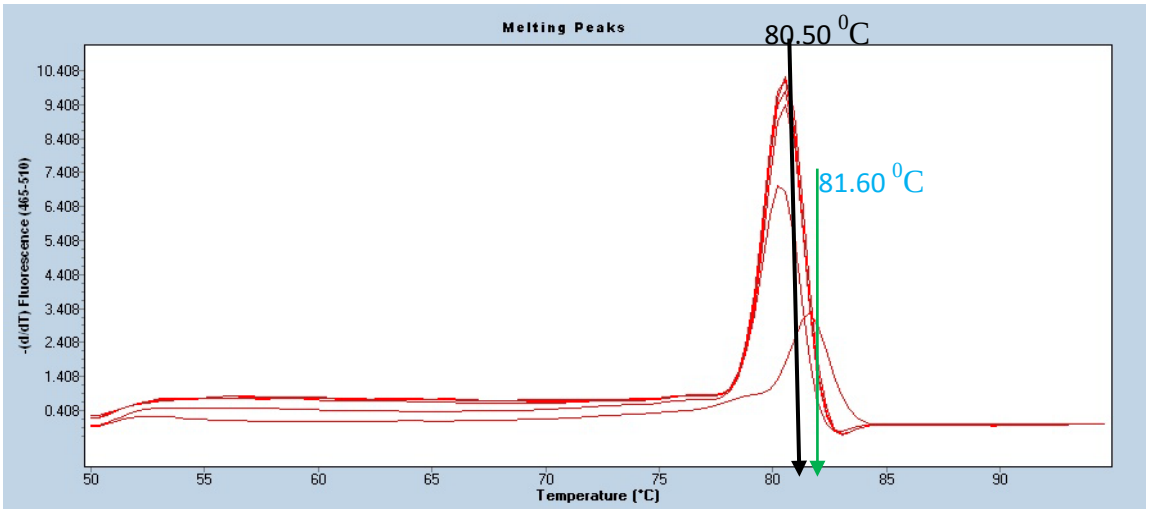


Figure 5.3 Melting peaks/peak temperatures for *Clostridium botulinum* serotype B neurotoxin gene. Melting of all serial dilutions of control DNA of serotype B peaks at 80.50 ^{0}C while the melting peak temperature of the serotype B isolate detected in dust sample 11 is 81.60 ^{0}C.

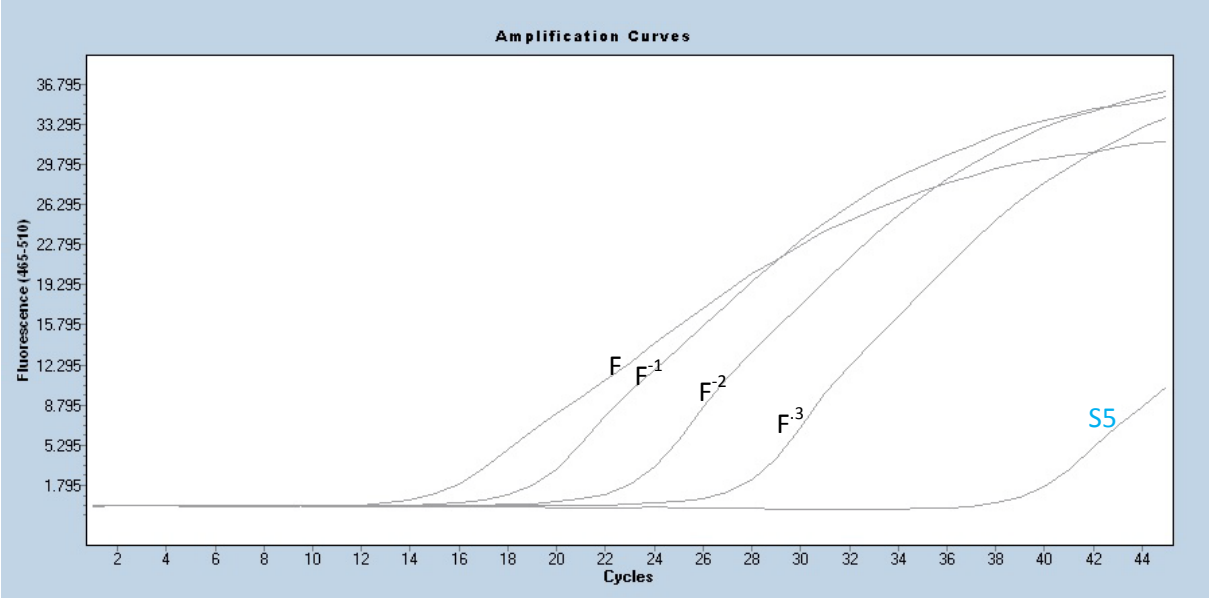


Figure 5.4 Amplification curves) for *Clostridium botulinum* serotype type F neurotoxin gene. F, F-^{1}, F^{-2} and F^{-3} are serially diluted control DNA FOR serotype F while **S5** is the detected serotype F neurotoxin gene in dust sample 5.

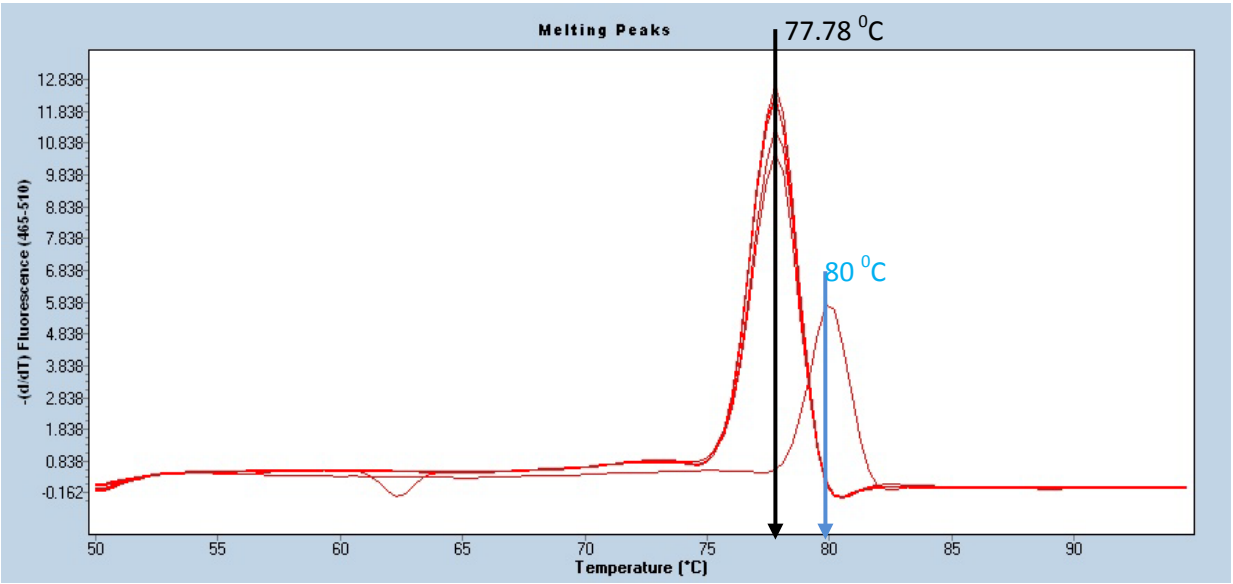


Figure 5.5 Melting peaks/peak temperatures for *Clostridium botulinum* serotype type F neurotoxin gene Melting of control DNAs for type B peaks at 77.78 ^{0}C while the melting peak of the B isolate in dust sample 11 is 80 ^{0}C.

Discussion

We detected *Clostridium botulinum* serotype F neurotoxin genes in dust from the rails at the entrance hall, and *Clostridium botulinum* serotype B neurotoxin genes in dust from the boxing area.

In real-time PCR analysis, melting peaks are generated and used to check that the targeted samples were amplified. Ideally, the melting peak temperature of a target DNA sample is expected to be the same with that of its corresponding control DNA. Melting temperature of DNA is dependent on the nucleotide composition and length of the DNA sequence. In classical situations, identical genes in two organisms of the same species should have the same melting peak temperatures. But this was not the case in our experiment. Melting of the targeted neurotoxin genes for type F and B detected in dust samples 5 and 11 respectively, did not peak at the same temperatures with melting of the neurotoxin genes from their corresponding control serotypes B and F. This raises concern on our claim that *Clostridium botulinum* is present in dust sample 5 and 11. It can be argued that these signals could have been from primer-primer dimers, given that the signals arrived quite late during the PCR run, or that the signals were as a result of nonspecific amplifications due to mispriming. However, a check of the primers against corresponding published genomes showed a perfect match Also, the high values of the melting peak temperatures ruled out the possibility that the signals could have been from primer-primer dimmers. The primers used in this experiment were just over 20 bp long and could not have melted at such high temperatures.

The cause of the difference in melting temperatures between the targeted neurotoxin genes in the dust sample 5 and 11 could be accounted for by the fact that C*lostridium botulinum* is very heterogeneous specie. Different subtypes of *Clostridium botulinum* serotypes A, B, E and F have been reported (Smith et al., 2005; Smith et al., 2007; Chen et al., 2007: Hills et al., 2007 and Carter et al., 2009), with a sequence variation of up to 31.6% among the different subtypes of the same serotype (Smith et al., 2005). Such a huge sequence variance amongst subtypes of the same serotypes would account for a slight difference in melting peak temperatures amongst them, as would have been the case in our results.

The failure to detect *botulinum* neurotoxin genes in the other dust samples in this experiment could not be interpreted as the absence of *Clostridium botulinum* spores in the sites from which these dust samples were collected. *Clostridium botulinum* spores have a unibiquitous

presence in the environment but at low numbers (Anonymous, 2008). Spore counts in the dust samples could have been below the detection limit of the PCR reaction in this study.

Due to the low numbers of *Clostridium botulinum* spores counts in the environment, large sample sizes are often required to be able to carry out an effective investigation on the incidence of these spores. The probability of finding *Clostridium botulinum* spores in a small sample is very low, due to its low spore counts in the environment. The necessity for a large sample size is further emphasized by the fact that other *Clostridium spp,* will inhibit *Clostridium botulinum* growth (Notermans et al., 1979), and the growth of *Clostridium botulinum* type E will be further inhibited by certain *Bacillus spp* (Lyver et al., 1998), given that these competitor bacteria to *Clostridium botulinum* had been detected in dust samples collected from the same sites as the dust investigated in this study (Section 2, this thesis). Unfortunately, only about 0.05-0.1 g of dust was analyzed for each of the dust samples investigated in this report. Such amounts could not have guaranteed the quality of an effective assessment on the incidence of *Clostridium botulinum,* and the absence of this bacterium in some of the dust samples in this study could have been false negative results.

It is thought that less than 1 spore in 1 million spores contaminating the low acid aseptic gable top carton of Elopak is a spore of *Clostridium botulinum*. This is based on the belief that there are no *Clostridium botulinum* spores within the convertor plant. With a carton sterilization system capable of a log 6 cycle reduction in spores of *Clostridium botulinum,* risk management experts at Elopak assume that the risk of *Clostridium botulinum* growth in the Elopak low acid aseptic gable top carton is effectively zero (Elopak, 2011). However, the results in this report showed that *Clostridium botulinum* spores might be contaminating Elopak low acid aseptic blanks at a higher frequency than is assumed. The presence of *Clostridium botulinum* type B in dust from the boxing area was critical. The boxing area is where side sealed carton blanks are packed into corrugated boxes for shipment to filling plants. Dust, which could be considered as an accumulation of airborne contaminants over time (Anonymous, 2004), would constitute a source of contamination in this area. Long and Tauscher (2006) suggested that dust in an area was the main source of airborne contamination in that area. The presence of *botulinum* spores in the boxing area indicates that the cartons handle in this area are probably contaminated with spores of *Clostridium botulinum* serotype B from the dust particles in the air around this area during the packing process. (Note that the

dust in this area was mostly from corrugated boxes) Toledo et al. (1973) identified airborne contamination as the main source of bacteria present on the blanks.

The dust from the rail near the entrance hall was most probably settling environmental dust particles, meaning that the *Clostridium botulinum* serotype F that was detected in this dust sample could have been coming from the soil around the rail. This area is the main entrance into the plant. Trucks and factory workers pass through this entrance into the plant, and would carry soil around this area on their tyres and shoes respectively, introducing it into the plant. Drying and aerolization of this soil would result in it being airborne contamination within the plant. This would increase the frequency of contamination on surfaces, including surfaces of flat and / or carton blanks by the *Clostridium botulinum* serotype F spores present in the soil (dust) particles. A consequence of this would be higher levels of contamination of Elopak low acid aseptic blanks by spores of *Clostridium botulinum* spores, more than is assumed to be present by packaging researchers, and the risk assessment committee at Elopak.

Recommendation

The gene products of from dust sample 11 and 5 should be sequence and identified to further confirm the identities of these genes.

SECTION 4

The ingression of bacteria through pinholes in packaging materials.

1 Introduction

Hermetic sealing of a package is very important in product sterility assurance. According to Gibney, (2000), aseptically packaging food does not guarantee the safety of the food during its shelf life. The safety assurance of food after post-processing is partially a responsibility of the package. An aseptic package should prevent external contamination, and should itself not serve as a source of contamination throughout the shelf life of its content. In packages with paperboard, such as the gable top food carton of Elopak, contamination of a food product by the paperboard is prevented by laminating the paperboard with thin sheets of polyethylene and aluminum (PE and Alu). The integrity of these layers is very crucial in enabling them to keep bacteria and other contaminants away from the stored food throughout its shelf life. Most cases of post-processing contamination in aseptically packaged food products have been blamed on defective packages (Michels and Schram, 1979). According to Fredrik; a packaging expert at Elopak, the Elopak gable top carton is has micro cracks and microscopic holes otherwise known as pinholes of approximately 20 μm in diameter in the layers laminating the paperboard. This paperboard is known to contain bacteria spores (This project), which might migrate through the pinholes to contaminate product. Determining when a pinhole or a crack will allow bacteria through it is an important aspect of package integrity analysis. For food safety assurance purposes, it is essential to investigate whether or not these defects in the gable top carton will allow bacteria found in the paperboard to pass through them into the product stored in the carton. Thus far, the presence of cracks and pinholes in the gable top carton presents a risk which has so far not been quantified. In order to use these cartons in the newly developed low aseptic processing chain, the quantification of such a risk an absolute necessity. Bacterial spores will easily germinate in low acid foods and or food products, posing spoilage problems, and even a public health hazard. The aim of this study was to determine the chances of bacteria spores in the paperboard of the Elopak gable top cartons to pass through worst case pinholes scenarios and contaminate products stored in these cartons. Worst case pinhole sizes were artificially created in the PE/Alu layers of the gable top carton in a challenge test to see if these defects will let allow bacteria ingression from paperboard through them into products stored

2 Literature Review

2.1 Pinhole studies

With the establishment of a direct link between the presence of a package defects (such as pinholes and cracks) and loss of package sterility (Kamei et al, 1991), much has been done to determine the minimum pinhole size that will allow microorganisms into packaged product. Virginia Chamberlain, a sterility expert with the US Food and Drug Administration once asked "If a package contains a pinhole, how big does it have to be to compromise product sterility? (Bryant, 1988). The question of when a pinhole begins to let bacteria pass through it might seem simple, but it has been a puzzle to packaging researchers over the years (Bix et al, 2004). Reported sizes at which bacteria begin passing through pinholes have ranged from 0.2μm to 80μm in diameter (Elopak 2010). By applying a vacuum, Horward and Duberstein (1980) found out that it was possible for bacteria present in water to cross 0.2μm membrane filters. They concluded their finding by speculating that pinholes of 0.2-0.4μm would let bacteria through. In 1998, Keller mathematically modeled the rate of evaporation on openings of tiny defects, and the rate of flow through these defects. He found out that for holes less that 2μm, the rate of evaporation exceeded the rate of flow. He then stated that at this point, plugging of the hole will result from deposited material left behind after evaporation, hence preventing ingression. These results seemed to tie with earlier suggestions by Lakes et al (1985) that ingression could only occur through pinholes significantly larger than 1μm. Chen et al (1991) demonstrated ingression through 5μm pinholes in packages containing juice. By imbedding metal and plastic discs with pinholes of different sizes into the closures of plastics vials containing different products, Sadhana et al (2005) determined that microbial ingression through the discs occurred in pinholes of sizes 5μm to 30μm, depending on the nature of the product as well as type of surface (metal or plastic). Other factors that have been documented to affect migration of bacteria through pinholes include;

- surface tension, viscosity, pH, chemical content of package product (Kamei et al 1991; Sadhana et al 2005)
- Bacterial morphology and motility (McEldowney and Fletcher 1990b).
- Differential pressure across defect (Keller 1998), Vacuum within package (Howard and Duberstein 1980).

- Length to diameter ratio of defect (Gnanasekharan and Floros 1994),
- Porosity of packaging components, thickness of packaging components, rigidity of the package at test, mechanism of microbial challenge (Elopak, 2010)

In 1979, Michels and Schram demonstrated post-process contamination through pinholes, and how storage environments impacted on the level of contamination. Using a needle of 100μm in diameter, they created pinholes in packages. These packages were withdrawn from the processing line after cooling, some stored wet, and others dried before storing. Contamination was documented in each case, but was ten times higher in packages that were stored wet (Michels and Schram 1979) Also by manually punching pinholes on paperboard packaging materials, Kamei et al (1991) showed that in the presence of pinholes, microorganisms in the paperboard contaminated packaged products. By filling packages with products of different constituents, they demonstrated that the nature of the product affected the rate of microbial ingression through pinholes. Keller (1998) stated that for microorganisms to migrate through a defect and contaminate a product, a flow of product through the defect was essential, and a threshold pressure was required to initiate this flow. Below this threshold pressure, no ingression occurred, and rate of ingression significantly increased at pressures above the threshold pressure (Keller et al, 2003). They also found no significant changes in ingression through pinhole sizes of 5μm, 10μm and 30μm with regards to bacteria morphology and motility. This contradicted the previous studies by McEldomney and Fletcher (1990a; 1990b) and. McEldomney and Fletcher (1990a) also noticed a fall in ingression with increasing product viscosity. A similar observation was made by Banks and Stringer (1988).

3. Materials and method.

3.1 Technical information.

The Elopak gable top carton is made up of 6 different layers (Figure 3.2a &3.2b); the paperboard, laminated with five other very tiny layers of different polymers.

The ***outer polyethylene (PE) layer*** is water resistant, and also protects against moisture and air. This layer bares the printing on the carton

The ***paperboard layer*** is the thickest layer of the carton. It is a network of cellulose fibres from wood. Its main function is providing the strength of the carton.

The ***Aluminium (alu) layer***. Aluminum is resistant to corrosion. Laminating paperboard with aluminum provides a highly effective barrier to the effects of air, temperature, moistures, light, as well as chemical attack. It also prevents microbes in the paperboard from accessing the food in the package. Its toughness and malleability also provides resilience to the food carton (Marsh and Bugusu, 2007).

The ***inner PE layer*** provides resistance to heat, mineral oils, solvents, and acids.

The ***Tie1*** **and** ***2 layers*** are so called because of the attachment roles. They are essential in holding adjacent layers to each other (Marsh and Bugusu, 2007).

Figure 3.1 Paperboard blank laminated with aluminium and polyethylene

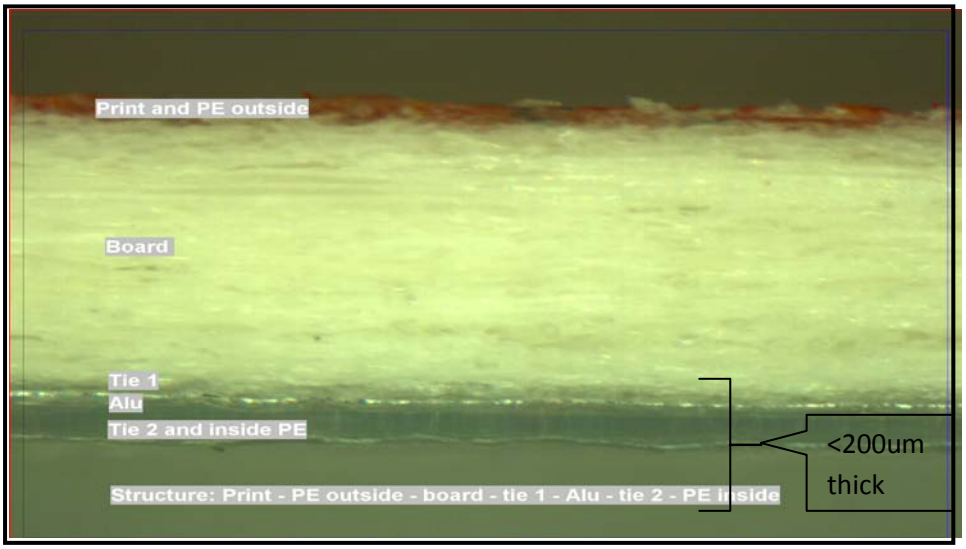


Figure 3.2a Cross section of the materials that make up the gable top carton

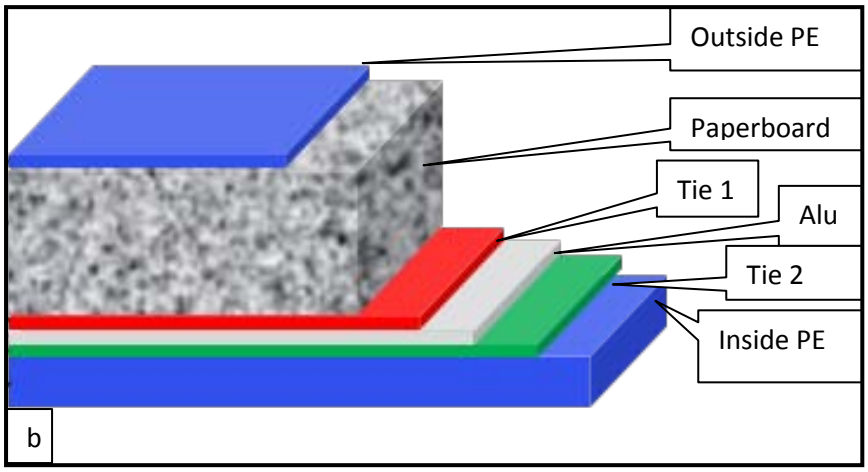


Figure 4.2b Illustration of the different layers in the Elopak gable top carton.

3.2 Making of pinholes

Pinholes were made in the PE/Alu layers using a special tool designed in the workshop at the Elopak Package Verification Center in Spikkestad, Norway. The tooling consisted of a metal plate, metal discs, needles, a metal rod and a "needle holder" (Figure 4.3 below). Three metal discs were placed on each other on the metal plate. The needle holder was then carefully placed on the arranged discs, holding it on top of the discs. A needle was then inserted through the cavity in the needle holder down to the metal plate surface. Then the metal rod was placed on the needle in the cavity to ensure that there was no upward movement of needle during fixing. The needle fixer was then screwed tight to hold needle in place. Care was taken not to fasten the fixer too tight as this could cause bending of the needle. After fixing, the rod was removed. The needle was now fixed in the holder with the tip of the needle extending approximately 300 μm at the bottom of the holder. The needle holder now with the needle extending 300μm below its bottom end was then placed standing inside semi-formed cartons, with open tops and bottoms. A gentle force was then applied for about 20 seconds on the needle holder to push the needle through the PE-ti2-Alu-Tie1 layers into the paperboard, creating pinholes in the process. 10 pinholes were created in each carton, with all panels having at least 2 pinholes. Pinholes were made at positions about 5cm above the bottom horizontal scorelines, and 10cm below the top horizontal scorelines on each panel of the carton. 53 parallels were used in this experiment.

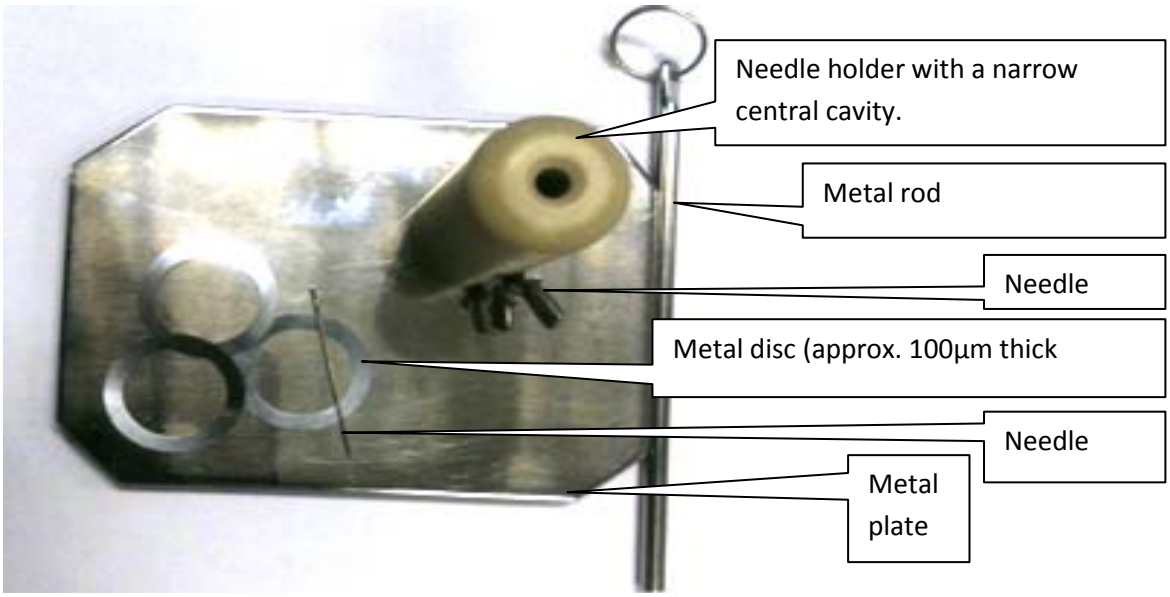


Figure 4.3: Pinhole tooling

3.3 Measuring Pinhole size

5 pinholes were created with a single needle and the sizes of the different pinhole were observed and measured using a biconcave microscope. The sizes of the holes on the tie1 layer, which is in direct contact with the paperboard, were also determined. The PE-tie2-Alu layers were peeled off to expose the inner Tie 1 layer by soaking the laminated board sheets containing pinholes in diluted solutions of Nitric acid for 18 h. Dilute nitric acid loosen bonding between the different layers in the carton. After 18 h, the inner PE, tie2 and Alu layers were carefully peeled off by hand. The remaining layers were rinsed with distilled water and dried. After drying, the size of the pinholes on the tie1 layer were observed and determined under biconcave microscopy.

3.4 Dye testing

5 pinholes were created in the carton layers of a semi-formed carton in the same way as described previous in 3.2. The semi-formed carton was opened along one of the vertical scorelines. Using a hot melt applicator (3M Scotch weld ^{TM,} U.S.A), melted wax was placed round the openings of the pinholes, creating small shallow wells of about 2cm in diameter. After solidification of the wax, the wells were filled with 2% methyl isobutyl ketone in 96% ethanol for 10mins, after which the dye was quickly washed away. The lamination (Inner PE,

tie2, Alu and Tie1 layers) on the paperboard was peeled off, and the diameter of the colored area of the paperboard, colored by the dye which had penetrated through the pinhole was determined. A similar test was done on the pinholes present in the cartons carrying AVM after the incubation period. Thoroughly washing the cartons with clean water was done in order to wash off sugars and other residues of AVM from the surface of the PE layer. The dye was allowed to stay for 20 min on the surface of the carton containing the pinhole opening.

3.5 Sterilization and Filling of cartons.

Cartons in which pinholes had been created were filled with media using the newly developed filling machine. Briefly, cartons were first bottomed sealed by the bottom sealer, then transported into the aseptic zone of the machine where they were sterilized with vaporized hydrogen peroxide (35%) and heat. After sterilization, the cartons were filled with Aseptic Validation Medium (AVM). Then top ends of the cartons were folded, and closures (corks) added. Cartons containing aseptically packaged AVM then exited the aseptic chamber and out of the filling machine. These cartons were carefully transported into an incubation room and stored for 5 weeks at 30^0C.

4 RESULTS

4.1 Measured pinhole sizes.

The sizes of the pinholes were determined both on the PE and the Tie 1 layers.

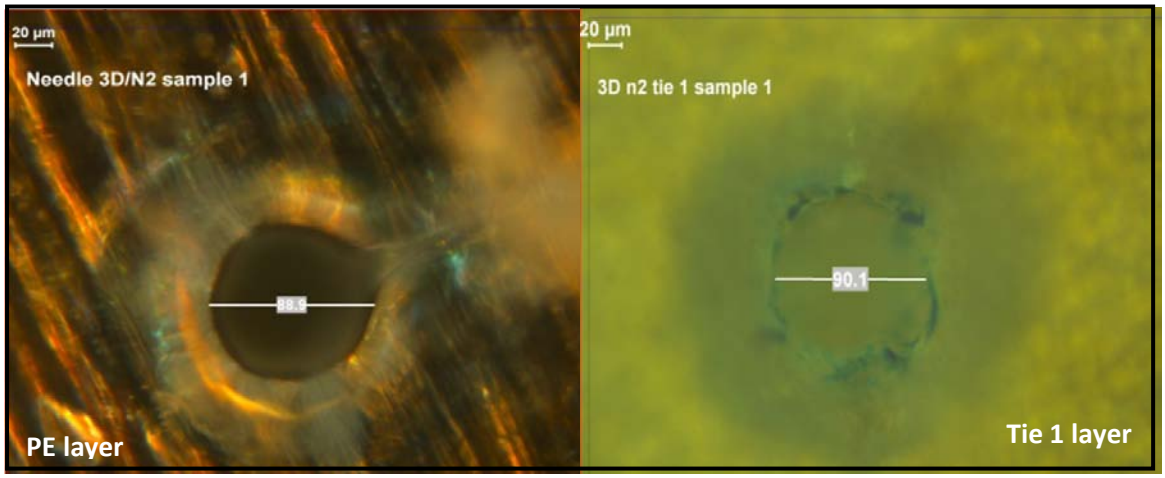


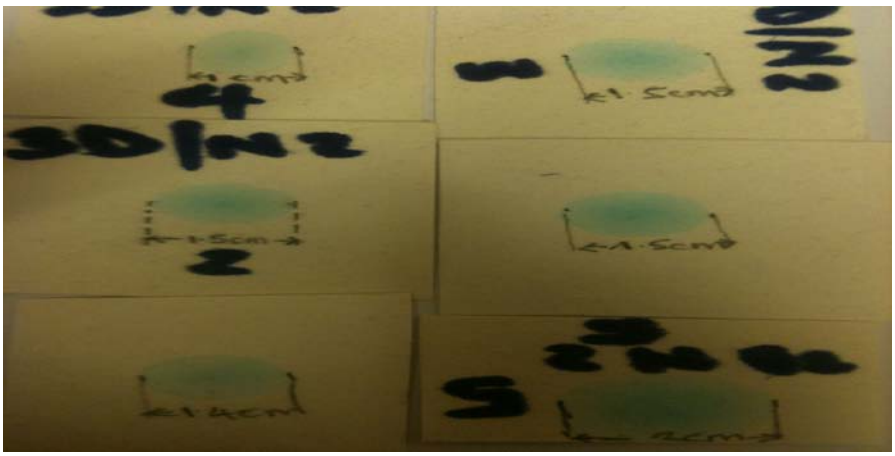
Figure 4.1 pinhole on surface of PE and Tie1 layers

Table 4.1 measured sizes of pinholes produced on inner PE and Tie1 surfaces.

Pinhole number	Diameter of pinhole on the inner PE layer (µm)	Diameter of pinhole on the tie1 layer (µm)
1	89	90
2	85	91
3	83	70
4	85	89
5	94	79
Mean	87.2	85.5
Standard deviation	4.4	9.1

4.2 Dye test.

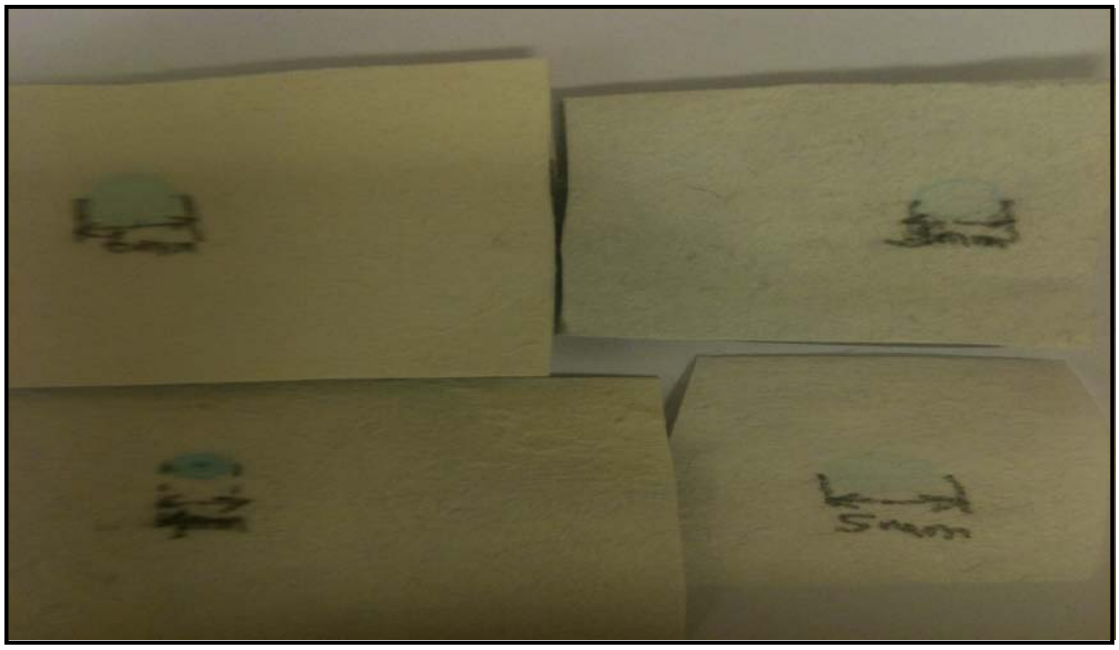
The diameter of dyed area of paperboard beneath 6 pinholes after dye testing for 10 minutes ranged from 1- 2cm (Table 4.2). The sizes of the dyed areas were greatly reduced, ranging from 0.4 cm to 0.6cm when dye testing was conducted in cartons that had been incubated with AVM in them for 5 weeks (see table 4.3)



Picture 4.1 Dye spread areas after in paperboard after 10minutes of dye testing on the pinholes.

Table 4.2 Diameters of areas covered by dye beneath pinholes after 10 minutes.

Pinhole number	Diameter of dyed area of board beneath (cm)
1	1
2	1.5
3	1.5
4	1.5
5	1.4
6	2
Average	1.48
Standard deviation	0.31



Picture 4.2 Dye spread areas in paperboard after 20minutes of dye testing on the pinholes in cartons with AVM incubated for 5 weeks.

Table 4.3 Diameters of areas covered by dye beneath pinholes from cartons containing AVM stored for 5 weeks.

Pinhole number	Diameter of dyed area of board beneath (cm)
1	0.5
2	0.6
3	0.5
4	0.4
Average	0.5
Standard deviation	0.08

Results from table 4.2 and table 4.3 showed an approximate 67% reduction in dyed area beneath pinholes of similar sizes when covered with AVM for 5 weeks.

4.3 Bacteria ingression into paperboard

AVM in all 53 cartons were completely free of turbidity after 5 weeks of incubation in cartons with pinholes, indicating the absence of bacteria growth

5 Discussion

A major problem associated with pinhole studies in packaging material is the creation of reproducible pinholes. Large variations in pinhole sizes have been document in many published reports. Axelson et al. (1990), reported variations of 5 µm -100 µm in needled-made pinholes while Maunder et al. (1968) reported 33µm to 160 µm. Reproducibility has also been a problem even in laser-made pinholes. Girlchrist et al (1989) reported variations between 17 µm -81 µm for laser drilled pinholes.

Hence the method of creating pinholes used in this study was much more reproducible, with a variation of 83-94 µm on the PE layer, and 79 µm to 94 µm on the Tie 2 layer. A mean size of 85.5 µm was determined on Tie 1 layer to represent the approximate size of all pinholes in

this study. The pinhole size was determined on the Tie one layer because it is the closest layer to paperboard where ingression would begin.

Several tests at Elopak using skive and unskived low cartons (not shown in this report) have shown that bacteria present in paperboard readily contaminates products stored in unskived paperboard cartons due to contact between product and paperboard, but did not contaminate the same products stored in skived paperboard cartons.

In this study, no bacteria were found to migrate from the paperboard through the 85.5µm pinholes in the Tie1-Alu-Tie2-PE layers into the Elopak low acid aseptic gable top cartons to contaminate AVM. This could imply that the size of pinholes in the Tie1-Alu-Tie2-PE layers of the Elopak low acid aseptic gable top carton that would allow bacteria ingression from the paperboard into the carton is greater than 85.5µm. The failure of bacteria to migrate across the laminating layers observed in this study could also have been due to the absence of bacteria in the vicinity of the pinholes. In addressing the short comings of microbial challenge testing involving pinhole studies, Morrical et al (2007) stated that in a microbial challenge test "one thing is having a pinhole of minimum size; another thing is having bacteria in vicinity of the pinhole.". If a bacterial spore was close to, but not in the vicinity of the pinholes, even if the pinhole sizes were large enough to tolerate bacteria ingression, no ingression would occur. The size of a pinhole will not matter if there are no bacteria to pass through it

The ability of bacteria present in the paperboard but not found in the vicinity of the pinhole, to migrate into the carton would depend on the ability of the product to diffuse in paperboard. Bacteria are said to either migrate across defects into packages (Banks and Stringer, 1988), or are being carried into the package by the product flowing through defect as a result of pressure differential across the defects (Keller et al, 1998). However, ingression of bacteria from paperboard into paperboard-based packages such as the low acid aseptic gable top cartons would only be due to transportation by the flowing product. This is because paperboard contains only spores (Vaisanen et al, 1991); Pirttijarvi et al 1996; Pirttijarvi, 2000: Raaska et al, 2002), which are non motile. Therefore, no ingression could also have been due to the fact that the product (AVM) in the cartons did not penetrate the pinholes, or did penetrate, but diffused only minimally in the paperboard after 5 weeks and did not reach spores.

To a packaging researcher, the findings of this study might still seem too optimistic. This is because previous studies have reported bacteria ingression through micro holes as small as 0.2µm in diameter (Horward and Duberstein, 1980). However, eventhough the principles of bacteria ingression through pinholes in packaging materials might be very similar, the dynamics involved in bacterial ingression from paperboard through pinholes into the packaged product are different from ingression that occurs in pinholes that traverse the entire packaging materials, as was the case with pinholes in documented pinhole studies. For the AVM that was filled in the cartons to access spores that were not in the vicinity of the pinholes, it needed to penetrate the pinholes and diffuse in the paperboard to reach these spores. Time could have been of essence for product to penetrate pinholes, diffuse in paperboard and reach spores for ingression to occur, given that the rate of product diffusion in paperboard was not known.

Also, the paperboard contained air, and the outer PE layer covering the outer surface of the paperboard and the Alu and inner PE layers (Figure 4.2a and 4.2b) were impervious to air. This meant that for product to penetrate pinholes to access the bacterial spores that were present in the paperboard, it needed to displace the air in the paperboard, a displacement which would most likely have occurred through the same pinholes. Outwards movement of air and inward movement of product might have created a “standoff” situation at the pinhole opening. As such, an extra force (threshold force) was required for product to displace the air, and the force would be directly proportion to the pressure exerted by the product, and the surface area of the pinhole. In pinholes with one opening such as those that were created in the PE/Alu layers, size was more of a limiting factor to penetration of product compared to pinholes with two opening, as was in pinholes that traversed the entire packaging material in reported literature. Air in the latter situation would have been displaced through the other end of the pinhole, and product was faced with a lesser resistance as it flowed into the pinhole.

Plugging of pinholes could have also been the reason why no microbial ingression into the cartons from the board was observed. Paperboard is a network of cellulose fibers from wood. Wetting of these fibers would have resulted to a close association between them, possibly leading to a blockade of the micro gaps between them. Deposition of sugars from the broth on the closely associating fibers, would have resulted in the plugging the pinholes. An evident of the effect of plugging on movement of substances in and out of the pinholes could be seen in the difference in dye penetration. In this study, incubation of AVM broth in cartons greatly

reduced the amount of dye that penetrated through pinholes, possibly due to plugging of pinholes.

The lack of bacterial contamination from the paperboard to the AVM in gable top cartons through pinholes of averagely 86 μm in diameter is quite a promising result with regards quality assurance of new low aseptic processing chain, as the most common pinhole sizes in the gable top carton have an average diameter of 20 μm.

However, no hasty decisions should be made based on this finding. Migration of bacteria though pinholes is affect by interplay of a combination of other factors besides pinhole size. This study did not test parameters such as viscosity of different products, pressure differentials created during transportation of product, Bacterial morphology and motility, etc. all of which have been reported to play pivotal roles in the ability of bacteria to penetrate pinholes.

The rate of diffusion of product through paperboard is not known. More time might be required for product to diffuse within paperboard to reach spores. A longer incubation time is recommended given that the shelf life of most shelf stable aseptic products exceeds 5 weeks.

Hence while this is a positive result regarding the risk posed by pinholes in the gable top carton, no definite conclusions can or should be made, without taking into account the effect of each one of the other factors that affect bacteria ingression into cartons.

General Discussion

Aseptic packaging of food in paperboard cartons has been on the rise in the past decade, and today, almost every type of food can be found in these “boxes” (Murphy, 2004). This technology has had a proud history so far on its contribution to food sanitation, thanks to its adherence to sanitary regulations and effective risk assessment studies, aspects which must be maintained for the continuous success of this technology.

Paperboard microbiological quality plays a very vital role in the overall robustness of this technology, and must be monitored in events of new developments. Our study has shaded light on the current microbiological conditions of the low acid aseptic blanks of Elopak.

This study has demonstrated that low aseptic made from paperboard produced at Stora Enso do contain *Bacillus spp.* (Table 4.1, section 2), a known pathogenic group of bacteria

(Granum et al., 1996: Ombui et al., 2001: Salkinoja-Salonen et al, 1999). However, the chances of these bacteria in the paperboard to access the product stored in low acid aseptic cartons is limited by lamination layers on the paperboard (Figure 4.2a&b, section 4). Although Kamei et al. (1991) demonstrated that microscopic holes in these layers would result to contamination of product by bacteria present in the paperboard; our study showed that none of these bacteria present in the paperboard could migrate through micro holes of about 90 µm in diameter on the laminating layers to contaminate products stored in these cartons (testing of other parameters in still required to effectively assess this; see 6, section 4). The infective dose of *Bacillus cereus* is quite high, between 10^5 and 10^8 cells, and toxin production has been determined to occur only at the end of the growth phase (Anonymous, 2005). Toxin production hence will most likely correspond with spoilage; hence reducing human exposure through consumption, with an overall reduction in the risk of human intoxication due to *Bacillus cereus*.

Bacteria found the carton blank surfaces have a direct impact on the sterilization efficiency, and the number of defective packages after carton sterilization by a filling machine. From our study, it could be deduced that Elopak low aseptic blanks produced at the converter plant in Terneuzen are contaminated with Spores of *Bacillus* spp, and also possible spores of *Clostridium botulinum*. Although Elopak is aimed at producing an efficient sterilization system, results from section 1 in this study showed that structures produced during bottom sealing of Elopak cartons were capable of preventing inactivating of some of the spores trapped in them during the process of carton sterilization (4.1 and 4.4, section 1). These spores would grow under favorable conditions in products filled in the cartons during storage and distribution. Unlike *Bacillus* spp., which have a very high infective dosage, with toxin production on at the later stages of growth, *Clostridium botulinum* toxins of 30–100 ng is potentially fatal (Peck et al., 2011), and toxin production occurs at the very early stages of growth (Peck et al, 1997). This implies that defective packages due to the failure of the system to inactivate trapped spores in the structures within the carton might easily result in human exposure, because visual and/or taste detection of spoilage which would prevent the product from being consumed would be unlikely.

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