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Evaluation of Concrete Sealant¥ for the Elimination of *Clostridium perfringens* and *Bacillus subtilis*: A Poultry Processing Plant Model

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Abstract: This study was conducted to determine the efficiency of BioSealed for Concrete™ against *C. perfringens* and *B. subtilis* on concrete blocks. Concrete blocks were divided into four different treatments: A) No Biosealed application; B) Biosealed applied before inoculation; C) Biosealed applied after inoculation; or D) Biosealed applied before and after inoculation with *C. perfringens* and *B. subtilis* individually (Ca. 10^9 CFU/mL). The *C. perfringens* inoculated concrete blocks were then incubated at 37°C for 48 h anaerobically; while the *B. subtilis* inoculated concrete blocks were incubated at 37°C for 24 h aerobically. External and internal surfaces of the treated concrete blocks were swabbed for microbiological analysis. Significantly lower ($p<0.05$) populations of both microorganisms were observed for treatment groups C and D as compared to A and B on the external surface of the concrete blocks whereas, no significant differences ($p>0.05$) were observed between treatment groups A, B and C on the internal surfaces of the concrete blocks. No significant differences ($p>0.05$) were found when comparing groups A and B, while a dual application of Biosealed for Concrete™; pre- and post-inoculation showed the greatest reduction ($p<0.05$) on the external and internal surfaces of the concrete blocks. Results from this study indicated that Biosealed for Concrete™ has an immediate bactericidal effect on *C. perfringens* and *B. subtilis* and has the potential to be used in combination with other GMP's and sanitation practices to control bacterial colonization on concrete surfaces in a poultry processing plant.

Key words: *Bacillus*, *Clostridium*, concrete, antimicrobial

INTRODUCTION

Clostridium perfringens is an important pathogen that causes a wide variety of diseases in humans and animals. The ubiquitous nature of this organism results in its frequent implication in a variety of foods (ICMSF, 1996). *C. perfringens* has two main characteristics that contribute to its ability to cause foodborne disease. Firstly, it's low generation time (reportedly <10 min for vegetative cells) allows *C. perfringens* to quickly multiply in foods (McClane, 2001; Setlow and Johnson, 2001) and secondly, its relatively higher heat tolerance enhances its ability to survive in undercooked foods. *C. perfringens* also has the ability to form spores which are resistant to environmental stresses such as radiation, desiccation and heat which facilitates survival of the pathogen in undercooked and/ or inadequately warmed foods (McClane, 2001). Meat and poultry products have been implicated in numerous outbreaks of foodborne disease (Doyle, 2002). The Centers for Disease Control and Prevention estimate more than 248,000 cases of foodborne illness due to *C. perfringens* infection occur annually in the United States. *Bacillus subtilis* is another spore forming pathogen which has been implicated in numerous foodborne outbreaks, although not typically associated with poultry and meat products The natural ability of this pathogen to transform under stress and form spores as well as an abundance of molecular, biological and genetic information has made *B. subtilis* the organism of choice for mechanistic studies on sporulation, spore germination and spore resistance (Setlow and Johnson, 2001). In addition, several researchers, while investigating the physiopathology, behavior, sporulation and resistance of *B. anthracis* have been using *B. subtilis* as a surrogate due to its low pathogenic profile.

It has been well documented that spores exhibit a higher resistance than vegetative cells when exposed to a variety of chemical compounds and physical treatments. Sporulation allows the organisms protection from cross-linking agents such as glutaraldehyde, oxidizing agents, phenols, formaldehyde, chloroform, octanol, alkylating agents such as ethylene oxide, iodine and detergents, as well as pH and temperature extremes and lytic enzymes such as lysozyme (Slepecky and Hemphill, 1992; Setlow and Johnson, 2001). The spore metabolic dormancy is undoubtedly one factor contributing to the survivability of these microbes during extended periods both in the absence of nutrients and hostile environments. When nutritive and environmental conditions become conducive, spores can germinate

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and return to a vegetative state, resulting in potentially causing serious problems to the food industry due to shelf life reduction and contamination of foods (Setlow and Johnson, 2001). Bacterial endospores can survive in the environment for an extended period of time and are resistant to a wide-variety of treatments such as heat, desiccation, radiation, pressure and chemicals (Nicholson *et al*., 2000).

During poultry processing, meat comes in direct and close contact with a variety of surfaces, including equipment, machines, tables and potentially walls and floors at multiple sites along the production line. These surfaces can potentially become contaminated and can serve as sources of contamination during subsequent processing (Gerats *et al*., 1981). Studies related to environmental cross-contamination have been carried out in dairy processing (Frank *et al*., 1990; Gabis *et al*., 1989; Lopes, 1986). Although a potentially important source of contamination, limited efforts and studies have been conducted to decrease cross-contamination from environmental factors such as conveyor belts, drains, floors, etc. during poultry processing (Thomas *et al*., 1987; Frank *et al*., 1990; Krysinski *et al*., 1992).

Concrete is present in the food industry especially in flooring, walls and ceilings. During processing, concrete receives a great amount of organic matter. The organic matter in the poultry industry is a result of usual processing steps such as bleeding, scalding, eviscerating and feather-picking. This organic matter has the potential to serve as an initial source of nutrients to microorganisms allowing them to colonize on and/or in concrete. Concrete is a microporous, microstructure-sensitive construction material and the pores in concrete are randomly sized, arranged and connected (Yang *et al*., 2004). These pores form capillary systems in concrete allowing water and other substances to traffic freely in concrete structures. When liquids flow freely on concrete, they may serve as carriers in the transport of microorganisms such as bacteria. Therefore, concrete or masonry walls in food processing and storage facilities require coatings or treatments that can be efficiently cleaned but remain impermeable to moisture, cleaning solutions, food acids, fats and other materials (Katsuyama and Slrachan, 1980).

To understand and interpret the behavior of composite element such as concrete, knowledge of the characteristics of its components is necessary. Disintegration of concrete due to cycles of wetting, freezing, thawing, drying, chemicals and the propagation of the resulting cracks is a matter of great importance for the food industry (Nawy, 1996). The disintegration of concrete will serve as great attachment sites for bacteria to form niches which in turn can work as permanent sources of contamination within a processing environment. Contamination of food may occur from direct contact of food to concrete surfaces or indirect contact during normal operating procedures (water splashing during sanitation, staff shoes and clothing). Inorganic interfaces are rapidly colonized by microorganisms sometimes posing serious problems for the industry and hygiene in general (Brisou, 1995). Therefore, the food industry has placed a great deal of effort on the reduction of the development of bacterial niches which can ultimately result in the formation of more resistant and protective biofilms. There is no single action which will reduce or eliminate bacterial niches from industrial environments and several actions must be taken collectively to prevent the formation and elimination of these chronic sources of contamination. The objective of this study is to determine the efficiency of BioSealed for Concrete™ (GreenSealed Solutions, Inc. -Georgia) as an antimicrobial and its ability to prevent colonization of *C. perfringens* and *B. subtilis*.

MATERIALS AND METHODS

Bacterial cultures: *Clostridium perfringens* and *Bacillus subtilis* were independently cultured in Brain Heart Infusion broth (BHI; Acumedia Manufacturers Inc., Lansing, MI) and incubated at 37°C for 24 h with *C. perfringens* incubated anaerobically (Bactron IV Anaerobic Chamber; Shel Lab, Cornelius, OR) prior to challenge. The length of incubation and inoculation of the concrete blocks were based on 24 h growth curves that were performed in the laboratory (data not shown).

Concrete bricks preparation: Quikrete® concrete mix #1101 (Quikrete®, Atlanta, GA), a 4000 psi compressive strength blend of portland cement, sand and gravel used for general concrete of floors and walls was reconstituted as per manufacturers' directions to produce concrete blocks in commercial sized ice cube trays. Ice cube sized bricks (total external surface area 40 cm^2) were made to simulate commercial concrete blocks for experimental purposes in the laboratory.

Application of Biosealed for Concrete™: Concrete bricks were divided into four treatment groups: A) bricks which were not treated with BioSealed for Concrete™ (control); B) bricks treated with BioSealed for Concrete™ before inoculation; C) bricks treated with BioSealed for Concrete™ after inoculation and D) bricks treated with BioSealed for Concrete™ before and after inoculation. The individual inoculum was divided in two equal parts and the treatments were challenged together as: (1) Groups A and C (bricks untreated before inoculation) and (2) Groups B and D (bricks which were treated before inoculation). BioSealed for Concrete™ was sprayed on the surface of the bricks using a paint sprayer (Wagner 5.4 GPH, Wagner Spray Tech Corporation, Plymouth, MN) according to the manufacturer's directions (using a fan spray nozzle held 6 in. from the concrete surface @ 200 ft^2/gal with an overlapping spray pattern of 20-30%).

Microbiological analysis: For these experiments, the concrete blocks were divided into groups and challenged with either *C perfringes* or *B subtilis*. To study the effects on vegetative cells, concrete blocks were submerged into inocula of *C. perfringens* (Ca. ~7.07 $\log_{10}$ CFU/ml) and *B. subtilis* (Ca. ~ 7 $\log_{10}$ CFU/ml) for 24 h at 37°C. Concrete blocks were removed and dried for 30 min in a sterile laminar flow cabinet (Nuaire Inc., Plymouth, MN). Moist sterile swabs (Solon Mfg. Co., Skowhegan, ME) were then used to sample the entire external surface of each brick. The swabs were then placed in tubes containing 10 ml sterile 0.1% peptone water (PW; Acumedia Manufacturers Inc., Lancing, MI), vortexed for 30 sec and serially diluted. After swabbing the external surface, bricks were broken in halves using a sterile chisel and hammer. The internal surfaces of both halves were swabbed and the swabs were placed in 10 ml sterile 0.1% PW tubes. Tubes were vortexed for 30 sec and serial dilutions were spread plated onto tryptose sulfite cycloserine agar (TSC; Oxoid Ltd., Hampshire, England) for *C. perfringens* analysis, or mannitol-egg-yolk-polymyxin agar (MYP; Becton, Dickinson and Company, Sparks, MD) for enumeration of *B. subtilis*. The TSC agar plates were incubated anaerobically in 7.0 L anaerobic chambers (Mitsubishi Gas Chemical, Japan) with Anaerogen gas sachets (Oxoid, Basingstoke, Hampshire, England) at 37°C for 48 h and the MYP agar plates were incubated aerobically at 37°C for 24 h. Results were recorded following incubation as $\log_{10}$ CFU/cm^2 with the exception of inocula samples, which were recorded as $\log_{10}$ CFU/ml. To evaluate the spore resistance of *C. perfringens* and *B. subtilis* in this study, after the bricks had been submerged in the respective inocula for 24 h at 37°C, they were subjected to a temperature of 70°C for 15 min to induce sporulation of the cells while still immersed. The concrete blocks were then removed from the inocula and dried for 30 min in a sterile laminar flow cabinet (Nuaire Inc., Plymouth, MN). Sporulation of the cells was confirmed by Gram staining and observation of these samples under a contrast microscope (unstained refractory structures). Sporulation was also verified by the Schaffer-Fulton spore staining technique using malachite green followed by microscopic observation.

Statistical analysis: A completely randomized design was used to assign concrete blocks to the four treatment groups. Three replications of this experiment were performed and within each replication the survival populations ($\log_{10}$ CFU/cm^2) of *C. perfringens* and *B. subtilis* were reported as a mean of three concrete blocks. Results were analyzed using analysis of variance (ANOVA) with SAS PROC GLM procedures (2002-03 SAS 9.1 Institute, Gary, NC) and statistical significance was reported at a p-value of less than or equal to 0.05 ($p \leq 0.05$).

RESULTS AND DISCUSSION

External brick surfaces: Analysis of Variance (ANOVA) of survival populations of *Bacillus subtilis* ($\log_{10}$ CFU/cm^2) did not show any significant difference ($p>0.05$) between treatment groups A and B (Table 1) on the vegetative cells and spores. Also, no significant difference ($p>0.05$) was observed when comparing treatment groups C and D. Significantly lower ($p<0.05$) survival of the vegetative cells was observed in treatment groups C (2.96 $\log_{10}$ CFU/cm^2) and D (2.7 $\log_{10}$ CFU/cm^2) as compared to those in treatment groups A and B suggesting a bactericidal effect of BioSealed for Concrete™. Similarly, the spores of *B. subtilis* were reduced ($p<0.05$) following treatments C and D as compared to treatment A. The survival populations of the *B. subtilis* spores were 1.09 $\log_{10}$ CFU/cm^2 and 1.53 $\log_{10}$ CFU/cm^2 for treatment groups C and D respectively, indicating the spores to be more resistant to BioSealed for Concrete™ as compared to the vegetative cells. No significant differences ($p>0.05$) between the treatment groups A and B indicate that prior application of BioSealed for Concrete™ does not prevent vegetative cell or spore attachment to the external surfaces of the concrete.

Results for *Clostridium perfringens* were similar to this of *B. subtilis* (Table 1). BioSealed for Concrete™ significantly reduced ($p<0.05$) the survival populations of the vegetative cells and spores of *C. perfringens* following treatments C and D. Surviving populations of 1.19 $\log_{10}$ CFU/cm^2 and 1.86 $\log_{10}$ CFU/cm^2 following treatment C and D respectively for vegetative cells; 1.04 $\log_{10}$ CFU/cm^2 and 1.7 $\log_{10}$ CFU/cm^2 for the spores following treatments C and D respectively indicate that the spores and vegetative cells responded similarly to the BioSealed for Concrete™. Throughout this study no significant differences ($p>0.05$) were observed between treatment groups A and B. Also, because survival populations of *B. subtilis* and *C. perfringens* were not significantly different ($p>0.05$) between treatment groups B and C, it suggests that treating concrete with BioSealed for Concrete™ prior to or post bacterial contamination does not change bacterial colonization behavior.

Internal brick surfaces: Analysis of Variance (ANOVA) of the survival populations ($\log_{10}$ CFU/cm^2) of vegetative cells and spores of *B. subtilis* on the internal surfaces of concrete blocks did not suggest any significant differences ($p>0.05$) between treatment groups A, B and C (Table 2). The populations of vegetative cells and spores of *B. subtilis* recovered from the internal surfaces of concrete blocks were below 1-$\log_{10}$ CFU/cm^2 for all four treatment groups, hence limiting the ability to determine the true magnitude of the efficacy of BioSealed for Concrete™. Similar results can be observed for the *C. perfringens* spores, where no

Table 1: Survival populations[@] (log_{10} CFU/cm^2) of *Bacillus subtilis* and *Clostridium perfringens* on the external surfaces of concrete blocks

	Bacillus subtilis		*Clostridium perfringens*	
Treatments	Planktonic	Spores	Planktonic	Spores
A	5.53(0.44)^a	2.23(0.31)^a	3.76(0.26)^a	2.59(0.25)^a
B	4.26(0.44)^a	1.37(0.31)^{ab}	2.97(0.26)^{ab}	1.87(0.25)^{ab}
C	2.57(0.44)^b	1.14(0.31)^b	2.57(0.26)^{bc}	1.55(0.25)^{bc}
D	2.83(0.44)^b	0.70(0.31)^b	1.90(0.26)^c	0.89(0.25)^c
p-value	0.0051	0.0243	0.0071	0.0082

[@]Least square means (standard error)
A = No BioSealed for Concrete™ application
B = BioSealed for Concrete™ applied before bacterial inoculation
C = BioSealed for Concrete™ applied after bacterial inoculation
D = BioSealed for Concrete™ applied before and after bacterial inoculation
Superscripts (a, b and c) indicate significant difference ($p<0.05$) within a column

Table 2: Survival populations[@] (log_{10} CFU/cm^2) of *Bacillus subtilis* and *Clostridium perfringens* on the internal surfaces of concrete blocks

	Bacillus subtilis		*Clostridium perfringens*	
Treatments	Planktonic	Spores	Planktonic	Spores
A	0.95(0.37)^a	0.81(0.21)^a	1.66(0.21)^a	0.70(0.10)^a
B	0.70(0.37)^a	0.70(0.21)^a	1.21(0.21)^b	0.70(0.10)^a
C	0.70(0.37)^a	0.70(0.21)^a	1.20(0.21)^b	ND(0.10)^b
D	ND(0.37)^b	ND(0.21)^b	0.83(0.21)^c	ND(0.10)^b
p-value	0.3806	0.4411	0.2036	0.0015

[@] Least square means (standard error)
A = No BioSealed for Concrete™ application
B = BioSealed for Concrete™ applied before bacterial inoculation
C = BioSealed for Concrete™ applied after bacterial inoculation
D = BioSealed for Concrete™ applied before and after bacterial inoculation
Superscripts (a and b) indicate significant difference ($p<0.05$) within a column

significant differences ($p>0.05$) were observed between treatment groups A, B, C and D (Table 2). On the other hand, significant reduction ($p<0.05$) of the vegetative cells were observed when comparing treatment groups A to B (Ca. 0.45 log_{10} CFU/cm^2), C (Ca. 0.46 log_{10} CFU/cm^2) and D (Ca. 0.83 log_{10} CFU/cm^2). Although, lower counts of *C. perfringens* spores recovered from the internal surfaces of the concrete blocks were a limiting factor to have a true estimation of the antimicrobial effect of the BioSealed for Concrete™, this also indicates a possibility that the topical spray of BioSealed for Concrete™ prevented any further penetration of vegetative cells and spores of *B. subtilis* and *C. perfringens*.

The major types of sanitizers used in the food industry over the years have been halogens, peroxygens, acids and quaternary ammonium compounds (Bower and Daeschel, 1999; Gandhi and Chikindas, 2007; Johnson *et al*., 1990). The bactericidal efficacy of these antimicrobials depends on several factors such as time of exposure/ length of contact, concentration of the antimicrobial and temperature, making direct comparisons difficult. Lindsay and Holy (1999) evaluated the responses of planktonic and attached (stainless steel and polyurethane) *B. subtilis* to different sanitizer treatments. In their study it was reported that a 170 ppm mixture of peracetic acid and hydrogen peroxide with a 5 min exposure time was the most effective sanitizer against planktonic *B. subtilis*, resulting in a 2-log reduction. However, when attached *B. subtilis* was exposed to 35 ppm of iodophor or 1000 ppm of chlorhexidine gluconate for 5 min, only a 1-log reduction was observed. Data from our study suggested an increased bactericidal activity of BioSealed for Concrete™ when applied as a topical spray post-inoculation, suggesting its application against pre-existing contamination on concrete in processing plants would likely decrease bacterial counts. Very limited information is available on the susceptibility of vegetative cells of *C. perfringens* to sanitizers. Taormina and Dorsa (2007) evaluated a hot water and sanitizer dip treatment against loosely attached cells of *C. perfringens* on stainless steel knives. In their study a 2.04 log reduction was reported after dipping the knives in 400 ppm of quaternary ammonium for 1 sec, while acid quaternary ammonium (440 ppm) and peracetic acid (700 ppm) resulted in a 1.96 and 1.50 log reduction, respectively. Shetty *et al*. (1999) evaluated the bactericidal activity of a mixture of oxidizing compounds with hypochlorous acid as the main component at a concentration of 144 mg/l. In their study they used a spore suspension of *C. difficile* in the presence and absence of organic matter. A 4-log reduction was reported when the spore suspensions alone were exposed for 2 min. while the organic load completely inactivated the sanitizing mixture.

In our study, although application of BioSealed for Concrete™ prior to contamination of *B. subtilis* and *C. perfringens* showed limited reduction of these pathogens, multiple factors such as contact time and concentration of the disinfectant need to be addressed. Further studies need to be conducted to establish application parameters taking into consideration factors like surface type and disinfectant concentration to further determine the antimicrobial potential of BioSealed for Concrete™ against sporeforming pathogens in food processing plants.

Conclusion: BioSealed for Concrete™ was effective in reducing the vegetative cell and spore populations of *B. subtilis* and *C. perfringens* on the external surfaces of the concrete blocks. Although its antimicrobial effectiveness was limited due to the lower populations recovered from the internal surface of the concrete blocks, results from this study indicate that BioSealed for Concrete™ can be used as an antimicrobial for plant sanitation and prevention of surface contamination on non food contact surfaces, particularly concrete. Results of this study are useful to further understand translocation of spores of *B. subtilis* and *C. perfringens* into concrete surfaces that are abundantly present in processing plants and can serve as potential sources of cross contamination.

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