

## I. INTRODUCTION

Over the past 12 years there have been several reports of foodborne illness outbreaks associated with the consumption of unpasteurized fruit juice, particularly with apple cider and orange juice. These outbreaks have raised the concern of those in the fruit juice industry, as well as those in the Food and Drug Administration (FDA). Historically, only rarely have fruit juice beverages been shown to be associated with illness. It is believed that recent outbreaks occurred, in part, due to the fact that persons at higher risks of life-threatening illness (children, the elderly, immunocompromised individuals) largely consume fruit juices. Recent outbreaks have been attributed to three organisms *Eschericia coli* O157:H7, *Salmonella typhimurum*, and *Cryptosporidium parvum*. Currently a great deal of research is being conducted on inhibiting *E. coli* O157:H7 in juices (Fisher and Golden, 1998; Linton et al., 1999); however, not much research has been done concerning potential contamination of juices by the protozoan parasite *Cryptosporidium parvum*.

*Cryptosporidium parvum*, an Apicomplexan protozoan parasite, is considered an emerging pathogenic microorganism that may cause severe wasting gastrointestinal illness in humans. *Cryptosporidium* is a highly infectious enteric pathogen, because it is resistant to chlorine, difficult to filter out of contaminated water due to its minute size, and ubiquitous in many common animals. Over the past decade, *Cryptosporidium* has become a major threat to the U.S. water supply (Guerrant, 1997). It has been estimated that up to 35% of the U.S. population is seropositive to *Cryptosporidium* (Upton et al., 1991). Sereprevalence is believed to be at similar levels throughout Europe and North

America, with higher rates in Asia, Africa, and South America, where there are as many as 500 million cases annually (Current and Garcia, 1991). *Cryptosporidium parvum* can cause illness lasting 1-2 weeks in previously healthy individuals or lasting indefinitely in immunocompromised individuals. Currently, there is no effective treatment for cryptosporidiosis, so it is best to minimize contact with the microorganism. Infection can occur by ingestion of the thick-walled oocysts (Adal, et al., 1995). These oocysts appear to be highly infectious, with the infectious dose ranging from only one oocyst up to 30 oocysts (DuPont et al., 1995; Haas and Rose, 1994). The development of the organism occurs within a single host. All stages seem to grow attached to the host cell, but they are located in an intracellular extracytoplasmic vacuole located at the host cell surface or brush border of epithelial cells. The organism undergoes several reproductive stages to produce more oocysts that are shed and responsible for transmission to new hosts. Unlike some protozoa, *Cryptosporidium* oocysts are infective immediately upon release into the environment. Approximately 20% are thin-walled oocysts that may re-infect the same host (Current W.L., 1985). This autoinfective form of the oocyst is likely responsible for the repeated infections seen in immunocompromised hosts, which increases the seriousness of this illness.

As with any food or waterborne pathogen, it is important to understand the possible origins of contamination. The FDA states that the most likely source of pathogenic microorganisms is fecal material (McLellan and Splittstoesser, 1996; CFSAN, 1997). Ruminants, like cattle, sheep, and deer, are common on or near farms and orchards and are reservoirs of pathogens, although they are often asymptomatic themselves. Birds, rodents, insects, and poor worker hygiene may also play a role in

contamination. Contamination is likely to occur during the fruit growing and harvesting phases. Fruit can be contaminated directly or indirectly by contacting microorganisms on the ground, in harvesting crates, or in water. Unless the water is contaminated, washing fruit often reduces the number of microorganisms (Adams et. al, 1989). In discussing *Cryptosporidium parvum*, water contamination is an important concern, due to the minute size of oocysts (4-5  $\mu\text{m}$ ) and their resistance to chlorine. All along the harvesting and processing route it is necessary to implement good manufacturing practices (GMPs), to ensure that pathogenic microorganisms are not introduced. However, problems do arise; therefore, it is important to treat the final juice products properly to inhibit and control microorganisms. Treatment can include, but is not limited to product pasteurization. In some juices, such as apple cider, pasteurization is not desirable since it can compromise flavor and product quality. The FDA has taken several steps to protect consumers from unpasteurized fruit juices. In 1998 the FDA required warning statements be placed on unpasteurized juices (Federal Register 63). In January 2001, the final juice Hazard Analysis Critical Control Point (HACCP) rule was formulated by the FDA which stated that all juice producers must implement a final kill step. Between 2002-2004 juice manufacturers of varying sizes must implement this final treatment step. Many smaller juice companies are unable to pasteurize due to the large expense required in purchasing equipment. The approximate retail price for pasteurization of apple cider is \$1.00/L (Kozempel et al., 1998). Additionally, in plant units where less than 150L/min are produced, the costs can increase sharply (Kozempel et al., 1998). For these reasons the use of non-thermal treatments as a final treatment step is a novel and desirable approach.

Most recently attention has been directed towards microbial contamination in apple cider; however, questions have been raised with regard to the safety of other unpasteurized juices. Orange and grape juices are available as unpasteurized, refrigerated juices. While it may be less likely that drop fruit is used in the manufacturing of these juices (citrus and grape) contaminated water and workers are still pertinent issues.

Over the past five years, the FDA has held various meetings in response to the concern of public safety over fruit juice consumption. On April 24, 1998 the FDA published a proposed Hazard Analysis and Critical Control Point (HACCP) regulation for the fruit juice industry and the final rule was proposed in January 2001 (21 CFR 120#). Additionally, industry and consumers should be educated about the risks associated with consuming untreated fruit juice. It is plausible and compliant with current regulation that effective non-thermal treatments be implemented by the fruit juice industry to better control the potential microbiological contamination problem. A treatment is considered effective with a five-log reduction in a specific microorganism.

The present study was undertaken to assess the activity of potential non-thermal treatments to inhibit microbial contamination by *Cryptosporidium parvum*. Technologies alternative to pasteurization that were evaluated include addition of organic acids, hydrogen peroxide, and ozone.

## II. REVIEW OF LITERATURE

### A. *Cryptosporidium parvum*

#### 1. Waterborne outbreaks

*Cryptosporidium parvum* is an Apicomplexan protozoan parasite that is well documented as a cause of large waterborne outbreaks (Richardson et al., 1991; MacKenzie et al., 1995; Frisby et al., 1997; Willocks et al., 1998; Girdwood and Smith, 2000). Waterborne outbreaks are caused by the ingestion of environmentally resistant oocysts that replicate within the gut. Three characteristics make *Cryptosporidium* oocysts ideal for transmission through water: 1) the low dose required for infection, 2) prolonged excretion of high numbers of oocysts, and 3) the high prevalence in ruminants with subsequent transmission to humans (Carpenter et al., 1999; Fayer et al., 1997). Oocysts are highly prevalent in the environment, found in most lakes and streams. *Cryptosporidium* becomes a greater problem in March through June as spring rains increase run-off and infant animals shed large amounts of oocysts (Casey, 1991). In general, neonate animals shed larger numbers of oocysts, while many adult animals produce oocysts. Calves can shed up to  $10^{10}$  oocysts per day in fecal material (Atwill, 1996). At least 152 species of mammals can be infected with *Cryptosporidium* and shed oocysts, including, humans, ruminants, cervids, swine, cats, and dogs (Fayer et al., 2000).

A survey of beef and dairy cattle farms across the US indicated that 59% of the animals were positive for *C. parvum* (Garber et al., 1994). Similar studies on infected calves in Europe and North America showed higher infection rates, exceeding 76% in

Spain, 92% in England and California, and 80% in Canada (Quilez et al., 1996; Morck et al., 1997; Scott et al., 1994; Atwill et al., 1998). This information introduces the idea of environmental contamination, which is often blamed for waterborne outbreaks of cryptosporidiosis. The concept of watershed contamination is supported by the large amount of cattle activity in ranching and farming, and assumes that cattle contribute significantly to waterborne outbreaks (Graczyk et al., 2000); however, there is no direct evidence supporting this assumption (Ong et al., 1996; Atwill et al., 1998).

Typing an outbreak is important in determining the cause of an outbreak. The current hypothesis is that *Cryptosporidium parvum* exists as two distinct genotypes. Genotype 1 or H (human) causes infection in humans and primates. Genotype 2 or C (calf) causes infection in a wide range of animals, including humans. Typing is particularly important when discussing watershed contamination related to the animal husbandry industry. Currently ten species of *Cryptosporidium* have been identified and are considered valid (Fayer et al., 2000). While human infections are generally caused by *C. parvum*, other species may cause illness in immunocompromised persons. *Cryptosporidium* isolates from HIV-infected persons have been identified by DNA as isolates of the *C. parvum* cattle and human genotypes (Pieniasek et al., 1999) as well as the possible dog genotype, *C. canis* (Fayer et al., 2001). Immunocompromised patients have also been infected with *C. meleagridis* from turkeys (Pedraza-Diaz et al., 2001) and *C. felis* from cats (Caccio et al., 2002).

While *C. parvum* was first recognized as a cause of human illness in 1976, it was not until the AIDS epidemic evolved in 1982 that the number of cases increased. Municipal water outbreaks have occurred across the world, and in the United States in

Texas (1984), Georgia (1987), and Oregon (1992), where the largest outbreak occurred in Wisconsin (1993) (Colley, 1995). A myriad of smaller outbreaks have occurred as well, linked to municipal water supplies and recreational waters (Rose et al., 1997). At least twelve documented outbreaks have occurred within the United States at drinking water systems during the late 1980s and 1990s (Craun, 1998; USEPA, 2000). *Cryptosporidium parvum* gained national attention in 1993, when the largest waterborne outbreak ever recorded occurred. Over 400,000 people in Milwaukee, Wisconsin became ill and at least 50 people died when the municipal water supply became contaminated (MacKenzie et al., 1995).

Outbreaks have also occurred with ground water. The largest recorded outbreak occurred in the United Kingdom in 1997 and was the first ever to be associated with filtered borehole water (Willocks et al., 1998). The source of the contamination was never identified; however, hypotheses exist based on changes in the environment and climatic conditions that may have effected the aquifer and allowed entry of oocysts through a defect in the wall (Willocks et al., 1998).

Recreational waters can also be affected by *Cryptosporidium* contamination. Since 1998 there have been over thirty outbreaks in recreational water facilities in the United Kingdom and the United States (Carpenter et al., 1999). Several factors increase the risks of *Cryptosporidium* outbreaks at these facilities, including having large numbers of children as patrons with many still in diapers, along with the use of overwhelmed chemical disinfectant and filtration systems which place these facilities at high risk. Not surprisingly, studies have shown that fecal material present in these facilities has a large

negative effect on chlorine inactivation of *Cryptosporidium* oocysts and on recreational water quality (Carpenter et al., 1999).

After the large outbreak in 1993 in Wisconsin, the US Environmental Protection Agency (EPA) implemented task forces and new stricter regulations on monitoring *Cryptosporidium* oocysts in water supplies (Colley, 1995). The Safe Drinking Water Act (SDWA) required the EPA to set enforceable standards to protect the public from contaminants that could occur in drinking water. Since *C. parvum* oocysts are very small (4-6  $\mu\text{m}$ ), highly resistant to typical concentrations of chemical disinfectants, like chlorine and chloramine, and are present in surface water, adequate filtration systems are essential. It was shown that *Cryptosporidium* is 240,000 times more resistant to chlorination than is *Giardia*, another protozoan parasite capable of causing gastrointestinal illness (Jakubowski, 1995). Physical removal is key to reducing cryptosporidiosis. The finalized Long Term 1 Enhanced Water Treatment Rule (LT1ESWTR) was recently published, January 14, 2002 (40 CFR 9. 141-142). Compliance with this rule started on March 15, 2002. This rule requires enhanced filtration systems that produce at least a 2-log removal of *Cryptosporidium* (99%) and a standard deviation of 0.63 logs. The filtered systems must comply with strengthened and combined filter effluent turbidity performance standards while continuously monitoring the turbidity of individual filters combined with regular follow-up activities including monitoring. Decreasing the number of waterborne outbreaks of cryptosporidiosis has economic importance as well. The monetary value of a case of cryptosporidiosis may range from \$796 to \$1,411 depending on the severity of the case and including losses for medical costs and loss of productivity (Harrington et al., 1985; USEPA, 2001).

## 2. Foodborne outbreaks

While *C. parvum* is best known as a water contaminant, over the past decade there has been a rise in foodborne cryptosporidiosis. It is likely that most foodborne cases of cryptosporidiosis are missed, since testing for *Cryptosporidium* is not routine across the US (Hoskin and Wright, 1991). Waterborne and foodborne *Cryptosporidium* contamination may be linked. For example, farmers can inadvertently contaminate their crops through irrigation or the use of non-potable water placed directly on fruits and vegetables. Farmers should avoid using non-potable water supplies and adequately filter water to remove oocysts. Travellers diarrhea can also be caused by *Cryptosporidium*, as has been the case in travellers returning from Egypt, Mexico, the Caribbean, or other tropical islands (Gatti et al., 1993; Sterling et al., 1986; Ma et al., 1985).

*Cryptosporidium parvum* oocysts were found on over 14% of randomly sampled vegetables in Peru and Costa Rica (Monge, 1996; Ortega et al., 1997). Foods may be contaminated by water or environmental sources, as mentioned above, or by infected food handlers or the parents of infected children (Nichols, 1999). Foodborne outbreaks attributed to *C. parvum* have been associated with various foods, inadequately pasteurized milk, raw milk, apple cider, uncooked green onions in salads, chicken salad, sausage, and tripe (Casemore et al., 1986; Smith, 1993; Millard et al., 1994; Besser-Wiek et al., 1996; Gelletlie et al., 1997; CDC, 1998).

Immunocompromised persons are at the greatest risk of cryptosporidiosis and should thoroughly cook and heat all foods. *Cryptosporidium parvum* oocysts are susceptible to moderate heat, pasteurization, and desiccation (Anderson, 1985,1986; Harp

et al., 1996). Oocysts have been shown to survive in water at pH 3-10, and can survive in beverages including milk, beer, and orange juice (Girdwood and Smith, 2000). Research currently underway is attempting to better evaluate the effects of processing treatments, preservatives, and pH on *Cryptosporidium* oocysts. Additionally research seeks to evaluate washing treatments for their efficacy in removal of oocysts.

In 1993, 230 people from an elementary school in Maine became ill after attending an agricultural demonstration at a fair (Millard et al., 1994). All those who became ill had consumed freshly prepared apple cider at the fair. Approximately 160 people showed symptoms of cryptosporidiosis. This was the first documented outbreak of cryptosporidiosis attributed to unpasteurized apple cider. Contamination was likely due to dairy cattle that were shedding oocysts near the orchard. During this outbreak, household transmission was documented for about 84% of the cases, and there were 53 identified secondary cases in family members (Millard et al., 1994). As shown by this episode, along with others, *C. parvum* is highly transmissible by person-to-person contact (Laberge et al., 1996). The second outbreak of cryptosporidiosis linked to fresh juice occurred in Connecticut and New York during October 1996 (CDC, 1997). During this outbreak a cluster of over thirty illnesses occurred as a result of unpasteurized apple cider containing *Escherichia coli* O157:H7 and *C. parvum*. The orchard owner reported only using picked apples for the cider, while drop apples were used in processed apple products. It is possible that contamination originated from the well water, although neither pathogen was identified in the well water. Cattle were not maintained on site, but the cider mill was located across the street from a dairy farm. There are several obvious means of contamination in both outbreaks associated with fresh juice.

Infection of farm livestock (calves, lambs, goats, swine) with *Cryptosporidium* sp. is becoming more widespread (Laberge et al., 1996), which puts produce at an increased risk for contamination. An estimated 50 people became ill after consuming chicken salad that was prepared by a woman who ran an in-home day care facility (CDC, 1996). The origin of *C. parvum* was never determined, and the woman herself rejected a fecal test. In a similar situation, nearly 100 students at a university in Washington, D.C. became ill after food was contaminated with *C. parvum* by a cafeteria worker (Quiroz et al., 2000). It is clear that *Cryptosporidium* can be transmitted by water, food, and person-to-person contact.

### **3. Life cycle**

The life cycle of *Cryptosporidium parvum* begins with the ingestion of a sporulated oocyst. *Cryptosporidium* is different from other protozoan parasites in that the oocysts are excreted already infective and sporulated, while the oocysts of other protozoa require a time period outside the host to sporulate. Each *Cryptosporidium* oocyst contains 4 infective sporozoites. These small worm-like stages excyst from the oocyst through a suture located along one side of the oocyst. Excystation occurs with exposure to reducing conditions, pancreatic enzymes, and bile salts; however, excystation can occur in warm aqueous solutions that do not contain these enzymes, including the conjunctiva of the eye, respiratory tract, gall bladder, and lymph nodes (Blagburn and Soave, 1997). The sporozoites preferentially penetrate intestinal epithelial cells in the ileum (Gut et al., 1991). The anterior end of the sporozoite adheres to the luminal surface of the epithelial cells. One adherence factor that has been identified as an agent involved

in attachment is a sporozoite-specific lectin (Keusch et al., 1995). Parasitic life stages develop on the luminal surface of the cells, and grow intracellularly yet remain extracytoplasmic from the host cells. Host microvilli surround the parasite contained within a parasitophorous vacuole, and a unique desmosome-like attachment organelle forms that is visible in electron micrographs (Dubey et al., 1990). This structure is referred to as the feeder organelle. The endogenous phase begins after the sporozoites excyst.

Each sporozoite differentiates into a spherical trophozoite. The trophozoite nucleus divides, and undergoes asexual reproduction called shizogony or merogony. *Cryptosporidium* species differ in the number of types of schizonts/meronts in the life cycle; for example, *C. parvum* has two types of schizonts, while *C. muris* has three types. Type I schizonts develop six or eight nuclei and each is incorporated into a merozoite. The mature merozoite leaves the schizont to infect another host cell, where it then develops into a type II schizont. Four merozoites emerge from the type II schizont and differentiate into gametes. Some merozoites enter host cells and enlarge to become female macrogamonts. Other merozoites undergo multiple fission inside host cells to form male microgamonts. The two gametes then initiate sexual reproduction leading to the formation of an immature zygote. A resistant oocyst wall forms around the zygote within which meiosis leads to the formation of four sporozoites. Sporulated oocysts are passed in the feces of the host and the cycle continues. The resistant oocyst wall forms in only about 80% of oocysts. The other 20% are termed thin-walled oocysts, which are responsible for the “autoinfective” cycle of cryptosporidiosis that cannot be cleared by

immunocompromised individuals. These thin-walled oocysts excyst while still in the gut, before exiting the host, and then infect new cells.

#### **4. Infection**

*Cryptosporidium* is transferred from an infectious individual to a new host by oocysts using the fecal-oral route. The infectious dose is thought to be quite small for *C. parvum*. To gain a better understanding of the infectious dose, twenty-nine volunteers consumed a number of oocysts ranging from thirty to one million oocysts (DuPont et al., 1995). The median infectious dose (ID<sub>50</sub>) was calculated to be 132 oocysts, while the infectious dose ranged at the lower end from only one oocyst up to 30 oocysts (DuPont et al., 1995). With further data the ID<sub>50</sub> was later recalculated to be 87 oocysts (Okhuysen et al., 1999). Using different isolated strains of *C. parvum* the ID<sub>50</sub> ranged from 9 oocysts to 1042 (Okhuysen et al., 1999). Additionally, the dose may vary depending on the immune status of the host.

After initial sporozoite attachment, it is believed that host mucosal cells secrete various cytokines that initiate host phagocytosis and the host immune response (Goodgame, 1996). These activated cells cause the secondary release of soluble factors (histamine, serotonin, prostaglandins, platelet-activating factor) which in turn inhibit absorption and alter intestinal secretion of water and chloride (Goodgame, 1996). It is further hypothesized that host cell damage occurs in two ways (Goodgame, 1996): 1) as a direct result of parasite invasion, multiplication, and extrusion and 2) through T-cell mediated inflammation which results in villus atrophy and crypt hyperplasia.

As the parasite invades the mucosal surface of the host cell, it establishes a unique intracellular compartment or niche within the cell where it divides and reproduces both asexually to produce meronts containing merozoites and sexually where gamogony can occur. For *Cryptosporidium*, this compartment is termed intracellular but extracytoplasmic, as it is separate from the apical host cell cytoplasm and protrudes into the lumen of the gut. During the stages of invasion and parasitic development, elements of both the host and parasite cytoskeletons are disrupted. As the invading organism moves into the host cell, various capping and binding proteins are transferred from the apical tip to the posterior pole of the parasite. These actions along with the colocalization of host actin and myosin, although not well understood, make it possible for the parasite to invade the cell and maintain motility. During infection, the microvilli become absent in the area of infection and normal epithelial cells are significantly shortened (Godwin, 1991).

Interactions at the site of host-parasite invasion are complex. Utilization of host cell actin is a common theme in microbial pathogenesis and has been observed in both prokaryotic and eukaryotic pathogens, including, *E. coli*, *Shigella*, *Salmonella*, *Listeria*, and *Toxoplasma* (Finlay and Cossart, 1997; Dobrowolski and Sibley, 1996). Each organism utilizes actin for a specific evolved purpose in a unique mechanism. It is postulated that *C. parvum* utilizes host actin in a similar manner (Elliot and Clark, 2000; Elliot et al., 2001). Attachment is complex and often modulated by local factors, including pH, divalent cations, and receptors (Hamer et al., 1994). The surface-associated lectin proteins of the sporozoite are postulated to regulate the initial parasite-host interaction (Joe et al., 1994). Once the initial attachment has occurred, the cell

penetration process is intricate, yet complete in only a few minutes (Lumb et al., 1988). After the development of a relatively large secretory vacuole, the outer membrane of the sporozoite fuses with the host cell membrane near the apical end. At this time, the sporozoite moves by rapid extension and contraction of the anterior end. After attachment, a dense band forms at the host-parasite interface of the host cell, just below the conoid (Lumb et al., 1988). This electron dense band, termed an actin plaque, is believed to be the accumulation of host actin, as indicated by FITC-phalloidin staining of filamentous actin in an infected cell line (Elliot and Clark, 2000). Differential interference microscopy (DIC) images indicate that this accumulation of host actin actually occurs quite early in the invasion process, even before the parasite is entirely internalized. A similar phenomenon occurs as the bacterial pathogen *E. coli* infects a cell, whereby the organism uses an accumulation of the host's actin as scaffolding to which it can attach (Finlay and Cossart, 1997). The similar utilization of host actin by *Cryptosporidium* seems to mimic normal cellular structures where actin is linked to membranes, as in focal adhesions or adherens junctions. In these instances, linker molecules such as cadherin or integrin proteins assist in the assembly of actin; however, these linkers were absent in the *Cryptosporidium* actin plaque and only host actin was present (Elliot and Clark, 2000).

Although experiments indicated that the actin plaque was void of parasitic-actin (Elliot and Clark, 2000), there is an important role for actin-polymerization in sporozoite motility (Forney et al., 1998). It is believed that *Toxoplasma gondii* tachyzoites use actin-powered motility to infect host cells (Dobrowolski and Sibley, 1996). Sporozoite motility is not a significant virulence factor, compared to other apicomplexans; however,

cell invasion is dependent on the dynamics of parasite actin polymerization. The invasive lifestages of *C. parvum*, sporozoites and merozoites, exhibit “gliding” motility that is further characterized by the shedding of surface antigens called “trails” which can be followed when studying this movement on an adhesive substratum (Russell and Sinden, 1981). Compared to other apicomplexans the trails of *Cryptosporidium* sporozoites are fairly short; however, the tails may seem long, measuring about one to two times the length of the sporozoite (Forney et al., 1998; Tilley and Upton, 1994).

Sporozoite gliding motility relies on the interaction of actin polymerization and the molecular motor myosin. Experiments treating actin and myosin with inhibitory compounds showed that *C. parvum* does not require locomotion to infect cells (Forney et al., 1998). Treated sporozoites did not elongate in the characteristic shape, indicating that actin-myosin contractile forces are necessary for maintaining cell shape and integrity (Mitchison and Cramer, 1996). This finding suggests an important role for actin complexes at the anterior end of the sporozoite. Anterior actin provides structural organization to the apical organelles, thereby ensuring apical orientation of sporozoites during attachment to the host cell as well as during invasion of the host cell (Peterson, 1993). It is clear that actin-myosin cytoskeleton arrangements influence sporozoite motility and are involved in host cell infection.

The role of actin microfilaments in host and parasite interactions can be described as a two-step infection process. In the first step, accumulation of host actin by the parasite ensures attachment through formation of an actin plaque. In the second step, sporozoites use parasite actin-based motility to invade the host cell. This intricate process of infection utilizes actin polymerization, actin-myosin complexes, and various

adhesion and binding proteins. Treatment development for cryptosporidiosis should concentrate on inhibiting these mechanisms of parasite actin polymerization, thereby inhibiting parasite attachment and host cell invasion. A thorough understanding of the infection mechanism and the role of the parasite cytoskeleton are beneficial to the development of cryptosporidiosis in *in vitro* and *in vivo* models.

## 5. Illness

*Cryptosporidium* progresses through its life cycle quite quickly. Each generation can develop and mature in approximately 12 hours. This fact combined with the autoinfective cycle allows *Cryptosporidium* to colonize the ileum within a few days. An infected individual can pass approximately  $10^5$  or more oocysts per gram of feces for several weeks or months, depending on the health status of the individual. Secondary infections can occur in the duodenum, large intestine, or stomach in severely immunocompromised patients.

Clinical signs of illness include severe wasting diarrhea, weight loss, and abdominal cramping. Severe electrolyte imbalance can occur in immunocompromised individuals. The interval between infection and the appearance of clinical symptoms is called the prepatent period and varies from 3-4 days. Oocysts are shed in the feces during the patent period, which lasts from 6 days to two weeks. This patent period can last much longer in immunosuppressed individuals due to the autoinfective cycle.

Currently no treatment is completely effective for cryptosporidiosis. Oral rehydration can include Gatorade, boullion, or a rehydration solution containing glucose, sodium bicarbonate, and potassium (Flanigan,1993). Previously healthy individuals

usually clear the infection after about two weeks of illness, but immunocompromised persons may try pharmacological therapies. Several antimicrobial agents have been evaluated for their effectiveness. The antimicrobial agent paromomycin, which is similar to neomycin, can result in a decrease in the intensity of infection and improves intestinal function and morphology (Goodgame, 1996). Unfortunately, paromomycin is poorly absorbed and the majority of patients do not have a complete response to therapy (Flanigan, 1993). Other antimicrobials tested include spiramycin and dicalzuril among others, all of which may decrease diarrhea or oocyst number but do not produce reliable results (Flanigan, 1993). Potential immunologic therapies are being evaluated as well. The host response to cryptosporidiosis is mostly T-cell mediated. Serum antibodies do not provide much protection; however, feeding infected humans bovine colostrum immunoglobulin has been shown to lessen symptoms of cryptosporidiosis (Heyworth, 1995).

Interestingly, *C. parvum* is highly immunogenic. The many surface proteins, glycoproteins, and phospholipids on the oocyst and other life stages are immunogenic. These glycoproteins or sporozoite surface lectins are essential in that they allow the initial attachment to the host glycocalyx covering the intestinal mucosa (Langer, 1999). The resulting infection and illness which ensue are so severe that if the parasite was not eliminated the host would die quickly through dehydration and electrolyte imbalance. Currently, the most effective therapy appears to be an active host immune response along with supportive care.

From a histopathologic perspective, cellular changes in animals with illness include villous atrophy, crypt hyperplasia, and inflammation of the lamina propria;

however, humans with cryptosporidiosis do not always show these signs and intestinal mucosa may remain intact (Marcial and Madara, 1986). Clinically, the loss of large volumes of watery diarrhea during a bout of cryptosporidiosis shows that the illness may be a result of an enterotoxin (Modigliani et al., 1985). In fact, it is hypothesized that this effect may be due to an enterotoxin similar to heat-stable enterotoxins as evaluated in an in vitro ileum cell-free system (Guarino et al., 1994). It is not well understood if this enterotoxic effect is time dependent or saturable. On the other hand, it was observed that the toxic response could be reduced in the absence of chloride or calcium ions, suggesting the mechanism is calcium-mediated and involves chloride secretion (Guarino et al., 1994). Finally, while the enterotoxigenic effect may be induced directly by *Cryptosporidium* it may also be induced by the increase of prostaglandins released by host cells during infection (Argenzio et al., 1993).

## **6. Detection Methods**

Various methods exist for detecting oocysts and for determining oocyst viability. For fecal smears, and food or water samples, standard methods include concentration and staining (Arrowood, 1997). Alternative concentration techniques to sample batch centrifugation include the use of continuous flow centrifugation, membrane filtration, cartridge filtration, and calcium carbonate flocculation. These systems can concentrate oocysts from large amounts of water. Then oocysts can be separated from sediment with immunomagnetic beads. Oocyst recovery varies depending on water turbidity and the physical-chemical properties of the sample (Casemore, 1985).

Both dye based and immunological-based stains are used. *Cryptosporidium* oocysts are identified by the use of differential stains, like safranin-methylene blue stain, acid fast stains like Kinyoun and Ziehl-Neelson, and DMSO-carbol fuchsin stain (Garcia et al., 1983). These stains color the oocysts red on a counterstained background. Other differential stains may be used which stain the oocysts green, but are not recommended due to problems with fecal background contamination. Immunological-based stains combine polyclonal antibodies with a fluorescent or colorimetric protein tag like the avidin-biotin system. Users must beware of potential cross-reactivity with other microorganisms. Since the oocysts are quite small (4-6  $\mu\text{m}$ ), even with the use of stains, identification requires an experienced microscopist (Fayer et al., 2000). Immunological-based stains or Enzyme-linked immunosorbent assays (ELISA) using polyclonal antibodies are also used to detect *Cryptosporidium* life stages.

Various techniques are used to detect oocysts and to determine viability. The polymerase chain reaction (PCR) can be used on both clinical and environmental specimens (Arrowood, 1997). However, some foods contain compounds which inhibit the enzymes necessary for PCR, likewise some environmental contaminants can interfere with the assay (Grigoriev et al., 1994). Reverse-transcriptase PCR can be used to measure the amount of mRNA and amyloglucosidase to determine viability (Wagner-Wiening and Kimmig, 1995). Dyes like propidium iodide (PI) and diamidino-2'-phenylindole (DAPI) have been used as indicators of viability. These dyes have been used for several years, but unfortunately are not an accurate measure of viability (Arrowood, 1997). The gold standard in viability assays was the mouse bioassay, but it is now thought by some that cell culture infectivity assays are more similar to human

infectivity (Chappell, 2000). Traditionally, neonatal mice are infected with *Cryptosporidium*, then euthanized 5 days post-inoculation, and intestines are observed for oocysts. Immunocompromised mice can be used as an alternative to neonates.

## **7. The *Cryptosporidium* oocyst**

Transmission of *Cryptosporidium* is widespread and rapid during an outbreak due to the hardy oocyst, the transmissive life stage. The oocyst wall provides the organisms inside with a safe and secure containment. This wall allows them to be unusually resistant to environmental pressures. It is hypothesized that *Cryptosporidium* oocysts evolved in mobile animal populations that exerted strong selective pressures for long-term survival; however, we now see concentrated, fixed animal populations that are exposed to higher levels of infective oocysts ((Blewett, 1988)).

The process of oocyst wall formation is not well understood, but is believed to be complex and essential to the inherent resistance of the organism. The oocyst wall of *Cryptosporidium* has distinct outer and inner layers. Transmission electron microscopy revealed the details of the trilaminar structure of the oocyst wall (Harris and Petry, 1999). The outer layer is composed of acidic glycoprotein, the central layer is more rigid and composed of a complex lipid material that is responsible for the oocyst acid-fast staining, and the thick inner layer is thought to be filamentous glycoprotein (Wolinsky, 1990; Bonnin et al., 1991). Two more inner layers provide rigidity with a unique elasticity to the oocyst wall (Harris and Petry, 1999). A unique suture is present at one end of the oocyst. The suture, which dissolves during excystation, lies along the inner layer and spans 1/3 to 1/2 of the oocyst circumference (Fayer, 1997). After the suture opens, the

sporozoites are released and an empty spherical wall is left behind (Reduker et al., 1985b). A residual body is also left behind, containing a lipid vacuole and amylopectin granules (Harris and Petry, 1999). The oocyst wall immediately surrounding the suture is slightly thicker (Reduker et al., 1985a). Parasites in the related genera, *Toxoplasma*, *Sarcocystis* and *Isospora* also have sutures, indicating their phylogenetic relationship (Fayer, 1997). The sutures of these parasites are a bit different from that of *Cryptosporidium* in that they have liplike thickenings around the suture (Speer et al., 1973; Dessler and Li, 1984). The relationships among these parasites may be important for developing effective therapies and for further biochemical studies. After all, the oocyst wall is what inhibits disinfection.

Prior to excystation, the exterior fluid acts on the outer layer of the oocyst wall and upon the suture. This causes the inner layer to cleave, as observed in transmission electron microscopy (Reduker et al., 1985a). This cleaving or folding inward creates an opening at one pole (Iseki, 1979). During in vitro excystation experiments, sodium hypochlorite perforates or removes the outer layer and the outer zone of the inner layer (Reduker et al., 1985a). It is also postulated that as oocysts age, the oocyst wall degrades (Reduker et al., 1985a) and this may explain why aged oocysts have lower excystation rates. While it appears necessary, the purpose and role of the outer wall has been questioned. The thin outer wall may be more of a vestigial wall (Reduker et al., 1985a) while the inner wall acts more like a sporocyst covering with the infectious sporozoites inside. Further analysis is needed to better evaluate this hypothesis.

As the organism proceeds through the *Cryptosporidium* life cycle, both thick and thin walled oocysts develop. Thin walled oocysts lack the two distinct types of wall

forming bodies that thick walled oocysts have (Current and Reese, 1986). These wall forming bodies contain a fibrillous glycoprotein-like material and are involved in the storage and release of the parasitophorous vacuole (Bonnin et al., 1991). There are also electron lucent vesicles involved in gametogenesis that may be separate from the wall-forming bodies described above. A hypothesis is that material contained within these electron-lucent vesicles provides resistance to sodium hypochlorite and has a strong interaction with the wall-forming bodies (Bonnin et al., 1991). These non-wall forming bodies may have a secretory role, involved in the formation of the parasitophorous vacuole (Entrala et al., 2001). As the oocyst develops, a two-unit membrane forms outside the plasmalemma, while the internal sporont forms are separated from the other organelles. The two membrane layers can be viewed with an electron microscope. The oocyst wall and membrane components are studied with monoclonal antibody- labeling combined with protein gel electrophoresis and electron microscopy.

Since no therapies or prophylaxis are proven to be effective consistently for humans, hygiene and disinfection are important management tools. The limits of oocyst survival have been measured under laboratory conditions. Oocysts can be made noninfective after substantial air-drying (Anderson et al., 1992). Also, oocysts can be made non-infective by freezing at  $-70^{\circ}\text{C}$  or lower with or without cryoprotectants, but remain infectious if frozen at  $-20^{\circ}\text{C}$  or higher (Fayer et al., 1991; Fayer and Nerad, 1996). Oocysts suspended in water or milk can be made non-infective in a pasteurizer held at  $71.7^{\circ}\text{C}$  for 5-15 seconds (Harp et al., 1996). Temperatures of  $64.2^{\circ}\text{C}$  for 5 minutes or  $72.4^{\circ}\text{C}$  for 1 minute can make oocysts noninfectious (Fayer, 1994). While traditional chemical disinfectants, like chlorine or iodophores used at normal levels are not useful,

other non-traditional disinfectants, like ultraviolet light, ozone, and hydrogen peroxide gas plasma may be useful. Hydrogen peroxide gas plasma can be used with 58% hydrogen peroxide to inactivate *Cryptosporidium* oocysts on endoscopic and surgical equipment (Vassal et al., 1998).

## **8. Importance of protease activity for protozoan infection**

Protease activity plays an important role in microbial infection. Perhaps the best known example is HIV, the virus believed to cause AIDS, which is now most successfully treated with protease inhibitors (Carrasco and Tying, 2001). Proteases are involved in parasite-host infection and protozoa are no exception. During infection, the apicomplexan parasite orients the apical complex towards the host cell, attaches, and penetrates the host cell membrane (Aikawa et al., 1978). This adhesion and invasion process appears to be mediated by at least one protease (Forney et al., 1996b). These protease-mediated or protease-dependent mechanisms have been studied in several parasites, including *Plasmodium knowlesi*, *Plasmodium falciparum*, *Eimeria tenella*, *Eimeria vermiformis*, and *Entamoeba histolytica* (Dluzewski et al., 1986; Adams and Bushell, 1988; Fuller and McDougald, 1990; Que et al., 2002). Protease-mediated events are believed to include host cell attachment (Adams and Bushell, 1988; Arroyo and Aldrete, 1989), host membrane penetration (Hadley et al., 1983; Fuller et al., 1990), host protein degradation used for parasitic growth (Chappell and Dresden, 1986), tissue migration (Moda et al., 1988; McKerrow et al., 1990; Moda and Doenhoff, 1994a; Morris and Sakanari, 1995; Yenbuter and Scott, 1995), parasite development (Richer et al.,

1993), and mechanisms used to fight and evade the host immune system (Malzels et al., 1993; Pupkis et al., 1986; Moda and Doenhoff 1994b).

Proteases are traditionally classified into three groups: cysteine, serine, and metalloproteases. Cysteine proteases, also called thiol proteases, have a thiol group of the amino acid cysteine in the active site of the protease. Serine proteases have a hydroxyl group in the active site, which is involved in binding and catalysis of the substrate at the active site. Metalloproteases contain a metal ion in the active site that is involved in substrate binding. Proteases work under various conditions, but do have optimum pH ranges. Serine and metalloproteases work best under neutral to slightly alkaline pH conditions (McKerrow, 1993). Cysteine proteases work at a broader range from pH 5.5-7.5, and require that reducing agent be present to keep the thiol group from being oxidized (McKerrow, 1993). Protease activity can be analyzed with a variety of biochemical assays. Localization can be observed with immunofluorescence and proteases can be purified from parasite homogenates. One of the most popular ways to evaluate protease activity is with protease inhibitors. Several serine, cysteine, and metalloprotease inhibitors are commercially available.

Cysteine proteinases are believed to be key agents in the cytopathology and degradation that allows parasite invasion. These proteinases appear to be so crucial to invasion that small changes in gene regulation or expression can differentiate between invasive and non-invasive species of the same genus, as is the case with *Entamoeba histolytica* and *Entamoeba dispar* (Que et al., 2002). It appears that the cysteine proteases are both surface-associated and membrane-associated on *Entamoeba* trophozoites, and those on the surface may be more important for invasion while others

act in digestion of nutrients (Que et al., 2002). The cysteine proteinase, cruzipain, found in *Trypanosoma cruzi*, has also been localized to the surface and lysosomes.

Interestingly, *Giardia*, the protozoa believed to have the earliest lineage to eukaryotic cells, has detectable cysteine proteinase activity that is directly involved in trophozoite excystation (Ward et al., 1997). Cysteine protease activity, from one of three genes, was detected as the major component of biochemical activity during excystation (Hare et al., 1989; Parenti, 1989; Werries et al., 1991). The *Giardia* protease is the most primitive known protease of the cathepsin B family, as identified by sequence analysis (Ward et al., 1997). The cysteine proteinase is localized in cytoplasmic vacuoles (Ward et al., 1997), reported previously to contain acid phosphatase and protease activity (Feely and Dyer, 1987; Lindmark, 1988). As with other protozoa, in *Giardia* the processing of cyst wall components may rely on cysteine proteinase activity (Ward et al., 1997).

Three different protease activities were recently identified in *Toxoplasma gondii* tachyzoites (Ahn et al., 2001). One protease is a metalloprotease, which has not been well characterized (Berthoneau et al., 2000). The other two are believed to have functional roles in the infection of host cells. One is a cysteine proteinase with an optimum pH of 6.0, and the other is a serine protease with an optimum pH of 8.5 (Ahn et al., 2001). Work conducted by Ahn et al. (2001) showed that enzyme activity was dependent on the presence of calcium ions. They also found that secretion of proteases was most likely involved in the recognition and binding of the parasite to the host-cell. It is most likely that proteases are secreted from *T. gondii* tachyzoites into the host cell membrane as the parasite is anchored.

## 9. Evidence for protease activity in *Cryptosporidium*

*Cryptosporidium* excystation may be enhanced in vitro by increasing temperature to 37°C, by preincubation in low pH, by addition of trypsin, and by the action of bile salts; however, excystation can occur without these additives as well. Various proteins play functional roles in excystation including proteinases.

An arginyl aminopeptidase and a cysteine proteinase were observed associated with the sporozoite outer membrane along with substrate adhesion proteins (Okhuysen et al., 1994; Nesterenko et al., 1995). A serine protease may also exist, acting similarly in function to trypsin, a pancreatic enzyme (Powers et al, 1993). While the cysteine protease appears to act similarly to the metalloactivated cysteine proteases calpain I and II (Sasaki et al., 1984). The calpains are calcium-dependent cysteine proteinases found in most mammalian and avian cells (Sasaki et al., 1984). The calpain proteases cleave a variety of cellular proteins, including cytoskeletal elements like those found in host ileal cells. Both serine and cysteine proteinases may function in excystation and cleave certain oocyst wall proteins as indicated by the increase in concentration during incubation at 37°C (Forney et al., 1996a). Additionally they may be involved in the invasion process as this process has been shown to be both pH and metallo-dependent (Hamer et al., 1994).

Several studies have evaluated the effects of varying concentrations of selected protease inhibitors (phenylmethylsulfonyl fluoride, antipain, leupeptin, aprotinin, soybean trypsin inhibitor,  $\alpha$ -antitrypsin) on *C. parvum* activity and reported an observed reduction in activity (Forney et al., 1996a,b). This author's work proposed that multiple proteolytic components exist in *Cryptosporidium* sporozoite homogenate, while the

serine protease may be more functionally important for excystation ((Forney et al.,1996a).

It was hypothesized that the protease activity is important in the early biochemical processes of infection dynamics (Forney et al., 1996a). Additionally, these authors suggested the use of anticryptosporidial protease inhibitors as a chemotherapeutic agent to counteract infection (Forney et al., 1996b). Forney et al (1996) evaluated *Cryptosporidium* homogenate for proteolytic activity that could associate with the human alpha-1-antitrypsin (AAT) human serine protease inhibitor. Human AAT is involved with protease-mediated tissue destruction during the elicited “acute phase” protein response (Travis and Salvesen, 1983; Potempa et al., 1994). This work lead to the belief that the serine-like proteases which may function like trypsin are enzymatically inactive before excystation occurs. Protease components may be upregulated as the parasite initiates the initial interaction and attachment with the host cell membrane.

## **B. Juice HACCP**

### **1. History of outbreaks associated with fruit juice**

In the United States fruit juices, including apple, orange, and grape, are consumed on a large scale. The total commercial production of apples, grapes, and oranges in the US on a yearly average was reported at roughly 5.3, 6.2, and 13.1 million tons respectively (USDA, 2001). Monetary value is also on a large scale, with apple, grape, and orange production ranging from 1.5 to 3 billion dollars annually (USDA, 2001). While most fruit is consumed whole, a good proportion is processed into juice: 23% of apples, 90% of oranges, 85% of grapes (USDA, 2001). Only a small proportion of fruit juices remains unpasteurized. Approximately 98% of juice produced in the U.S. is pasteurized, while the remaining 2% equals approximately 40 million gallons of unpasteurized product (Meskin, 1998). Unpasteurized fruit juices continue to have a strong following, perhaps in part due to their perceived superior flavor, aroma, or nutrient content.

Several outbreaks of foodborne illness associated with unpasteurized juices have been attributed to microbial contaminants, like *Escherichia coli* O157:H7 and *Salmonella* species. Between 1923 and the Spring of 2000, twenty-one recorded outbreaks were reported, and over 85% of these occurred after 1980 (Steele et al., 1982; Parish et al., 1997; Cook, 1998; FDA, 2001d). Two reported outbreaks associated with *Cryptosporidium parvum* have occurred. It is possible that sporadic cases have also occurred and were not reported.

## **2. Overview of Regulation**

The long awaited final juice HACCP rule is available in the Federal Register, Code of Federal Regulations, Title 21, part 120, entitled “Procedures for the safe and sanitary processing and importing of juice, the final rule” (FDA, 2001d). The juice HACCP rule became effective on January 22, 2002, with installment in smaller plants on January 21, 2003 and January 20, 2004. HACCP is a system of hazard control, directed specifically towards the juice industry in this case. This final rule was the last step in a series of actions taken to increase the safety of fresh juices and decrease the likelihood of microbial contamination. While the final rule provides a specific framework for juice processing, it remains important that industry and consumers are educated about the risks associated with consuming untreated fruit juice. The rule provides flexibility in the final processing step in that non-thermal treatments may be implemented by the fruit juice industry to control potential microbial contamination.

From 1997 through 2001, the FDA and other regulatory agencies worked in response to the concern of public safety over fruit juice consumption. One of the first steps occurred in August 1997, with a recommendation from the National Advisory Committee on Microbiological Criteria for Foods (NACMCF). The FDA issued a notice of intent to develop a juice HACCP program, warning label statement, and an educational program for fruit and juice beverage processors. The HACCP regulation for the fruit juice industry was formally proposed in April 1998.

In July 1998, the FDA published the warning statement rule (FDA, 1998), which required the labeling of juices not pasteurized or processed to a level necessary for a 5-log reduction in the most persistent pathogen. This rule went into effect in November

1998. This rule affected all juice manufactures by the end of the year 2000, allowing time for small manufacturers to comply. After further analysis, the FDA stated that there was insufficient evidence to warrant exclusion of citrus fruits from the warning label, although there is minimal contact with the peel. While it is unlikely that drop fruit is used in citrus and grape juices, compared to apple juices, contaminated water or workers are a pertinent issue.

As mentioned above, the final ruling requires the implementation of HACCP principles in the production of juice or juice products. Within the HACCP plan, juice must be treated with a process capable of obtaining a 5-log reduction of the most pertinent pathogen. The FDA states that historical association should determine the pertinent pathogen, while the NACMCF recommends that *E. coli* O157:H7 be used as a benchmark target microorganism. The HACCP rule does not mandate pasteurization, and therefore alternative methodologies such as UV, ozone, or chemical treatments may be used. The rule states that surface treatments may be part of the 5-log reduction protocol, but cannot be used without an acceptable end processing treatment.

The time frame for implementation of these regulations is three tiered, based on size and sales of the processor. Processors with more than 500 employees are given 12 months for implementation. Those with between 100 and 500 employees have 24 months to comply. Very small processors, defined as those with less than \$500,000 annual sales, greater than \$500,000 annual sales but less than \$50,000 from food, or less than 100 full time employees or less than 100,000 units sold per year, have 36 months before necessary compliance. During this interim period, small processors will likely be looking at non-thermal options in order to avoid the high capital costs of pasteurization equipment.

## **C. Antimicrobials as alternatives to traditional juice pasteurization**

### **1. Organic acids**

Plants accumulate organic acids within their tissues and they may be found at highest concentrations in the fruit. In general, organic acids are water-soluble, colorless liquids that lend their unique tastes and flavors to many foods. Acidic properties of organic acids are due to the presence of the carboxylic group (-COOH). In a food system, organic acids can act as flavor enhancers, defoaming agents, emulsifiers, and antimicrobials (Fennema, 1996). In fruit juices, organic acids impart a sour taste, act to balance the sugar/acid ratio (Arthey and Ashurst, 1996) as well as acting to provide necessary antimicrobial attributes.

In general, the inhibitory antimicrobial effects of organic acids is a factor of the amount of acid in its undissociated form, along with its ability to donate hydrogen ions in an aqueous system (Uljas and Ingham, 1998). The degree of dissociation for a particular acid is related to its dissociation constant and the acidity of the product. The dissociation constant (pKa) indicates the pH at which there is an equal distribution of undissociated (50%) and dissociated (50%) forms. At a pH lower than the pKa, more undissociated acid is present (Bruice, 1995). In this form, the microbial cell membrane is generally more permeable to the acid, allowing it to enter the cell where it dissociates and lowers the internal pH of the cell thereby disrupting cellular functions (Lück and Jager, 1997). In this system, organic acids act on microorganisms by affecting both the external and internal pH. This system inhibits the growth and survival of bacterial cells as lipophilic

organic acids can enter bacterial cells; however, the effect on protozoa may be quite different since these organisms are less susceptible to chemical inhibitors and acidity. Mild acid treatment seems to enhance excystation of *C. parvum* sporozoites (Kato et al., 2001). Acid may act on oocyst or sporozoite membrane proteins and proteolytic enzymes and affect infectivity through protein modification.

Organic acids are commonly added to foods, including fruit juices. Three common organic acids that were used in this study and discussed in more detail below are: citric, malic, and tartaric.

#### **a. Citric acid**

Citric acid is a major component of orange, lemon, strawberry, blackcurrent, and gooseberry fruits (Harborne, 1980). Citric acid ( $C_6H_8O_7$ ) is a tricarboxylic acid that is commonly used to acidify a wide variety of foods, including fruit juices, carbonated beverages, canned vegetables, and dairy products (Davidson and Branen, 1993). As a tricarboxylic acid, citric acid has three pKa values: 3.1, 4.8, and 6.4. Citric acid imparts a sour but fairly mellow flavor to juices (Lück and Jager, 1997). Citric acid lowers the pH of the food and has a secondary effect of chelating cations and essential minerals. Citric acid is approved as a generally recognized as safe (GRAS) food additive (FDA, 2002)

#### **b. Malic acid**

Malic acid is a dominant acid in several fruits, including grapes, apples, plums, and cherries (Harborne, 1980). Malic acid is the dominant acid in apples (Nagy et al., 1993). It is used for its flavoring and acidification properties in beverages, jams, jellies,

and sherbets (Davidson and Branen, 1993). Malic acid imparts a less tart flavor compared to citric acid and is fairly smooth in taste (Lück and Jager, 1997). As a dicarboxylic acid, malic acid ( $C_4H_6O_5$ ) has two pKa values: 3.4 and 5.1. As a food additive, malic acid has a GRAS status (FDA, 2001c).

### **c. Tartaric acid**

While both citric and malic acids are found in many fruits, tartaric acid is present primarily in grapes (Harborne, 1980) (Nagy et al., 1993). As a dicarboxylic acid, tartaric acid ( $C_4H_6O_6$ ) has two pKa values: 3.0 and 4.3. Tartaric acid has a strong tart taste compared to other organic acids, and is most useful in supporting grape-like flavors (Lück and Jager, 1997). As an antimicrobial agent, tartaric acid is believed to act only by lowering the pH of the product (Davidson and Branen, 1993). As a food additive, tartaric acid has a GRAS status (FDA, 2001c).

## **2. Hydrogen peroxide**

Hydrogen peroxide ( $H_2O_2$ ) is widely used for its strong oxidizing and bleaching powers. It is used in many industries including textile and chemical production, medicine, and food processing. Widely produced by mammalian metabolism, hydrogen peroxide is produced as an intracellular (i.e. by macrophages) and extracellular (i.e. in the eye) protective mechanism and antimicrobial with concentrations ranging from  $10^{-8}$  to  $10^{-3}$  M (ECETOC, 1993). Hydrogen peroxide is produced by the body as a result of oxidative stress and a byproduct of cellular respiration. Hydrogen peroxide does not accumulate within a biological system, since it rapidly decomposes to water and oxygen

in the presence of organic compounds. Additionally, several enzymes react with and reduce hydrogen peroxide within biological systems, including: catalases that are present in most cells at a wide range of concentrations, peroxidases that are present in cellular peroxisomes, and glutathione peroxidase also found in most cells (DeSesso et al., 2000). In fact hydrogen peroxide can decompose in biological fluids in 6-10 seconds (Ward, 1975; DeSesso, 1979) and do so within 2-4 nm from the site of production or placement (Myers, 1973; Marnett, 1987).

As hydrogen peroxide decomposes, reactive intermediates are formed, including: superoxide anions ( $\bullet O_2^-$ ) and hydroxyl radicals ( $\bullet OH$ ). The powerful antimicrobial action of hydrogen peroxide is not due to its oxidative properties as a molecule, but primarily due to in the production of these powerful oxidants (Davidson and Branen, 1993). These reactive oxygen species (ROS) can cause irreversible damage to cellular components such as enzymes, cellular membrane constituents, and DNA. Interestingly, aqueous solutions of hydrogen peroxide alone do not cause protein, lipid, or nucleic acid modification without the presence of radical formation catalysts (Juven, 1996). Through the well known Fenton reaction (below), unchelated ferrous iron (nuclear  $Fe^{2+}$ ) acts to promote the production of the hydroxyl radical.



Hydrogen peroxide can also react with cellular cuprous ions ( $Cu^+$ ) in a similar manner.



Hydroxyl radicals ( $\bullet OH$ ) most likely play the largest role in the cell toxicity due to hydrogen peroxide (Imlay and Linn, 1987). Hydroxyl radicals act on nucleic acids in two

ways, by adding to the nucleic acid bases and by removing hydrogen atoms from the DNA helix during the initial formation of hydroxyl radicals (Cadenas, 1989). Hydroxyl radicals react directly with cell membrane components and have been found to increase lipid peroxidation along with the ion permeability of model membrane systems (Anzai et al., 1999).

Hydrogen peroxide has been used as an antimicrobial agent and antiseptic for medical applications since the early 1800's (Davidson and Branen, 1993). Hydrogen peroxide is a weak acid, and the efficacy of hydrogen peroxide appears to increase with decreasing pH (Davidson and Branen, 1993). In food systems, hydrogen peroxide was used as a disinfectant in milk as early as 1904 (Lück and Jager, 1997). As a food additive, hydrogen peroxide has GRAS status (FDA, 2001b), and is currently approved by the FDA for packaging and surface sterilization in the food industry (FDA, 2001a). Hydrogen peroxide has limited use as a direct additive to foods. For example, hydrogen peroxide is used to treat milk for cheese production at 0.05%, thermophile free starch production at 0.15%, and the preparation of modified whey at 0.4% (FDA, 2001b). Additional applications include use as an oxidizing and reducing agent in wine, dried eggs, and corn syrup, along with use as a bleaching agent in tripe, beef feet, instant tea, colored cheese whey, and certain emulsifiers.

### 3. Ozone

Ozone gas is the strongest disinfectant and oxidizing agent available for use in the food industry. It is recognized as safe and has GRAS status for treatment of foods (21 CFR 184.1563). It is an unstable gas that is partially soluble in water and must be generated and used on-site. Ozone is generated when energetic ultraviolet light encounters oxygen in the air. Oxygen molecules are ruptured, which produces oxygen fragments that then unite with other oxygen molecules ( $O_2$ ) to produce ozone ( $O_3$ ). Ozone can be generated for commercial applications by the corona discharge technique, where air is passed across the discharge gap and ozone created where air is exposed to the gap (Hill and Rice, 1982).

As ozone comes in contact with organic compounds it readily decomposes and produces hydroxyl radicals ( $\bullet OH$ ) which, as stated above, are strong oxidizing agents. Molecular ozone is more effective at controlling microorganisms since hydroxyl radicals have a microsecond half-life. The half-life of ozone depends on the amount of ozone-demanding (organic) compounds in the aqueous environment. The half-life of molecular ozone can be as long as hours in very clean water or as short as seconds in waste-water (Graham, 1997). Ozone-treated products, like water, have no residual odor or taste.

Ozone antimicrobial activity is directly related to the intermediate reactive species that are formed (Kanofsky and Sima, 1991). Ozone causes damage to unsaturated lipids within cell membranes, intracellular enzymes, and nucleic acids. The degree of injury varies with microorganism, ozone concentration, and contact time. Henry's law states that ozone solubility in an aqueous solution is directly proportional to the pressure the gas exerts above the liquid (Bablon et al., 1991). Based on the Henry-Dalton constants,

solubility of ozone in water is higher at lower temperatures. A decrease in temperature increases ozone solubility and stability (Achen, 2000). The effectiveness of ozone against *Escherichia coli* and Hepatitis A virus decreased as the temperature was increased from 10°C to 20°C (Herbold et al., 1989). Additionally as the pH of a solution increases the rate of ozone decomposition increases, for example ozone decomposes instantly in a solution with a pH of 10 (Graham, 1997).

Ozone is currently being evaluated for increased use in the food industry. The effectiveness of ozone is dependent on food composition and surface structure. The use of ozone over chlorine is desirable due to the lack of harmful byproducts that are found after ozonation. Ozone can be used at different stages during processing.

Ozone is used by the food industry to tenderize meats, eliminate bacteria and spores during cheese ripening, increase the storage life of eggs, purify water, suppress souring of milk, and sterilize beer and soda processing equipment (Graham, 1997).

Ozone is being investigated for use on raw poultry and meats, in chill tanks, and on fresh fruits and vegetables.

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**IMMUNOHISTOCHEMISTRY BASED ASSAY TO  
DETERMINE THE EFFECTS OF TREATMENTS  
ON *CRYPTOSPORIDIUM PARVUM* VIABILITY**

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Key words: Cell culture, Viability, HCT-8 cells, Immunoperoxidase

Journal of Eukaryotic Microbiology, supplement vol 48:40S-41S

## ABSTRACT

Cell culture infectivity assays can provide an accurate means of detecting viable *Cryptosporidium parvum* oocysts from environmental samples or to test the effects of various treatments on oocyst infectivity. Cell culture assays can also be used to test candidate chemotherapeutic agents. The use of a human cell line provides a situation close to human infection. The present assay uses an anti-*Cryptosporidium* primary antibody, combined with a biotinylated secondary antibody, and an immunoperoxidase detection system. *Cryptosporidium parvum* oocysts excysted in vitro when placed on monolayers of HCT-8 cells and developmental stages including schizonts and merozoites were visualized using light microscopy of the immunoperoxidase stained slides and by transmission electron microscopy of infected HCT-8 cell cultures. Because the immunoperoxidase system used gives a permanent preparation, the cell cultures can be retained and examined later. Dose titration of oocysts indicated that as few as 50 inoculated oocysts could be detected. The activity of paromomycin was evaluated in this system and 500 µg/ml produced a 97.8% reduction in infection.

## INTRODUCTION

*Cryptosporidium parvum* is an Apicomplexan protozoan parasite that is a cause of waterborne and foodborne outbreaks of diarrheal disease [2-10, 14]. Infection occurs by ingestion of the thick-walled oocysts. Clinical disease caused by *C. parvum* is manifested by gastrointestinal illness lasting 1-2 weeks in previously healthy individuals or lasting indefinitely in immunocompromised individuals. Currently, there is no uniformly successful treatment for cryptosporidiosis. An effective in vitro assay is essential for assessing the efficacy of potential therapies. Viability assays using vital dyes and oocyst excystation are not reliable and the use of animal models is often expensive and time-consuming [1, 11, 12]. Infectivity for cell cultures has been determined by immunofluorescence, but may have high background staining and slides cannot be held indefinitely and observed at a later date. The present study describes a reliable infectivity assay using cell culture and an immunoperoxidase staining procedure to rapidly detect *C. parvum* developmental stages.

## MATERIALS AND METHODS

Human illeocecal adenocarcinoma cells (HCT-8 cells) (ATCC CCL-244) were maintained in RPMI 1640 media (Mediatech Cellgro) supplemented with L-glutamine (300 mg/L), and HEPES (25 mM), 5% fetal bovine serum for maintenance and 10% fetal bovine serum for infection [13]. Stock HCT-8 cells were maintained in 75-cm<sup>2</sup> tissue culture flasks in 5% CO<sub>2</sub> atmosphere, at 37°C, and passaged every 3-5 days. Collected cells were grown on sterile glass coverslips in 6-well plates at 1x10<sup>6</sup> cells per well and grown to ~95% confluency (48 hours).

*Cryptosporidium parvum* oocysts (Beltsville isolate) were treated with 0.525% sodium hypochlorite at 4°C, washed twice with Hanks Balanced Salt Solution (HBSS), incubated with the treatment of interest, and washed twice with HBSS.

Cells were then incubated with treated or non-treated (positive control) oocysts for 90-120 minutes and washed twice with HBSS. Cells were then grown for 48 hours with maintenance media.

Coverslips were fixed with 100% methanol for 20-30 minutes. Coverslips were then processed using a rabbit anti-*Cryptosporidium parvum* primary antibody (courtesy of C. Dykstra, Auburn University) and a biotinylated anti-rabbit secondary antibody (Vectastain ABC Kit, Vector Laboratories). Life stages were visualized with an immunoperoxidase stain using hydrogen peroxide, diaminobenzidine tetrahydrochloride (DAB, Sigma), with hematoxylin used as a counter stain.

As a negative control, oocysts were frozen in liquid nitrogen for two hours, prior to host cell inoculation. Selected cultures were fixed in 3% gluteraldehyde in 0.1M

sodium phosphate buffer 2 days after inoculation of oocysts, and processed for transmission electron microscopy (TEM).

Paromomycin (Sigma, St. Louis, MO) was used as a positive anti-*C. parvum* drug to determine the ability of our assay to detect reduction in developmental stages in treated cell cultures. Infected cell monolayers were incubated with media containing 5% fetal bovine serum and 500 µg/ml paromomycin. Paromomycin-treated cultures were compared to infected non-treated cultures. Treatment effectiveness was determined by counting the number of positive fields out of one hundred total fields. Percent reduction compared to control untreated oocysts was determined using the following equation:  
$$[(\text{Control} - \text{Treated}) / \text{Control}] \times 100.$$

## RESULTS AND DISCUSSION

Immunoperoxidase staining of HCT-8 cell cultures inoculated with viable oocysts demonstrated dark brown Immunoperoxidase staining of HCT-8 cell cultures inoculated with viable oocysts demonstrated dark brown stained developmental stages of *C. parvum* against a light blue host cell cytoplasm background produced by the hematoxylin counter stain. Developmental stages were usually observed in clusters indicating that development occurred at the site of excystation. No brown staining stages were observed in HCT-8 cells inoculated with frozen oocysts and processed using the immunoperoxidase procedure. This indicates that residual non-excysted oocysts or nonviable oocysts are washed off in our procedure and not detected by the immunoperoxidase stain. Based on examination of one hundred 40x microscopic fields, infection by  $1 \times 10^6$  untreated oocysts consistently produced between 90-98% infection. Detection is possible with an initial infection of as few as 50 oocysts (1% infection) or 100 oocysts (1%-20% infection), but detection is enhanced when  $\geq 10,000$  oocysts are used. Experiments and treatments were evaluated a minimum of three times.

Schizonts and merozoites were observed 48 hr after infection using TEM (Fig. 1). They were located in a parasitophorous vacuole within the microvilli of HCT-8 cells. The TEM results clearly demonstrate that development of *C. parvum* occurred in the HCT-8 cells.

This assay demonstrated that treatment with 500  $\mu\text{g/ml}$  paromomycin produced a 97.8% reduction in developmental stages based on five experiments. Treatment with paromomycin serves to validate the assay.

This method provides a relatively simple and reliable means for determining the effects of treatments on *Cryptosporidium parvum* viability in an in vitro assay. This method provides permanent slide preparations that can be examined independently and in a blinded fashion by a single or several evaluators.

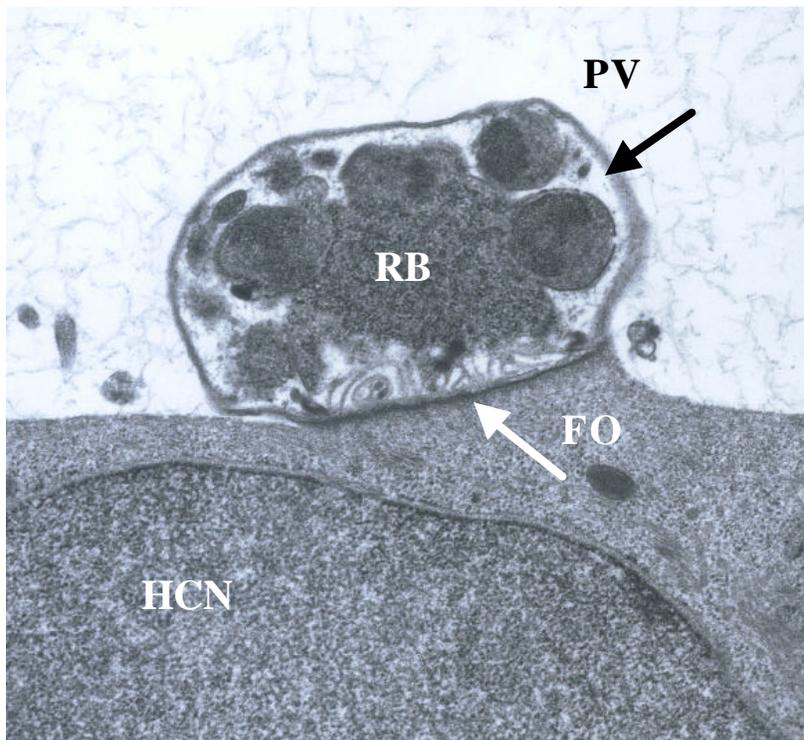
## **ACKNOWLEDGMENT**

Supported in part by CSREES USDA special food safety grant No. 98-34382-6916 and VPI & SU HATCH project #135563.

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**Figure 1.** Transmission electron microscopy of a *Cryptosporidium parvum* schizont growing in an HCT-8 cell. Parasitophorous vacuole (PV), feeder organelle (FO), residual body (RB), and host cell nucleus (HCN) are indicated.

## APPENDIX I

### DETERMINATION OF THE INFECTIVITY THRESHHOLD AND NUMBER OF FIELDS COUNTED FOR A CELL CULTURE-IMMUNOPEROXIDASE ASSAY

This assay is used to evaluate the effectiveness of various treatments on *Cryptosporidium parvum* infectivity. In order to best evaluate these treatments it is important to understand the assay, including the determining the threshold of detection for infectivity. Various assays, including Polymerase Chain Reaction (PCR), membrane filtration, fluorescent antibody detection, and magnetic bead separation, boast of detection of few (<10) oocysts (Carreno et al., 2001; Hsu and Huang, 2001; Karasudani et al., 2001; Linndquist et al., 2001; McCuin et al., 2001); however, this seems unlikely since only 5% of inoculated oocysts are infective (personal communication Jan Mead, 2001). Cell monolayers were infected with bleach-treated oocysts as previously described. Infection was routinely detected with >100 oocysts. *Cryptosporidium* life stages were detected after infection with 50 oocysts, but not routinely (75% of cell layers analyzed). No life stages were detected after infection with as few as 10 oocysts. Since infection most likely occurs with approximately 5% of oocysts inoculated, infection of HCT-8 cells as monitored in this assay by immunohistochemistry occurs with approximately 10 oocysts.

The percent reduction is determined by counting fields containing life stages visualized by an immunoperoxidase stain. Other cell culture assays evaluate infectivity in a similar fashion by counting fluorescent objects or foci within a certain number of

fields (Slifko et al., 1997). The number of infected fields was determined by counting life stages visible in 25, 100, and 300 fields. There was no statistical difference between the percent infectivity determined between these three counts (t-test, n=8, P <0.05). It seems that *C. parvum* infection is fairly evenly spread throughout the coverslip. This fact seems obvious given the life cycle of *C. parvum*.

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## APPENDIX II

### EVALUATING THE EFFECTIVENESS OF DRUGS ON *CRYPTOSPORIDIUM PARVUM* USING A CELL CULTURE ASSAY

*Cryptosporidium parvum* is a causative agent of diarrhea in humans and young dairy and beef calves. No effective treatment is available at this time. Drugs routinely used on various protozoan parasites were evaluated on *C. parvum* using the cell culture assay with immunoperoxidase staining as described previously. These drugs vary in effectiveness, depending on concentration, but those tested are not completely effective (Fig. 1). Paromomycin is most effective against *C. parvum*, but varies in real-time use with infected individuals.

Oocysts of the Beltsville isolate of *C. parvum* were used to infect human illeocecal epithelial cells (HCT-8 cell, ATCC CCL 244) grown on 22 mm<sup>2</sup> coverslips. The efficacy of treatments was evaluated after two days post-inoculation (pi) of monolayers. Oocysts were cleaned by bleach treatment, washed in sterile water and resuspended in cell culture medium. Each cell monolayer was infected with  $1 \times 10^6$  oocysts. Oocysts were allowed two hours to excyst and initiate infection of cultured cells. The infection medium was then removed and replaced with test medium containing ponazuril, irgasan, or paromomycin. Negative control wells were incubated with an equal number of nonviable (frozen/thawed) oocysts. Paromomycin (500µg/ml from stock made in distilled water) medium was used as a positive treatment control. Ponazuril (from stock 1000 µg/ml in DMSO) at concentrations of 5.0, 1.0, and 0.1 µM and Irgasan(from stock) at concentrations of were tested for activity against *C. parvum*.

The assay was stopped two days after infection was initiated. Coverslips were removed from plates, fixed in methanol, and developed using an immunohistochemistry stain. Coverslips were first treated with a *Cryptosporidium* polyclonal rabbit antibody (1:100) and then treated with a secondary antibody based on an avidin/biotin horseradish peroxidase system (VECTASTAIN<sup>®</sup> ABC Kit). The numbers of stained parasites in 100 40x fields was determined and a percent reduction calculated.

<b><i>Treatment</i></b>	<b><i>Concentration</i></b>	<b>% Reduction (n=20, ± SEM)</b>
Paromomycin	500 µg/ml	97.8% ± 9.2%
Freeze / Thaw	N/A	100% (no infectivity detected)

<b><i>Treatment</i></b>	<b><i>Concentration</i></b>	<b>% Reduction (n=18, ± SEM)</b>
Ponazuril	0.1 µg/ml	7.9% ± 1.9%
	1.0 µg/ml	18.4% ± 1.0%
	5.0 µg/ml	39.0% ± 16.0%
	10.0 µg/ml	45.6% ± 24.4%

<b><i>Treatment</i></b>	<b><i>Concentration</i></b>	<b>% Reduction (n=5, ± SEM)</b>
Irgasan DP 300	1 µg/ml	11.84% ± 4.1%
	5 µg/ml	21.64% ± 12.8%
	10 µg/ml	92.65% ± 5.6%
	100 µg/ml	toxic to cells

Figure 1. Evaluation of drugs on *C.parvum* infectivity of HCT-8 cells.

Percent reduction was determined for treated cells compared to control cells which received media plus the same ratio of chemical in which the stock was dissolved (0.4% DMSO for Ponazuril, 60:40 Ethanol:Distilled water for Irgasan). The following calculation was used to determine percent reduction:

$$(\text{Control} - \text{Treatment} / \text{Control}) \times 100.$$

**EFFECT OF ORGANIC ACIDS AND  
HYDROGEN PEROXIDE  
ON *CRYPTOSPORIDIUM PARVUM*  
VIABILITY IN FRUIT JUICES**

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Key words: Cell culture, HCT-8 cells, immunoperoxidase, viability, oocyst wall

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## ABSTRACT

*Cryptosporidium parvum* has historically been associated with waterborne outbreaks of diarrheal illness. While more recently, foodborne cryptosporidiosis has been associated with unpasteurized apple cider. Infectious oocysts are shed in the feces of common ruminants like cattle and deer in and/or near orchards. The addition of organic acids or hydrogen peroxide ( $H_2O_2$ ) to fruit juice to inhibit survival of *C. parvum* was analyzed in this study. Oocyst viability was analyzed with a cell culture infectivity assay, using a human ileocecal cell line (HCT-8) that is most similar to human oral infectivity. Cell monolayers were infected with  $10^6$  treated oocysts or a series of 10-fold dilutions. Parasitic life stages were visualized using immunohistochemistry and 100 microscope fields counted per monolayer. *In vitro* excystation assays were also used to evaluate these treatments. Organic acids and  $H_2O_2$  was added on a wt/vol basis to apple cider, orange juice, and grape juices. Malic, citric, and tartaric acids at concentrations from 1%-5% inhibited *C. parvum* infectivity of HCT-8 cells by up to 88%. Concentrations ranging from 0.025%-3%  $H_2O_2$  were evaluated. The addition of 0.025%  $H_2O_2$  to each juice resulted in a >5 log reduction of *C. parvum* infectivity as determined with an MPN-based cell culture infectivity assay. As observed with differential interference contrast and scanning electron microscopy, reduced infectivity may be mediated through effects on the oocyst wall caused by the action of  $H_2O_2$  or related oxygen radicals. Addition of low concentrations of  $H_2O_2$  can offer a valuable alternative to pasteurization.

## INTRODUCTION

*Cryptosporidium parvum* commonly causes a gastrointestinal illness that can vary from a self-limiting diarrhea to a severe wasting disease (37). Cryptosporidiosis may persist in immunocompromised persons, especially those with AIDS or undergoing chemotherapy (8), and in malnourished children (18). Cryptosporidiosis is associated with waterborne outbreaks and is frequently becoming associated with foodborne outbreaks (14). Within the past ten years several outbreaks of gastro-intestinal illness have been associated with fresh fruit juice and cider (30,1). Most juice outbreaks have been caused by pathogenic bacteria, like *Escherichia coli* O157:H7 and *Salmonella* sp.; at least two outbreaks were attributed to *C. parvum* (27,6).

The life cycle of *C. parvum* begins with the ingestion of the environmentally resistant oocyst which excysts within the host intestine. Infective sporozoites enter microvilli of enterocytes and replicate there until oocysts are formed and shed in the feces (13). *Cryptosporidium* oocysts are fairly resistant to most chemical compounds, due to their relative metabolic dormancy and impermeability of the oocyst wall (22). Organic acids such as citric, malic, and tartaric are known to possess antimicrobial activity and have been used in many food applications (10). It is believed that organic acids act on microbial cells by lowering the pH of the environment, disrupting the functions of membranes and key enzymes (11). Unlike bacteria, the environmentally resistant oocyst can resist changes in pH. The components of pH ( $H^+$  and  $OH^-$ ) are charged; therefore, they remain outside the oocyst wall and cannot inactivate oocysts alone (23).

Pretreatment with acidified media was previously shown to enhance excystation indirectly by increasing oocyst wall permeability (24). Additionally, the ability of some

organic acids to chelate metal ions, like calcium ( $\text{Ca}^{2+}$ ) and magnesium ( $\text{Mg}^{2+}$ ), may increase the ability of organic acids to inhibit oocyst viability, as these ions are essential for oocyst maintenance.

In many cases, food additives are added to enhance and modify the flavor of a food, as well as to act as a preservative and decrease microbial survival. In this study, food additives are added only for the latter and a physical change is not desired. The addition of a preservative can affect the complex components of the juice system. Sugars, organic acids, and phenols all play important roles in the character and quality of fruit juice flavor and can attribute to the antimicrobial effect of the juice (10). When food additives are used in an attempt to increase the antimicrobial properties of a juice, the balance between sweetness, acidity, and astringency should be retained (41). These qualities all lend sensory and chemical attributes to the final juice product. For these reasons fruit juice characteristics were noted in unamended juices as well as those treated with varying concentrations of organic acids and  $\text{H}_2\text{O}_2$ .

In accordance with the fruit juice hazard analysis critical control point (HACCP) rule (1), treatment of the final juice product is not limited to heat pasteurization.

Alternatives to pasteurization may be implemented in juice processing if they provide a 5-log reduction in the pertinent pathogen. One alternative may be the addition of  $\text{H}_2\text{O}_2$ , the most widely used of the inorganic peroxides, that exerts a strong antimicrobial effect through the production of reactive oxygen species, like hydroxyl radicals (3,10).

Hydrogen peroxide gas plasma at 58% has been shown previously to have an inhibitory effect on *C. parvum* oocysts, as the use of a medical sterilizing agent (39). Additionally,

treatment with H<sub>2</sub>O<sub>2</sub> was shown to increase the permeability of the oocyst wall altering the acid-fast staining pattern (12).

Treatments like changes in pH or oxidation affect oocysts in different ways that can be gauged by excystation and infection assays. For example, oocysts that have been killed by heat or formaldehyde will not excyst demonstrating that live sporozoites may be required for excystation (22,24). It is possible that some treatments do not fully inhibit excystation, but allow significant DNA damage has occurred so there is no development post-sporozoite release within host cells. It has also been noted that treatments that kill sporozoites may induce changes within the oocyst wall and suture, and inhibit excystation (24).

The objectives of this study were to identify the minimum treatment levels of H<sub>2</sub>O<sub>2</sub> and organic acids added to fruit juices to reduce or inhibit the infectivity of *C. parvum*. An HCT-8 cell culture infectivity assay (31) was used to evaluate the amended fruit juice samples on oocyst excystation and sporozoite viability. This assay provides information on the inhibition of *C. parvum* viability as a function of treatment concentration. Cell culture is an excellent tool used to study parasite development and the cell type used in this assay (HCT-8 cells) most closely mimics human infection (5). *In vitro* excystation assays were used to further evaluate the effects of juice treatments on oocyst wall structure, suture condition, and sporozoite viability.

## MATERIALS AND METHODS

**Oocyst preparation.** *Cryptosporidium parvum* oocysts (bovine Beltsville isolate, Genotype C) were obtained originally from infected dairy calves and processed at the Parasite Biology Laboratory, United States Department of Agriculture, Beltsville, MD (16). Oocysts were purified from fecal material first by washing through a series of graded sieves down to a pore size of 45  $\mu\text{m}$ , and second by density centrifugation over cesium chloride (25). Residual cesium chloride was removed by three cycles of centrifugation at 1,000 x g for 10 minutes each. Oocysts were resuspended in distilled water and quantified on a hemocytometer.

**Oocyst treatment.** *Cryptosporidium parvum* oocysts ( $1 \times 10^6$  per treatment) were treated with 0.525% sodium hypochlorite at 4°C for less than 5 minutes and washed twice with Hanks Balanced Salt Solution (HBSS). The oocysts were then incubated with 1.5 ml of treated or untreated fruit juice at 4°C for a specific length of time, and washed twice with HBSS after treatment. Treatment with sodium hypochlorite did not effect juice treatment (data not shown). No difference was observed between oocysts treated in juice incubated at 4°C or at 25°C (data not shown). Varying levels of food grade H<sub>2</sub>O<sub>2</sub> (FMC, Philadelphia, PA) and organic acids (Presque Isle Wine Cellars, North East, PA) were added to apple cider, orange juice, and grape juices on a wt/vol basis. The organic acids added corresponded to the dominant organic acid of the particular juice: malic acid-apple cider, citric acid-orange juice, tartaric acid-grape juices. Untreated juices and oocysts treated with HBSS served as controls.

**Juice preparation.** Apple cider was obtained unpasteurized from a local processor, stored frozen, and thawed at 4°C before use. Orange juice (Kroger Original Premium Pasteurized Orange Juice; Kroger, Cincinnati, OH) was obtained from a local supermarket and stored frozen until use. Purple grape juice (Welch's 100% Grape Juice; Welch's, Concord, MA) and white grape juice (Santa Cruz Organic White Grape Juice; Santa Cruz, Chico, CA) were stored at room temperature prior to use. All juices were preservative free.

**Juice Analysis.** No precipitates formed upon addition of acids to juice. Juice samples were stored at 4°C. Indicated levels of H<sub>2</sub>O<sub>2</sub> may not be exact due to dissipation, but residual H<sub>2</sub>O<sub>2</sub> could not be determined (Merk peroxide test strips). Physical and chemical measurements were determined as follows: pH, titratable acidity using 1M NaOH titration to pH 8.2, total phenolics, and color using a Minolta color analyzer. Phenol components were measured by spectral estimation. Absorption readings were used to estimate the concentration of total phenols [ $A_{(280\text{ nm})} - 4$ ]. The concentration of dominant organic acids (malic, citric, tartaric) and sugars (sucrose, glucose, fructose) were determined using liquid chromatography (data not shown). Sugars and organic acids were separated and collected using an anion exchange resin. The fractions were then run separately and concentrations determined by comparison with standard sugars and acids on a Phenomenex Resex ROA-organic acid column with a carbohydrate guard column using 0.01 N sulfuric acid as the mobile phase at 22°C and with refractive index detector.

**Cell culture media.** Human illeocecal adenocarcinoma cells (HCT-8 cells) (ATCC CCL-244, American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium (Mediatech Cellgro, Herndon, VA) supplemented with L-glutamine (300 mg/L; Mediatech Cellgro), and HEPES (25 mM; Mediatech Cellgro). For normal cell maintenance medium was supplemented with 5% fetal bovine serum (Biofluids, Inc., Rockville, MD) and increased to 10% fetal bovine serum for parasite infection (38).

**Cell maintenance.** Stock HCT-8 cells were maintained in 75-cm<sup>2</sup> tissue culture flasks in a 5% CO<sub>2</sub> atmosphere, at 37°C and 100% humidity, and passaged every 3-5 days. Cells were lifted from the surface with a solution of 0.25% (wt/vol) trypsin and 0.53 mM EDTA in Phosphate Buffered Saline (PBS) (Mediatech Cellgro). Trypsinization required 10-12 minute incubation in the solution at 37°C to assist in disruption of the cell monolayer. The cells were collected and pelleted by centrifugation for 10 minutes at 1000 x g, resuspended in maintenance medium, and split 1:10. Cell viability was assessed with trypan blue exclusion (0.02% in PBS) and cells counted with a hemocytometer.

**Inoculation of monolayers with parasites.** Collected cells were seeded on sterile 22-mm<sup>2</sup> glass coverslips in 6-well cluster plates (Corning, Corning, NY) at 1x10<sup>6</sup> cells per well and grown to ~95% confluency in maintenance medium (48 hours). *Cryptosporidium parvum* oocysts were treated as stated above. For infection of monolayers and prior to inoculation with oocysts, maintenance medium was removed and

2-3 ml growth medium added to each well in 6 well cluster plates. Cells were then incubated with treated or non-treated (positive control) oocysts ( $10^6$  oocysts in 1 ml growth medium) for 90-120 minutes. Then unexcysted oocysts, oocyst walls, and other materials that may have been liberated from the excysted oocysts were washed from the monolayers by washing each inoculated well twice with HBSS. Cells in cluster plates were then placed back in the incubator for 48 hours with 3-4 ml maintenance medium per well.

**Immunohistochemistry of viable parasitic lifestages.** Parasite infection was assessed 48 hours post infection using an immunohistochemistry stain (31). Coverslips in 6 well cluster plates were fixed with 100% methanol for 20-30 minutes and washed twice for 5 minutes each with PBS. Coverslips were removed from cluster plates and processed on slides, first with a rabbit anti-*Cryptosporidium parvum* primary antibody (courtesy of C. Dykstra, Auburn University), followed by a biotinylated anti-rabbit secondary antibody (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) and an avidin biotinylated complex (ABC reagent; Vectastain ABC Kit, Vector Laboratories). Life stages were visualized with an immunoperoxidase stain using  $H_2O_2$  (Sigma, St. Louis, MO), diaminobenzidine tetrahydrochloride (DAB, Sigma), with hematoxylin (Fisher Scientific) used as a counter stain. With this system, *Cryptosporidium* life stages are colored brown on a blue and purple background. Oocysts that were frozen in liquid nitrogen for two hours, prior to host cell inoculation served as a negative control. Paromomycin (500  $\mu$ g/ml ; Sigma) was used as a positive anti-*C. parvum* drug to

determine the ability of our assay to detect reduction in developmental stages in treated cell cultures (data not shown).

Treatment effectiveness was based on scoring the presence or absence of living stages (sexual gamonts and asexual meronts) in 50 (MPN-type assays) or 100 sequential and nonoverlapping fields visualized at a 400x magnification using phase contrast microscopy. Fields containing one or more *Cryptosporidium* life stages were scored as positive, while fields containing no stages were scored as negative. Each individual experiment was performed in triplicate. Data are shown two ways, as percent inhibition and as a log reduction.

i) Percent inhibition compared to control untreated oocysts was determined using the following equation:  $[(\text{Control} - \text{Treated}) / \text{Control}] \times 100$ . Data are expressed as the mean percent reduction followed by the standard deviation.

ii) The oocyst infectivity titer was determined for the most effective treatments using the Most Probable Number (MPN) method (35,34). Calculations were based on the number of positive and negative fields (test units) using the Thomas equation for obtaining the most probable number. At least 100 cell culture fields were counted in three sequential dilutions using  $10^6$ ,  $10^5$ , and  $10^4$  oocysts.

**Excystation assays.** Approximately  $1 \times 10^5$  *C parvum* oocysts were washed and treated as those used for cell culture infectivity assays described above. Then oocysts were incubated in 0.75% taurochloric acid (Sigma) for 30 minutes at 37°C. The excystation solution was observed at 400x and 1000x magnification using Differential Interference Contrast microscopy (DIC). A total of 100 shells and oocysts was counted.

A minimum of duplicate samples was evaluated. Oocysts containing sporozoites were considered unexcysted. Oocysts containing no sporozoites, also called shells, were considered excysted. Excystation rates were calculated by the Woodmansee method (40,17):  $\text{excystation} = ([\text{oocysts excysted}/\text{total oocysts counted}] \times 100)$ . The log reduction for excystation was determined by an adaptation from Chicks law for log inactivation using survivors (N) and initial applied dose (N<sub>0</sub>)(17):  $\log N/N_0 = \log (\% \text{excystation}_{\text{treated}}/\% \text{excystation}_{\text{control}})$

**Scanning electron microscopy.** Scanning electron microscopy (SEM) was performed to evaluate the oocyst surface topography after treatment with H<sub>2</sub>O<sub>2</sub>. *Cryptosporidium parvum* oocysts (~5.0 x 10<sup>5</sup>) treated with apple cider + 0.03% H<sub>2</sub>O<sub>2</sub> for 2 hr were gently mixed with HCT-8 cells (~1.0 x 10<sup>4</sup>) fixed in 2.5% gluteraldehyde in 0.1M sodium phosphate buffer, and coated with gold particles for SEM. Oocysts were examined with a JOEL 35-C scanning electron microscope.

**Statistical analysis.** Data from individual experiments were considered independent. Experiments and controls were analyzed for significant differences using PROC GLM in SAS statistical software (SAS Institute, Cary NC). Effects of each treatment on parasite viability were considered to be significant when P = ≤0.05. Treatments were separated using Tukeys test for significant differences.

## **RESULTS AND DISCUSSION**

### **Characteristics of amended juice**

The major sugars, acids, and phenols were evaluated in amended and unamended fruit juices; including, apple cider, orange juice, white and purple grape juices. It was determined that the addition of organic acids and/or H<sub>2</sub>O<sub>2</sub> resulted in a significant change in acidity as shown by pH and titratable acidity values (Table 1). The physical color of the juice was altered by some of the treatments (Table 1). Liquid chromatography was used to identify dominant sugars and acids. No significant differences were determined in sucrose, glucose, or fructose levels in amended juices compared to controls ( $P < 0.05$ ; data not shown). Significant differences in the dominant juice acids (malic, citric, tartaric) were detected only in acidified samples ( $P < 0.05$ ). The addition of organic acids and/or H<sub>2</sub>O<sub>2</sub> caused significant changes in juice phenol components. The addition of 5% acid was significantly different from all controls, while the addition of 0.03% H<sub>2</sub>O<sub>2</sub> did not significantly alter phenolics compared to controls. While all quantities of H<sub>2</sub>O<sub>2</sub> and organic acids added to juices were not evaluated, greater quantities added were evaluated, so that it is understood that lower quantities would show changes to a lesser degree.

### **Effect of organic acids on infectivity**

Infection and reproduction by *Cryptosporidium* life stages was evident by life stages stained with the *C. parvum* polyclonal antibody (Figure 1). Life stages were identified and visualized by transmission electron microscopy (31). Infection in HCT-8

cells by *C. parvum* oocysts incubated in fruit juices acidified by addition of the dominant organic acid was evaluated and compared to untreated oocysts incubated in HBSS.

Figure 2 shows the inhibition of *C. parvum* infectivity in apple cider untreated or acidified with 1%, 3%, or 5% malic acid (A); in orange juice untreated or acidified with 1%, 3%, or 5% citric acid (B); in purple grape juice (C) or white grape juice (D) untreated or acidified with 1%, 3%, or 5% tartaric acid. All graphs show the general trend with an increase in inhibition of infectivity with increased acid added and resulting decreased pH. While acid concentrations were not sufficient to completely inhibit *C. parvum* viability, these acidified juices were not palatable, as determined by sensory evaluation which indicated that 0.3% acid added to juice was out of specification by >65% of panelists (data not shown) (29). The addition of 1% or 3% acid was not significantly different in any juice, while the addition of 5% acid was significantly different from unamended juices; with the exception of white grape juice where no differences were detected in infectivity among the juice samples. As anticipated, change in pH was not completely effective at inhibiting infectivity of *C. parvum*. Preincubation in acids has been shown previously to enhance excystation (24). This was observed with oocysts incubated in apple cider prior to excystation (Table 3). The addition of acids to the juices most likely does not influence infectivity at lower amounts, which is why larger amounts of acids were added to juices in this study before an inhibition in infection was observed. The effect of pH can be seen on an intracellular level as sporozoite attachment to host cells was pH dependent to some degree, with an optimal pH at 7.2 to 7.6 (20). Oocysts incubated in acidified juices may become permeabilized and slight damage to the oocyst wall may reduce infectivity; however, oocysts were not visibly

damaged as observed by phase contrast or differential interference contrast (DIC) microscopy (data not shown). Citric acid chelates cations and has been shown to exert an inhibitory influence in this manner against bacteria and molds (4). The citric acid present in largest quantities in orange juice and to a lesser extent in the other juices may inhibit infection by this mechanism, as sporozoites showed enhanced attachment to host cells in the presence of calcium, zinc, and magnesium (20) (38). It was also observed that the toxic response of cells to *Cryptosporidium* was reduced by lowering the calcium concentration of the environment, and that the toxin may be mediated by calcium ions (19).

### **Effect of hydrogen peroxide on infectivity**

Hydrogen peroxide inhibited *C. parvum* infectivity in the cell culture assay. Tables 2 and 3 show the inhibition by H<sub>2</sub>O<sub>2</sub> in apple cider (A), orange juice (B), purple grape juice (C), and white grape juice (D) as measured by infectivity and excystation. In comparison to the cell culture assay, which measures the potential of oocysts to complete their life cycle within the host, excystation is a measurement of the oocyst's response to a biochemical stimulus. The two assays are useful in interpreting treatment effects on oocysts. They do not necessarily correlate, because oocysts that excyst may not be able to complete their life cycle within the host. Excystation overestimates oocyst viability (2), in particular after chemical inactivation compared with infectivity (2). Infectivity as evaluated with the cell culture assay was completely inhibited by incubation of oocysts in apple cider + 0.025% H<sub>2</sub>O<sub>2</sub> for 6 or more hours or in apple cider + 0.03% H<sub>2</sub>O<sub>2</sub> for 2 or more hours. No signs of infection were observed with oocysts incubated in these juices,

while the limit of detection with this assay is infection with  $\geq 50$  oocysts (31). The percent inhibition strongly correlates with the  $\log_{10}$  reduction as determined with the MPN method,  $> 5$  logs. It should be noted that identical results in the cell culture assay in terms of percent inhibition and  $\log_{10}$  reduction were found for orange juice, white grape juice, and purple grape juice with 0.03%  $\text{H}_2\text{O}_2$  added and oocysts treated for two hours prior to infection of cell monolayers.

Interestingly, these same treatments showed low excystation rates compared to unamended apple cider, while oocysts still excysted but were unable to infect the cells. In this case, it is likely that sporozoites are unable to infect cells due to DNA damage from reactions with hydroxyl radicals. Hydrogen peroxide readily breaks down into reactive oxygen species, including the reactive hydroxyl radical, which is well known to cause DNA damage by reacting with thymidine and other nucleic acids. Hydrogen peroxide has been shown to affect calcium intake both intracellularly and extracellularly of the internal parasite *Leishmania donovani*, resulting in an apoptosis-like death (9). Das et al. noted the inhibitory action of  $\text{H}_2\text{O}_2$  was in part due to a significant increase of calcium after a short incubation of parasite life stages in  $\text{H}_2\text{O}_2$  (2001). Free radical production by  $\text{H}_2\text{O}_2$  decomposition along with changes in calcium ion concentration is a common feature of programmed cell death as observed in eukaryotic cells (26). As noted above, calcium is necessary for proper infection by *C. parvum* (20) (38). The influx of calcium ions from the environment may be another mechanism by which  $\text{H}_2\text{O}_2$  inactivates *C. parvum* oocysts.

As the addition of  $\text{H}_2\text{O}_2$  did not change the pH of the amended juice samples compared to the control (Table 1), changes in infectivity cannot be attributed to pH.

Oocysts incubated in juices treated with these low levels of H<sub>2</sub>O<sub>2</sub> were analyzed for possible reactivation. After treatment, oocysts were washed with HBSS and were found to remain non-infective after two weeks stored at 5°C. Additionally, apple cider + 0.03% H<sub>2</sub>O<sub>2</sub> was found acceptable to >65% of panelists in sensory analysis using the In/Out method (29) (data not shown); thus may likely offer an alternative to heat pasteurization. While taste was not affected, color as analyzed with a colorimeter for Lab values, indicated slight differences showing a slight decrease in yellow color.

### **Oocyst morphology**

Differential interference contrast microscopy and scanning electron microscopy were used to evaluate *C. parvum* oocysts incubated in apple cider + 0.03% H<sub>2</sub>O<sub>2</sub>. Some oocysts incubated in apple cider + 0.03% H<sub>2</sub>O<sub>2</sub> looked shriveled or injured. These oocysts lacked their characteristic round shape. Under scanning electron microscopy these alterations in the oocyst wall were still visible. This may be evidence of reaction with reactive oxygen species formed from the breakdown of H<sub>2</sub>O<sub>2</sub>.

Hydrogen peroxide was previously shown to alter normal oocyst excystation (32). Acid fast staining and contrast was increased and improved after treatment with 10% H<sub>2</sub>O<sub>2</sub> (12). In some cases staining sensitivity was enhanced up to 40-times post-treatment. Sodium hypochlorite treatment also enhanced acid-fast staining, but to a lesser extent (7). Interestingly, while H<sub>2</sub>O<sub>2</sub> treatment presumably oxidizes or alters oocyst wall proteins, H<sub>2</sub>O<sub>2</sub> treatment did not effect monoclonal antibody staining and does not seem to remove antigenic sites (12). However, the formation of crystals post-chlorine

treatment interfered with antibody staining (12) and reduced antibody in Western analysis and flow cytometry (28).

Cryptosporidiosis is resistant to the majority of chemotherapeutic agents used to treat parasitic diseases. This is in part due to the fact that the majority of chemotherapeutic agents have been directed against the intracellular or zoite stages rather than oocysts (21). This may as well be a reflection of the strength of the trilaminar oocyst wall, and while no effective treatment has been found to date to damage the oocyst wall (21), the relatively low concentrations of  $H_2O_2$  used in this study appear to do so. Other treatments like freezing which reduce infectivity and kill oocysts do not alter oocyst morphology (15). While it is not clear if the inactivation of *C. parvum* is based on one target- the oocyst, or multiple targets- 4 sporozoites (36), it is most likely that concentrations of  $H_2O_2$  used in this study have a combined effect, where hydroxyl radicals first oxidize and weaken the oocyst wall and then effect the DNA of sporozoites rendering them non-infective. It is likely that  $H_2O_2$  could be used alone or in combination with other inactivation methods like ultraviolet light, freezing, or ozone to control *Cryptosporidium* contamination of unpasteurized fruit juices.

## **ACKNOWLEDGMENT**

This study was funded by a CSREES USDA Special Food Safety Grant No. 98-34382-6916 VPI&SU HATCH project #135563.

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**Table 1. Changes in pH, Titratable Acidity, Total Phenols and Color Values for Juice Samples**

Average pH value from 5 measurements with standard deviation <0.05 for all averages. Average TA value from 5 measurements with standard deviation <0.5 for all averages. TA is expressed in milliequivalents of the dominant acid per unit of juice. Within the same juice pH and TA values are significantly different from one another as (P < 0.05).

The L\*a\*b\* color values (n=3) were determined for each juice sample using a Minolta Colorimeter. The size of a color change in a modified juice sample compared to the unmodified juice is expressed as  $\Delta E^*ab$ . This value indicates the size of the change only and not what way the colors are different.  $\Delta E^*ab$  is defined by the following equation:  $\Delta E^*ab = \sqrt{[(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]}$ .

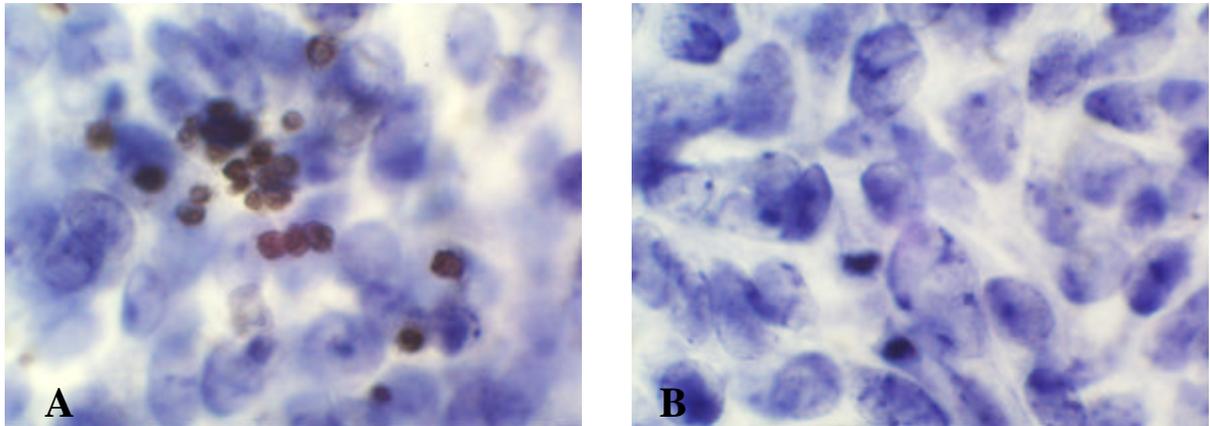
Phenolic compounds are particularly prominent in fruits, influencing color, flavor, and antimicrobial properties. Total phenolics were measured spectrophotometrically in absorbance units for each juice sample. Those samples not significantly different from the control juice sample remain in solid color. Total phenolics are expressed as gallic acid equivalents (mg/L GAE), using the following equation:  $y = 29.5(\text{tot. pH. in au}) + 210$ .

**Table 1. Changes in pH, Titratable Acidity, and Color Values for Juice Samples**

<b>Sample</b>	<b>pH</b>	<b>TA (meq. acid)</b>	<b>Phenolics (mg/L GAE)</b>	<b>D E*ab</b>
<b>Apple Cider</b>	3.90	0.39	1239.55	N/A
+3% Malic Acid	2.90	3.23	1180.55	1.07
+5% Malic Acid	2.43	4.91	897.35	0.82
+3% H <sub>2</sub> O <sub>2</sub>	3.78	0.22	1266.10	1.64
+1% Malic Acid + 1.5% H <sub>2</sub> O <sub>2</sub>	3.62	1.22	1230.70	1.54
+0.03% H <sub>2</sub> O <sub>2</sub>	3.90	0.36	1236.66	0.15

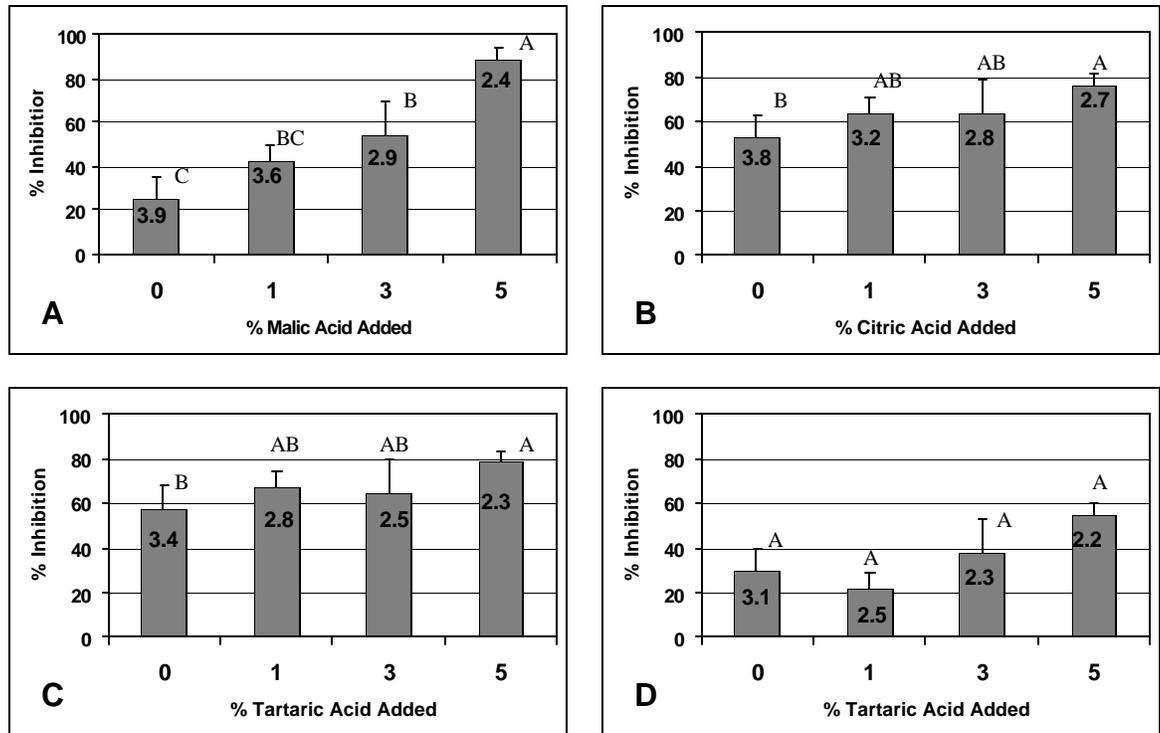
**Table 1.Continued**

<b>Sample</b>	<b>pH</b>	<b>TA (meq. acid)</b>	<b>Phennolics (mg/L GAE)</b>	<b>D E*ab</b>
<b>Orange Juice</b>	3.82	0.63	1481.45	N/A
+3% Citric Acid	2.88	3.56	1331.00	0.54
+5% Citric Acid	2.76	5.27	1213.00	0.92
+3% H <sub>2</sub> O <sub>2</sub>	3.74	0.51	1245.45	1.07
+1% Citric Acid + 1.5% H <sub>2</sub> O <sub>2</sub>	3.20	1.66	1221.85	1.03
+0.03% H <sub>2</sub> O <sub>2</sub>	3.89	0.62	1404.75	0.10
<b>White Grape Juice</b>	3.14	0.47	1183.50	N/A
+3% Tartaric Acid	2.33	3.44	1239.55	1.77
+5% Tartaric Acid	2.20	4.89	1561.10	1.60
+3% H <sub>2</sub> O <sub>2</sub>	3.08	0.61	1171.70	3.85
+1% Tartaric Acid + 1.5% H <sub>2</sub> O <sub>2</sub>	2.50	1.43	1419.50	1.76
+0.03% H <sub>2</sub> O <sub>2</sub>	3.10	0.44	1272.00	0.10
<b>Purple Grape Juice</b>	3.41	0.52	1295.60	N/A
+3% Tartaric Acid	2.54	3.43	1254.30	0.22
+5% Tartaric Acid	2.30	5.21	1207.10	0.27
+3% H <sub>2</sub> O <sub>2</sub>	3.36	0.62	1215.95	2.02
+1% Tartaric Acid + 1.5% H <sub>2</sub> O <sub>2</sub>	2.80	1.40	1198.25	1.15
+0.03% H <sub>2</sub> O <sub>2</sub>	3.39	0.54	1272.00	0.65



**Figure 1. Cell Culture Infectivity**

HCT-8 cell monolayers were infected with  $1 \times 10^6$  untreated oocysts (A) or  $1 \times 10^6$  oocysts frozen in liquid nitrogen (B). Sexual meront and asexual gamont life stages are observed with an immunohistochemistry stain with hemotoxylin as the counter stain. Note the clustering in panel A indicates reproduction has occurred.



**Figure 2. Inhibition of *C. parvum* infectivity in HCT-8 cells**

The dominant organic acid of each juice was added on a w/v basis to A) apple cider, B) orange juice, C) purple grape juice, and D) white grape juice. The % inhibition is the average mean of at least 3 experiments. Error bars indicate the standard deviation. The number on each bar represents the average pH of the juice sample. Treatments with different letters within the same graph are statistically different from one another ( $P \leq 0.05$ ).

**Table 2. Inactivation of *C. parvum* oocysts in fruit juices treated with H<sub>2</sub>O<sub>2</sub> based on cell culture infectivity.**

HCT-8 cells infected with 1x10<sup>6</sup> treated or control (HBSS) oocysts were compared to determine the percent inhibition. Log<sub>10</sub> reduction for cell culture was determined using an MPN-based analysis for cell culture infection with dilutions of 10<sup>6</sup>, 10<sup>5</sup>, and 10<sup>4</sup> oocysts. While both assays are based on cell infectivity, the results in the 2 columns are different and do not directly correlate. Each juice is shown individually for 0.025% H<sub>2</sub>O<sub>2</sub> and 0.03% H<sub>2</sub>O<sub>2</sub> for selected time periods: (A) apple cider, (B) orange juice, (C) purple grape juice, and (D) white grape juice.

<sup>a</sup>A “greater than” sign indicates that no living stages were observed in the sample evaluated. For calculation purposes it was assumed that one living stage was detected in the sample, and was used to calculate the log reduction for infectivity adapted from (34).

<sup>b</sup>Log reductions were based on the averages from at least three experiments

**A. Cell culture infectivity of *C. parvum* oocysts in apple cider + H<sub>2</sub>O<sub>2</sub>**

Sample	% Inhibition (SD) <sup>a</sup> 1 x 10 <sup>6</sup> initial infection	Log <sub>10</sub> reduction <sup>ab</sup> MPN assay
HBSS	0.00	0.00
Apple cider unamended (2 hr)	25.4% (3.8)	1.4
Apple cider + 0.025% H <sub>2</sub> O <sub>2</sub> (1 hr)	59.0% (10.3)	3.0
Apple cider + 0.025% H <sub>2</sub> O <sub>2</sub> (2 hr)	69.2% (9.7)	3.5
Apple cider + 0.025% H <sub>2</sub> O <sub>2</sub> (≥ 6 hr)	>99.9% (0)	> 5.9
Apple cider + 0.03% H <sub>2</sub> O <sub>2</sub> (1 hr)	64.6% (10.6)	3.5
Apple cider + 0.03% H <sub>2</sub> O <sub>2</sub> (≥ 2 hr)	>99.9% (0)	> 5.9

**B. Cell culture infectivity of *C. parvum* oocysts in orange juice + H<sub>2</sub>O<sub>2</sub>**

Sample	% Inhibition (SD) 1 x 10 <sup>6</sup> initial infection	Log <sub>10</sub> reduction <sup>ac</sup> MPN assay
HBSS	0.00	0.00
Orange juice unammended (2 hr)	52.5% (12.5)	2.8
Orange juice + 0.025% H <sub>2</sub> O <sub>2</sub> (≥ 6 hr)	>99.9% (0)	> 5.9
Orange juice + 0.03% H <sub>2</sub> O <sub>2</sub> (1 hr)	77.5% (3.9)	4.4
Orange juice + 0.03% H <sub>2</sub> O <sub>2</sub> (≥ 2 hr)	>99.9% (0)	> 5.9

**C. Cell culture infectivity of *C. parvum* oocysts in purple grape juice + H<sub>2</sub>O<sub>2</sub>**

Sample	% Inhibition (SD) 1 x 10 <sup>6</sup> initial infection	Log <sub>10</sub> reduction <sup>ac</sup> MPN assay
HBSS	0.00	0.00
Purple grape juice unammended (2 hr)	57.6% (9.6)	3.0
Purple grape juice + 0.025% H <sub>2</sub> O <sub>2</sub> (≥ 6 hr)	>99.9% (0)	> 5.97
Purple grape juice + 0.03% H <sub>2</sub> O <sub>2</sub> (1 hr)	75.3% (5.6)	4.1
Purple grape juice + 0.03% H <sub>2</sub> O <sub>2</sub> (≥ 2 hr)	>99.9% (0)	> 5.97

**D. Cell culture infectivity of *C. parvum* oocysts in white grape juice + H<sub>2</sub>O<sub>2</sub>**

Sample	% Inhibition (SD) 1 x 10 <sup>6</sup> initial infection	Log <sub>10</sub> reduction <sup>ac</sup> MPN assay
HBSS	0.00	0.00
White grape juice unammended (2 hr)	29.9% (13.8)	1.4
White grape juice + 0.025% H <sub>2</sub> O <sub>2</sub> (≥ 6 hr)	>99.9% (0)	> 5.97
White grape juice + 0.03% H <sub>2</sub> O <sub>2</sub> (1 hr)	70.5% (10.0)	4.0
White grape juice + 0.03% H <sub>2</sub> O <sub>2</sub> (≥ 2 hr)	>99.9% (0)	> 5.97

**Table 3. Inactivation of *C. parvum* oocysts in fruit juices treated with H<sub>2</sub>O<sub>2</sub> based on *in vitro* excystation.**

Excystation rates are based on at least 50 oocysts, scored as excysted or not excysted.

Rates are derived from excysted oocysts/total oocysts observed. Log<sub>10</sub> reduction for excystation was determined from excystation rates of control oocysts treated with HBSS prior to excystation. Each juice is shown individually for 0.025% H<sub>2</sub>O<sub>2</sub> and 0.03% H<sub>2</sub>O<sub>2</sub> for selected time periods: (A) apple cider, (B) orange juice, (C) purple grape juice, and (D) white grape juice.

<sup>a</sup> Log reductions were based on the averages from at least three experiments

**A. Excystation of *C. parvum* oocysts in apple cider + H<sub>2</sub>O<sub>2</sub>**

Sample	% Excystation (SD)	Log <sub>10</sub> reduction <sup>a</sup>
HBSS	78.9% (8.4)	0.00
Apple cider unammended (2 hr)	84.9% (5.6)	+0.03
Apple cider + 0.025% H <sub>2</sub> O <sub>2</sub> (≥ 6 hr)	29.7% (5.1)*	0.46
Apple cider + 0.03% H <sub>2</sub> O <sub>2</sub> (1 hr)	39.4% (6.3)	0.33
Apple cider + 0.03% H <sub>2</sub> O <sub>2</sub> (≥ 2 hr)	23.6% (4.3)*	0.56

**B. Excystation of *C. parvum* oocysts in orange juice + H<sub>2</sub>O<sub>2</sub>**

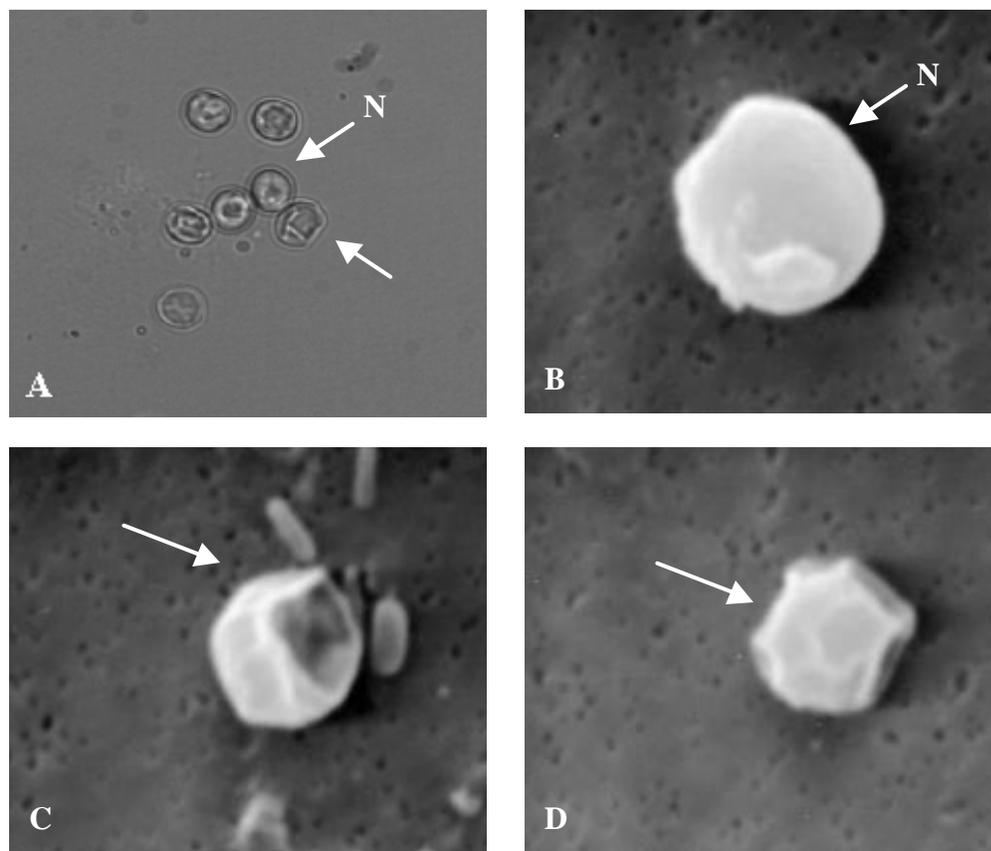
Sample	% Excystation (SD)	Log <sub>10</sub> reduction <sup>c</sup>
HBSS	78.9% (8.4)	0.00
Orange juice unammended (2 hr)	88.0% (5.6)	+0.04
Orange juice + 0.025% H <sub>2</sub> O <sub>2</sub> (≥ 6 hr)	24.7% (5.1)*	0.51
Orange juice + 0.03% H <sub>2</sub> O <sub>2</sub> (1 hr)	29.0% (6.3)	0.44
Orange juice + 0.03% H <sub>2</sub> O <sub>2</sub> (≥ 2 hr)	23.6% (4.3)*	0.53

**C. Excystation of *C. parvum* oocysts in purple grape juice + H<sub>2</sub>O<sub>2</sub>**

Sample	% Excystation (SD)	Log <sub>10</sub> reduction <sup>c</sup>
HBSS	78.9% (8.4)	0.00
Purple grape juice unammended (2 hr)	88.0% (2.5)	+0.04
Purple grape juice + 0.025% H <sub>2</sub> O <sub>2</sub> (≥ 6 hr)	22.6% (4.0)*	0.55
Purple grape juice + 0.03% H <sub>2</sub> O <sub>2</sub> (1 hr)	26.0% (6.3)	0.48
Purple grape juice + 0.03% H <sub>2</sub> O <sub>2</sub> (≥ 2 hr)	20.2% (5.0)*	0.60

**D. Excystation of *C. parvum* oocysts in white grape juice + H<sub>2</sub>O<sub>2</sub>**

Sample	% Excystation (SD)	Log <sub>10</sub> reduction <sup>c</sup>
HBSS	78.9% (8.4)	0.00
White grape juice unammended (2 hr)	93% (2.7)	+0.06
White grape juice + 0.025% H <sub>2</sub> O <sub>2</sub> (≥ 6 hr)	23.0% (3.5)	0.54
White grape juice + 0.03% H <sub>2</sub> O <sub>2</sub> (1 hr)	27% (3.0)	0.47
White grape juice + 0.03% H <sub>2</sub> O <sub>2</sub> (≥ 2 hr)	21.0% (3.3)	0.54



**Figure 3. Hydrogen peroxide damage to *C. parvum* oocyst wall**

Oocysts incubated with apple cider + 0.03%  $\text{H}_2\text{O}_2$  for 2 hrs at  $5^\circ\text{C}$  observed with differential interference contrast (A) and scanning electron microscopy (B, C, D).

Arrows pointed towards oocysts may indicate areas of damage to oocyst walls perhaps caused by radicals from  $\text{H}_2\text{O}_2$  decomposition compared to oocysts that appear more normal (N) in shape (A, B).

## APPENDIX I

### SENSORY EVALUATION OF AMENDED FRUIT JUICES

To establish flavor quality standards, taste evaluations were performed using the In/Out method (29) on apple cider and orange juice with added H<sub>2</sub>O<sub>2</sub> and malic or citric acid respectively. Twenty five untrained panelists tasted 5 juice samples (control, juice with 0.1% H<sub>2</sub>O<sub>2</sub>, 0.03% H<sub>2</sub>O<sub>2</sub>, 0.3% malic/citric acid, or 0.1% malic/citric added) and rated the samples as either “In” or “Out” of personal specification, according to their individual perception of flavor quality. If deemed “Out”, taste descriptors were requested. A minimum of 65% agreement was required to consider a particular H<sub>2</sub>O<sub>2</sub> or organic acid treatment as “In” specification. Unpasteurized apple cider and orange juice were used for describing changes in color and appearance as a result of H<sub>2</sub>O<sub>2</sub> additions.

Taste panels on H<sub>2</sub>O<sub>2</sub> and organic acid additions to apple cider and orange juice demonstrated that on the basis of taste alone, H<sub>2</sub>O<sub>2</sub> levels used in this study (< 0.03%) were within panelists perception of flavor quality (33). Panelists that perceived H<sub>2</sub>O<sub>2</sub> treated samples as out of personal specification provided descriptors such as “bitter”, “astringent”, and a generally unappealing aftertaste. Adding 0.1% of the dominant organic acid to apple cider and orange juice were within specification, but 0.3% additions were considered unqualified for consumption for most individuals.

**Evaluation of Organic Acid and Hydrogen Peroxide Added to Apple Juice**

Name: \_\_\_\_\_

Number: \_\_\_\_\_

Date: 4/27/01

**Instructions:**

Rinse your mouth with water before beginning. Expectorate the water into the container provided. You will review 5 samples. For each sample please indicate your acceptance for the taste by circling "In" or rejection for the taste by circling "Out". Only evaluate the taste (not the color or odor). Rinse your mouth with water between samples and expectorate all samples and the water into the cup provided.

Thank you for your participation! It is much appreciated!

**Comments**

**Sample #:**    In    Out

# **EFFECT OF OZONE TREATMENT ON *CRYPTOSPORIDIUM PARVUM* VIABILITY IN FRUIT JUICES**

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Key words: Cell culture, viability, HCT-8 cells, immunoperoxidase

Journal of Food Protection

## ABSTRACT

*Cryptosporidium parvum* is a protozoan parasite associated with both waterborne and foodborne outbreaks of diarrheal illness, that causes a severe gastrointestinal illness in both previously healthy and immunocompromised individuals. Particularly relevant to this study, cryptosporidiosis has been associated with consumption of contaminated apple cider and other foods. The infective life-stage is a small environmentally resistant oocyst (4 to 5  $\mu\text{m}$  in size) that can be transmitted through water and food. Oocysts have a relatively thick wall, which allows them to be resistant to chlorine and most environmental pressures, making oocysts difficult to kill. In this study ozone was used to inhibit *C. parvum* oocyst viability. Oocysts excyst within the small intestine and liberated sporozoites infect the microvilli of host cells. Infectivity of treated samples was monitored using a human illeocecal (HCT-8) cell culture system. Treating apple cider, orange juice, and grape juice with ozone (0.9 g/L flow rate) inhibited *C. parvum* viability to > 90% reduction as monitored in the cell culture assay. Juice samples were treated for 30 seconds up to 15 minutes at 6° and 22°C. The percent reduction is determined relative to control untreated oocysts. It is hypothesized that membrane proteins, which are necessary for infection, are oxidized by ozone treatment. Ozone, used here is a batch system, may be a potential alternative to traditional pasteurization for fruit juices to successfully inhibit *C. parvum* viability.

## INTRODUCTION

*Cryptosporidium parvum* is undoubtedly an important etiological agent of waterborne and more recently foodborne disease (11). Cryptosporidiosis can manifest itself as a severe gastrointestinal illness in previously healthy individuals or a life-threatening wasting disease in immunocompromised persons (7). Transmission of *Cryptosporidium* is widespread and rapid during an outbreak due to the hardy oocyst, the transmissible life stage. The oocyst wall provides the sporozoites inside with a safe and secure containment. The thick trilaminar oocyst wall allows the sporozoites, the infective life stage, to be unusually resistant to environmental pressures (15).

Control of *C. parvum* by disinfection is difficult for several reasons. Oocysts are highly prevalent in the environment (21). For example, surface water running through or near cattle pastures was shown to contain up to  $6 \times 10^3$  oocysts per liter of water (24). The prevalence of this organism in cattle pastures may lead to foodborne outbreaks of cryptosporidiosis as well, since this water is often used for crop irrigation. Another important factor is that *C. parvum* oocysts are resistant to normal levels of chlorine routinely used to control microorganisms in water (19). Adequate filtration is necessary to control *C. parvum* oocysts in water supplies (3). While ozone is currently an effective chemical disinfectant for the control of *C. parvum* oocysts in drinking water (31), there appears to be some discrepancy concerning the concentrations and times needed to achieve disinfection (19,13,32,8,9,31).

In addition to the waterborne outbreaks of cryptosporidiosis (11), there has been an increase in reported foodborne outbreaks of cryptosporidiosis (26), including those associated with fresh unpasteurized fruit juices (27). While pathogenic bacteria

(*Escherichia coli* O157:H7 and *Salmonella* Typhimurium) have caused the majority of these outbreaks in unpasteurized apple cider and orange juice; for at least two documented outbreaks (1993 and 1996) apple cider was contaminated by *C. parvum* (25,6). These outbreaks raised the concern of the Federal Drug Administration, who recently enacted a Hazard Analysis Critical Control Point system of prevention for the fruit juice industry (2). This regulation states that juices not pasteurized by traditional means using heat must be treated in such a way to cause a 5-log reduction of the pertinent pathogen.

Ozone antimicrobial activity is directly related to the intermediate reactive oxygen species that are formed (16). Ozone is successful in inhibiting *C. parvum* oocysts in that it most likely causes damage to lipids and proteins within oocyst membranes, intracellular enzymes, and sporozoite nucleic acids. The degree of injury due to ozone treatment varies with the microorganism, ozone concentration, and contact time.

Ozone was recently given the title as generally recognized as safe (GRAS) and approved for food processing applications (14); and is currently being investigated for several new food processing applications. This study seeks to evaluate the use of ozone on the inactivation of *C. parvum* in apple cider, orange juice, white grape juice, and purple grape juice using an HCT-8 cell culture infectivity assay. While the solubility of ozone in water is higher at lower temperatures (4), fruit juices were treated using a batch ozone process at 6°C and 22°C to investigate two possible processing temperatures.

## MATERIALS AND METHODS

**Oocyst preparation.** *Cryptosporidium parvum* oocysts (bovine Beltsville isolate, Genotype C) were obtained originally from infected dairy calves and processed at the Parasite Biology Laboratory, United States Department of Agriculture, Beltsville, MD (12). Oocysts were purified from fecal material first by washing through a series of graded sieves down to a pore size of 45  $\mu\text{m}$ , and second by density centrifugation over cesium chloride (17). Residual cesium chloride was removed by three cycles of centrifugation at 1,000 x g for 10 minutes each. Oocysts were resuspended in distilled water. Oocysts were quantified on a hemacytometer.

**Oocyst treatment.** *Cryptosporidium parvum* oocysts ( $1 \times 10^6$  per treatment) were treated with 0.525% sodium hypochlorite at 4°C for less than 5 minutes and washed twice with Hanks Balanced Salt Solution (HBSS). The oocysts were then added to 50 ml juice and treated with ozone (generated by corona discharge; 0.9 g/L) using a portable ozone generator (Activated Oxygen Generator, Golden Buffalo) at a pre-determined flow rate of 2.4 L/minute for variable lengths of time. Experiments were conducted with constant stirring in borosilicate glass reactors covered loosely with parafilm with ozone bubbled continuously into the juice at a rate predetermined for the desired time period. Ozone was pumped through a diffuser stone. Ozone concentrations varied from 3.125 mg/L to 93.75 mg/L. The experiments were terminated and residual ozone neutralized with 0.5 ml 10% sodium thiosulfate immediately after treatment. Treated juice samples were centrifuged at 1,000 x g for 15-20 minutes to collect oocysts. Oocysts treated with HBSS served as controls. In select experiments without oocysts, juice samples were

treated with ozone and residual ozone was determined using Accuvac ampuls ozone test kits (Hach, Loveland, CO) based on the indigo method of determination (5). Residual ozone could not be detected in fruit juice samples; compared to clean water samples (data not shown).

**Juice preparation.** Apple cider was obtained unpasteurized from a local processor, stored frozen, and thawed at 4°C before use. Orange juice (Kroger Original Premium Pasteurized Orange Juice; Kroger, Cincinnati, OH) was obtained from a local supermarket and stored frozen until use. Purple grape juice (Welch's 100% Grape Juice; Welch's, Concord, MA) and white grape juice (Santa Cruz Organic White Grape Juice; Santa Cruz, Chico, CA) were stored at room temperature prior to use. All juices were preservative free. The pH of each juice sample (treated and untreated) was evaluated using a pH meter (Fisher Scientific, Pittsburgh, PA).

**Cell culture media.** Human ileocecal adenocarcinoma cells (HCT-8 cells) (ATCC CCL-244, American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium (Mediatech Cellgro, Herndon, VA) supplemented with L-glutamine (300 mg/L; Mediatech Cellgro), and HEPES (25 mM; Mediatech Cellgro). For normal cell maintenance medium was supplemented with 5% fetal bovine serum (Biofluids, Inc., Rockville, MD) and increased to 10% fetal bovine serum for parasite infection (36).

**Cell maintenance.** Stock HCT-8 cells were maintained in 75-cm<sup>2</sup> tissue culture flasks in a 5% CO<sub>2</sub> atmosphere, at 37°C and 100% humidity, and passaged every 3-5 days. Cells were lifted from the surface with a solution of 0.25% (wt/vol) trypsin and 0.53 mM EDTA in Phosphate Buffered Saline (PBS) (Mediatech Cellgro). Trypsinization required a 10-12 minute incubation in the solution at 37°C to assist in disruption of the cell monolayer. The cells were collected and pelleted by centrifugation for 10 minutes at 1000 x g, resuspended in maintenance medium, and split 1:10. Cell viability was assessed with trypan blue exclusion (0.02% in PBS) and cells counted with a hemocytometer.

**Inoculation of monolayers with parasites.** Collected cells were seeded on sterile 22-mm<sup>2</sup> glass coverslips in 6-well cluster plates (Corning, Corning, NY) at 1x10<sup>6</sup> cells per well and grown to ~95% confluency in maintenance medium (48 hours). *Cryptosporidium parvum* oocysts were treated as stated above. For infection of monolayers and prior to inoculation with oocysts, maintenance medium was removed and 2-3 ml growth medium added to each well in 6 well cluster plates. Cells were then incubated with treated or non-treated (positive control) oocysts (10<sup>6</sup> oocysts in 1 ml growth medium) for 90-120 minutes. After 2 hours, unexcysted oocysts, oocyst walls, and other materials that may have been liberated from the excysted oocysts were washed from the monolayers by washing each inoculated well twice with HBSS. Cells in cluster plates were then placed back in the incubator for 48 hours with 3-4 ml maintenance medium per well.

**Immunohistochemistry of viable parasitic lifestages.** Parasite infection was assessed 48 hours post infection using an immunohistochemistry stain (30). Coverslips in 6 well cluster plates were fixed with 100% methanol for 20-30 minutes and washed twice for 5 minutes each with PBS. Coverslips were removed from cluster plates and processed on slides, first with a rabbit anti-*Cryptosporidium parvum* primary antibody (courtesy of C. Dykstra, Auburn University), followed by a biotinylated anti-rabbit secondary antibody (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) and an avidin biotinylated complex (ABC reagent; Vectastain ABC Kit, Vector Laboratories). Life stages were visualized with an immunoperoxidase stain using hydrogen peroxide (Sigma, St. Louis, MO), diaminobenzidine tetrahydrochloride (DAB, Sigma), with hematoxylin (Fisher Scientific) used as a counter stain. With this system, *Cryptosporidium* life stages are colored brown on a blue and purple background. Oocysts that were frozen in liquid nitrogen for two hours, prior to host cell inoculation served as a negative control. Paromomycin (500 µg/ml ; Sigma) was used as a positive anti-*C. parvum* drug to determine the ability of our assay to detect reduction in developmental stages in treated cell cultures (data not shown).

Treatment effectiveness was based on scoring the presence or absence of living stages (sexual gamonts and asexual meronts) in 50 to 100 sequential and nonoverlapping fields visualized at a 400x magnification using phase contrast microscopy. Fields containing one or more *Cryptosporidium* life stages were scored as positive, while fields containing no stages were scored as negative. Each individual experiment was performed in triplicate. Data are shown two ways, as percent inhibition and as a log reduction.

i) Percent inhibition compared to control untreated oocysts was determined using the following equation:  $[(\text{Control} - \text{Treated}) / \text{Control}] \times 100$ . Data are expressed as the mean percent reduction followed by the standard deviation.

ii) The oocyst infectivity titer was determined for the most effective treatments using the Most Probable Number (MPN) method ((34,33). Calculations were based on the number of positive and negative fields (test units) using the Thomas equation for obtaining the most probable number. At least 100 cell culture fields were counted in three sequential dilutions using  $10^6$ ,  $10^5$ , and  $10^4$  oocysts.

**Excystation assays.** Approximately  $1 \times 10^5$  *C. parvum* oocysts were washed and treated as those used for cell culture infectivity assays described above. Then oocysts were incubated in 0.75% taurochloric acid (Sigma) for 30 minutes at 37°C. The excystation solution was observed at 400x and 1000x magnification using Differential Interference Contrast microscopy (DIC). A total of 100 shells and oocysts were counted. A minimum of duplicate samples was evaluated. Oocysts containing sporozoites were considered unexcysted. Oocysts containing no sporozoites, also called shells, were considered excysted. Excystation rates were calculated by the Woodmansee method (37,13):  $\text{excystation} = ([\text{oocysts excysted} / \text{total oocysts counted}] \times 100)$ . The log reduction for excystation was determined by an adaptation from Chicks law for log inactivation using survivors (N) and initial applied dose ( $N_0$ ) (13):  $\log N/N_0 = \log (\% \text{excystation}_{\text{treated}} / \% \text{excystation}_{\text{control}})$ .

**Statistical analysis.** Data from individual experiments were considered independent. Experiments and controls were analyzed for significant differences using PROC GLM in SAS statistical software. Effects of each treatment on parasite viability were considered to be significant when  $P = \leq 0.05$ . Treatments were separated using Tukeys test for significant differences.

## RESULTS AND DISCUSSION

Figure 1 shows HCT-8 cells infected with oocysts that were untreated in HBSS (A) or in orange juice treated with ozone for 15 minutes (B). Infectivity as determined by this cell culture assay was used to determine the percent inhibition over time of *C. parvum* oocyst viability in fruit juices exposed to ozone using a batch ozone system. Figure 2 shows the decline in infectivity, increasing inhibition, over time for all four juices. Ozone treatment was evaluated at 22C and 6C. As ozone is more soluble and more stable at lower temperatures, and inactivation by ozone most likely occurs through the release of reactive intermediates from the decomposition of ozone (1), there is less inactivation of oocysts at this temperature. It appears that ozone inactivation of *C. parvum* oocysts occurs quite quickly at first and then slows after the first 5 minutes. It is not correct to assume zero or first order kinetics due to the many oxidation reactions required to break down the oocyst wall (13). Changes in the oocyst during ozone treatment are proposed to occur in three stages: injury, kill, and lysis (13). The varying degrees of infectivity associated with these stages can be observed within the graphs shown in figure 2. Oocyst injury appears to occur within the first minute, with the initial decrease in infectivity, as some sporozoites remain active. Within the second stage at 3-10 min there is a further increase in inhibition, as an increasing number of oocysts become injured and some are killed. The third stage, oocyst lysis, occurs from 10-15 minutes where there is a near complete inactivation of all oocysts. Lysed oocysts have no active sporozoites, but appear excysted as monitored in the *in vitro* excystation assay (Table 3) (13).

Inactivation of oocysts in all juices reached  $\geq 90\%$  after 10 minutes of ozone treatment, with the exception of apple cider treated at 6C. Inactivation rates differed somewhat for each juice. This may be due in part to the rate of ozone decomposition related to the total soluble solids of each juice. This may also be affected by juice pH. Ozone increased the pH of each juice gradually over the 15 min treatment (Table 1). It had been shown previously that ozone stability and lethality decrease with increasing pH, as well as that there is a smaller ozone residual as the pH increases (18). In general, oocyst inactivation occurred at a slightly faster rate at 22C than at 6C. This statement is most apparent for apple cider, where inactivation at 3 min at 22C was not significantly different from that for 15 min at 6C and inactivation after 30 seconds at 22C was the same after 5 min at 6C. For orange juice, there was no statistical difference after 5 min of ozone treatment at 22C. Oocyst inactivation in white grape juice occurred at the same rate within the first 30 seconds at both temperatures; while inactivation in purple grape was different for all time points for both temperatures.

Table 2 shows the percent inhibition of infectivity and log reduction corresponding to the inhibition of viable oocysts after ozone treatment as determined by the MPN assay. After 15 minutes of ozone treatment, there was a reduction of at least 5 logs of oocysts in each juice. The greatest reduction was observed in apple cider ( $\log_{10}$  reduction = 5.98). This may be due to the increased number of hydroxyl radicals formed as ozone molecules reacted with the large number of organic molecules in the cider.

Excystation was performed on oocysts treated with ozone in each juice for 15 min at 22C, the treatment correlated to the highest decrease in infectivity. Excystation rates were also determined for oocysts treated with ozone in apple cider at 6C due to the

significant difference between changes in infectivity at the two temperatures. There was a great deal of organic material in each sample, with the highest visible amount in apple cider and orange juice. In general, ozone treatment did not have a visible effect on oocyst morphology compared to control untreated samples. While ozone treatment in each juice significantly reduced the excystation rate compared to the control, *in vitro* excystation likely overestimates the viability of treated oocysts. The sporozoites observed in treated juice samples were not moving compared to those in the untreated samples. It is likely that oxygen radicals formed during ozone decomposition damaged the DNA of sporozoites and rendered them non-infectious. Another possibility is that exposure to ozone for prolonged periods of time, as in the case of these oocysts exposed to ozone for 15 min, may cause sporozoites to lyse within the oocyst rendering them non-infectious (13). The non-moving sporozoites become evident when the suture opens upon exposure to excystation fluid.

It is generally believed that the reactivity of ozone is due to the oxidizing power of the free radicals formed through a chain of events during ozone decomposition (20). The free radicals react with various targets on the oocyst wall including lipids and proteins and with the nucleic acids of sporozoites. The final applied ozone concentrations used in this study range from 3.1 mg/L to 93.7 mg/L, much greater than that applied to water to achieve 2-3 log reductions in viable oocysts (28). While it was not possible to determine the residual ozone dose in the fruit juice, due to the presence of large amounts of organic material and oxidized compounds, the applied dose is known based on the applied dose of 6.25 mg min/L. Even in the presence of the myriad of organic compounds in juices, there is a strong oxidizing effect that leads to oocyst

inactivation. Further, it is possible that as the number of reactive oxygen species increases in the presence of sucrose (20) so does the killing power on the oocysts. This so-called quenching effect of sucrose may actually aid in the final inactivation of *C. parvum* oocysts, since it is most likely that these reactive compounds react with oocyst wall proteins and lipids. The solutes in each juice may influence the radicals formed during ozone composition (18), and may result in the various inactivation rates observed in the four juices.

The reactive molecules produced at the treatment interface influence ozone toxicity (35), and these are influenced by the type of organic material present in the juice. Ascorbic acid may be a key reactant for ozone reactivity in juices, as it is in plants and within the human respiratory tract (35). *Cryptosporidium* oocysts were inactivated at a faster rate in orange juice, perhaps due to the high amount of ascorbic acid compared to the other fruit juices evaluated. As ozone reacts with ascorbic acid various radicals are produced that react with the oocysts, while simultaneously antioxidants are removed from the liquid. Ozone-derived oxygen radicals are scavenged by ascorbic acid and other antioxidants and reactions among these compounds form more radicals along the propagating chain (29). This chain of reactions, beginning with ascorbic acid may increase the rate of lipid peroxidation by oxygen radicals (35). In turn this may be responsible to oocyst inactivation, in that it is believed that lipids within the oocyst wall may be necessary for maintaining mechanical rigidity and resistance disruption by environmental factors (23,15).

Ozone appears to be effective at inactivating *C. parvum* oocysts in fruit juices due to the oxygen radicals generated through ozone reactions combined with the inherent

reactivity of the organism. Compared to other intracellular parasites *C. parvum* has a poor ability to scavenge reactive oxygen molecules and avoid oxidative burst (10). Within the host, *C. parvum* life stages are able to avoid macrophage-derived reactive oxygen molecules due to their unique niche at the luminal surface of intestinal cells (22) but oocysts subjected to oxygen radicals in vitro are inactivated. Ozone may be an effective alternative to heat pasteurization, as it was shown to reduce viable *C. parvum* oocysts by 5 logs. It is difficult to predict how ozone will react in the presence of organic material (18), which differs greatly among juices. This point makes it more likely that ozone could be used in conjunction with other treatments like addition of chemical preservatives, including hydrogen peroxide and organic acids. Additionally, the effectiveness of ozone has been shown to vary with only minor changes in the experimental design (18), and this may effect the final log reduction obtained by different ozone units.

## **ACKNOWLEDGMENT**

This study was funded by a CSREES USDA Special Food Safety Grant No. 98-34382-6916 VPI&SU HATCH project #135563.

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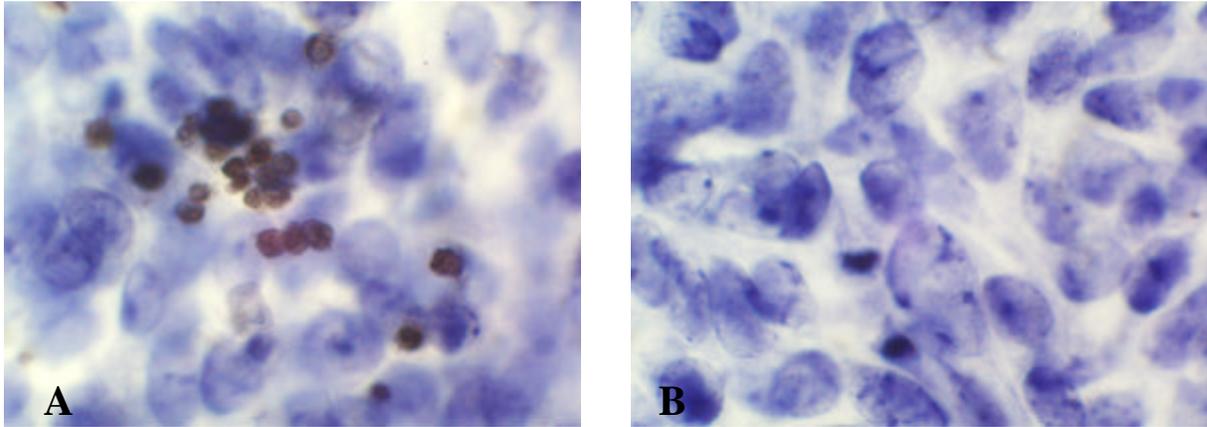
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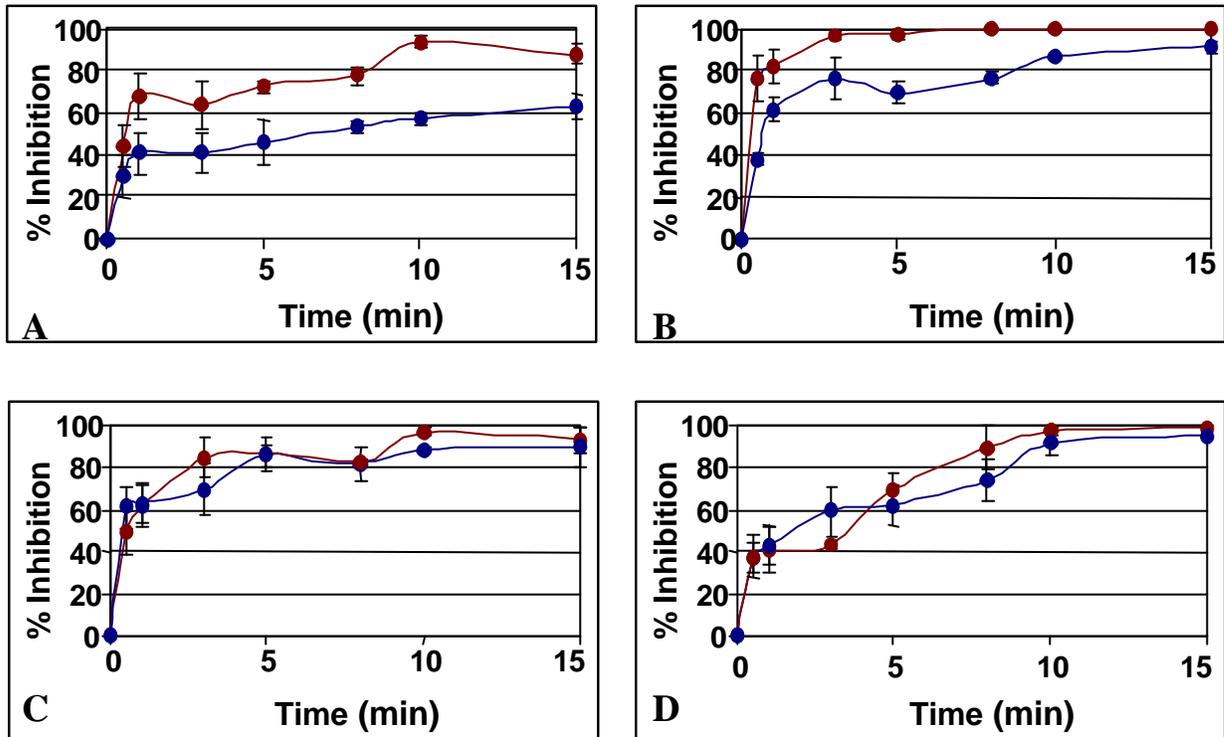
**Figure 1. Cell Culture Infectivity**

HCT-8 cell monolayers were infected with  $1 \times 10^6$  untreated oocysts in HBSS (A) or  $1 \times 10^6$  oocysts in orange juice treated with ozone for 15 minutes (B). Asexual meront and sexual gamont life stages are observed with an immunohistochemistry stain with hemotoxylin as the counter stain. Note the clustering in panel A indicates reproduction has occurred.

Fruit Juice	Time of Ozone Treatment (min)							
	0.0	0.5	1.0	3.0	5.0	8.0	10.0	15.0
Apple Cider	3.72	3.70	3.70	3.75	3.80	3.85	3.87	3.89
Orange Juice	3.81	3.82	3.83	3.83	3.83	3.84	3.86	3.89
Purple Grape Juice	2.47	2.49	2.50	2.53	2.55	2.57	2.60	2.66
White Grape Juice	3.15	3.08	3.08	3.10	3.12	3.15	3.17	3.20

Table 1. The pH Values of Fruit Juices Treated with Ozone

Treatment of fruit juices with ozone for 0.5, 1, 3, 5, 8, 10, and 15 min caused changes in the pH of all fruit juice samples as indicated. pH values shown are averaged  $\pm 0.05$ .



**Figure 2. Ozone inactivation of *C. parvum* oocysts in fruit juices**

Apple cider (A), orange juice (B), purple grape juice (C), and white grape juice (D) samples were inoculated with  $1 \times 10^6$  *C. parvum* oocysts and treated with ozone for 0.5, 1, 3, 5, 8, 10, and 15 min at 22°C (red) and at 6°C (blue). The infectivity of HCT-8 cells was determined for each sample and compared to control oocysts treated with HBSS and the percent inhibition by ozone treatment determined. Each time point is represented by the average of at least three replicate experiments with the standard deviation shown. For some samples, the standard deviation may be within the data point marker.

Cell Culture Infectivity Assay		
Sample	% Inhibition (SD) Initial Infection $1 \times 10^6$	$\text{Log}_{10}$ Reduction <sup>b</sup> MPN assay
HBSS	0.00	0.00
Apple Cider Ozone 15 min 6C	63.27 (5.65)	5.69
Apple Cider Ozone 15 min 22C	88.00 (4.60)	5.98
Orange Juice Ozone 15 min 22C	>99.00 <sup>a</sup> (0.05)	5.54
Purple Grape Juice Ozone 15 min 22C	93.10 (6.33)	5.11
White Grape Juice Ozone 15 min 22C	99.48 (0.70)	5.11

**Table 2. Inactivation of *C. parvum* oocysts in fruit juices treated with ozone based on cell culture infectivity.**

HCT-8 cells infected with  $1 \times 10^6$  treated or control (HBSS) oocysts were compared to determine the percent inhibition.  $\text{Log}_{10}$  reduction for cell culture was determined using an MPN-based analysis for cell culture infection with dilutions of  $10^6$ ,  $10^5$ , and  $10^4$  oocysts. While both assays are based on cell infectivity, the results in the 2 columns are different and do not directly correlate.

<sup>a</sup>A “greater than” sign indicates that no living stages were observed in the sample evaluated. For calculation purposes it was assumed that one living stage was detected in the sample, and was used to calculate the log reduction for infectivity adapted from (33).

<sup>b</sup>Log reductions were based on the averages from at least three experiments

<i>In Vitro</i> Excystation Assay		
Sample	% Excystation (SD)	Log <sub>10</sub> reduction <sup>a</sup>
HBSS	90.9 (8.5)	0.00
Apple Cider Ozone 15 min 6C	37.3 (4.6)	0.41
Apple Cider Ozone 15 min 22C	31.8 (3.0)	0.46
Orange juice Ozone 15 min 22C	15.7 (3.2)	0.76
Purple Grape Juice Ozone 15 min 22C	21.2 (3.0)	0.63
White Grape Juice Ozone 15 min 22C	24.2 (4.2)	0.58

**Table 3. Inactivation of *C. parvum* oocysts in fruit juices treated with ozone based on *in vitro* excystation.**

Excystation rates are based on at least 50 oocysts, scored as excysted or not excysted. Rates are derived from excysted oocysts/total oocysts observed. Log<sub>10</sub> reduction for excystation was determined from excystation rates of control oocysts treated with HBSS prior to excystation.

<sup>a</sup> Log reductions were based on the averages from at least three experiments

**EFFECT OF OXIDATION BY  
HYDROGEN PEROXIDE ON  
*CRYPTOSPORIDIUM PARVUM*  
PROTEASE ACTIVITY**

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Key words: Protease, Cysteine, HCT-8 cells

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## ABSTRACT

*Cryptosporidium parvum* oocyst infectivity and excystation are inhibited by treatment with hydrogen peroxide. This may be a result of oxidation of oocyst wall proteins by the reactive oxygen species formed during the breakdown on hydrogen peroxide. Hydroxyl radicals can oxidize sulfhydryl groups, inactivating cysteine proteases, which are known to be important for oocyst excystation. Treatment of oocysts with hydrogen peroxide inhibited protease activity up to 50% compared to controls. Similarly treatment of oocysts with chemicals that affect sulfhydryls, like NEM and DTT, inhibited protease activity by > 90%. Treatment of oocysts with these chemicals along with known protease inhibitors, like PMSF, EDTA, and cystatin, inhibited protease activity as anticipated, as well as excystation and infection in HCT-8 cells. Successful inhibition of infection and excystation by hydrogen peroxide treatment may be due to oxidation of essential surface proteins. Other mechanisms of action include chelating cations necessary for infection and excystation or radical-induced DNA damage to sporozoites thereby inhibiting infection.

## INTRODUCTION

*Cryptosporidium parvum* is an intestinal Apicomplexan protozoan parasite, which can cause severe illness in immunocompetent individuals (Tzipori, 1988), or a wasting illness in immunocompromised individuals (Current et. al., 1991) and malnourished children (Griffiths et.al., 1994). Outbreaks of cryptosporidiosis are both water- and food-borne, where it is transmitted by the small (4 to 5  $\mu\text{m}$ ) environmentally resistant oocyst (Fayer et. al., 2000). Once ingested, oocysts undergo excystation within the intestine, sporozoites are released, which then generate further life cycle stages including meronts and gamonts through asexual and sexual reproduction (Fayer, 1997). While the life cycle is well documented, the intricacies of excystation have not yet been completely elucidated. *Cryptosporidium* excystation may be enhanced in vitro by increasing temperature to 37C, by preincubation in low pH, by addition of trypsin, and by the action of bile salts; however, excystation can occur without these additives as well (Fayer and Leek, 1984) (Kato et. al., 2001).

Specific proteins called proteases appear to be involved in excystation (Forney et. al., 1996). Proteases are involved in parasite-host infection where at least one step during the invasion-infection process appears to be mediated by protease activity (Forney et. al., 1996b). These protease-mediated or protease-dependent mechanisms have been studied more extensively in related coccidian parasites, including: *Plasmodium knowlesi*, *Plasmodium falciparum*, *Eimeria tenella*, and *Eimeria vermiformis* (Dluzewski et. al., 1986; Adams and Bushell, 1988; Fuller and McDougald, 1990; Que et. al., 2002). Protease-mediated events are believed to include: host cell attachment (Adams and

Bushell, 1988; Arroyo and Aldrete, 1989); host membrane penetration (Hadley et. al., 1983; Fuller and McDougald, 1990); host protein degradation used for parasitic growth (Chappell and Dresden, 1986); tissue migration (Moda et. al., 1988; McKerrow et. al., 1990; Moda and Doenhoff, 1994a; Morris and Sakanari, 1995; Yenbuter and Scott, 1995); parasite development (Richer et. al., 1993); and mechanisms used to fight and evade the host immune system (Malzels et. al., 1993; Pupkis et. al., 1986; Moda and Doenhoff, 1994b). Proteases are classified by the active group and are divided into three groups: cysteine, serine, and metalloproteases. Both serine and cysteine proteinases may function in excystation of *Cryptosporidium* oocysts and cleavage of oocyst wall proteins as indicative by their increase in concentration during incubation at 37C prior to excystation (Forney et. al., 1996a). Additionally they may be involved in the invasion process that has been shown to be both pH and metallo-dependent (Hamer et. al., 1994).

While *C. parvum* oocysts are resistant to normal levels of chlorine, other oxidizing agents like ozone and hydrogen peroxide are more successful at inhibiting viability. It was observed that treatment of *C. parvum* oocysts with low concentrations of hydrogen peroxide (0.03%) inhibited excystation regardless of pretreatment with sodium hypochlorite. The purpose of this study was to better understand the action of hydrogen peroxide in terms of protease activity. While the current model is that protease activity is associated with sporozoite membranes, hydrogen peroxide may exert its effect intra-oocyst or perhaps oxidize proteins on the oocyst wall. It is generally regarded that cysteines among the amino acids most susceptible to oxidation, which can inactivate cysteine dependent enzymes due to the loss of the free sulfhydryl group (reduced). Cysteine proteases are potential targets for chemotherapeutic attack, and can function

with sufficient reducing agent present (McKerrow, 1993). Hydrogen peroxide readily oxidizes sulfhydryl groups, and could potentially inhibit *C. parvum* excystation this way by affecting cysteine proteases known to be involved in excystation (Forney et. al., 1996a). In this study, the protease activity of hydrogen peroxide treated oocysts was compared to that of untreated oocysts along with known and potential protease inhibitors in three assays: HCT-8 cell culture infectivity assay, *in vitro* excystation assay, and an *in vitro* proteolytic activity assay.

## MATERIALS & METHODS

**Oocyst preparation.** *Cryptosporidium parvum* oocysts (bovine Beltsville isolate, Genotype C) were obtained originally from infected dairy calves and processed at the Parasite Biology Laboratory, United States Department of Agriculture, Beltsville, MD (Fayer et. al., 2001). Oocysts were purified from fecal material as previously described (Kilani and Sekla, 1987). Oocysts were quantified on a hemacytometer.

**Oocyst treatment.** *Cryptosporidium parvum* oocysts ( $5 \times 10^5$  per treatment) were treated with 0.525% sodium hypochlorite at 4°C for less than 5 minutes and washed twice with Hanks Balanced Salt Solution (HBSS). Oocysts were then treated with protease inhibitors or related chemicals at specified concentrations for 30 min at 22°C or with specified concentrations of hydrogen peroxide in water or apple cider for 2 hr at 4°C. Oocysts were washed twice with HBSS after treatment. Oocysts washed with sodium hypochlorite and HBSS served as controls. Chemicals used included known protease inhibitors, sulfhydryl reagents, and other chemicals: hydrogen peroxide (Sigma); N-ethyl maleimide (NEM, Sigma); dithiothreitol (DTT, Sigma); EDTA; cystatin (Sigma); phenylmethylsulfonyl fluoride (PMSF, Sigma). Chemicals were dissolved in phosphate buffered saline (PBS) and made to specified concentrations. PMSF was first dissolved in dimethylsulfoxide (DMSO) and diluted to specified concentrations in PBS. Control assays were prepared in both PBS and DMSO (final concentrations equal to those of diluted PMSF).

**Cell Culture maintenance.** Human illeocecal adenocarcinoma cells (HCT-8 cells) (ATCC CCL-244, American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium (Mediatech Cellgro, Herndon, VA) supplemented with L-glutamine (300 mg/L; Mediatech Cellgro), and HEPES (25 mM; Mediatech Cellgro). For normal cell maintenance medium was supplemented with 5% fetal bovine serum (Biofluids, Inc., Rockville, MD) and increased to 10% fetal bovine serum for parasite infection (Upton et. al., 1995). Stock HCT-8 cells were maintained in 75-cm<sup>2</sup> tissue culture flasks in a 5% CO<sub>2</sub> atmosphere, at 37°C and 100% humidity, and passaged every 3-5 days. Cells were lifted from the surface with a solution of 0.25% (wt/vol) trypsin and 0.53 mM EDTA in phosphate buffered saline (PBS) (Mediatech Cellgro). Trypsinization required 10-12 minute incubation in the solution at 37°C to assist in disruption of the cell monolayer. The cells were collected and pelleted by centrifugation for 10 minutes at 1000 x g, resuspended in maintenance medium, and split 1:10. Cell viability was assessed with trypan blue exclusion (0.02% in PBS) and cells counted with a hemacytometer.

**Inoculation of monolayers with parasites.** HCT-8 cells were seeded on sterile 22-mm<sup>2</sup> glass coverslips in 6-well cluster plates (Corning, Corning, NY) at 1x10<sup>6</sup> cells per well and grown to ~95% confluency in maintenance medium (48 hours). *Cryptosporidium parvum* oocysts were treated as stated above. For infection of monolayers and prior to inoculation with oocysts, maintenance medium was removed and 2-3 ml growth medium added to each well in 6 well cluster plates. Cells were then incubated with treated or non-treated (positive control) oocysts (10<sup>6</sup> oocysts in 1 ml

growth medium) for 90-120 minutes. After 2 hours, unexcysted oocysts, oocyst walls, and other materials that may have been liberated from the excysted oocysts were washed from the monolayers by washing each inoculated well twice with HBSS. Cells in cluster plates were then placed back in the incubator for 48 hours with 3-4 ml maintenance medium per well.

**Immunohistochemistry of viable parasitic lifestages.** Parasite infection was assessed 48 hours post infection using an immunohistochemistry stain (Phelps et. al., 2002). Coverslips in 6 well cluster plates were fixed with 100% methanol for 20-30 minutes and washed twice for 5 minutes each with PBS. Coverslips were removed from cluster plates and processed on slides, first with a rabbit anti-*Cryptosporidium parvum* primary antibody (courtesy of C. Dykstra, Auburn University), followed by a biotinylated anti-rabbit secondary antibody and an avidin biotinylated complex (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA). Life stages were visualized with an immunoperoxidase stain using hydrogen peroxide (Sigma), diaminobenzidine tetrahydrochloride (DAB, Sigma), with hematoxylin (Fisher Scientific) used as a counter stain. Treatment effectiveness was determined by counting the number of positive fields out of one hundred total fields visualized with 400x magnification. Each individual experiment was performed in triplicate. Percent reduction compared to control untreated oocysts was determined using the following equation:  $[(Control - Treated) / Control] \times 100$ . Data are expressed as the mean percent inhibition followed by the standard deviation.

**Protease assay.** *Cryptosporidium parvum* homogenate was prepared from treated and untreated oocysts as described by Forney et al. (1996a). Oocysts were snap frozen in liquid nitrogen after treatment then thawed in a 37°C water bath twice, and then sonicated 6 x 1min (50 mW) in a ice-cooled water-bath sonicator to produce the homogenate. The homogenate was used in an assay for protease activity using azocasein (Sigma) as the substrate (Plantner, 1991; Forney et. al., 1996a). Briefly, 2 mg/ml azocasein in 100 mM sodium phosphate buffer was prewarmed to 37°C for 15 min, and 50 µl of *C. parvum* homogenate added, incubated at 37°C for 10-11 hours. After incubation, 40 µl of cold 50% trichloroacetic acid (Sigma) was added to each sample to precipitate any undigested azocasein. Samples were incubated on ice for 60 min and then centrifuged at 2,000 x g for 10 min. The supernatant was removed to a microtiter plate already containing 200 µl of 10 N NaOH in each well. Absorbance was measured at 450 nm with an automated microtiter plate reader. Diluted trypsin was used as a control, as suggested by Forney et al. (1996a). Protease activity readings from treated and untreated oocysts were compared and the percent inhibition determined.

**Excystation assays.** Approximately  $1 \times 10^5$  *Cryptosporidium parvum* oocysts were washed and treated as those used for cell culture infectivity assays described above. Then oocysts were incubated in 0.75% taurochloric acid (Sigma) for 30 minutes at 37°C. The excystation solution was observed at 400x and 1000x magnification using differential interference contrast microscopy (DIC). A total of 100 shells and oocysts were counted. A minimum of duplicate samples was evaluated. Oocysts containing sporozoites were considered unexcysted. Oocysts containing no sporozoites, also called

shells, were considered excysted. Excystation rates were calculated by the Woodmansee method (Woodmansee, 1987; Finch et. al., 1993): excystation = ([oocysts excysted/total oocysts counted] x 100).

**Statistical analysis.** Data from individual experiments were considered independent. Experiments and controls were analyzed for significant differences using PROC GLM in SAS statistical software. Effects of each treatment on parasite viability were considered to be significant when  $P = \leq 0.05$ . Treatments were separated using Tukeys test for significant differences.

## RESULTS AND DISCUSSION

HCT-8 cells support complete development of *C. parvum* (Upton et. al., 1995) and are most similar to human infection (Chappell, 2000). Figure 1 shows the percent inhibition of cell culture infectivity by various treatments, all compared to infection with untreated oocysts ( $94\% \pm 2.8$ ). No viable life stages were observed for infection by oocysts treated with any concentration of hydrogen peroxide (2 hrs, 4°C). In the absence of any other treatment these oocysts were not excysted prior to infection of the HCT-8 cells. The inhibition of treatment with EDTA was closest to that caused by hydrogen peroxide. EDTA chelates cations ( $\text{Ca}^{2+}$ ) and inhibits metallodependent proteases (Nesterenko et. al., 1995). Hydrogen peroxide also affects calcium cations (Lipton and Nicotera, 1998); and more importantly calcium has been noted to be necessary for proper infection by *C. parvum* (Hamer et. al., 1994; Upton et. al., 1995). All treatments are statistically significant ( $P < 0.0001$ ), with two exceptions. The hydrogen peroxide treatments are identical to each other regardless of concentration. No viable life stages were observed in cells infected with hydrogen peroxide treated oocysts. Inhibition by 1.0 mM DTT and 25  $\mu\text{g/ml}$  cystatin were not significantly different, which is interesting since both treatments effect cysteines. DTT reduces sulfhydryls and can inhibit cysteine proteases at relatively high concentrations and cystatin is a cysteine protease inhibitor.

Protease activity was significantly affected by all treatments compared to control levels. Table 1 shows the percent inhibition of treated oocysts. Protease activity was significantly reduced in *C. parvum* homogenates prepared from treated oocysts. This is different from what was done previously where homogenates were treated (Forney et. al.,

1996a). Interestingly inhibition levels were similar for oocysts and homogenate treated with 10 mM PMSF, which is a serine protease inhibitor. It is possible that PMSF can act intra-oocyst through hypochlorite-permiabilized oocysts. While it is possible that weak protease activity is present on the oocyst surface, it remains unlikely that serine protease activity may be present on the oocyst exterior, as Nesterenko et al detected protease activity localized to the surfaces of sporozoites and not oocyst walls (Nesterenko et. al., 1995). Inhibition of protease activity by hydrogen peroxide (0.5 mg/ml and 10 mg/ml) was similar to of EDTA (1.5 mM). Again both chemicals may chelate calcium ions and affect oocyst activity that way. Inhibition by NEM (5.0 mM) and DTT (5.0 mM) were similar, and both treatments affect sulfhydryls and inhibit cysteine protease activity.

Oocysts did not excyst after chemical treatment prior to incubation with taurochloric acid at 37°C for 30 min. Table 2 shows the excystation rates of oocysts treated with inhibitory chemicals with the corresponding log reduction determined from the control excystation rate ( $89 \pm 5.0$ ). Intact oocysts, shells, and sporozoites were observed with DIC microscopy under 1000x magnification. Sporozoites from oocysts treated with hydrogen peroxide were not moving, possibly due to DNA damage by oxygen radicals generated from the decomposition of hydrogen peroxide. Sporozoites derived from oocysts treated with inhibitory compounds other than hydrogen peroxide were moving. Excystation rates of treated oocysts were all significantly different from one another, with the exception of the following treatments: hydrogen peroxide, 5.0 mM NEM, and 10 mM PMSF. EDTA (1.5 mM) inhibited excystation by 70%, close to the inhibition by hydrogen peroxide. As in the other assays, these two chemical treatments gave similar inhibitory actions on *C. parvum* oocyst viability. The cysteine protease

inhibitor cystatin did not significantly effect excystation, supporting what was observed previously (Forney et. al., 1996a).

It is clear that hydrogen peroxide penetrates or affects the oocyst wall and thereby inhibits sporozoite viability. While the mechanism of action is not clear; it may be two-fold, first by sulfhydryl oxidation of proteins on the oocyst surface and secondly by altering the movement of cations. This information was obtained by comparing the inactivation rates of cell culture infection, protease activity, and excystation with known sulfhydryl (NEM, DTT, cystatin) and protease (PMSF, EDTA) reactive agents.

Interestingly, *Giardia* has detectable cysteine proteinase activity that is directly involved in excystation and is localized in cytoplasmic vacuoles (Ward et. al., 1997). As may be the case with *Cryptosporidium* and other protozoa, in *Giardia* the processing of cyst wall components may rely on cysteine protease activity (Ward et. al., 1997). Hydrogen peroxide may oxidize the cysteine proteases necessary for oocyst wall maintenance thereby reducing wall strength and lessening oocyst viability.

## **ACKNOWLEDGMENT**

This project was supported in part by CSREES USDA Special Food Safety Grant No. 98-34382-6916, VPI&SU HATCH project #135563, and a New Initiative Grant from the Virginia-Maryland Regional College of Veterinary Medicine.

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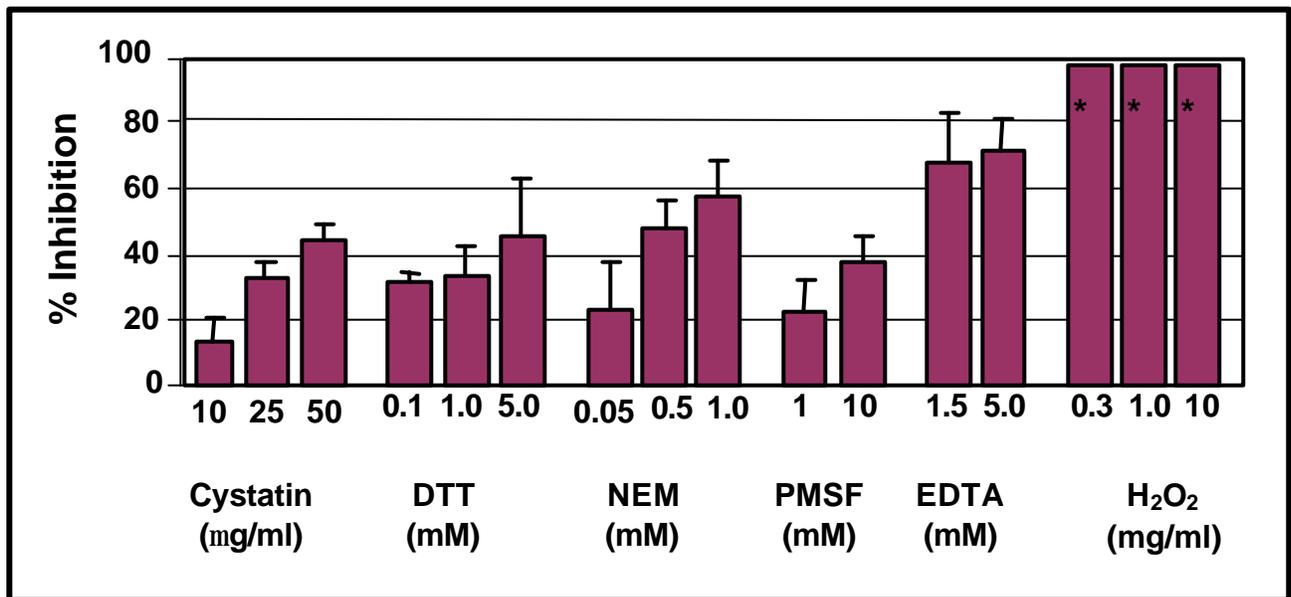
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### Figure 1. Effect of chemicals on HCT-8 cell culture infectivity

Percent inhibition of infection by treated oocysts was determined compared to control oocysts. The percent inhibition is represented by an average of three replicate experiments with standard deviation shown. No viable life stages were observed in cells infected with oocysts treated with hydrogen peroxide (\*). Treatments were significantly different from each other with two exceptions: hydrogen peroxide treatments; 1.0 mM DTT and 25 µg/ml Cystatin.



Chemical Inhibitor	Concentration	% Inhibition (Std. Dev.)
H <sub>2</sub> O <sub>2</sub>	0.3 mg/ml	24.1 <sup>d</sup> (1.53)
	0.5 mg/ml	40.4 <sup>c</sup> (5.94)
	10 mg/ml	47.7 <sup>c</sup> (5.09)
NEM	1.0 mM	90.4 <sup>b</sup> (17.1)
	5.0 mM	98.4 <sup>a</sup> (6.36)
DTT	5 mM	90.8 <sup>ab</sup> (12.2)
Cystatin	50 µg/ml	88.8 <sup>b</sup> (7.56)
EDTA	1.5 mM	40.1 <sup>c</sup> (5.43)
PMSF	10 mM	90.3 <sup>ab</sup> (2.10)

**Table 1. Effect of chemicals on *in vitro* protease activity**

Protease activity was determined using azocasein as described in the Materials and Methods. The % inhibition was obtained by comparing protease activity of control oocysts to treated oocysts and is represented by the average (standard deviation) of three replicate experiments. Treatments with the same letter were not significantly different from each other ( $p < 0.001$ ).

Chemical Inhibitor	Concentration	% Excystation (Std. Dev.)	Log <sub>10</sub> Reduction
H <sub>2</sub> O <sub>2</sub>	0.3 mg/ml	24.4 <sup>e</sup> (5.2)	0.56
	0.5 mg/ml	22.2 <sup>e</sup> (2.5)	0.60
	10 mg/ml	17.5 <sup>e</sup> (6.3)	0.70
NEM	1.0 mM	56.7 <sup>c</sup> (6.8)	0.19
	5.0 mM	24.5 <sup>e</sup> (2.0)	0.56
DTT	5 mM	68.2 <sup>a</sup> (7.5)	0.11
Cystatin	50 µg/ml	61.9 <sup>b</sup> (3.0)	0.15
EDTA	1.5 mM	30.7 <sup>d</sup> (6.4)	0.46
PMSF	10 mM	21.0 <sup>e</sup> (4.1)	0.62

**Table 2. Effect of chemicals on *in vitro* excystation**

The excystation rate is represented by the average (standard deviation) of three replicate experiments. Treatments with the same letter were not significantly different from each other ( $p < 0.001$ ). The log<sub>10</sub> reduction was determined by comparing protease activity of control oocysts to treated oocysts.

## VII. VITA

Kalmia Elisabeth Kniel was born and raised in Rockville, Maryland, where she received her primary and secondary education. She received her undergraduate education and training in Biology at Virginia Tech, where she first learned to appreciate and love the laboratory while doing undergraduate research in Microbiology and Cell Biology. She continued her graduate education and training at Virginia Tech, where she received her Masters in Science in Molecular Cell Biology and Biotechnology. This is where her love for teaching first developed. After teaching pathogenic bacteriology, she ventured to the applied sciences and continued her graduate experience in Food Science and Technology. She is an active member of several professional associations including, International Association for Food Protection (IAFP), Institute of Food Technologists (IFT), Sigma Xi, The Scientific Research Society, Phi Sigma, Biological Honor Society, American Society of Parasitologists (ASP), American Association for Veterinary Parasitologists (AAVP), and The Society of Protozoologists.