

**EFFECT OF LACTOFERRIN DIGEST AND OZONE TREATMENTS
ON *Penicillium* spp. ISOLATED FROM BOTTLED WATER**

by

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ABSTRACT

Recently, consumers have complained about visible fungi contamination in bottled water; this has caused the industry a series of rejected products. These fungi seem to be resistant to the common sterilization processes, such as ozonation, used for bottled water. In this study, the main objective was to investigate the effect of an antimicrobial peptide and ozone treatments on a *Penicillium* spp. isolated from bottled water.

Foreign bodies were isolated from bottled water and plated on potato dextrose agar. A loopfull from a visible colony was examined by phase contrast microscope for identification based on its morphology. Fungi isolated from the bottled water were identified as *Penicillium* sp. using three specialized media. A stock suspension was obtained by washing the spores after 7 days incubation at 25°C on PDA.

Lactoferrin (5% w/v), isolated from cheese whey, was digested at 37°C for 4 hrs using porcine pepsin (3% w/w) at pH 2.3. Digestion was terminated by increasing the temperature to 80°C for 15 min and the pH to 7.0. The digest was centrifuged and the supernatant filtered through a 0.45 µ cellulose acetate filter. Digestion was confirmed by SDS-PAGE electrophoresis. Lactoferrin digest (LFD), containing lactoferricin, was used without further purification.

The antimicrobial assay was performed using a 96 well microplate containing peptone yeast glucose medium (PYG) and known concentrations of LFD. Each well was inoculated with the spore suspension (7×10^3 mL⁻¹ spores); bovine serum albumin digest was used as negative control. Culture optical density was monitored at 595 nm over time.

LFD at concentrations of 0.06 and 0.3 mg/mL inhibited spore germination and mycelial fusion for up to 9 and 21 days at 30°C, respectively.

Ozonation experiments were carried out at 0.1-0.4 ppm ozone in 100 mL of distilled deionized water with or without LFD (0.03, 0.06 0.30 mg/mL). Two experimental set ups were used; one consisted of adding ozonated water (65 mL) to 35 mL of spore suspension ($3 \times 10^6 \text{ mL}^{-1}$ spores). Samples (1 mL) of ozonated spores were retrieved at 0, 4, 8 and 12 min and plated on potato dextrose trypan blue agar using the hydrophobic grid membrane filtration (HGMF) method. The second set up consisted of directly ozonating 100 mL inoculated water (0.9 mL spore suspension with 99.1 mL d-d water). Samples (1 mL) were retrieved every 30 seconds for a period of 3 minutes and plated as described above. Ozone concentrations in the range of 0.1-0.4 ppm stimulated spore germination, mycelial fusion and growth. LFD when added, led to slower development of mould colonies on the HGMF filters.

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LIST OF ABBREVIATIONS

ACC	aerobic colony counts
ANOVA	analysis of variance
BSA	bovine serum albumin
BSAD	bovine serum albumin digest
°C	degree Celsius
CYA	Czapek yeast extract agar
Da	daltons
d-d water	distilled deionized water
EDTA	ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
g	gravitational force
GRAS	generally recognized as safe
G25N	glycerol nitrate agar
GLM	general linear model
h	hours
HCl	hydrochloric acid
HGMF	hydrophobic grid membrane filter
LFD	lactoferrin digest
lactoferricin B	bovine lactoferricin
lactoferricin H	human lactoferricin
LPS	lipopolysaccharide

log	logarithmic
mA	milliAmperes
MEA	malt extract agar
min	minutes
MPNGU	most probable number of growth units
μL	microliter
nm	nanometers
NA	not applicable
NM	not measured
NaOH	sodium hydroxide
Na ₂ SO ₃	sodium sulfite
O ₃	ozone
PDA	potato dextrose agar
PDTB	potato dextrose trypan blue
pI	isoelectric point
ppm	parts per million
PYG	peptone yeast glucose medium
sec	seconds
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
UHP	ultra high purity
v	volts

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I. INTRODUCTION

This thesis is compelled by two different aspects: cheese whey utilization and the bottled water industry. The disposal of cheese whey is a problem that the dairy industry is facing as the production of cheese keeps increasing at a rate of about 3% per year (Zall, 1992). The new regulations on the maximum amount of solids whey may not exceed before being disposed in municipal treatments plants are very strict. These disposal problems have encouraged the dairy industry to employ waste treatment methods like activated sludge, trickling filters, aerated lagoons and irrigation. Another possible solution for dealing with the disposal of whey from cheese processing is isolation and conversion of whey proteins into value-added products with interesting properties from a biological and functional point of view.

Among these proteins, lactoferrin is one that has been intensively studied for its characteristic antimicrobial effect; the intensive research on lactoferrin led to the first International Symposium on Lactoferrin Structure and Function, held from September 19-24, 1992. Recently, a potent peptide generated from the pepsin digestion of lactoferrin was discovered. The peptide, lactoferricin, has broad spectrum antimicrobial activity which is different from the activity of the native molecule. The only drawback on the utilization of lactoferricin is the inhibiting effect that some food components have on it, limiting its application as a potential food additive or supplement in infant formulas. From recommendations by other researchers, the uses of lactoferricin should be in less demanding environments than foods, such as water.

So how does lactoferricin connect to the bottled water industry? For starters, it is important to say that everyday more consumers are demanding that the bottled water they consume be of the highest quality. Visible contamination in some production lots of the bottled water has led to recalls and lawsuits. A potential exists for incorporating lactoferricin in bottled water during processing, to prevent microbial growth arising from contamination after the purification treatments have been used. Of the purification treatments available today, ozonation is one of the most commonly used. The ability of ozone to destroy toxic or noxious industrial impurities and to inactivate bacterial contaminants has made it an attractive alternative to chlorination.

After reviewing the issues mentioned above, the main objective of this thesis was established: to study the possibility of utilizing lactoferricin prepared from cheese whey as a part of the treatment process used in bottled water production, along with ozonation.

The research was focused on three more specific objectives which consisted of (1) determining the overall effect of the bovine lactoferricin (LFD) on fungal spores isolated from bottled water; (2) investigating the possibility of a synergistic effect between ozone and lactoferricin; and (3) assessing whether the antifungal activity of lactoferricin was affected when exposed to ozone.

II. LITERATURE REVIEW

A. The Bottled Water Industry

The bottled water industry in North America has experienced a full expansion rate (Wilson 1991). This increase in consumption is mainly due to the consumer's concern about water pollution, objection to offensive tastes or odours, to fluoride, chlorine and other additives as well as microbiological quality (Warburton, 1993; Warburton et al., 1998). The Council Directive of the European Community (European Community 1980), defines natural mineral water as a "microbiologically wholesome water, originating in an underground water deposit and emerging from a spring tapped at one or more natural or bore hole exits" (Massa et al., 1998).

The Food and Drug Administration (1995) defines bottled water as any potable water that is manufactured, distributed or offered for sale, which is sealed in food grade bottles or other containers, and is intended for human consumption. According to Warburton et al. (1998), the source water for bottled water may be springs, municipal systems, or other sources that have been inspected and analyzed, and found to be of safe and sanitary quality. The water may be subjected to a number of treatments that include distillation, carbonation, ozonation, and/or filtration. The treatment should be selected on the basis of the initial quality of the source water, the type of bottled water being manufactured and location (Warburton et al., 1998).

Recently, consumers have complained about visible contamination in bottled water; this has caused the industry to initiate a series of recalls and rejection of products. In a study performed by Fujikawa et al. (1997), a total of 292 bottles of mineral water

(representing 90 brands) were tested. Of these, 109 (48 brands) were produced domestically (Japan) and 183 (42 brands) were imported. The results showed that 59 bottles (30 brands) contained foreign bodies, which were microbial in nature; 45 (20 brands) had moulds and 14 (10 brands) had bacteria. Of water samples that tested positive, 41 (22 brands) had been imported; furthermore, of those countries whose water had tested positive, Canada was the most predominant with 11 brands followed by the USA (3 brands), Australia and New Zealand (2 brands each). The fungi encountered in the mineral water seemed to be resistant to the common sterilization processes used for bottled water. Among the moulds isolated, the most predominant genus was *Penicillium*, followed by *Acremonium* and *Cladosporium*.

Bottled water is generally not sterile and can contain bacteria from naturally occurring sources as well as the bacteria introduced during manufacturing and consumer handling. It is known that storage of bottled water can induce the growth of indigenous bacteria (Warburton et al., 1992). These indigenous microorganisms remain at low numbers while water is in its natural environment but begin growth after water is bottled. The reason for this is yet not clear but it may be due to oxygenation of the water during the bottling process, an increase in surface area provided by the bottle, and trace amounts of nutrients arising from the bottle (Schmidt-Lorenz, 1976; Warburton et al., 1998). In addition to indigenous bacteria, bottled water may contain a wide range of microorganisms such as pathogenic bacteria, viruses and parasites. The sources of contamination are equipment used to pump or transport water, equipment used in processing or bottling water and the bottles and caps used; even the brief exposure of water to air during bottling can be an additional mode of contamination (Schmidt-Lorenz,

1976; Warburton et al., 1992). Until now there has not been much literature available on the occurrence of mould in bottled water; in the work previously mentioned by Fujikawa et al. (1997), all the fungi they isolated from the bottled water are commonly found in the environment (*Penicillium*, *Cladosporium*, *Aureobasidium* and *Paecilomyces*). The authors suggest that the reasons why these mineral waters were contaminated could be that the sterilization processes may not have been effective, contamination may have occurred during some part of the process used in producing the bottled water, the bottles used to hold the water may have been already contaminated and finally, during the filling of the bottles the water may have become contaminated from the surrounding environment. These are similar reasons to those given by Warburton et al. (1992).

Because of the increased consumption of bottled water, standards have been established in order to protect consumers from waterborne disease outbreaks. From the surveys conducted by Warburton et al. (1992) and Warburton (1993) during the 1980s it was clear that potential problems existed in the microbial content of bottled water. About 40% of bottled water on the Canadian market, between 1981 and 1989, had aerobic colony counts (ACC) exceeding the standards established which, in Division 12 of Canadian regulations, state that mineral or spring water must be potable water from an underground source, not from a public water supply, and should not contain any coliform bacteria. Other bottled waters should not contain any coliform bacteria or more than 100 aerobic bacteria per milliliter (Warburton, 1993).

Between 1992 and 1997, Warburton et al. (1998) conducted two surveys. One of them consisted of routine monitoring of bottled water, with 3460 sample units (SU) cultured for ACC, with 2086 SU cultured for coliforms and faecal coliforms, 2720 SU for

Aeromonas hydrophila, and 2820 SU cultured for *Pseudomonas aeruginosa*. The second survey consisted of random sampling of 267 SU of bottled water sold across Canada. The results from the two surveys indicated an improvement in that the lowering of ACC, coliforms and pathogen counts started to occur in the last decade. The same authors suggest that disinfection processes can substantially decrease the bacterial bioload; they encourage the use of ozonation in the manufacturing process of bottles as a method of destroying any bacteria or biofilms that are present in the new or recycled bottles.

From the information provided above, it is apparent that regulations mainly focus on the presence of bacterial contamination; there is no specific regulation and not enough information regarding the incidence of mould in bottled water. Fujikawa et al. (1997) presented an interesting challenge regarding mould contamination as an important factor that must be controlled in the bottled water industry regardless of the absence of regulations on this particular subject.

B. Whey utilization

Cheese whey, the yellow-green liquid that separates from the curd during the manufacture of cheese and casein, is a major by-product of the dairy industry (Chen and Wang, 1991; Smithers et al., 1996). The volumes of whey production continue to grow and as much as half of the world's production (approximately 130 million tonnes) continues to be disposed as waste. Whey is comparatively concentrated compared to normal municipal waste and therefore adds a great demand on municipal sewage systems. New and strict environmental regulations are now encouraging the dairy industry to deal with waste management and whey disposal more specifically (Zall, 1992; Smithers et al.,

1996). The traditional methods for handling whey have been by drying and using it as feed for livestock, or as fertilizer. Both methods have a handling cost and excessive feeding may lead to digestive disturbances in some animals (Gillies, 1974). All these factors are focusing research into the recovery of whey solids and transforming them into value-added products.

Whey contains essentially 100% of the total milk carbohydrate (lactose) and 14-24% of the total milk protein whose main constituents are two small globular proteins β -lactoglobulin and α -lactalbumin, that account for approximately 70 to 80% of total whey protein. The rest are minor protein components that include bovine serum albumin, lactoferrin, immunoglobulins, phospholipoproteins, and several enzymes (Marshall, 1990; Smithers et al., 1996). All of these proteins are soluble at the pH conditions used to precipitate casein proteins. After removal of the casein proteins, the whey proteins can be removed from the whey and purified from the lactose and mineral components by ultrafiltration, microfiltration with affinity chromatography, ion exchange, adsorption or heat precipitation techniques (Marshall, 1990; Chen and Wang, 1991; Akita and Li-Chan, 1998) and isolated as spray-dried whey powder and, in more limited quantities, as whey protein concentrate (Smithers et al., 1996).

The separation of whey by reverse osmosis or ultrafiltration systems, produces a permeate and a whey protein concentrate. Permeate can be used in products such as glues in plywood manufacture, fermentation media, and in the production of ethanol or lactic acid. Whey protein concentrate on the other hand, is currently used in dairy and bakery products, soups, soft drinks and meat products (Clark, 1987) with increasing usage of encapsulated whey protein concentrate to replace fat in diet products (Zall, 1992).

Whey represents a rich and heterogeneous mixture of secreted proteins with wide ranging functional attributes for nutritional, biological, and food purposes. These attributes make whey utilization an interesting field for future research and development on its utilization.

C. Lactoferrin

Lactoferrin, also known as lactotransferrin, is an iron-binding glycoprotein which belongs to the transferrin family. Other members of the transferrin family are serum-transferrin which occurs in blood plasma and other extracellular fluids, and ovotransferrin which is present in avian and reptile egg white (Hambraeus and Lönnerdal, 1993). Milk contains two kinds of iron binding proteins; transferrins, which seem to be virtually identical to serum transferrin, and lactoferrin, which is closely related to transferrin but more characteristic of milk and exocrine glands. Lactoferrin, a cationic protein (pI 8.5) with an approximate molecular weight of 75,000 to 88,000 Da, is synthesized in the mammary gland and also in other exocrine glands. Consequently lactoferrin occurs in all external secretions such as tears, saliva, sweat, semen, bronchoalveolar lavage fluid as well as the specific granules of polymorphonuclear leukocytes (Hambraeus and Lönnerdal, 1993; Ellison, 1994). Lactoferrin is found in substantial amounts in milk. Human milk is particularly rich in lactoferrin, ranging from 5-10 mg/mL in colostrum with concentrations decreasing during lactation to about 1 to 2 mg/mL in mature milk (Masson and Heremans, 1971; Harzer and Bindels, 1985). Lactoferrin is also present in milk of some, but not all, mammalian species. Lactoferrin concentration varies from 0.1 to 0.3 mg/mL in bovine milk and about 0.05 to 0.15 mg/mL in whey (Harzer and Bindels, 1985). The cationic

property of lactoferrin has been used to advantage in procedures for isolation from whey, since the major and many of the minor whey protein constituents are acidic, with pI < 7.0 (Smithers et al., 1996).

The lactoferrin molecule is folded into two lobes, each with a binding site for iron. Lactoferrin has broad spectrum antimicrobial properties against a wide range of microorganisms such as gram-positive, gram-negative bacteria, rods and cocci, facultative anaerobes, and aerotolerant anaerobes. The antimicrobial activity was initially thought to be a function of its ability to chelate iron, inhibiting microbial growth through nutritional deprivation of iron (Arnold et al., 1980; Finkelstein et al., 1983). Some selected bacterial pathogens have evolved techniques by having specific iron-regulated cell surface receptors that abolish this effect and use the protein (lactoferrin and transferrin) as an iron source. Examples of these pathogens are *Neisseria meningitidis*, *N. gonorrhoeae*, *Branhamella catarrhalis*, and *Hemophilus influenzae*. In these bacteria, the receptors for lactoferrin and transferrin are separate bacterial proteins, giving them a selective advantage in causing invasive disease (Ellison, 1994).

More recently, several investigators (Arnold et al., 1980; Yamauchi et al., 1993; Ellison, 1994) suggested that lactoferrin, in its iron-free state, has other effects on microorganisms by directly damaging the outer membrane of gram-negative bacteria causing the release of lipopolysaccharides (LPS) that sensitize the cell to antibiotic action (Figure 1).

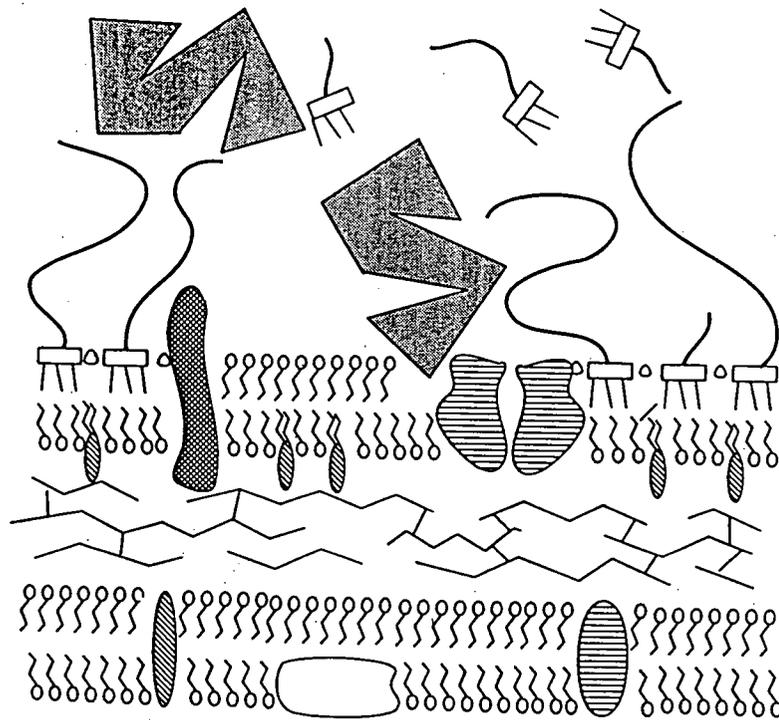


Figure 1. Effect of lactoferrin on outer membrane of gram-negative bacteria. Lactoferrin, indicated by large shaded angular structure with two clefts, binds to the outer membrane causing displacement of LPS molecules (Source: Ellison, 1994).

D. Lactoferricin (LFD)

In 1991, Tomita and co-workers generated a potent broad spectrum antimicrobial peptide from lactoferrin which completely inhibited the growth of *E. coli* O111 at concentrations of 0.25 mg/mL or more and it retained activity in the presence of added iron. This peptide was obtained from the enzymatic digestion of bovine lactoferrin with gastric pepsin. Apparently, the potent antibacterial peptides of bovine lactoferrin are generated by enzymes having cleavage site specificity to porcine pepsin, which cleaves at the carboxyl terminus of phenylalanine and leucine residues in the substrate (Tomita et al., 1991). This low molecular weight peptide (approximately 3,000 Da) is 25 amino acids long, having the sequence Phe-Lys-Cys-Arg-Arg-Trp-Gln-Trp-Arg-Met-Lys-Lys-Leu-Gly-Ala-Pro-Ser-Ile-Thr-Cys-Val-Arg-Arg-Ala-Phe. This region seems to have exact homology with an amino-terminal segment of the whole lactoferrin sequence and has a quasicyclic structure due to the presence of a disulfide bond between its two cysteine residues (19 and 36 of bovine lactoferrin); however, it lacks detectable carbohydrate (Bellamy et al., 1992a; Wakabayashi et al., 1992; Yamauchi et al., 1993). From the amino acid sequence, lactoferricin contains five arginine and three lysine residues, making it strongly cationic. Lactoferricin has been compared to other cationic peptides, including magainins, from frog skin (Bevins and Zasloff, 1990); cecropins, from the haemolymph of insects (Boman and Hultmark, 1987); and defensins, from mammalian neutrophils (Lehrer et al., 1991). Those cationic peptides, known for their high affinity for negatively-charged phospholipids and lipopolysaccharides, have a direct effect on outer membranes of microorganisms, leading to disruption of energy metabolism or other essential functions of the target microorganism.

The antimicrobial sequence of lactoferricin is located away from the iron-binding residues of undigested lactoferrin since it lacks tyrosine and histidine residues which are essential for the metal chelating functions of the protein (Tomita et al., 1991; Bellamy et al., 1992a), indicating that the peptide's antimicrobial effect is different from the iron deprivation functions of the native protein. Low molecular weight peptides generated by pepsin cleavage of human lactoferrin and bovine lactoferrin, exhibited greater effectiveness against *E.coli* O111 than undigested lactoferrin in each instance, and lactoferricin B (bovine) was about 9-fold more effective than lactoferricin H (human) (Bellamy et al., 1992a).

Apparently, the overall mechanism of antimicrobial action of lactoferricin is similar to that of the native lactoferrin molecule which entails disruption of the cell wall and release of the membrane's lipopolysaccharide (LPS) molecules (Figure 2). That makes the cell permeable by disrupting the inner membrane, causing immediate development of electron-dense membrane blisters and subsequent aggregation of cytoplasmic material (Yamauchi et al., 1993; Bellamy et al., 1994; Ellison, 1994).

Optimal activity of lactoferricin was reported to be at a pH range of 5.5 to 7.5 (Wakabayashi et al., 1992; Bellamy et al., 1992b). Jones et al. (1994) reported that 50% of the antibacterial activity of lactoferricin was lost when the pH was adjusted to 11.0 and neutralized without heat treatment. The loss of antibacterial activity was thought to be due to the formation of inactive aggregates from insoluble peptide components, which were formed under the alkaline conditions.

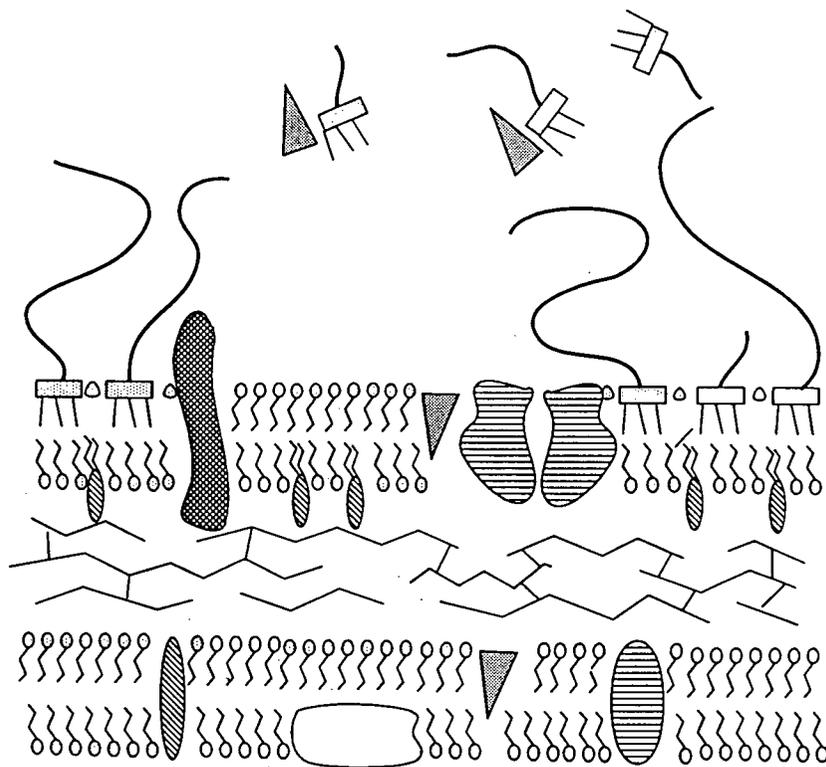


Figure 2. Lactoferricin (indicated by shaded triangles) binds to the outer membrane causing LPS release and altered outer membrane permeability. Due to lactoferricin's smaller size, it is likely that it penetrates through the outer membrane (OM) and murein sacculus reaching and damaging the bacterial cytoplasmic membrane, thereby causing bacterial cell death. (Source: Ellison, 1994)

The effectiveness of lactoferricin has been demonstrated against a wide range of gram-positive and gram-negative bacteria (Bellamy et al., 1992b; Yamauchi et al., 1993; Ellison, 1994), and to a lesser extent yeast and mould. Yeast strains appeared to be more sensitive to inhibition by lactoferricin than the filamentous fungi tested (Bellamy et al., 1993; Bellamy et al., 1994).

Bellamy et al. (1992b) tested 28 bacterial species for susceptibility to inhibition by lactoferricin B (bovine) at concentrations varying from 0.3 to 150 µg/mL, depending on the strain and culture medium used. Lactoferricin B was lethal causing rapid loss of colony forming capability in most of the species tested. The only bacteria that were highly resistant to the peptide were *Pseudomonas fluorescens*, *Enterococcus faecalis* and *Bifidobacterium bifidum*. Lactoferricin seems to have a higher lethal effect on gram-negative than on gram-positive bacteria. The reason for this could be based on the absence of an outer membrane in the gram-positive bacteria and the presence of certain negatively-charged cell wall components which could potentially bind the cationic peptide, blocking its access to lethal target sites on the cytoplasmic membrane (Bellamy et al., 1992b).

Wakabayashi et al., (1992) studied the susceptibility of *Listeria monocytogenes* to lactoferricin B. Complete inhibition of four strains of *L. monocytogenes* was obtained using low concentrations of lactoferricin (0.3 to 9 µg/mL). The effectiveness of lactoferricin was not affected by the presence of various carbohydrates or proteins, but the authors did notice that the presence of various salts diminished the effectiveness.

Another study, performed by Yamauchi et al. (1993), tested the effectiveness of bovine lactoferrin and its pepsin-derived peptide (lactoferricin) fragment on three bacteria, *Escherichia coli* CL99 1-2, *Salmonella typhimurium* SL696, and *Salmonella montevideo*

SL5222. Their results indicated that more membrane LPS were released when in contact with lactoferricin, demonstrating a higher bactericidal effect. Lactoferrin mostly exhibited a bacteriostatic activity.

In 1994, Tomita et al. examined the effect of lactoferricin B against a wide range of gram-negative, gram-positive bacteria, yeasts and moulds. Concentrations required to cause complete inhibition of growth varied within the range of 0.3 to >60 µg/mL depending on the strain and medium used (either 0.1% peptone or peptone yeast glucose medium). The lethal effect of lactoferricin was thought to result from the ability of the peptide to disrupt essential functions of the cytoplasmic membrane of target microorganisms. Similar to previous studies, *Pseudomonas fluorescens* was not inhibited.

Bellamy et al. (1993) demonstrated the high susceptibility of *Candida albicans* to bovine lactoferricin. The lethal effect was obtained at lactoferricin concentrations that varied in the range of 18 to 150 µg/mL. By means of electron microscopy, the authors were able to see the morphological changes induced by lactoferricin. The peptide induced an autolytic response in *C. albicans* causing an increase in cytoplasmic debris.

A number of yeasts, dermatophytes and other filamentous fungi have also been tested for susceptibility to inhibition by lactoferricin B. In 1994 Bellamy et al. noticed that lactoferricin inhibited fungal uptake of ³H-glucose suggesting that it may target the cell membrane. The effective concentrations varied from 3 to >60 µg/mL. When the cell's morphological changes were evaluated from electron micrographs, they showed similar observations to those reported in the previous work with *C. albicans*.

All the studies mentioned reported that the effectiveness of lactoferricin is influenced by the medium, microbial strain and presence of salts. Among other

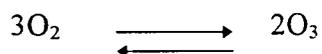
observations, Facon and Skura (1996) noticed a synergistic effect between lactoferricin B and lysozyme, EDTA or both, against *Salmonella enteritidis*, whereas the three agents alone had little or no activity against the target agent. The activity of lactoferricin B seemed to be sensitive to the addition or presence of calcium, magnesium, citrate, succinate, lactate or acetate and other complex media like tryptic soy broth or milk-based and soy-based infant formula (Bellamy et al., 1992a; Bellamy et al., 1992b; Facon and Skura, 1996). However, addition of glucose, fructose, mannose, xylose, sucrose, lactose, starch, gelatin or bovine serum albumin at concentrations up to 10 mg/mL had no effect on the antimicrobial activity of bovine lactoferricin (Jones et al., 1994; Wakabayashi et al., 1992; Bellamy et al., 1992b).

One advantage of using bovine lactoferrin is that it can be recovered from fresh skim milk or cheese whey on an industrial scale and is now available as a product of the dairy industry (Tomita, 1993). This protein could be used as a starting material for the large-scale manufacture of lactoferricin. Bellamy et al. (1992b) and Tomita et al. (1991) suggest that considerable potential exists for the widespread commercial use of lactoferricin as a “natural” antimicrobial agent in a variety of product applications such as food and cosmetics. However, from the information provided above, we can see that in order to prevent a diminished antimicrobial effect of lactoferricin, the peptide’s concentration has to be optimized or used in a less demanding environment than food (Facon and Skura, 1996).

E. Ozone

In 1840, Christian Friedrich Schönbein noticed an “odour of electrical matter” by subjecting oxygen to electrical discharges. Schönbein concluded that this odour was due to a gas which he named ozone, from Greek *ozein* (to smell). The true nature of ozone as another form of oxygen was first established by Auguste Arthur de la Rive and Jean-Charles Galinard de Marignac in 1845. By the indirect determination of its density, Jean Luis Soret proved in 1865 that ozone is triatomic oxygen (Sartori and Yüan, 1994).

Ozone is an unstable allotropic form of oxygen, one and one half times as dense, with a molecular formula O_3 and molecular weight of 48.00 g/mole (Evans, 1972). Ozone is formed by the excitation of molecular oxygen by ultraviolet radiation or an electrical discharge. The overall reaction of ozone formation is described by an endothermic reaction (Wickramanayake 1991):



Ozone can be commercially generated by ultraviolet radiation, electrolysis of perchloric acid, or by electrical discharge. Of these methods, the corona (electrical) discharge is the most common technique (Figure 3). In this method, ozone is generated by passing oxygen or dried air through a field of a silent electrical discharge created by a high voltage alternating power source. This electrical discharge occurs in a narrow gap between electrodes separated by a glass or ceramic dielectric medium (Bott, 1991).

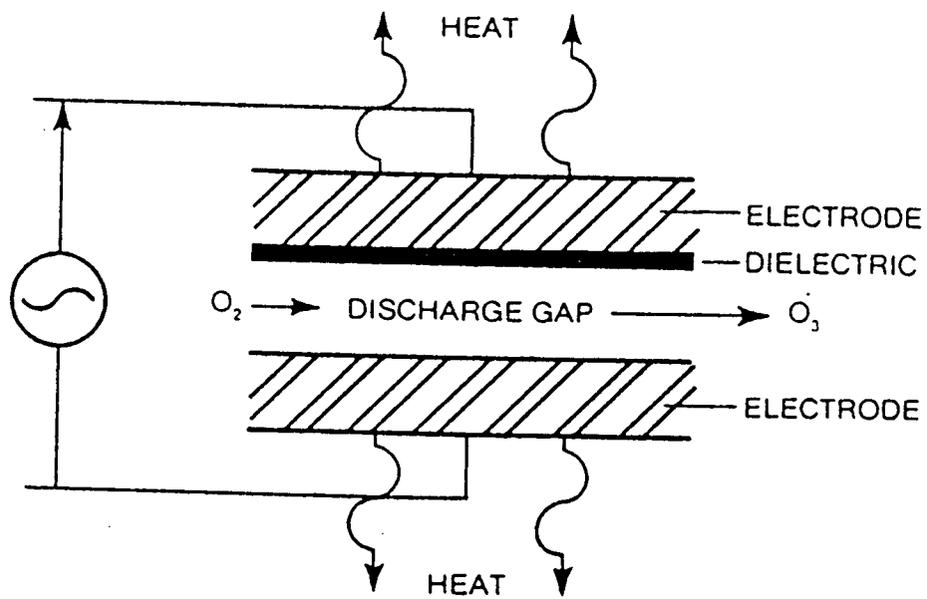


Figure 3. Schematic diagram of corona discharge ozone generator (Source: Rice and Browning, 1981).

For water treatment, the generated ozone is pumped into the water through a stone of fine porosity, creating very small bubbles which rise slowly through the water. The slower the bubbles rise through the water, the greater the amount of ozone transferred to the water (Hess machine, 1999).

Ozone is a gas at ambient and refrigerated temperatures and has the unique property of autodecomposition producing numerous free radical species like the hydroxyl free radical (OH^*). Like oxygen, ozone is a supporter of combustion and is a powerful oxidizing agent, fifth in thermodynamic potential behind elemental fluoride, chlorine trifluoride, atomic oxygen, and hydroxyl radical (Graham, 1997). Ozone is capable of attacking almost all organic compounds, making it the most potent oxidizing agent available for water and wastewater treatment and disinfection (Evans, 1972; Graham, 1997). One factor that needs special attention is temperature. Heat will accelerate ozone's decomposition, and decomposition is instantaneous at temperatures of several hundred degrees Celsius. Figure 4 depicts the theoretical solubility of ozone in water; as the temperature increases, ozone solubility decreases. Moisture, high pH, silver, platinum, manganese dioxide, sodium hydroxide, chlorine and nitrogen pentoxide catalyze decomposition (Kinman, 1972).

Several methods exist for the measurement of residual ozone: the indigo trisulfonate method, iodometric method, the leuco crystal violet method, carmine indigo procedure, UV absorption, and electrochemical devices. The iodometric method was one of the most commonly used methods for ozone measurements in water.

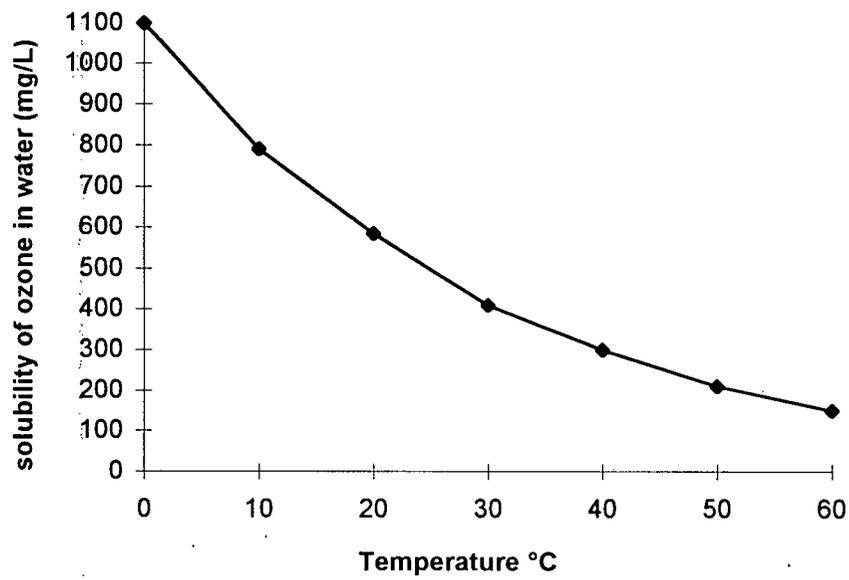


Figure 4. Solubility of ozone in water at different temperatures
(Adapted from Kinman, 1972).

Considering the absence of a one to one stoichiometric reaction (as a result of the pH, buffer composition, iodide ion concentration, sampling techniques and reaction time) the iodometric method has become inappropriate (Evans, 1972). The carmine indigo procedure has been in use for at least 15 years in water treatment plants of Quebec, Canada. It is a very simple, rapid method for water treatment plants; nevertheless, more information is needed on its accuracy. The UV absorption consists of measurements at wavelengths of 258-260 nm. The uncertainty with respect to the molar absorptivity for aqueous ozone makes it a disadvantage and generally unacceptable for the measurement of residual ozone in drinking water (Bablon et al., 1991). Of all the methods, the indigo trisulfonate colorimetric method is the most sensitive, precise, rapid, selective, and commonly used. In this method, the direct stoichiometric reaction (1:1) of ozone with the double bond of indigo trisulfonate results in the discoloration of the latter under acidic conditions (Bader and Hoigne, 1982). The reaction is followed by the change in absorbance measured spectrophotometrically at 600 nm. The decrease in absorbance at 600 nm is linear with increasing ozone concentration; this method has a detection limit of 2 µg ozone /L (Bader and Hoigne, 1982).

Ozone has two main advantages. One, it is an even more powerful oxidizer, as mentioned previously, than chlorine. The second advantage is that in its reaction with organic material it does not produce compounds having the toxicity of chlorinated by-products (trihalomethanes). Furthermore, ozone in solution is unstable and in a short period of time it reverts back to oxygen. This means that due to its short half-life (about 20 to 30 min in distilled water at 20°C) it would not persist in the environment and therefore, it does not remain a potential hazard for long after its application.

The main disadvantage of ozone is precisely its short-life, which creates the need to generate the ozone on site, and prevents it from being stored. The other disadvantage is due to ozone's high chemical reactivity, which makes it corrosive to many materials such as copper, rubber, and some plastics (Bott, 1991).

The use of ozone in the food industry has been investigated for food preservation, shelf life extension, equipment sterilization, and improvement of food plant effluents. Ozone has been used to treat ground and surface water in many European cities for years, with Paris, France opening its first ozone treatment plant in 1906. Now, there are more than 2,000 municipal water treatment plants worldwide using ozone. Ozone is now becoming the industry standard for treating bottled water (Langais et al., 1991). It wasn't until July, 1997 that ozone was generally recognized as safe (GRAS) in the United States for the treatment of bottled water and as a sanitizer for process trains in bottled water plants (Graham, 1997).

Mignot (1973) suggested that the use of ozone for drinking water treatment should be conducted on water that is limpid and must not contain any trace of iron or manganese which may cause re-coloration. The author also reported that ozone residual levels for drinking water treatments should be 0.4 mg/mL for 6 to 12 minutes. Nevertheless, this concentration is dependent on the presence and the nature of the organic matter involved. It appears that a contact time exceeding 8 minutes will generally not improve the decreasing rate of removable matter and micropollutants. The implementation of longer treatment times (longer than 8 min at 0.4 mg/mL) can be an expensive operation since additional ozone supply of at least 20% is required to maintain a free residual dose of 0.4 mg/mL for 12 minutes instead of 6 minutes (Mignot, 1973).

There are a number of studies regarding the role of ozone in different food applications. Bott (1991) examined the use of ozone as a disinfectant in a process plant. Ozone at relatively low dose concentrations (ranging from 0.069 to 0.81 ppm) was able to completely remove biofilms from the test surface. After contact with ozone, the cells of *Pseudomonas fluorescens* were not recognizable, being shriveled and mis-shapen, indicating destruction of the cells.

In Israel, Sarig et al. (1996) have studied the possibility of treating table grapes with ozone as a measure to prevent post-harvest decay. The authors concluded that exposing table grapes to ozone was almost as effective as sulphite fumigation for the control of storage decay caused by *Rhizopus stolonifer*; the only difference was the overall appearance of the ozonated grapes, which did not look as fresh as the grapes exposed to sulphite.

In a study performed by Kötters et al. (1997), the effect of ozonation on rockfish (*Sebastes* spp.) was evaluated. When ozonated intermittently during transport, the fish shelf-life was extended by about 36 h. Ozonated water decreased total bacterial count of skin samples by 90% and ozonation appeared to ease the separation of slime and its bacterial flora from the fish.

The ability of ozone to oxidize organic compounds makes it a powerful agent for removing colour, odour, and tastes. This same ability is used to kill microorganisms in water, making ozone a strong disinfecting agent (Kinman, 1972). With reference to the reaction of ozone towards chemical compounds, in the case of amino acids and proteins, Mudd et al. (1969) concluded that sulfhydryl groups were most susceptible to oxidation, followed by tryptophan and methionine, then by tyrosine and histidine, and finally cystine.

Apparently, none of the other amino acids are affected by ozone. Bablon et al. (1991) explained that the preferred sites of attack are the nitrogen atom or the R group consisting of sulfur or unsaturated bonds; consequently, the effect of ozone on polypeptides and proteins depends on the nature of their constituent amino acids. Ozone is also capable of reacting with nucleobases, in particular with thymine, guanine and uracil as well as their nucleotides (Bablon et al., 1991). Saturated fatty acids seem to react slightly with ozone (Bablon et al., 1991) although the reactivity seems to increase when ethylenic bonds are present in the carbon chain. From these reactions, by-products such as aldehydes, acids and hydrogen peroxide are formed. The effect of ozone on carbohydrates is based on its reactivity with polysaccharides, leading to the breakage of glucosidic bonds and production of monosaccharides (Bablon et al., 1991) followed by the oxidation of the alcoholic sites on the monosaccharides, and the eventual formation of aldehydes and aliphatic acids.

From the information provided above, it is clear that ozone will react with the main cell components of microorganisms: carbohydrates, proteins (amino acids), fatty acids and nucleobases. This has led to numerous studies on the effect of ozone on microorganisms. Bacteria, including *E. coli*, *Staphylococcus aureus*, *Bacillus cereus*, *B. megaterium*, *Salmonella typhimurium*, *Shigella flexneri* and *Vibrio cholerae* are sensitive to ozonated water under various conditions (Broadwater et al., 1973; Burleson et al., 1975; Restaino et al., 1995) including the presence of organic matter, temperature, pH and substrate. Apparently, gram-negative organisms are more sensitive to ozone than gram-positive organisms, with the exception of *Listeria monocytogenes*. The most sensitive organism appears to be *E. coli*, while the gram-positive *Staphylococcus* spp. and *Streptococcus* spp.

are more resistant to ozone. Yeast cells exposed to ozonated water displayed a 4.5 log decrease instantaneously after 5 min exposure to ozone concentrations ranging from 0.15 to 0.2 ppm; whereas fungal spores were highly resistant with less than one log reduction after 5 min of exposure at the same ozone concentrations (Restiano et al., 1995).

There is also some information on the efficiency of ozone against eukaryotic pathogens including *Cryptosporidium parvum*, *Giardia lamblia* and *G. muris* (Finch et al., 1993; Korich et al., 1990). By treating these parasites with 1 ppm ozone for 5 min, a 90% inactivation was observed. Chlorine dioxide (1.3 ppm) required 1 h of exposure to yield the same percent of inactivation, while 80 ppm of chlorine and 80 ppm of monochloramine required approximately 90 min for the same inactivation. Based on these results, the authors stated that with the possible exception of ozone, the use of other disinfectants alone should not be expected to inactivate *C. parvum* oocysts in drinking water.

III. MATERIALS AND METHODS

A. Experimental design

Hypothesis:

H₀: There is no difference in observations on the test organism (fungal spores) between treatment conditions, the treatments being the use of LFD, ozone or both at different concentrations.

H_a: LFD is effective; ozone is effective; ozone and LFD effects are not independent

Factors: LFD, ozone

Response: Inhibition of fungal spore growth, measured as OD₅₉₅ and Log MPNGU/mL.

Levels LFD: 0, 0.03, 0.06 and 0.3 mg/mL

Ozone: 0, 0.1 and 0.4 ppm

(Temperature was held constant at 25°C)

<u>Response</u>	<u>Factor levels</u>	
	<u>LFD (mg/mL)</u>	<u>ozone (ppm)</u>
OD ₅₉₅	0, 0.06, 0.3	NA*
MPNGU/mL	0, 0.03, 0.06, 0.3	0, 0.1-0.4

*NA, not applicable

B. Mould culture and identification

As foreign bodies were observed in the bottled water, the bottle was shaken and 1 mL was inoculated into potato dextrose agar (Difco, Detroit, MI) by the pour plate method. This was done in triplicate and the plates were incubated at 25°C for 7 days. From a visible colony growth, a loopfull was examined under phase contrast microscope at 100x under oil immersion for identification based on its morphology. The identification was further accomplished by using specialized identification media using a standardized regime described for the identification of *Penicillium* species by Pitt (1979). For this, a sample culture was grown on three standard media at 25°C, and one of these at 5°C and 37°C for a period of 7 days. The three media used were Czapek Yeast Extract Agar (CYA), used at all three temperatures, Malt Extract Agar (MEA) and 25% Glycerol Nitrate Agar (G25N) used at 25°C (Pitt and Hocking, 1985). For inoculation of the media, a needle point of spores and mycelium was transferred into a vial containing sterilized molten agar (0.2%) and polysorbitan 80 (0.05%). The vial was mixed slightly. Then, before flaming the needle, it was used to stab inoculate the 5°C plate. Next, with a sterile loop the vial was thoroughly mixed and the rest of the plates inoculated. Observations were made on the morphology and diameters of the colonies (refer to Appendix A).

C. Culture growth conditions

Cultures from PDA plates were transferred every 30 days. In order to produce a spore “stock” suspension, spores were washed from 7-day old cultures on PDA with 20 mL of sterile distilled deionized water. The plates were washed three times and the

suspension was stored in a sterile dilution bottle at 4°C. Before each test run, a sample of the stock suspension was examined under the microscope to make sure that spores were present. Serial dilutions were prepared from the stock suspension and plated in duplicate on potato dextrose-trypan blue agar (PDTB) using the ISO-GRID HGMP, followed by incubation at 30°C for 48 hours. The most probable number of growth units (MPNGU) was calculated (Lin et al., 1984; Spotts and Cervantes, 1992).

D. Preparation of the lactoferrin digest (LFD)

Bovine lactoferrin PT716LF (50 g) was obtained from Inovatech Labs (Abbotsford, B.C.). The stated purity of lactoferrin in the powder containing 90% protein was 70% (% protein). Lactoferrin was dissolved in water at a concentration of 5% (w/v), weight of protein per volume, and the pH adjusted to 2.3 with HCl (2 N). The concentration of lactoferrin was determined by spectrophotometry at 280 nm using an extinction coefficient (at 1% and 1 cm) of 15.1 (Fasman, 1976); the concentration was corroborated by the Biuret method using BSA as standard. Porcine pepsin (E.C. 3.4.23.1) (Sigma P-7012, 2,500-3,500 U/mg protein) was added at a concentration of 3% (w/w) enzyme:substrate basis (Tomita et al., 1991). The mixture was incubated at 37°C for 4 h (Bellamy et al., 1992a) in a water bath. Digestion was terminated by heating at 80°C for 15 min. NaOH (1 M) was added to bring the pH to 7.0 and the digest was centrifuged at 10,000 x g for 25 min. The supernatant was filtered through a 0.45µ cellulose acetate filter (Millipore, Bedford, MA) and stored frozen at -80°C. The digestion was confirmed by SDS-PAGE (see section G) using a Phastsystem electrophoresis unit (Pharmacia LKB Biotechnology, Uppsala, Sweden). The lactoferrin digest (LFD) was used without any

further purification. Its concentration (% weight) was calculated from the original lactoferrin concentration assuming a molecular weight for bovine lactoferrin of 83,000 and 3,126 for lactoferricin B (LFD) (Bellamy et al., 1992a).

E. Preparation of bovine serum albumin digest (BSAD)

Bovine serum albumin (Sigma Chemicals) was used as a negative control. The protein was digested, centrifuged and filtered under the same conditions described above for lactoferrin. BSAD was stored at -80°C and used without further purification.

F. Antimicrobial assay

A 96 well microplate (Fisher Scientific) containing 100 µL of PYG medium, sterilized distilled water and a standard concentration (0.06 and 0.3 mg/mL) of either LFD or BSAD in each well, was inoculated with 25 µL of a spore suspension containing 7×10^4 mL⁻¹ spores. Nine replicates, within one plate, were used for each test agent (LFD, BSAD, and the respective controls). The total volume in each well was 250 µL. The plates were placed on a stand inside sterilized glass dissectors containing sterilized d-d water in order to prevent the microplate-wells from drying, and incubated separately at 30°C over time. One plate contained only spores in PYG medium supplemented with LFD, and the second plate contained the spore controls and the spores in PYG medium supplemented with BSAD. From each microplate the culture optical density was monitored at 595 nm using a microplate reader (Labsystems iEMS Reader MF, Helsinki). The antimicrobial assay was used to monitor the concentration of the test agent (LFD, BSAD) that caused complete inhibition of spore germination and mould growth over time.

The statistical significance of observed differences of experimental results among samples was evaluated by analysis of variance (ANOVA) using a general linear model (GLM) followed by Tukey's pairwise comparison of means. The statistical software MINITAB version 12.21 (Minitab Inc., State College, PA) was used.

G. SDS-PAGE

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed on lactoferrin, BSA, LFD and BSAD. Samples were diluted to a final protein concentration of 1 to 6 mg/mL in a sample buffer consisting of 2 μ L 2-mercaptoethanol, 20 μ L 10% SDS and 5 μ L tracking dye (0.05% bromophenol blue) in 10 mM Tris/HCl, 1 mM EDTA, pH 8.0. The final volume was adjusted to 100 μ L. The samples were heated for 10 min in a boiling water bath and centrifuged at 13,000 x g for 1 min. The electrophoresis was performed on a PhastSystem (Pharmacia, Biotech Inc., Uppsala, Sweden) with a PhastGel gradient high density polyacrylamide gel. The gel was run at 500 V, 10 mA, 15°C for 184 volt h. The gel was stained with Coomassie blue (0.1% PhastGel Blue R solution in 30% methanol and 10% acetic acid), destained with 30% methanol and 10% acetic acid and preserved in 10% acetic acid and 5% glycerol. The approximate molecular weights were determined using peptide range molecular weight standards (Sigma Chemicals, St. Louis, MO).

H. Ozone generation

Ozonated water was produced using a Sander 301 ozonator (Sander, Uetze-itze, Germany). Pure U.S.P. grade oxygen containing < 3 ppm of water was used as the feed

gas. The ozone levels were measured by the indigo colorimetric method (Bader and Hoigné, 1982). The antimicrobial effect of ozone was assayed in distilled deionized water with or without added LFD. All solutions were ozonated at 25°C under neutral pH conditions. The samples were ozonated at 0.1-0.4 ppm (the FDA has stipulated that ozone levels be maintained at or below 0.4 ppm; White et al., 1991) by constantly stirring with a magnetic stirrer in order to thoroughly mix the ozone.

Two experimental set ups were used:

The first one consisted of ozonating 1 L of d-d water to approximately 2 ppm then pouring 65 mL (within 15 sec) of the ozonated water into a beaker containing 35 mL of spore suspension (3×10^6 spores mL^{-1}). This was done in order to work with a smaller volume (100 mL) once the LFD was incorporated. Samples were collected at 0, 4, 8 and 12 min after incorporation of the ozonated water. Immediately after addition of ozonated water, 1 mL was transferred into sterile test tubes with 9 mL of 0.1% peptone water (Difco, Detroit, MI) containing 15 mg/L of sodium sulphite added to neutralize the ozone (Domingue et al., 1988). Serial dilutions were made, with each tube being vortexed followed by plating on PDTB agar using the ISO-GRID HGMP to enumerate the survival of spores by the MPNGU. The plates were incubated at 30°C for a period of 4 to 7 days. The set up for this series of experiments is presented in Figure 5a.

The second experimental set up consisted of directly ozonating for 15 seconds 100 mL of d-d water containing 0.9 mL of spore suspension. This procedure was done in order to decrease the starting spore count to 3×10^4 spores mL^{-1} . Samples were collected every 30 sec for a period of 3 min and plated in the same manner as described above (Figure 5b).

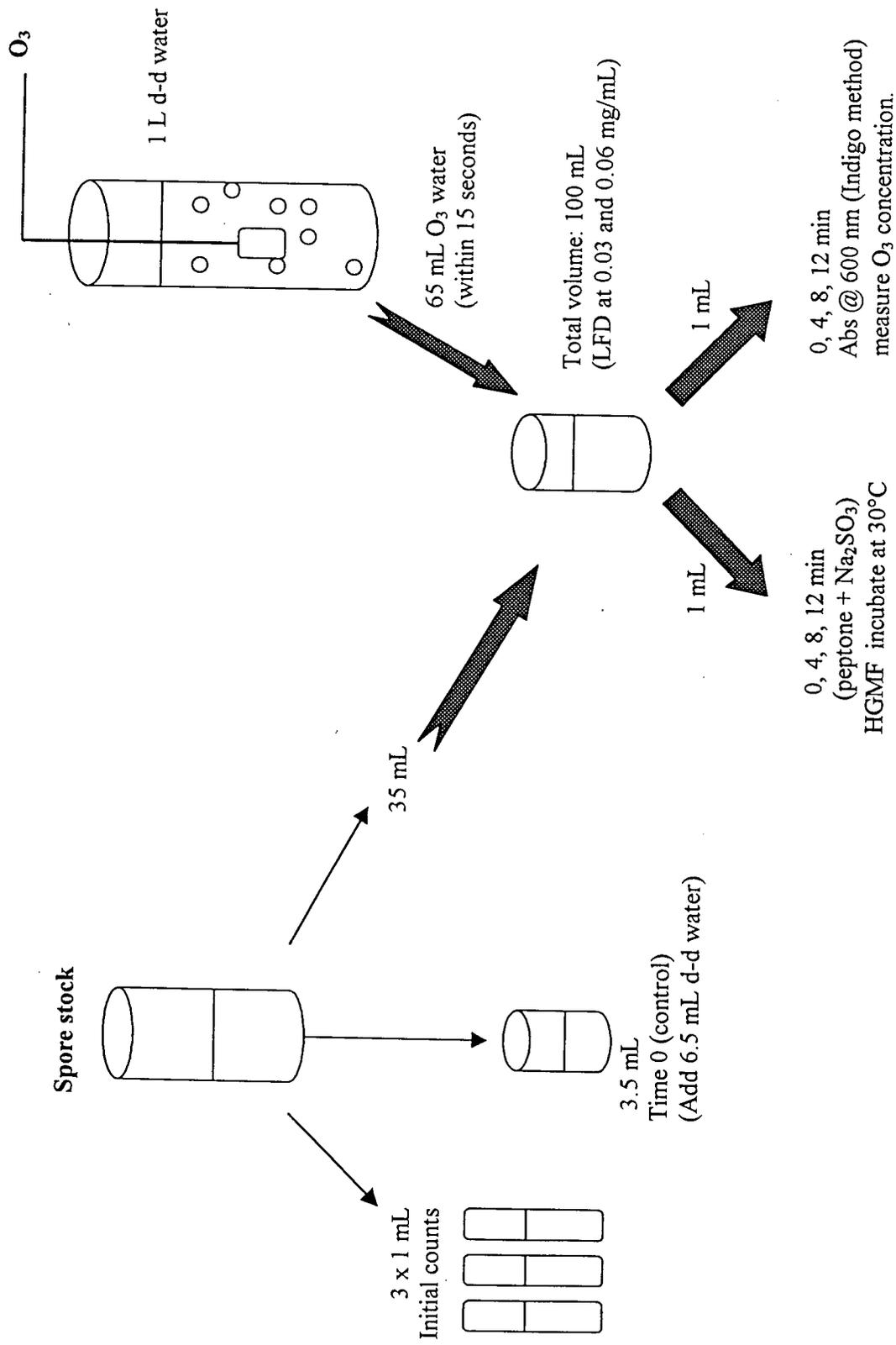


Figure 5a. Flow chart describing the procedure for the first experimental set up.

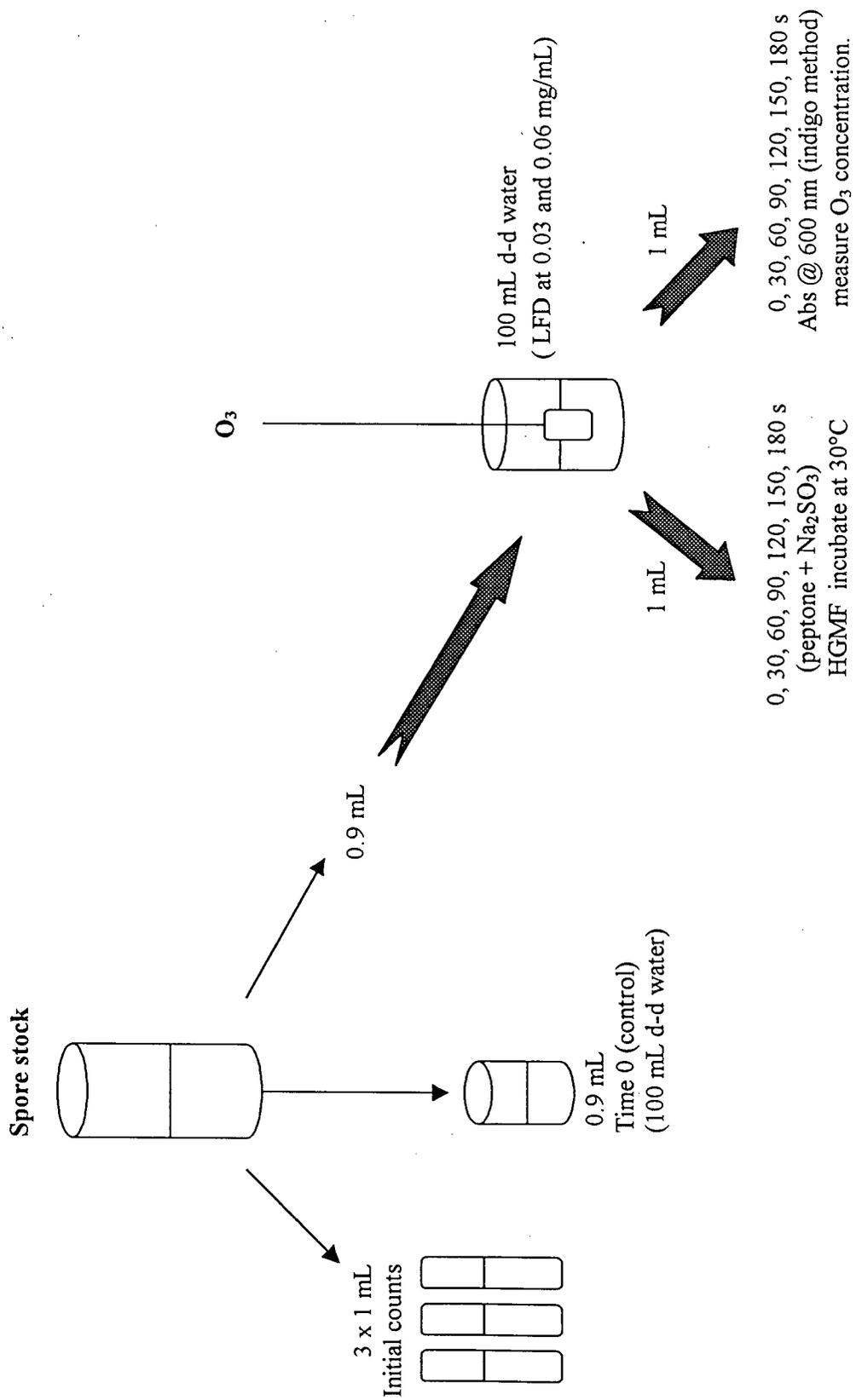


Figure 5b. Flow chart describing the procedure for the second experimental set up.

I. Ozone measurements

At the same time 1 mL samples were being taken to determine the number of surviving spores exposed to ozone, 1 mL samples were taken to measure ozone concentration (Bader and Hoigne, 1982). The sample (1 mL) was carefully added to 1 mL of indigo trisulfonate solution, followed by 8 mL of ozone demand free water. These 10 mL suspensions were vortexed and filtered using a syringe fitted with a 0.45 μ m filter (Millipore Products Division, Bedford, MA). The filter removed any suspended spores without removing the indigo trisulfonate solution. Absorbance was read at 600 nm with a Shimadzu UV-Visible (UV-160) spectrophotometer (Kyoto, Japan).

The ozone concentration was calculated from the absorbance difference (Δ Abs) between blank and sample, using the following equation (Bader and Hoigne, 1982):

$$\text{Ozone concentration (mg O}_3\text{/L)} = (\Delta\text{Abs} \times 10) / (f \times b \times v)$$

b = the path length of the cuvette (cm)

v = volume of the sample added (mL)

f = slope of calibration curve at 600 nm [$0.42 \pm 0.1 \text{ cm}^{-1}$ per mg/L, with ϵ (molar absorptivity) = $20,000 \text{ M}^{-1} \cdot \text{cm}^{-1}$].

A problem was encountered while measuring the ozone concentration in samples that had LFD. When 1 mL of the sample was added to the indigo solution, a reaction occurred changing the colour of the solution from indigo blue to a turquoise blue. After adding 8 mL of ozone demand free water, the solution colour remained very different from the other sample solutions (ozonated water with no LFD). When filtration of the 10 mL solution was attempted, the 0.45 μ filter absorbed the indigo dye (turning blue in

colour) indicating that somehow the peptide (LFD) had reacted with the indigo solution to form a complex that was retained by the filter. Therefore, ozone measurements could not be taken for samples containing LFD. The same test was performed using 1 mL of indigo solution and 1 mL of d-d water. Again, 8 mL were added and the 10 mL solution was filtered as described above. There was no absorption of the indigo dye by the filter.

Nunez (1998) recommended filtering the microbial sample from the indigo solution prior to measuring the ozone concentration. In order to measure if there was any interference of the fungal spores with the indigo solution, 1 mL of the spore suspension was added to 1 mL of the indigo trisulfonate solution. A volume of 8 mL of ozone demand free water was added and the 10 mL vortexed. Spectrophotometric measurements were taken before and after filtration of the indigo solution. There was very little change in absorption values (before and after filtration) indicating that there was basically no interference of the spores with the indigo dye. However, as a precautionary step, the samples were filtered in order to remove the spores from the indigo solution. This step would avoid errors in determination of ozone concentrations by any turbidity created by the spores that could cause the absorbance of the solution to increase leading to an underestimation of ozone concentration.

J. Effect of ozone and lactoferricin

First experimental set up:

LFD (0.03, 0.06 mg/mL) was added to 35 mL of spore suspension (3×10^6 spores mL⁻¹). After 60 min, 1 mL sample was taken (prior to adding the 65 mL of ozonated water), serially diluted in 9 mL of 0.1% peptone containing 15 mg/L of sodium sulphite and plated

in duplicate on PDTB agar using the ISO-GRID HGMF. Plates were incubated at 30°C for a period of 4 to 7 days. Once the ozonated water was added, samples were withdrawn in the same manner as described in the section on ozone generation.

Second experimental set up:

LFD (0.06 mg/mL) was added to 100 mL of d-d water containing 0.9 mL of spore suspension (3×10^4 spores mL⁻¹). A 1 mL sample was taken after 60 min (prior to direct ozonation) and diluted and plated as previously described. After the 60 min the stainless steel ozone aerator was introduced in the beaker in order to ozonate the sample solution. Immediately a foam was formed which prevented continued ozonation of the solution.

Microscopic observation of treated spores:

Another experiment was carried out in order to observe the changes or effect of LFD (0.30 mg/mL) and ozone on spores of *Penicillium* spp. Because of the foaming capacity of LFD, the first experimental set up (previously described) was followed with some differences, including the LFD concentration which was now 0.30 mg/mL and samples were retrieved every 30 seconds for a period of 3 min instead of every 4 min. Slides were taken from each of the PDTB plates containing the HGMF filters, where each grid-cell measures 1.25 by 1.25 mm.. The equipment used was a Wild Leitz (MP552) dissecting photomicroscope (Herbrugg, Switzerland) with a Tungsten 160T professional colour slide film. Photographs were taken from different dilution plates (10^{-4} and 10^{-3}) at 12x magnification.

K. Test of spore sensitivity to sodium sulphite

In order to study the effect of sodium sulphite on fungal spores, a sensitivity test (Restiano et al., 1995; Domingue et al., 1988) was performed using the same starting population of spores which were serially diluted using either 0.1% peptone or 0.1 % peptone containing 15 mg/L of Na_2SO_3 . Both samples were plated on PDTB and incubated at 30°C. Counts were made after 48 h. The germination and outgrowth of fungal spores was not affected by the presence of Na_2SO_3 .

L. Preparation of Glassware

All the glassware used for the ozone experiments was washed with tap water and soap, followed by soaking overnight in a 2% FL-70 detergent solution (Fisher Scientific). The glassware was then rinsed thoroughly with tap water, followed by a final rinse with distilled-deionized water. Clean glassware was filled with distilled-deionized water and placed in a plastic pail. The pail had already been filled with distilled-deionized water allowing the glassware to be submerged. The glassware was ozonated for 20 min at an ozone residual level of at least 2.0 ppm. Once ozonated, the glassware was removed, wrapped in aluminum foil and sterilized by dry heat at 180°C for 7 hr. This procedure ensured that no oxidizable residuals remained on the glassware (Domingue et al., 1988).

M. Preparation of water

Distilled-deionized water was used for all the experiments. For the preparation of the indigo trisulfonate stock solutions and for ozone measurement purposes, the water was rendered ozone demand free. This was done by ozonating the water for 20 min, until

residual levels of 1.0 ppm were obtained, followed by boiling the water for one hour in order to remove residual ozone. Once the ozone demand free water was cooled, spectrophotometric measurements at 600 nm with indigo trisulfonate were performed to make sure there was no trace of ozone left in the water (Domingue et al., 1988).

IV. RESULTS AND DISCUSSION

A. Mould identification

The observations made with the microscope (Figure 6) indicated morphology similar to the description, given by Pitt and Hocking (1985), for the genera *Penicillium* spp. This genera is characterized by having conidia in a structure termed a *penicillus* (Latin, little brush) which consists of a well defined cluster of phialides bearing chains of small, single-celled, conidia. These phialides are either attached to a stipe directly or through one or more stages of branching. The *Penicillium* taxonomy is not easy, and as recommended by Pitt and Hocking (1985) the identification can be accomplished by growing isolated colonies under standardized conditions of growth medium and incubation temperature. The specialized media (CYA, MEA and G25N) used in this experiment allowed fruiting structures and colony colours to be easily identified with the specified key given by Pitt and Hocking (1985). The colony diameters were 18-22 mm in diameter on G25N medium within 7 days of incubation at 25°C. According to Pitt and Hocking (1985) nearly all the commonly encountered species in *Penicillium* characteristically grow to 18 mm or more in diameter on G25N at 25°C in 7 days and present a coloration that ranges from shades of blue, green and/or gray (refer to Appendix I).

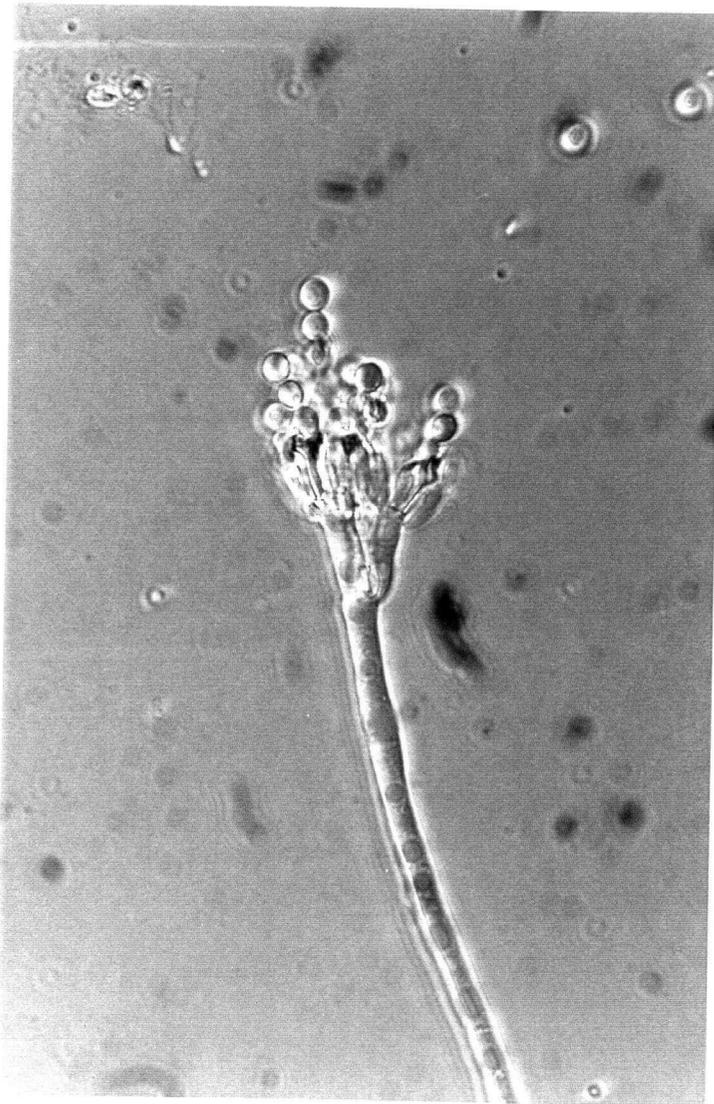


Figure 6. Microscopic observations of fungi isolated from bottled water (100 x).

B. Lactoferrin digestion

After 4 hours at 37°C in the presence of porcine pepsin, the band corresponding to intact lactoferrin could no longer be observed by SDS-PAGE (Figure 7 and Appendix III). The SDS-PAGE profile of the reaction products (Figure 7, lane 2) indicates the presence of peptides in the hydrolysate with a molecular weight below 6,000 Da. The peptide corresponding to the major band is likely lactoferricin, having an approximate molecular weight of 3,162 Da as calculated by the Rf value. The original protein preparation was not 100% pure lactoferrin, as indicated by the presence of various bands (lane 5), however we decided to use it as the startup material for production of lactoferricin to avoid increasing its cost from purification methods. By using the lactoferrin digest without further purification, it will be more economically viable for the industry.

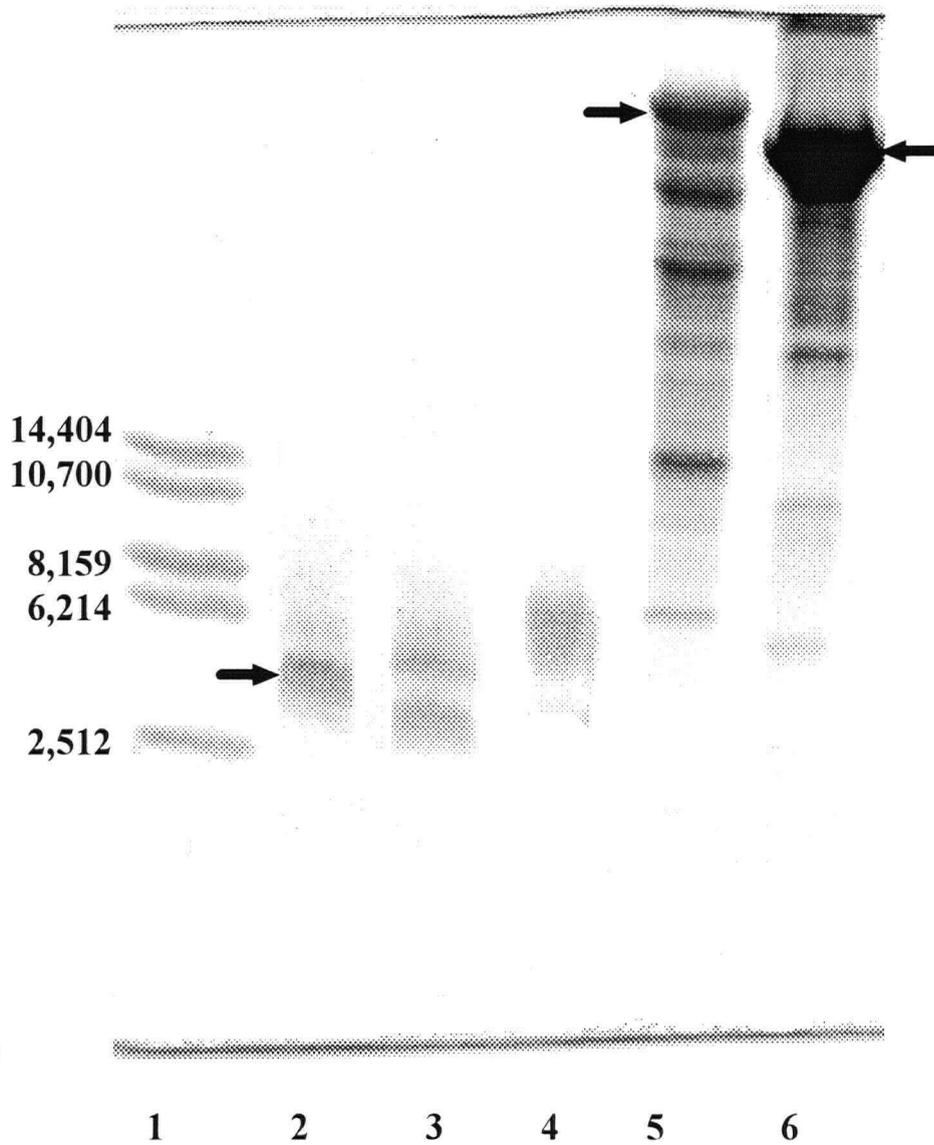


Figure 7. SDS-PAGE profiles on a high density gel of LFD (lane 2), BSAD (lane 3), lactoferrin (lane 5), and BSA (lane 6). Lane 1 contains low range (peptide) molecular weight markers (Sigma Chemicals), while lane 4 contains fish protein hydrolysate (not used in this study). Arrows in lanes 2, 5 and 6 indicate bands assigned to lactoferricin, lactoferrin and BSA, respectively, based on molecular weight.

C. Antimicrobial assay

LFD at a concentration of 0.30 mg/mL, inhibited fungal growth for a period of at least 504 hours or 21 days (Figure 8). At this time growth began to develop in 2 of the 9 wells in the microplate. The wells containing BSAD at the same concentration had no inhibition effect as shown in Figure 8 where the mould began to grow after 36 hours, indicated by the significant increase in optical density. The wells containing LFD remained stable, as did the controls without spores containing PYG medium, LFD and BSAD (data not shown).

The ANOVA results indicated significant difference between wells containing LFD and BSAD, as well as between LFD and the spore control ($p < 0.05$). There was no significant difference between wells containing BSAD and the spore control ($p > 0.05$) and there was no significant difference in wells containing LFD over time ($p > 0.05$), indicating complete inhibition of fungal growth in the presence of LFD.

The inhibition effect using a concentration of 0.06 mg/mL of LFD was inferior to the effect of LFD at a concentration of 0.30 mg/mL (Figure 9). LFD at 0.06 mg/mL inhibited mould growth for only 9 days (216 hours). Nevertheless, the wells containing LFD presented an inhibitory effect when compared to the wells with BSAD at the same concentration as well as the spore control. Both, BSAD and spore control were significantly different ($p < 0.05$) from the LFD after 36 hours. Again, the BSAD and spore control were not significantly different ($p > 0.05$) from each other. Wells containing LFD presented significant growth ($p < 0.05$) by 336 hours.

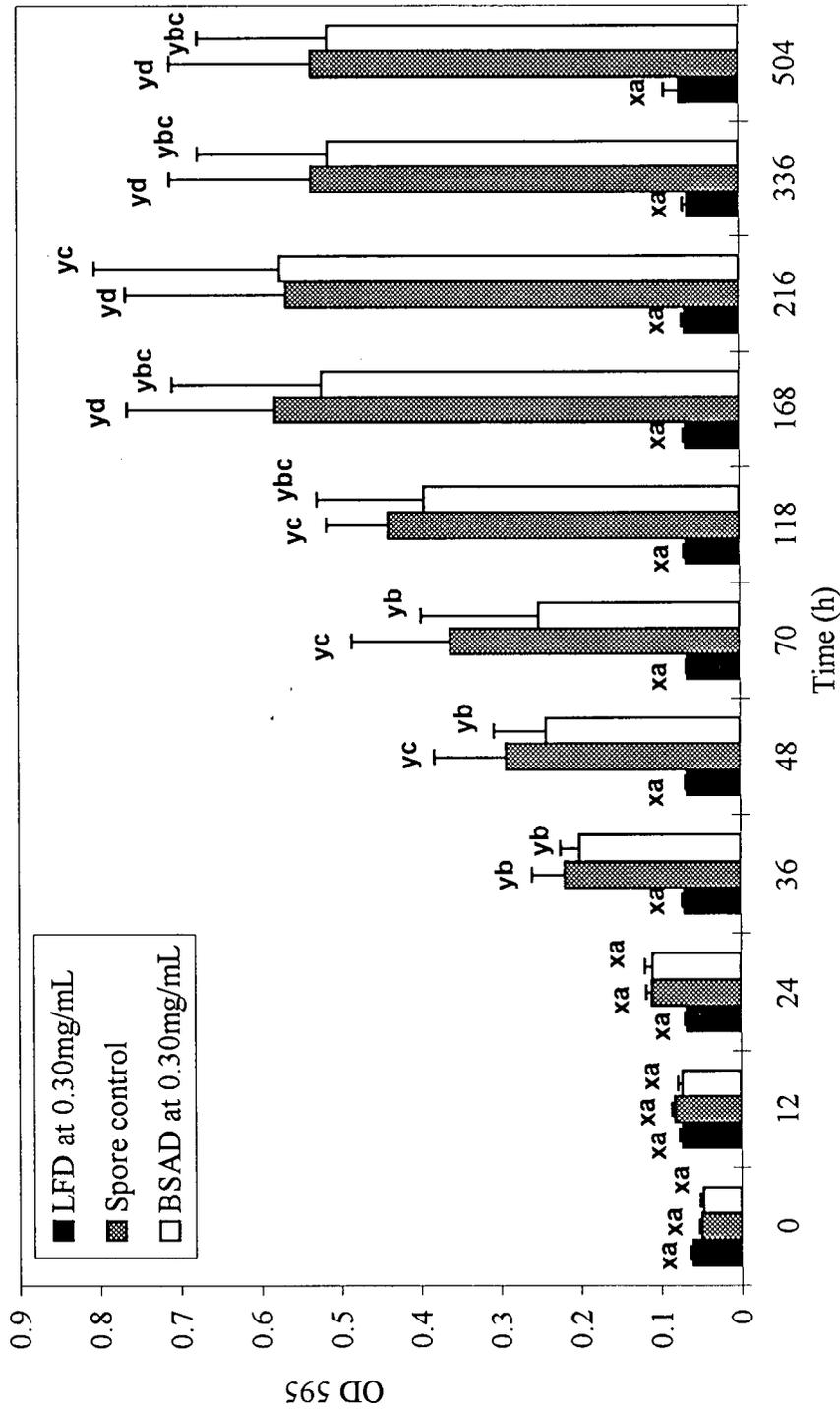


Figure 8. Optical density (OD595) of spores (3×10^4 /mL) treated with 0.30 mg/mL lactoferricin (LFD), in black bars; 0.30 mg/mL bovine serum albumin digest (BSAD), in white bars; and spores with no treatment, in gray bars. Spores were incubated in PYG medium at 30°C over time.

x, y bars not sharing the same letters are significant differences ($p < 0.05$) between samples at a given time. a,b,c,d bars not sharing the same letters are significant differences ($p < 0.05$) within each sample at different times.

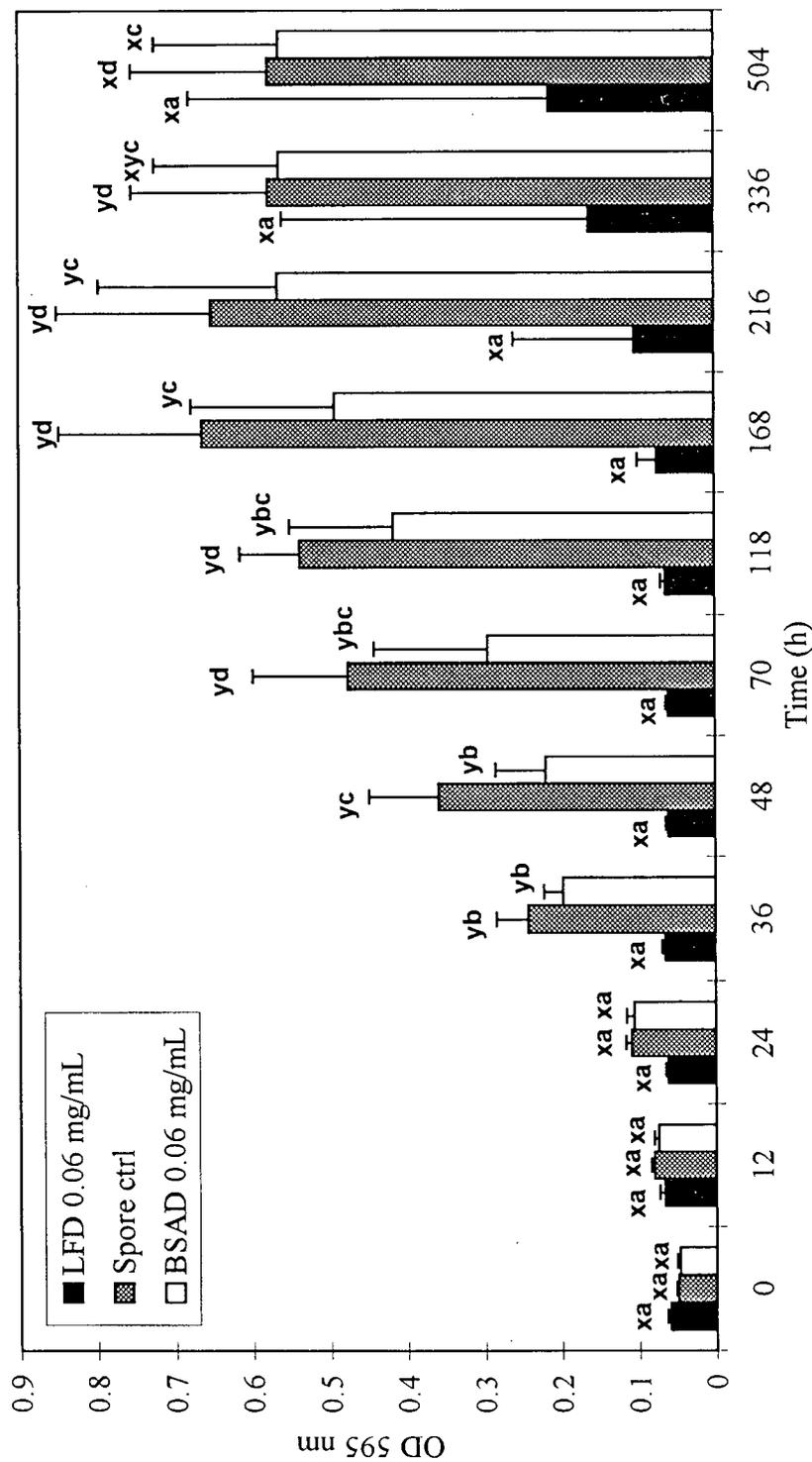


Figure 9. Optical density (OD595) of spores (3×10^4 /mL) treated with 0.06 mg/mL lactoferricin (LFD), in black bars; 0.06 mg/mL bovine serum albumin digest (BSAD), in white bars; and spores with no treatment, in gray bars. Spores were incubated in PYG medium at 30°C over time.

x, y bars not sharing the same letters are significant differences ($p < 0.05$) between samples at a given time.

a,b,c,d bars not sharing the same letters are significant differences ($p < 0.05$) within each sample at different times.

D. Ozone experiments

The reason for using two experimental set ups was in order to use a spore population high enough to see a decrease in the viable spore population. Therefore, an initial count of 10^6 spores/mL was thought to be enough to see the population gradually decline. After examining the results from the first experiment the decision was made to work with a lower initial population of 10^4 spores/mL. Also, in order to more closely resemble the actual set up used in the ozonation process that takes place in the bottled water industry, the water along with the spore suspension was ozonated directly rather than adding pre-ozonated water to the sample.

FIRST EXPERIMENTAL SET UP

In this set up, 1 L of d-d water was ozonated for approximately 5 min until a concentration of about 2 ppm was reached. Once ozonated to the required concentration, 65 mL were poured into a beaker containing 35 mL of the spore suspension (10^6 spores/mL). The total working volume was now 100 mL and the ozone concentration dropped to a range between 0.1-0.4 ppm as measured by the indigo method (Bader and Hoigne, 1982). Once the ozonated water had been added, the spore suspension lost its characteristic green colour, becoming a clear solution. This observation indicated the powerful decolourizing effect that has been attributed to ozone.

Because of the known fact that fungal spores tend to be more resistant to ozone than other microorganisms (Restiano et al., 1995) measurements were not taken every 30 seconds, but instead every 4 min. The observations on the HGMF plates indicated a strange behaviour of the spores. At 4, 8 and 12 min after exposure to ozone, the mycelia

began to develop more rapidly on the HGMF than the control (non ozonated spores). As shown on Figure 10, the fungi were at a later stage of growth. They had most probably germinated, the mycelia elongated and formed new spores. It seemed that something was happening to the spores when in contact with ozone at concentrations ranging from 0.1-0.4 ppm. The results from Tables 1, 2 and 3 indicate that ozone at concentrations ranging from 0.09 to 0.4 ppm did not decrease the logarithmic count (Log MPNGU/mL) over a period of 12 minutes.

There is hardly any literature on the subject of stimulation of fungal growth by ozone. There are only a few references that date to the 1940s and are mentioned below.

In 1948, Kuss concluded that ozone may be used to induce earlier and increased sporulation of certain fungi. The author also refers to work by Schomer and McCollock (1948) where in an effort to establish the recommended use of ozone in apple storage, the authors noted that aggregation of spores (clumping) prevented ozone from penetrating the cells, while very dilute ozone concentrations had an inhibitory and even lethal effect on microorganisms if they occurred singly and unprotected. The work by Kuss (1948) and Schomer and McCollock (1948) agrees with the observations in the present study that ozone may act as a stimulant rather than as an inhibitor to certain fungi.

In a brief note by Richards (1949) it was reported that certain fungi which produced few or no spores in culture responded in a stimulatory manner when exposed to ozone. *Mycosphaerella citrulina* produced conidia in greater numbers and in less time than the control cultures. Also, sporulation was increased in *Alternaria* spp. with no impairment of germination. Another study by Harding (1968) indicated that ozone at 1.0 ppm was insufficient to have any significant effect on blue or green *Penicillium* spp. In

that study, ozone was introduced in the atmosphere of storage rooms that contained lemons and oranges in open wooden storage boxes and fiberboard cartons.

Rich and Tomlinson (1968) reported that sporulating cultures of conidiophores of *Alternaria sonali* began to germinate while still attached to the conidiophores when exposed to 1 ppm of ozone for 30 min. They suggest that ozone may stimulate germination of attached conidia either by destroying a germination inhibitor or by altering the permeability of the cell wall to gases.

Hibben and Stotzky (1969) reported that detached spores of 14 fungi varied in germination after exposure on agar to 10-100 p.p.hm. (parts per hundred million) ozone for 1-6 h. They worked with spores in agar, spores in liquid and dried spores. Spores in agar were divided into 3 groups based on their reaction to ozone: insensitive, moderately sensitive and most sensitive. They noticed that low concentrations of ozone sometimes stimulated germination, especially spores of *Trichodeerma viride*, *Aspergillus terreus*, *Penicillium egyptiacum*, *Rhizopus stolonifer* and *Verticillium dahliae*. Apparently, the toxic effects of ozone were related to the amount of ozone in contact with the spores. A low concentration for a longer time appeared to be about as efficient in decreasing germination as a shorter exposure to a higher concentration.

For spores in liquid medium, ozone at 50 p.p.hm. did not decrease germination for *Penicillium egyptiacum* and *Aspergillus terreus* and in fact, germination of *P. egyptiacum* appeared to be stimulated. The authors mentioned that the most resistant spores were large, pigmented and multicellular and that the thick walls of the larger spores may have decreased the penetration of ozone. Hibben and Stotzky (1969) made observations (in the

stimulatory effect of ozone on germination of some spores) similar to those made by Kuss (1948) and Rich and Tomlinson (1968).

In the present study, when LFD was added to the 35 mL spore suspension, the mixture was allowed to stand for 60 min before the 65 mL of ozonated water was poured. The reason for allowing the LFD to “stand” for 60 min was based on the work by Bellamy et al. (1992a) and Soukka et al. (1992) where the fungi were incubated with LFD for 60-75 min prior to plating.

The results from the LFD control (prior to adding ozonated water) showed no decrease in the logarithmic count (Table 4 and Table 5). This observation appears to be contradictory with the results obtained in the antimicrobial assay. However, here a much higher spore population (10^6 spores mL^{-1} versus 10^4 spores mL^{-1}) was used and the spores were in contact with the LFD for only 60 min after which time the LFD was most probably washed away during filtration through the HGMF. The 60 min contact time prior to HGMF filtration did not lower the population of viable spores. Hence, it appears that constant contact with LFD inhibited spore germination in the antimicrobial assay where the inhibition was seen after 36 h which was the time when the spores began to grow on the BSAD and spore control (Figures 8 and 9). Because of the interference of LFD with the indigo trisulfonate dye it was not possible to measure the ozone concentration at which the spores were exposed at the time of the experiment; nevertheless, it was apparent that LFD in combination with ozone did in fact slow the rate at which the mould colonies began to develop on the HGMF filter (see following results on microscopy observations).

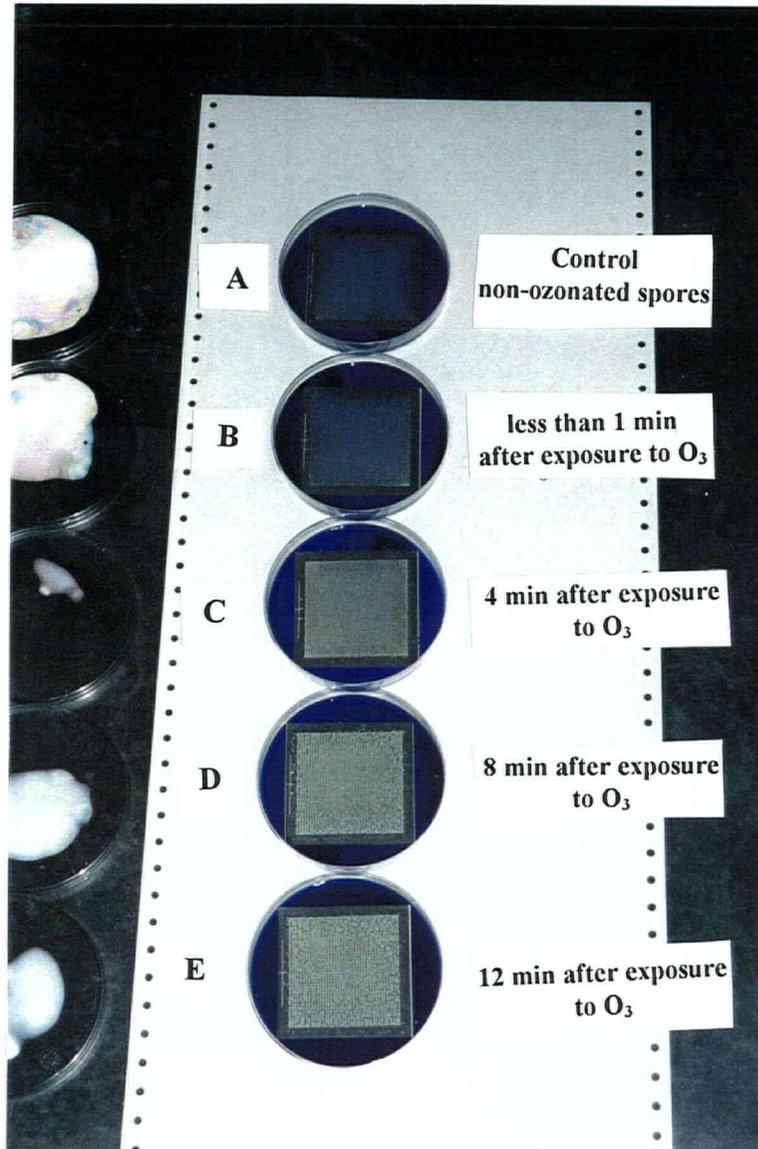


Figure 10. Photograph showing the growth stimulation of *Penicillium* spp. exposed to 0.1 ppm ozone at 25°C. The HGMF filters were plated on PDTB and incubated at 30°C for a period of 48 hours. **A,B** filters show the characteristic growth of blue colonies caused by the trypan blue dye. (**A**, no spore formation; **B**, initial formation of fruiting structures). **C,D,E** filters reveal the stimulation of colony growth, indicated by the increased formation of green conidia as a result of longer contact of spores with ozonated water prior to plating on PDTB.

Table 1. Effect of 65 mL of ozonated distilled-deionized water added to 35 mL of spore suspension (3×10^6 /mL) of *Penicillium* spp. (Trial 1).

time (min)	Log	
	MPNGU/mL ¹	Ozone (ppm)
0	5.8	0.13
4	5.8	0.09
8	5.6	0.02
12	5.6	0.02

¹Spores seem to be stimulated to grow faster on the HGMF filter over time of exposure to ozone. Observations were made after 48 h of incubation at 30°C.

Table 2. Effect of 65 mL of ozonated distilled-deionized water added to 35 mL of spore suspension (3×10^6 /mL) of *Penicillium* spp. (Trial 2).

time (min)	Log	
	MPNGU/mL ¹	Ozone (ppm)
0	5.8	0.09
4	6.5	0.05
8	5.8	0.07
12	5.8	0.02

¹Spores seem to be stimulated to grow faster on the HGFMF filter over time of exposure to ozone. Observations were made at 48 h of incubation at 30°C.

Table 3. Effect of 65 mL of ozonated distilled-deionized water added to 35 mL of spore suspension (3×10^6 /mL) of *Penicillium* spp. (Trial 3).

time (min)	Log MPNGU/mL ¹	Ozone (ppm)
0	5.9	0.40
4	6.2	0.36
8	6.2	0.34
12	6.5	0.26

¹Spores seem to be stimulated to grow faster on the HGMF filter over time of exposure to ozone. Observations were made at 48 h of incubation at 30°C.

Table 4. Effect of 65 mL of ozonated distilled-deionized added to 35 mL of spore suspension (3×10^6 /mL) of *Penicillium* spp. with 0.03 mg/mL LFD.

time (min)	Log MPNGU/mL	Ozone ^a (ppm)
0	6.3 ¹	NM
4	6.0 ²	NM
8	6.3 ²	NM
12	6.3 ²	NM

^aOzone could not be measured due to absorption of the indigo dye by the LFD.

¹Full covered grid-cell.

²LFD seemed to slow the development of colonies on the HGMF filter. The colonies on each grid-cell appear as pin points barely elongating.

Observations were made at 48 h of incubation at 30°C.

NM = not measured

Table 5. Effect of 65 mL of ozonated distilled-deionized water added to 35 mL of spore suspension (3×10^6 /mL) of *Penicillium* spp. with 0.06 mg/mL LFD.

time (min)	Log MPNGU/mL	Ozone ^a (ppm)
0	5.9 ¹	NM
4	5.6 ²	NM
8	5.6 ²	NM
12	5.8 ²	NM

^aOzone could not be measured due to absorption of the indigo dye by the LFD.

¹Full covered grid-cell.

²LFD seemed to slow the development of colonies on the HGFM filter. The colonies on each grid-cell appear as pin points barely elongating.

Observations were made at 48 h of incubation at 30°C.

NM = not measured

SECOND EXPERIMENTAL SET UP

By ozonating directly, the experiment was more similar to the ozonation process that takes place in the bottled water industry. This time, the spore population used was similar to the population used in the antimicrobial assay (3×10^4 spores/mL). In order to observe any rapid decline in spore population the samples were taken every 30 s for a period of 3 minutes.

When the initial ozone concentration was 0.24 and 0.47 ppm (Figures 11 and 12) the viable spore count remained constant over 180 seconds (3 min), indicating once more that the ozone concentrations established for the experiments (0.1-0.4 ppm) were not sufficient to kill the spores. In Figure 13, the logarithmic count of the ozonated spore suspension decreased 4 log cycles within 3 minutes of exposure to 1.71 ppm of ozone. A lower ozone concentration of 0.62 ppm (Figure 14) was enough to cause about 2 log cycle decrease of the initial spore count. These results indicate that at least 0.6 ppm of ozone will be required for lowering the spore count. However, as reported by White et al. (1991) the levels of ozone stipulated by the FDA for use in production of bottled water should not be above 0.4 ppm. In the study performed by White et al. (1991) levels above 0.3 ppm increased the plastic off-taste of water due to low molecular weight compounds formed through ozonation (oxidation) of the high density polyethylene (HDPE) bottles used in that study; these compounds are believed to be a mixture of oxygenated hydrocarbons which tend to migrate from the plastic into the water.

It seems that there should be a time interval that would allow the ozone to diffuse through the water in order to penetrate the microbial cell. Watson (1942), while working with fungal spores, reported that as the ozone concentration increased, the speed at which

the spores were killed was not increased proportionally. Moreover, as the ozone concentration decreased, the time of treatment increased more than the calculated value of concentration (ppm) and time (hours). Watson (1942) concluded that there is a minimum concentration below which the killing of the spores will not occur regardless of the duration of the treatment.

Another important observation occurred when introducing the ozone diffuser to the spore suspension containing LFD (0.06 mg/mL). As soon as the diffuser entered in contact with the water a foam was immediately created, impeding ozone diffusion through the suspension and the diffuser had to be removed. Unless, there is a way to aseptically add the LFD after ozonating prior to capping, LFD cannot be used in water that is going to be ozonated due to its foam capacity and foam stability (data not shown). Nevertheless, in Figure 15 there was a one log cycle decrease of the spore population within three minutes, yet it is difficult to say if the desired ozone concentration had been reached due to the interference with the foam and the inability to conduct ozone concentration measurements by the indigo trisulfonate method.

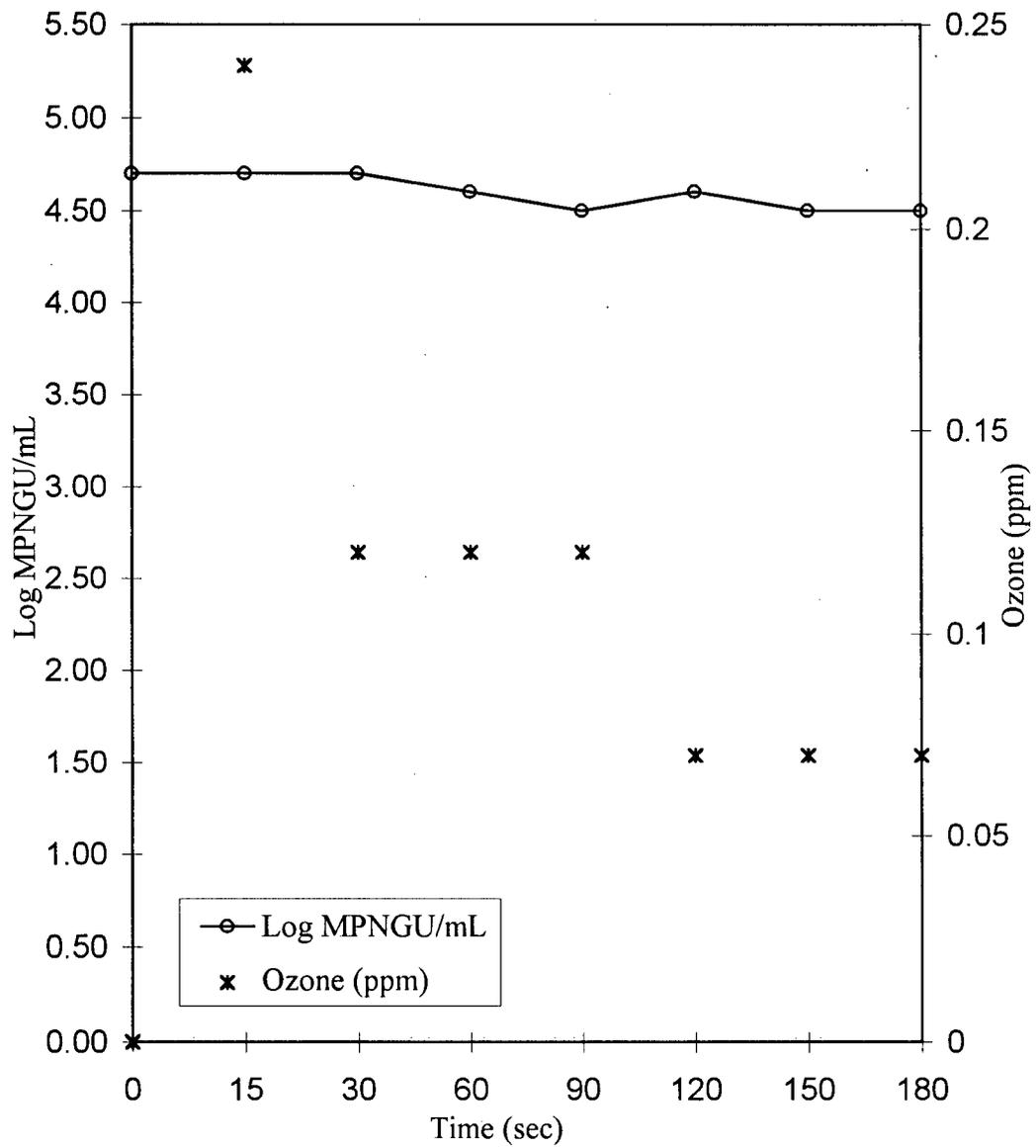


Figure 11. Effect of 0.24 ppm initial ozone concentration on spore suspension of *Penicillium* spp. The experiment was carried out at 25°C.

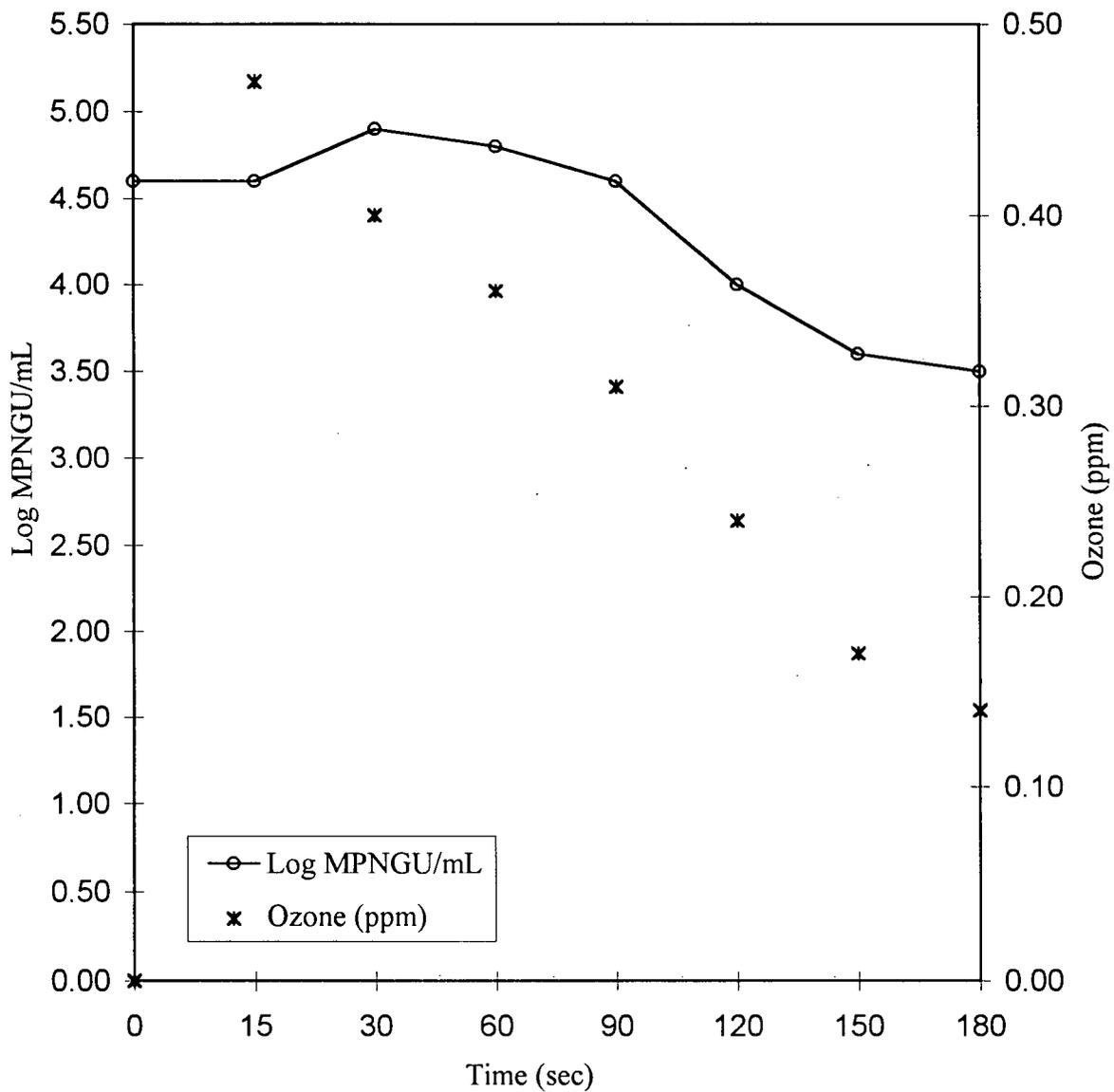


Figure 12. Effect of 0.47 ppm initial ozone concentration on spore suspension of *Penicillium* spp. The experiment was carried out at 25°C.

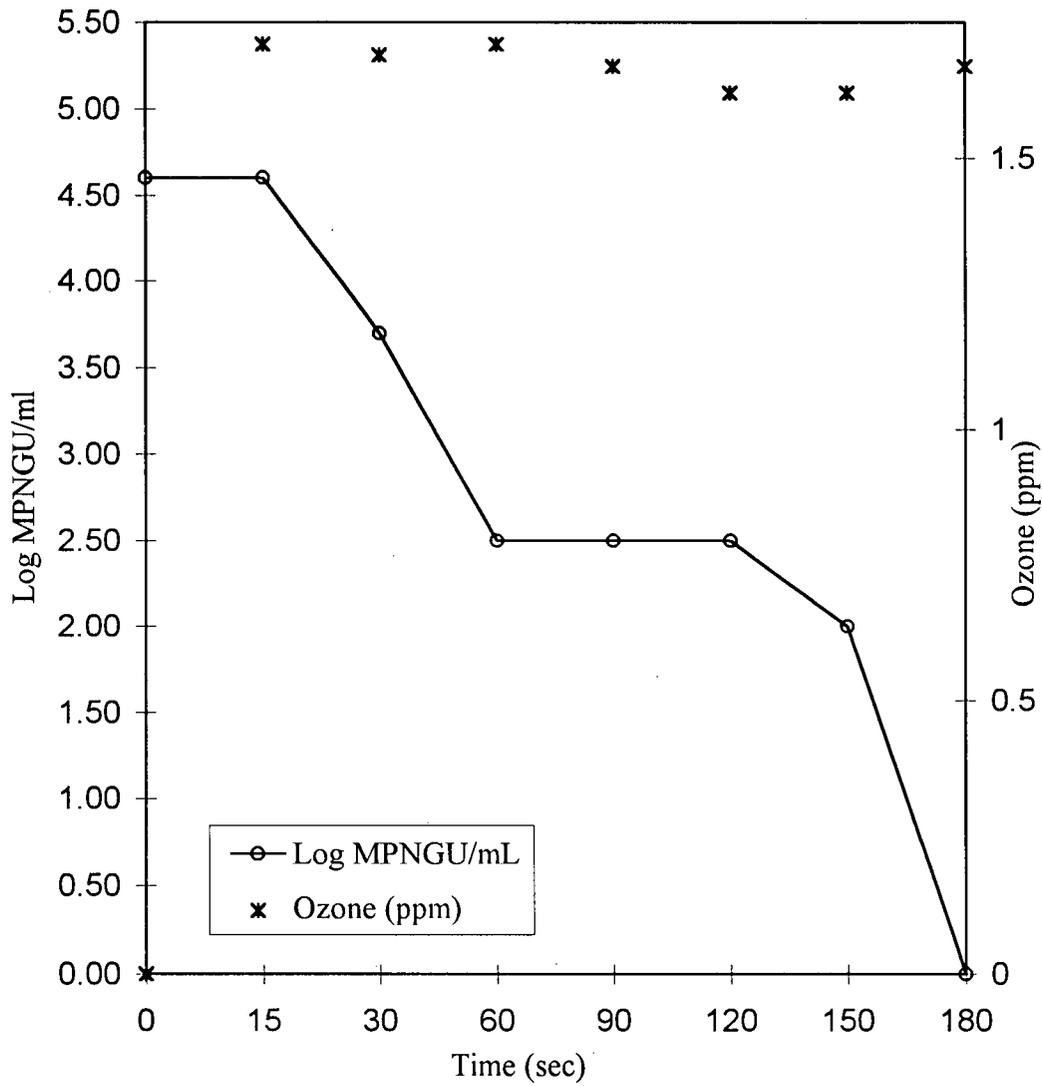


Figure 13. Effect of 1.71 ppm initial ozone concentration on spore suspension of *Penicillium* spp. The experiment was carried out at 25°C.

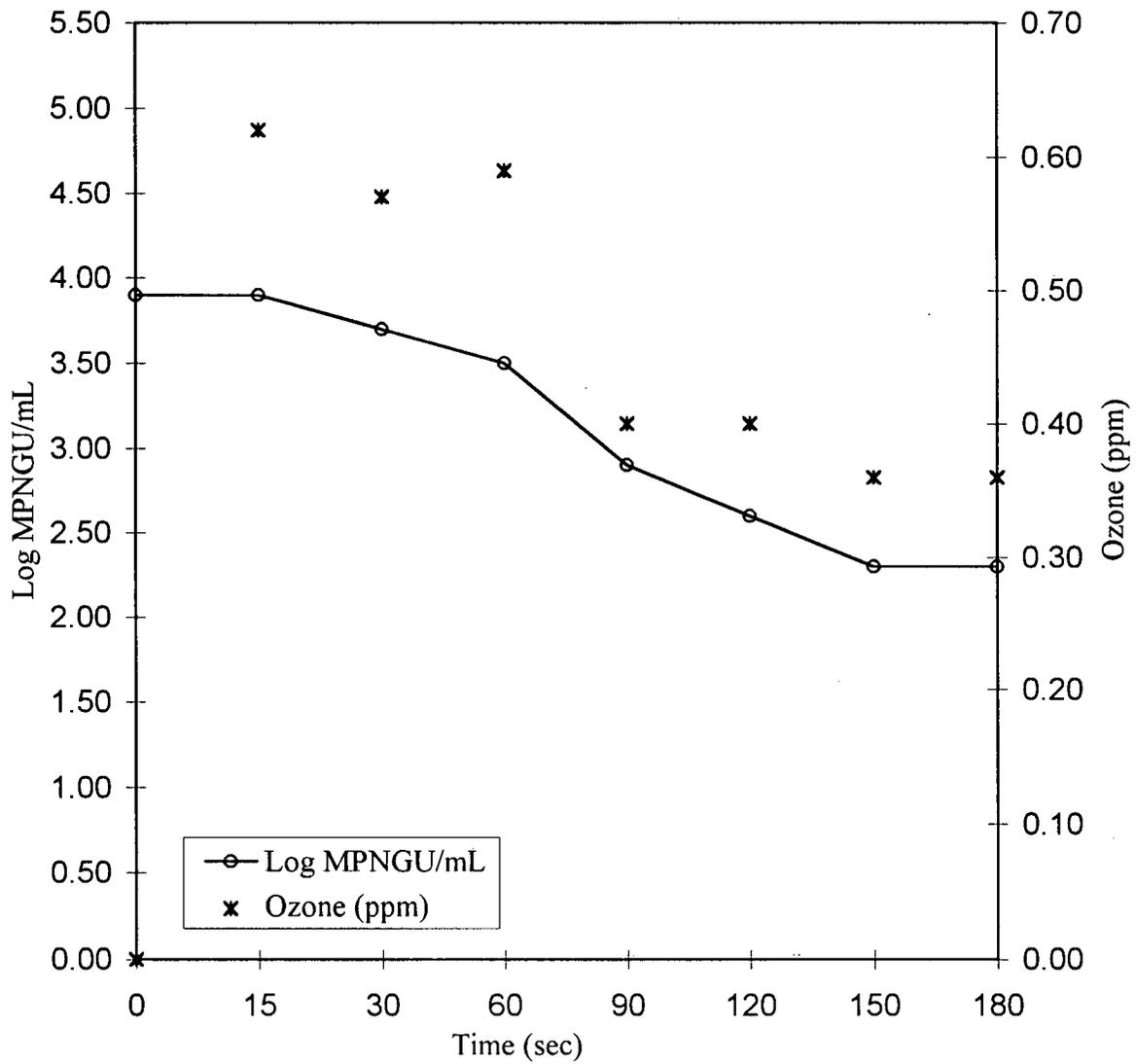


Figure 14. Effect of 0.62 ppm initial ozone concentration on spore suspension of *Penicillium* spp. The experiment was carried out at 25°C.

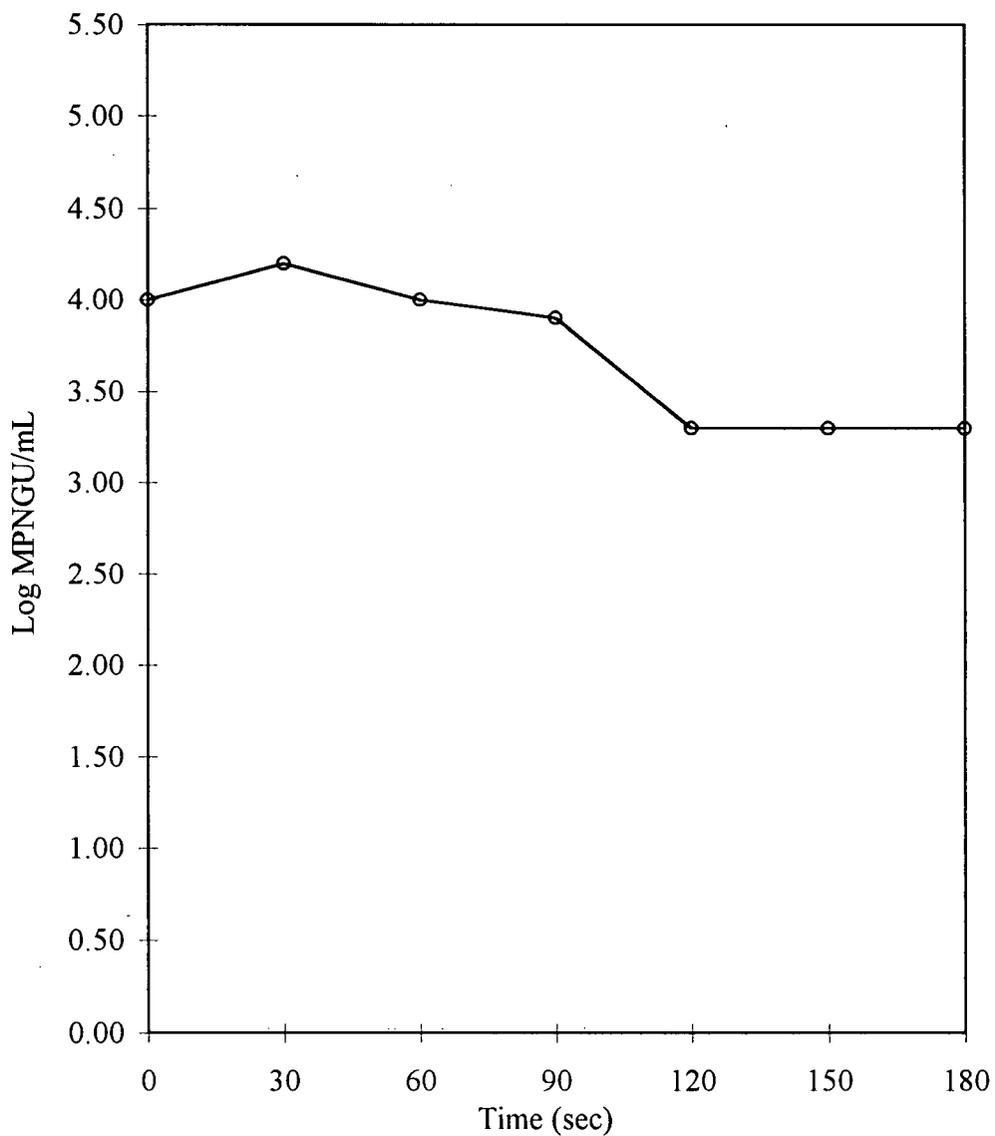


Figure 15. Effect of ozone and LFD (0.06 mg/mL) on spore suspension of *Penicillium* spp. The experiment was carried out at 25°C. Ozone concentration could not be measured due to interference of LFD with the indigo trisulfonate dye.

MICROSCOPY OBSERVATIONS ON TREATED SPORES

Another experiment was carried out in order to observe the effect of both LFD and ozone on spores of *Penicillium* spp. This time, the concentration of LFD was higher (0.30 mg/mL). The first experimental set up was followed because of the foaming properties of LFD. Non treated spores (control) germinated and produced colonies on the HGMF filter within 72 h of incubation at 30°C (Figure 16). There is more than one colony per grid-cell at the 10^{-3} dilution. Figure 17 represents colonies from spores treated for 60 min with LFD (0.30 mg/mL). In this figure the grid-cells appear less covered than the grid-cells in Figure 16; however, there was also more than one colony per grid-cell. In Figures 18 and 19, at 15 and 30 sec after the ozonated water was added to the spore suspension containing LFD, the results indicate some initial stimulation of the spores because the colonies appear bigger, almost covering the whole grid-cell compared to the control (Figure 16). This could indicate some of the stimulation effect discussed before (seen in Figure 10) where treatment of *Penicillium* spores with ozone at low concentrations appeared to accelerate the development of colonies on the HGMF grid-cells.

It was not until one min after the treatment of the spores with LFD and ozone that the colony size was decreased. Figures 20 to 24 show the grid-cells on the filters with very small colonies, pin point in size; particularly at 2 and 3 min after the ozonated water was added. These observations demonstrate that somehow the combination of LFD (0.30 mg/mL) and ozone slowed down the rate at which the colonies appeared on the grid-cells of the HGMF filter, either by delaying the time of onset of spore germination and/or by slowing down the rate of mycelium fusion produced from the germinated spores.

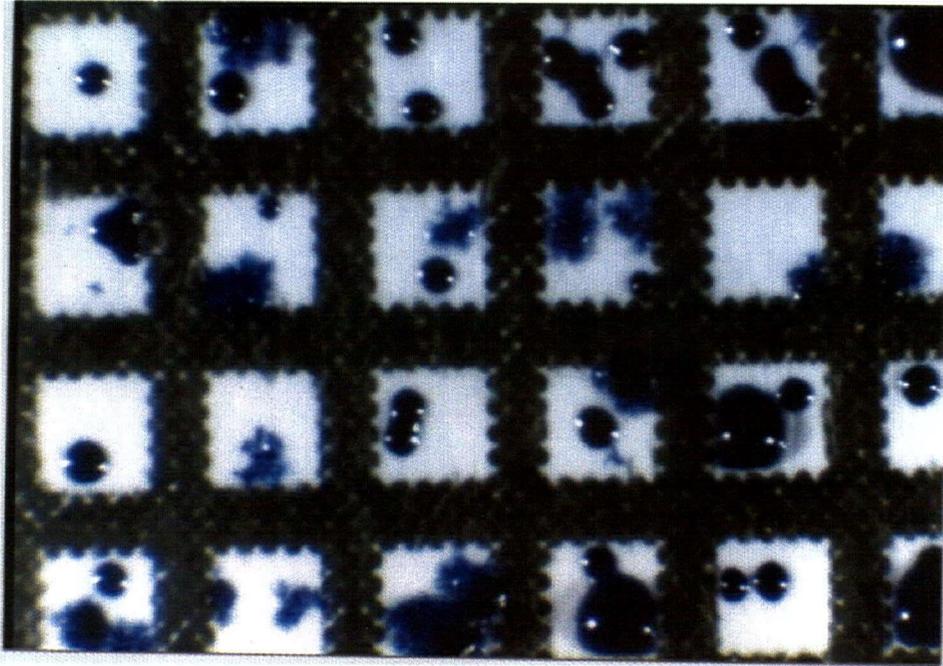


Figure 16. Control (non treated) spores. Observations on 10^{-3} dilution filter are after 72 h incubation at 30°C.

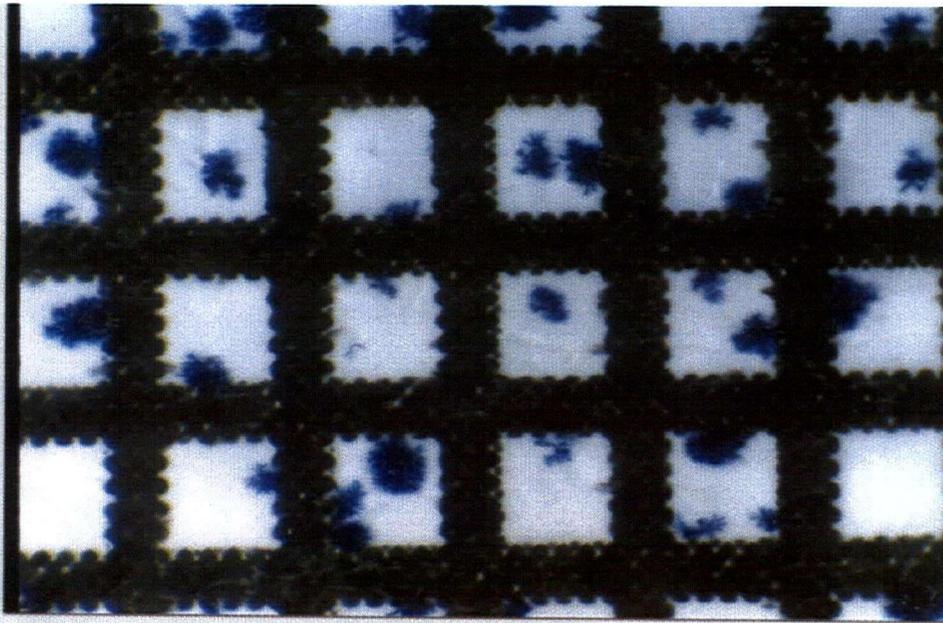


Figure 17. Effect of LFD (0.30 mg/mL) after 60 min incubation at room temperature. Observations on 10^{-3} dilution filter are after 72 h incubation at 30°C.

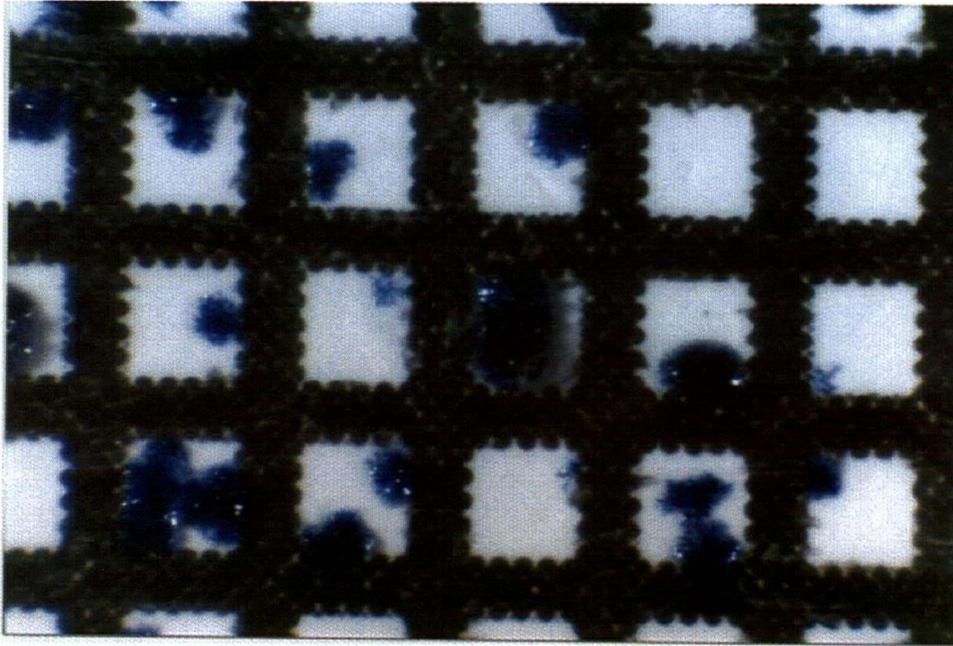


Figure 18. Effect of ozone and LFD (0.30 mg/mL) 15 seconds after addition of ozonated water. Observations are on 10^{-3} dilution filter after 72 h incubation at 30°C.

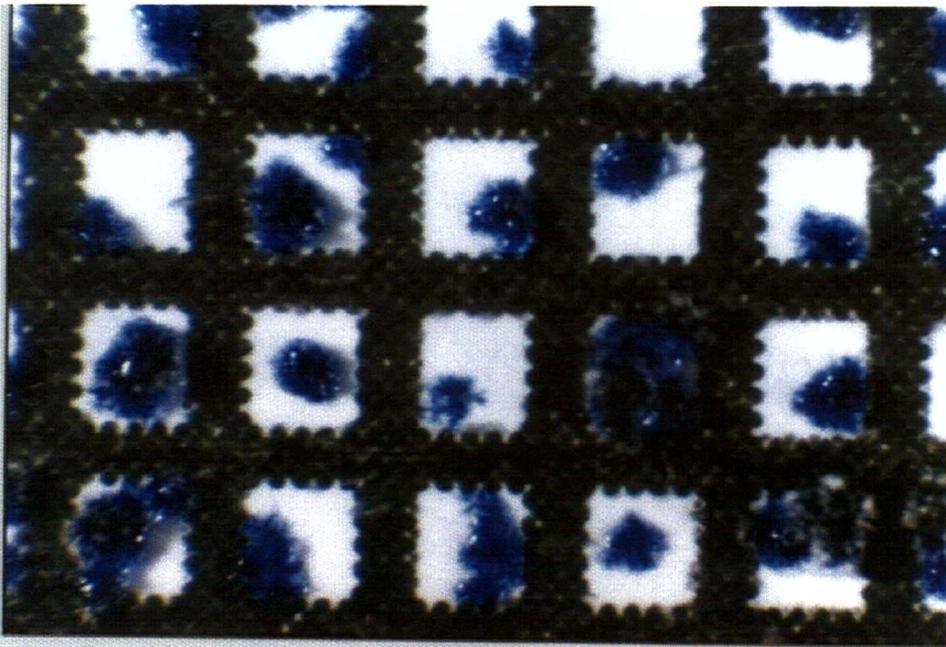


Figure 19. Effect of ozone and LFD (0.30 mg/mL) 30 seconds after addition of ozonated water. Observations are on 10^{-3} dilution filter after 72 h incubation at 30°C.

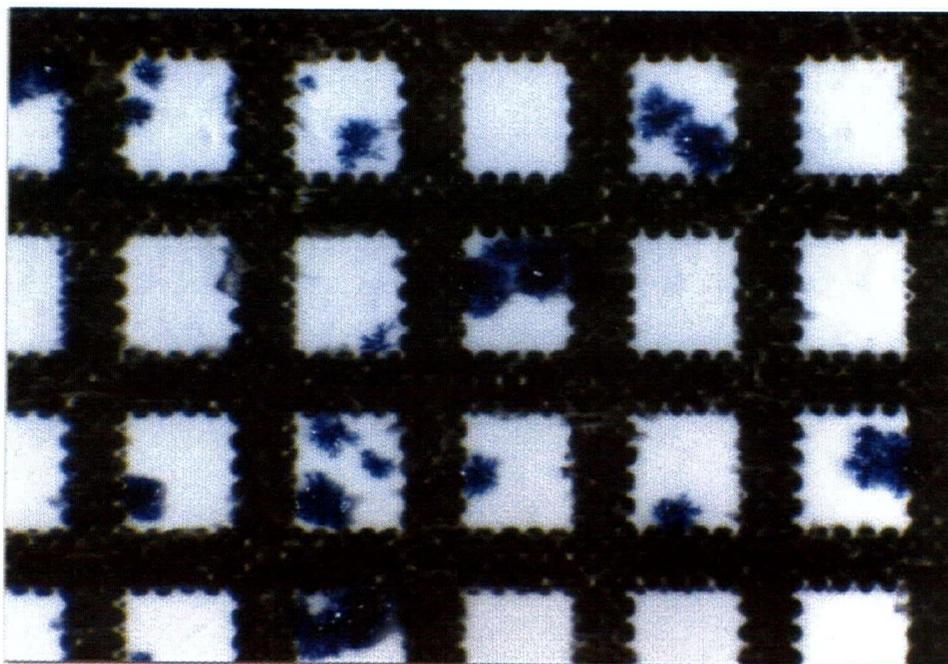


Figure 20. Effect of ozone and LFD (0.30 mg/mL) 1 min after addition of ozonated water. Observations are on 10^{-3} dilution filter after 72 h incubation at 30°C.

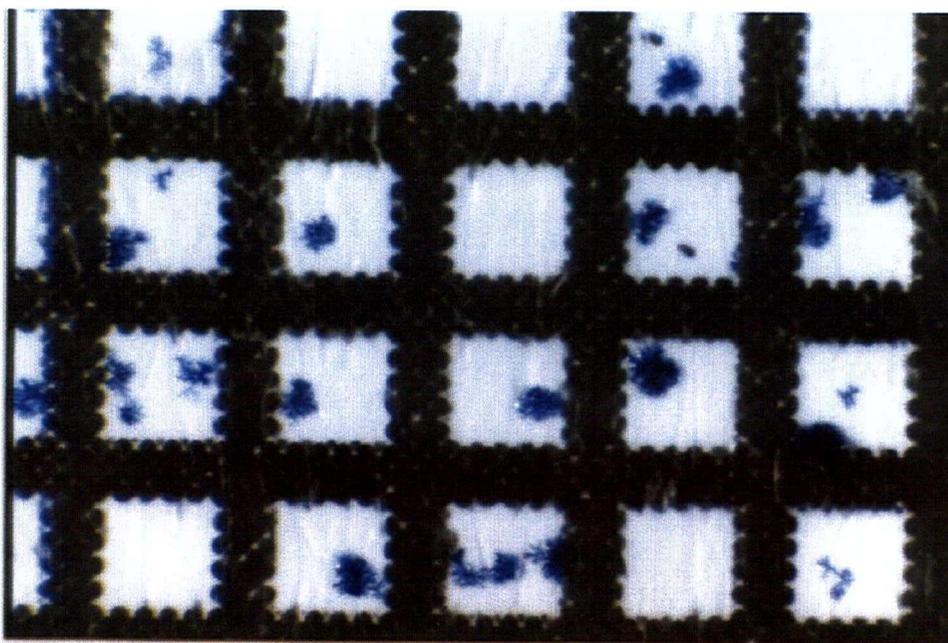


Figure 21. Effect of ozone and LFD (0.30 mg/mL) 1.5 min after addition of ozonated water. Observations are on 10^{-3} dilution filter after 72 h incubation at 30°C.

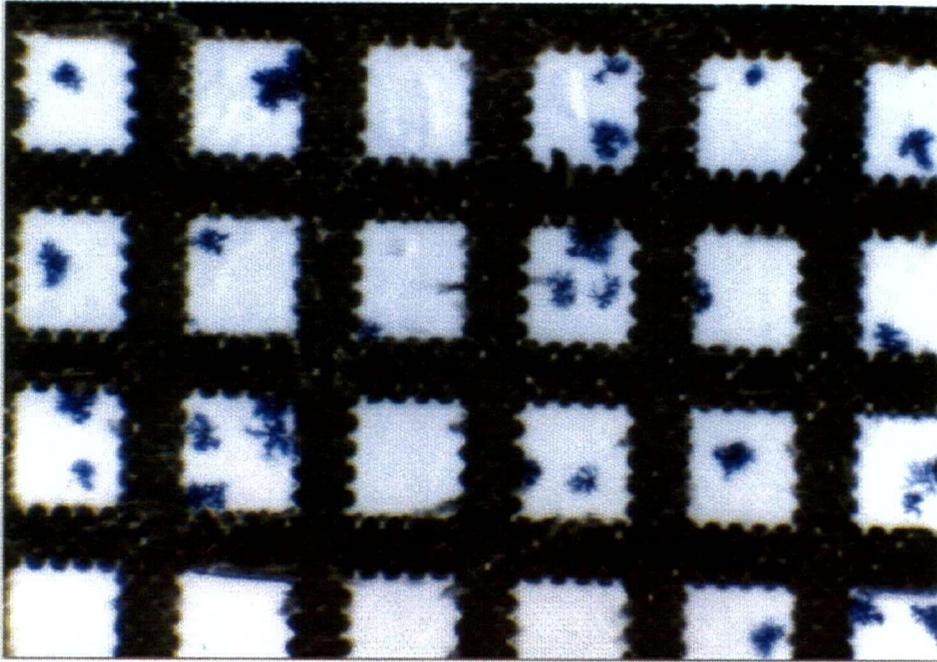


Figure 22. Effect of ozone and LFD (0.30 mg/mL) 2 min after addition of ozonated water. Observations are on 10^{-3} dilution filter after 72 h incubation at 30°C.

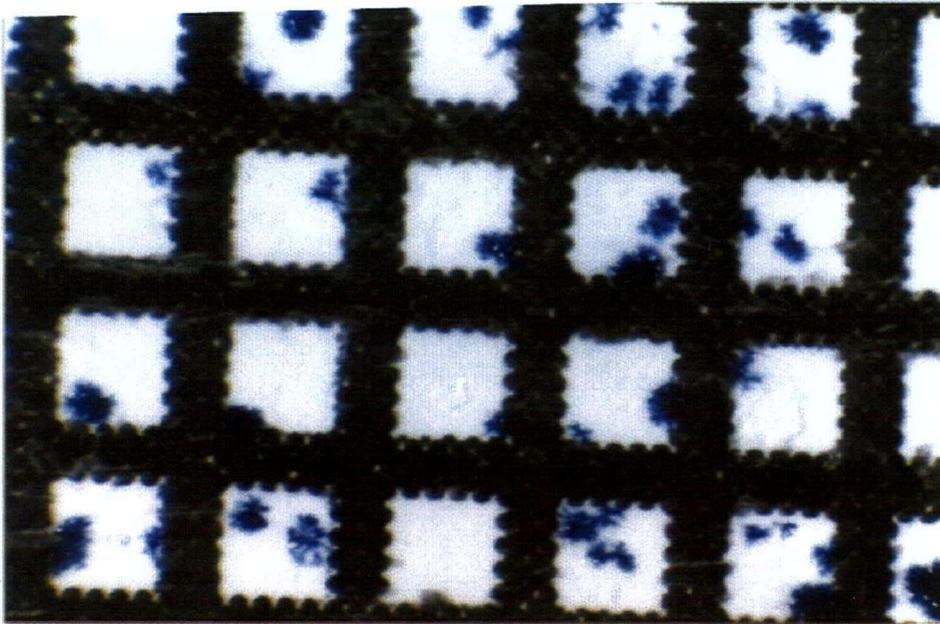


Figure 23. Effect of ozone and LFD (0.30 mg/mL) 3 min after addition of ozonated water. Observations are on 10^{-3} dilution filter after 72 h incubation at 30°C.

In order to determine how long the delayed spore germination time and/or mycelium fusion lasted, the plates were incubated at 30°C for a period of 8 days. At this time, photo-micrographs of the same HGMF filters were taken again. Figure 24 indicates the advanced growth stage of the colonies derived from the control (non treated) spores. The colonies were already sporulating and growing upwards (due to prevention of lateral spreading beyond the margins by the hydrophobic grid lines). The filter inoculated with spores treated only with LFD (Figure 25) had colonies that also began to spread or elongate as compared to Figure 17.

In Figures 26 to 28, the colonies have begun to slowly sporulate; however, colonies on those filters remained at a smaller size compared to the control (no treatment) spores, which implies a slower rate of growth. Figures 29 to 31 represent the treatment effect at 1.5, 2 and 3 min after the addition of ozonated water. In these treatments, the colonies on the grid-cells are smaller than those for LFD treated spores exposed to ozone for 15 s, 30 s and 1 min. The combination of ozone and LFD (0.30 mg/mL) was capable of preventing the stimulation effect that appeared for spores that were ozonated only with low ozone concentrations. The combination of the two treatments slowed the growth development of the mould colonies for up to 8 days as the colonies changed little since the observations made at 72 hours.

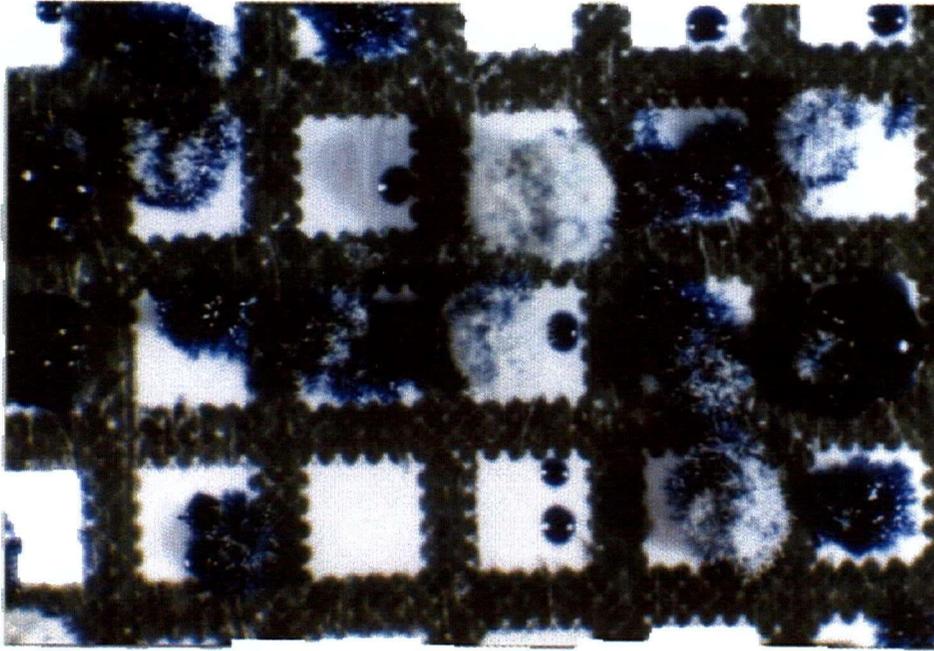


Figure 24. Control (non treated) spores. Observations on 10^{-3} dilution filter are after 8 days incubation at 30°C .

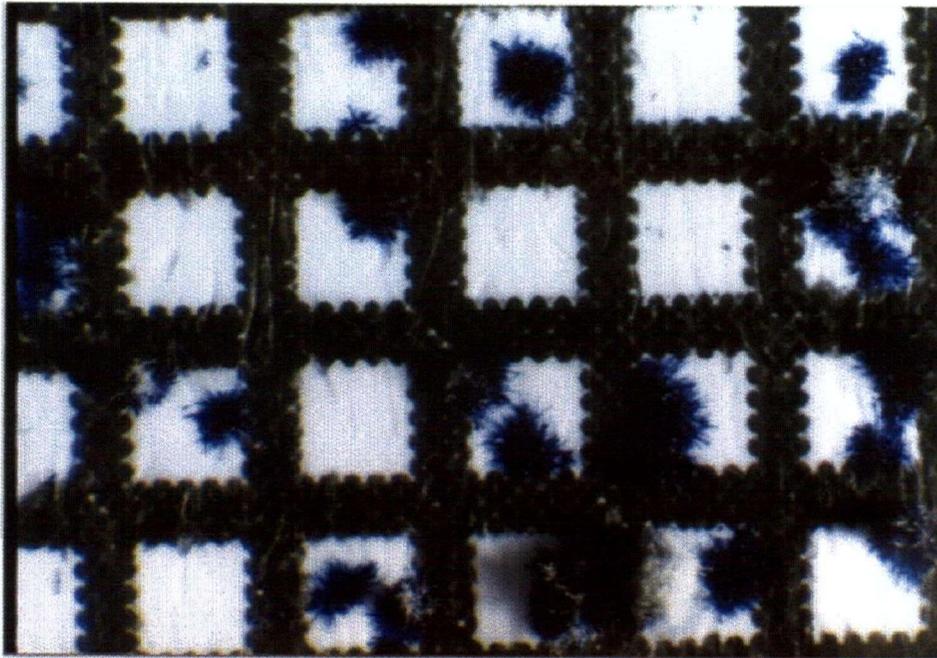


Figure 25. Effect of LFD (0.30 mg/mL) after 60 min incubation at room temperature. Observations are on 10^{-3} dilution filter after 8 days incubation at 30°C .

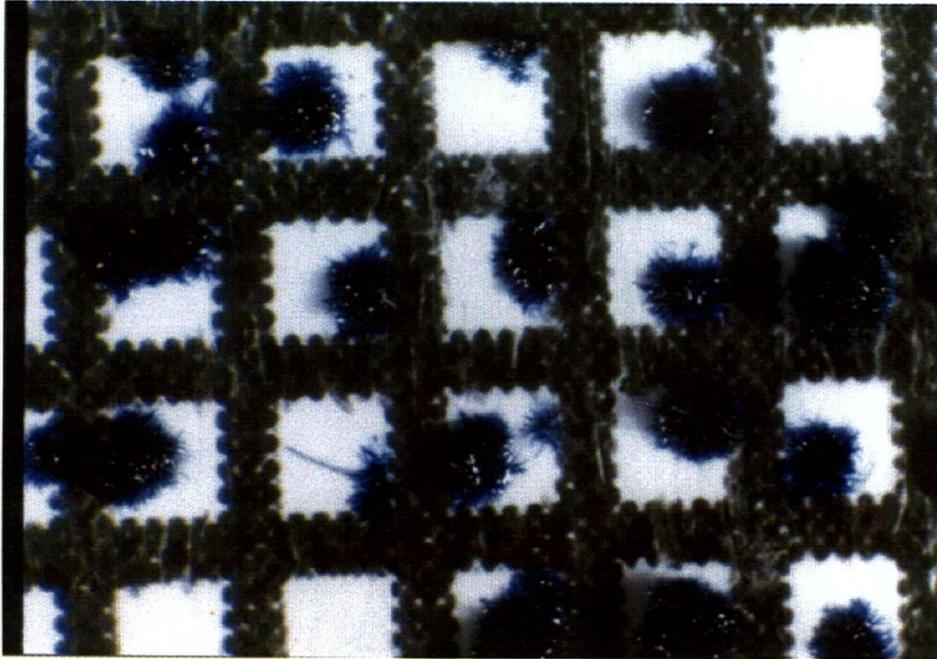


Figure 26. Effect of ozone and LFD (0.30 mg/mL) 15 seconds after addition of ozonated water. Observations are on 10^{-3} dilution filter after 8 days incubation at 30°C .

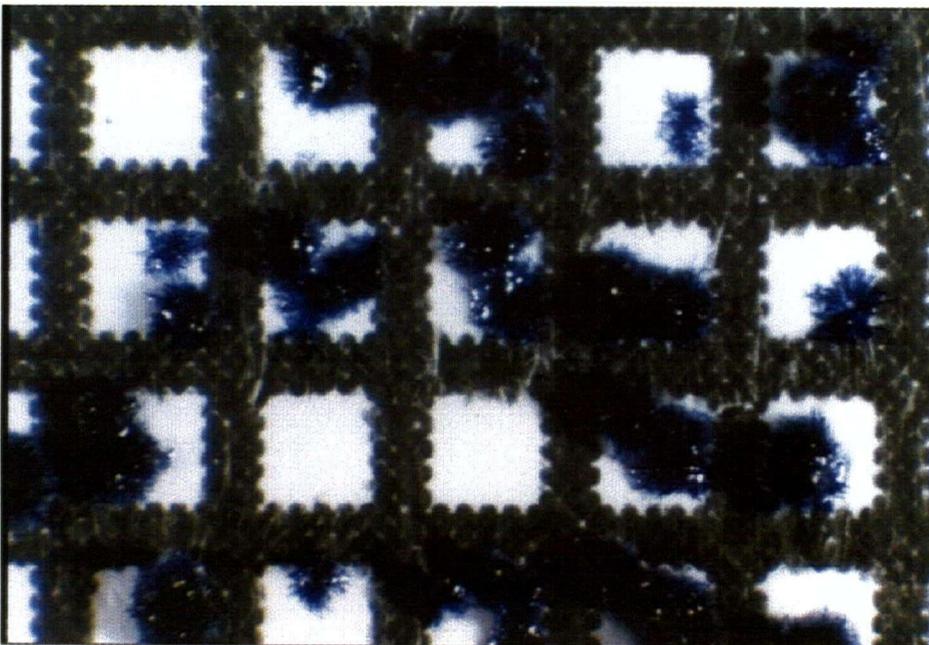


Figure 27. Effect of ozone and LFD (0.30 mg/mL) 30 seconds after addition of ozonated water. Observations are on 10^{-3} dilution filter after 8 days incubation at 30°C .

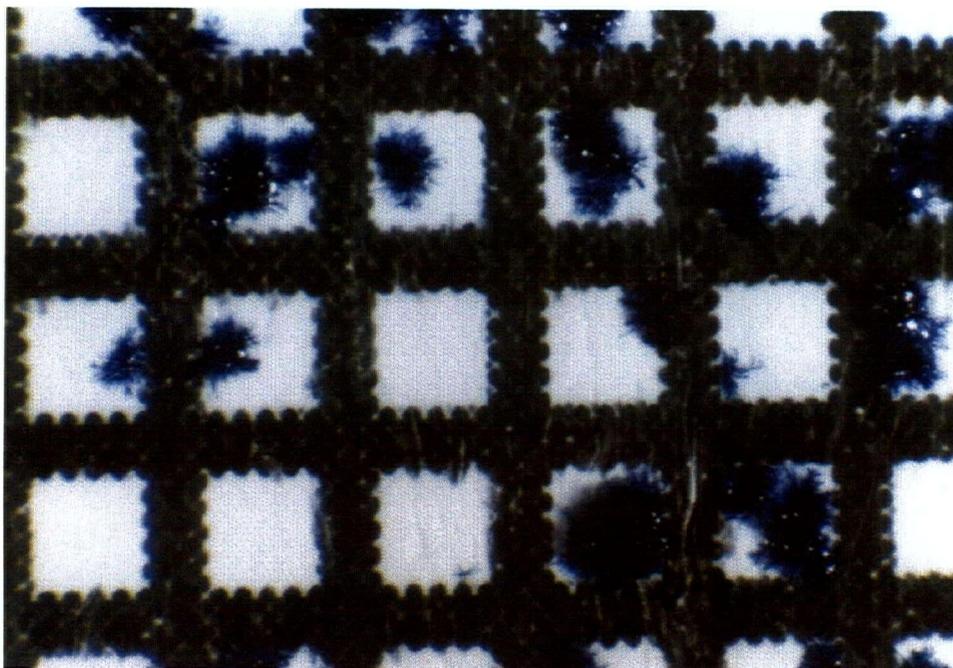


Figure 28. Effect of ozone and LFD (0.30 mg/mL) 1 min after addition of ozonated water. Observations are on 10^{-3} dilution filter after 8 days incubation at 30°C.

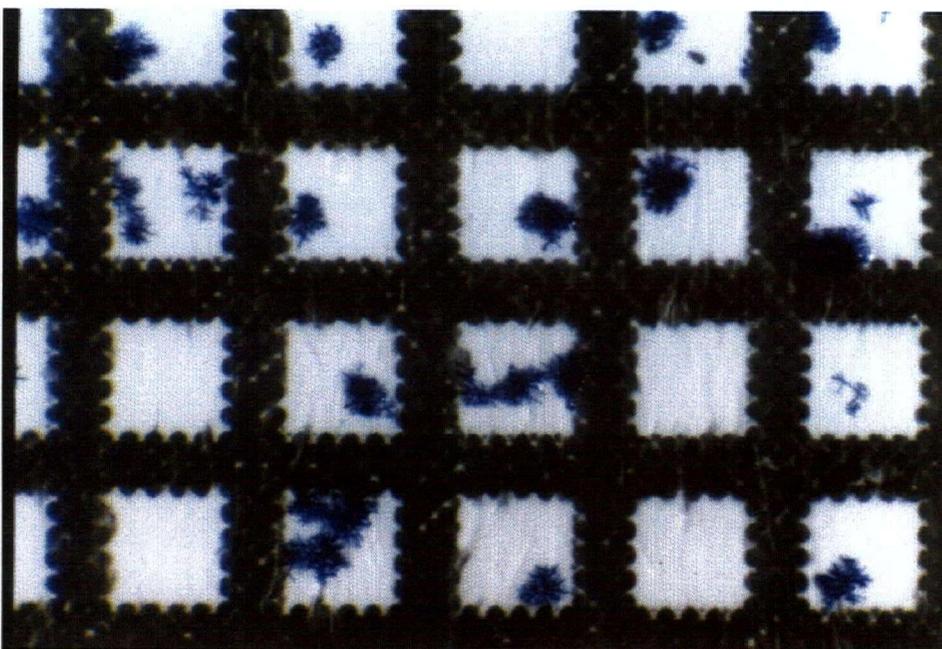


Figure 29. Effect of ozone and LFD (0.30 mg/mL) 1.5 min after addition of ozonated water. Observations are on 10^{-3} dilution filter after 8 days incubation at 30°C.

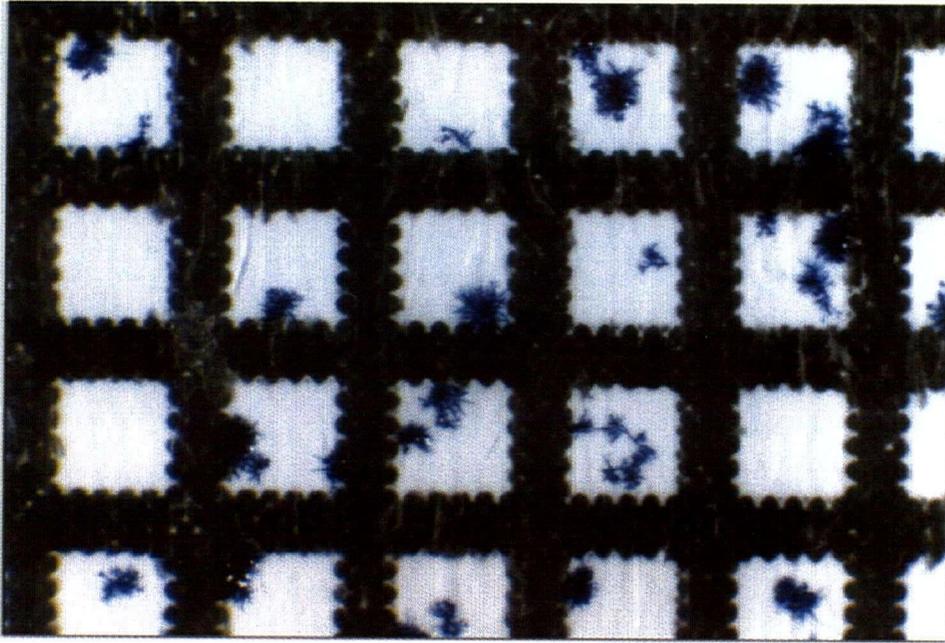


Figure 30. Effect of ozone and LFD (0.30 mg/mL) 2 min after addition of ozonated water. Observations are on 10^{-3} dilution filter after 8 days incubation at 30°C .

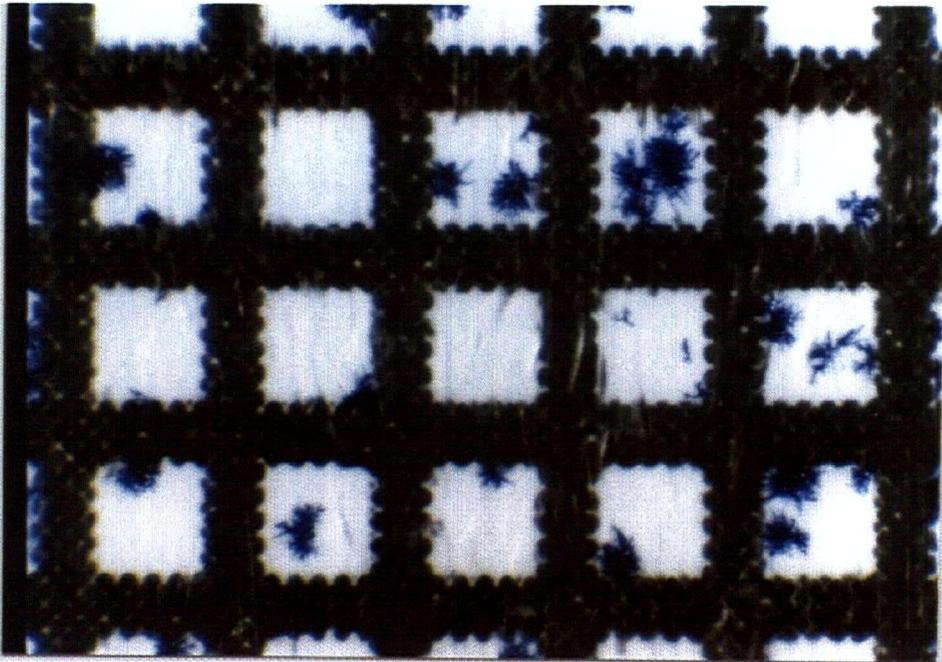


Figure 31. Effect of ozone and LFD (0.30 mg/mL) 3 min after addition of ozonated water. Observations are on 10^{-3} dilution filter after 8 days incubation at 30°C .

Finally, the photo micrographs taken from the 10^{-4} dilution filters, after 6 days incubation, proved once more that the control (no treatment) samples (Figure 32) did not prevent the colonies from developing on the HGMF filters. Figures 33 and 34 (30 sec and 1 min after adding ozonated water) and Figure 35 (1.5 min after adding ozonated water), show the advanced growth stage of the colonies, which are already forming spores in the grid-cells. It is at 2, 2.5 and 3 min after adding the ozonated water (Figure 36 to 38) that the growth rate was lowered leading to small pin point size colonies on each grid-cell. These observations indicate that during the first 1.0 min of exposure to ozone (in combination with LFD) the spores were not affected to an extent that was noticeable in colony development, and further more, they could have been stimulated to grow. It was not until 1.5 min after the treatments that combination of ozone and LFD was effective in lowering the rate at which the colonies were developing on the grid-cells, leading to development of small pin point colonies that had not began to sporulate, compared to the filters prepared from the control spores.

An important observation is that the number of viable spores was not decreased by the combination of LFD and ozone treatments. The impact of both treatments (especially after 1.5 min) was manifested by pin point colonies appearing after 72 h of incubation. Colony size of the treated spores did not increase to any great extent between 72 and 192 hours (8 days) at 30°C. This suggests that although spores germinated, the mycelium produced from those treated spores had impaired growth as a consequence of the combined LFD-ozone treatment of the spores.

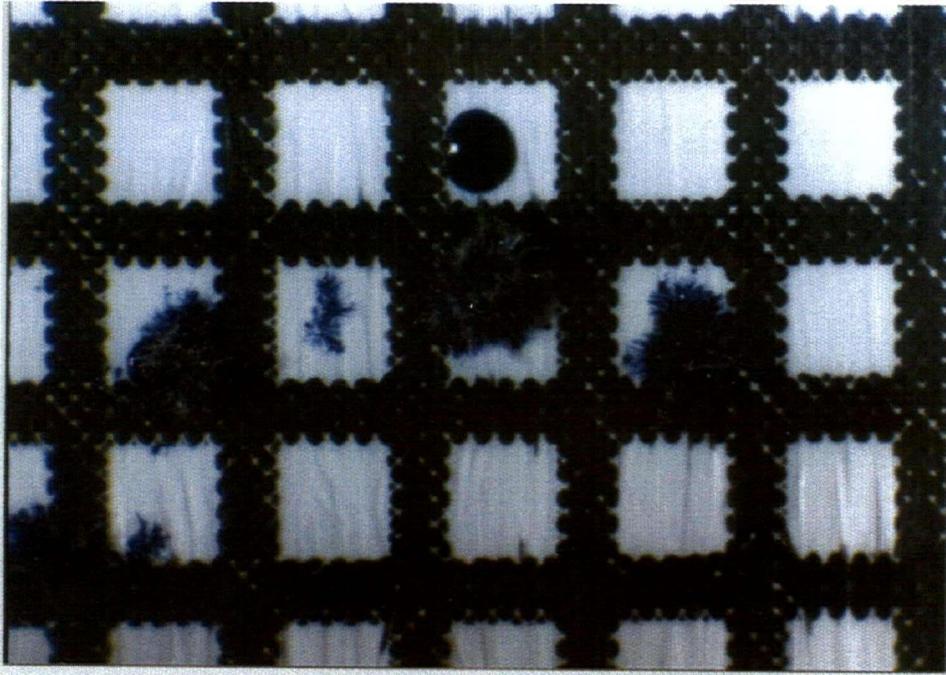


Figure 32. Control, no treatment of spores. Observations on 10^{-4} dilution filter are after 6 days incubation at 30°C .

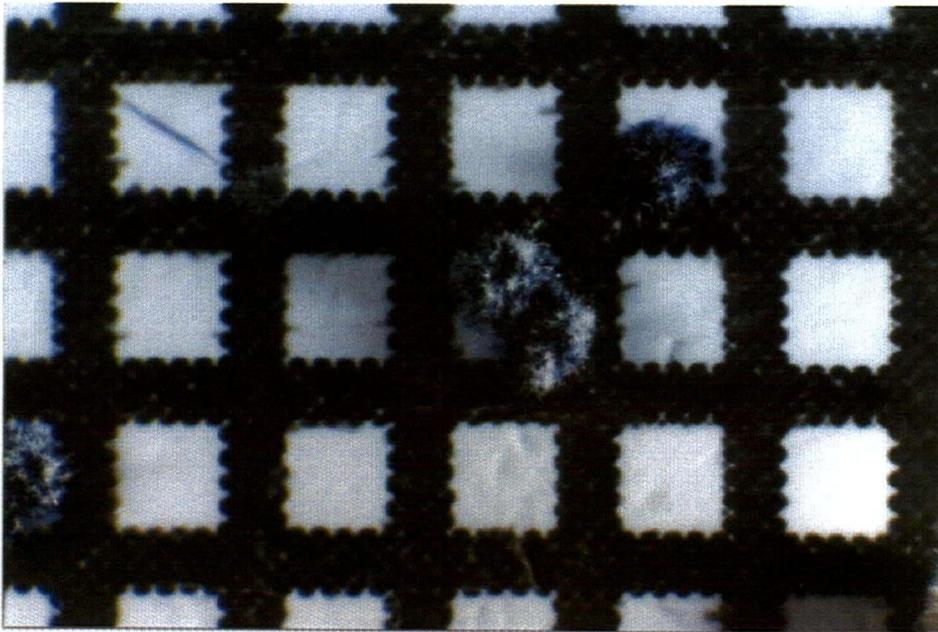


Figure 33. Effect of ozone and LFD (0.30 mg/mL) 30 seconds after addition of ozonated water. Observations are on 10^{-4} dilution filter after 6 days incubation at 30°C .

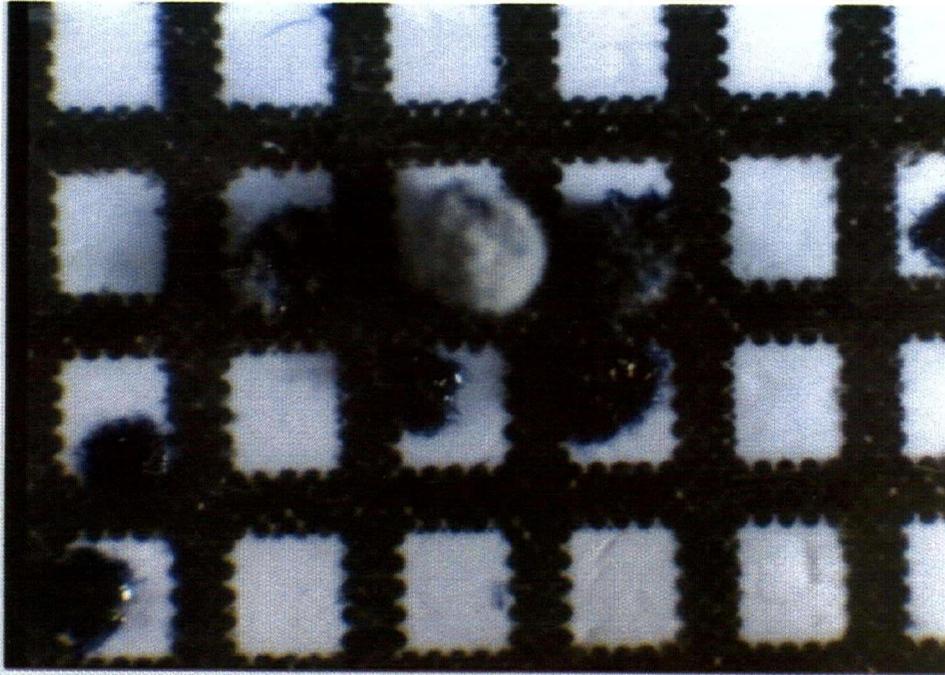


Figure 34. Effect of ozone and LFD (0.30 mg/mL) 1 min after addition of ozonated water. Observations on 10^{-4} dilution filter are after 6 days incubation at 30°C.

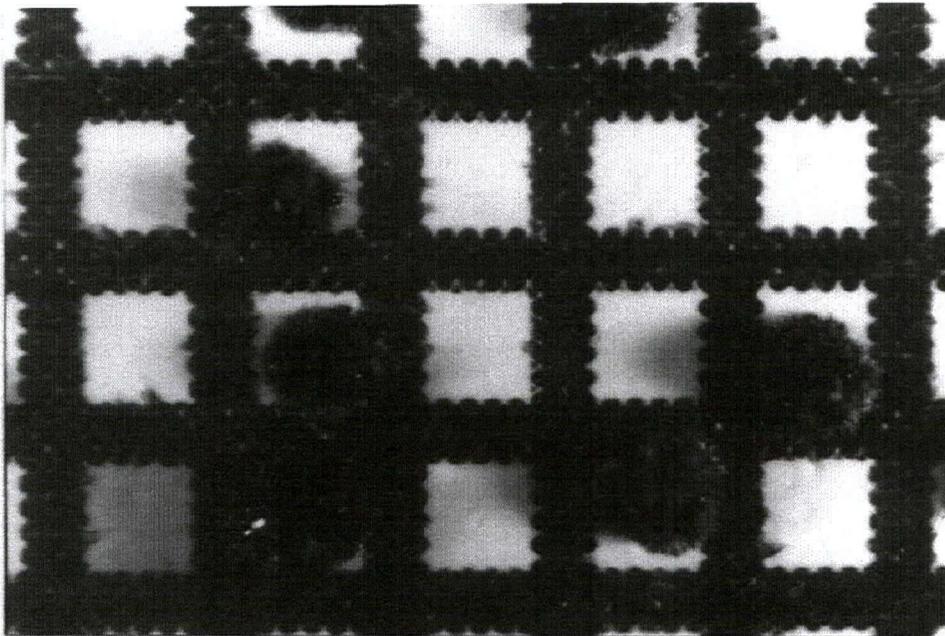


Figure 35. Effect of ozone and LFD (0.30 mg/mL) 1.5 min after addition of ozonated water. Observations on 10^{-4} dilution filter are after 6 days incubation at 30°C.

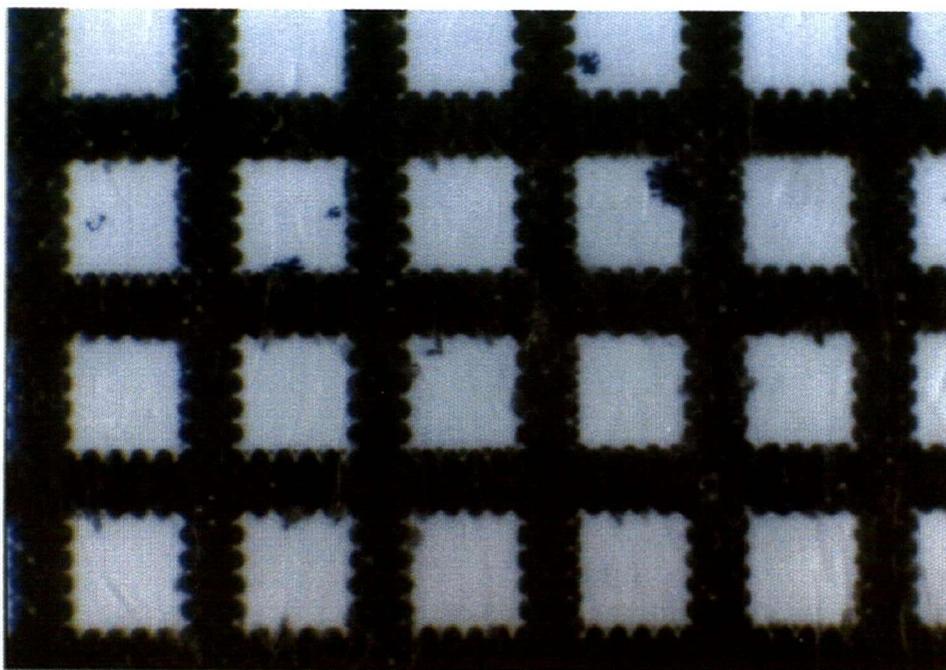


Figure 36. Effect of ozone and LFD (0.30 mg/mL) 2 min after addition of ozonated water. Observations on 10^{-4} dilution filter are after 6 days incubation at 30°C.

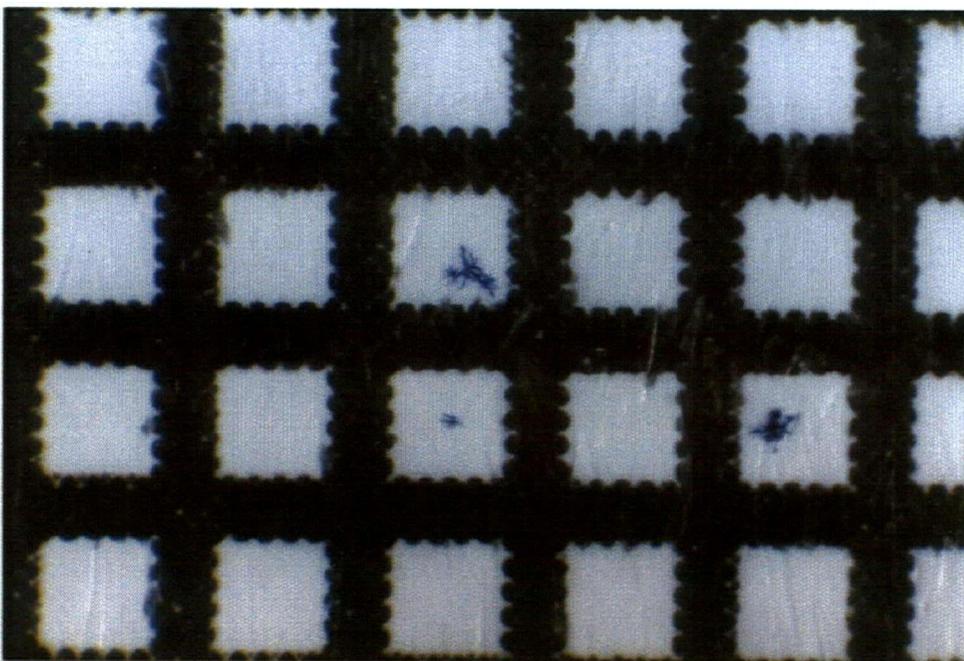


Figure 37. Effect of ozone and LFD (0.30 mg/mL) 2.5 min after addition of ozonated water. Observations on 10^{-4} dilution filter are after 6 days incubation at 30°C.

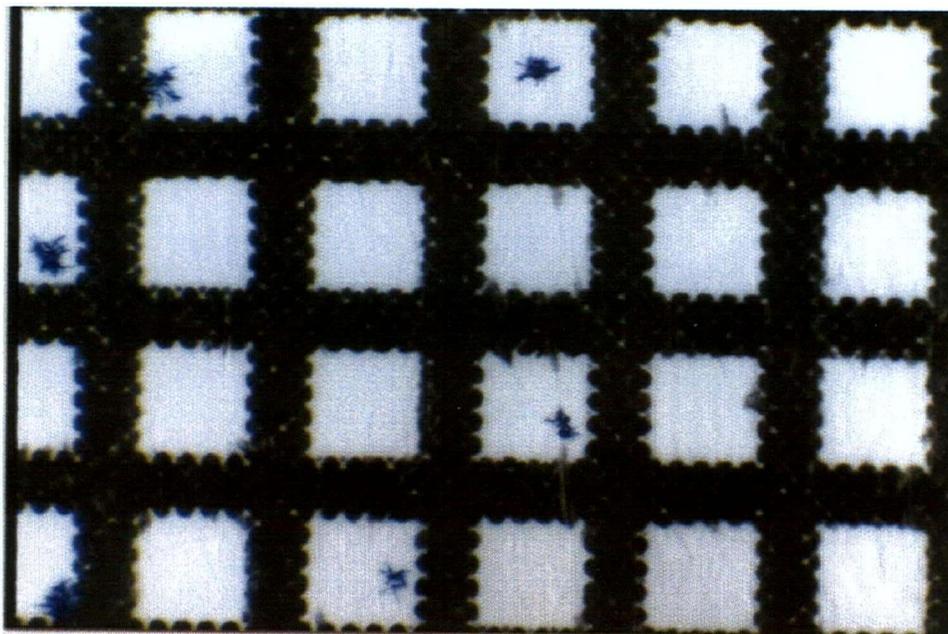


Figure 38. Effect of ozone and LFD (0.30 mg/mL) 3 min after addition of ozonated water. Observations on 10^{-4} dilution filter are after 6 days incubation at 30°C.

E. Ozone decay experiments

In order to determine the stability of ozone during the time frame used for the experiments, ozone decay curves were performed every time an experiment was carried out. Figure 39 shows the results from three different trials, indicating how ozone concentration slowly declined over a period of 10 minutes. It is important to note that the experiments were conducted at 25°C and ozone becomes less stable as the temperature increases. However, over a period of 10 min ozone concentration declined gradually. The same procedure for measuring the decay of ozone was performed in a time frame of three minutes at 25°C with measurements taken every 30 seconds (Figure 40).

F. Spore control

Figure 41 illustrates the effect of distilled-deionized water at 25°C on fungal spores. Distilled-deionized water had no effect on the initial spore count over three minutes. Four different trials were performed using only d-d water. The samples (1 mL) were taken at the same time intervals as in the ozone and ozone-LFD experiments. As expected, the initial population was constant over the three minutes suggesting that any decrease in population would have been due to the presence of LFD, ozone or a combination of LFD and ozone.

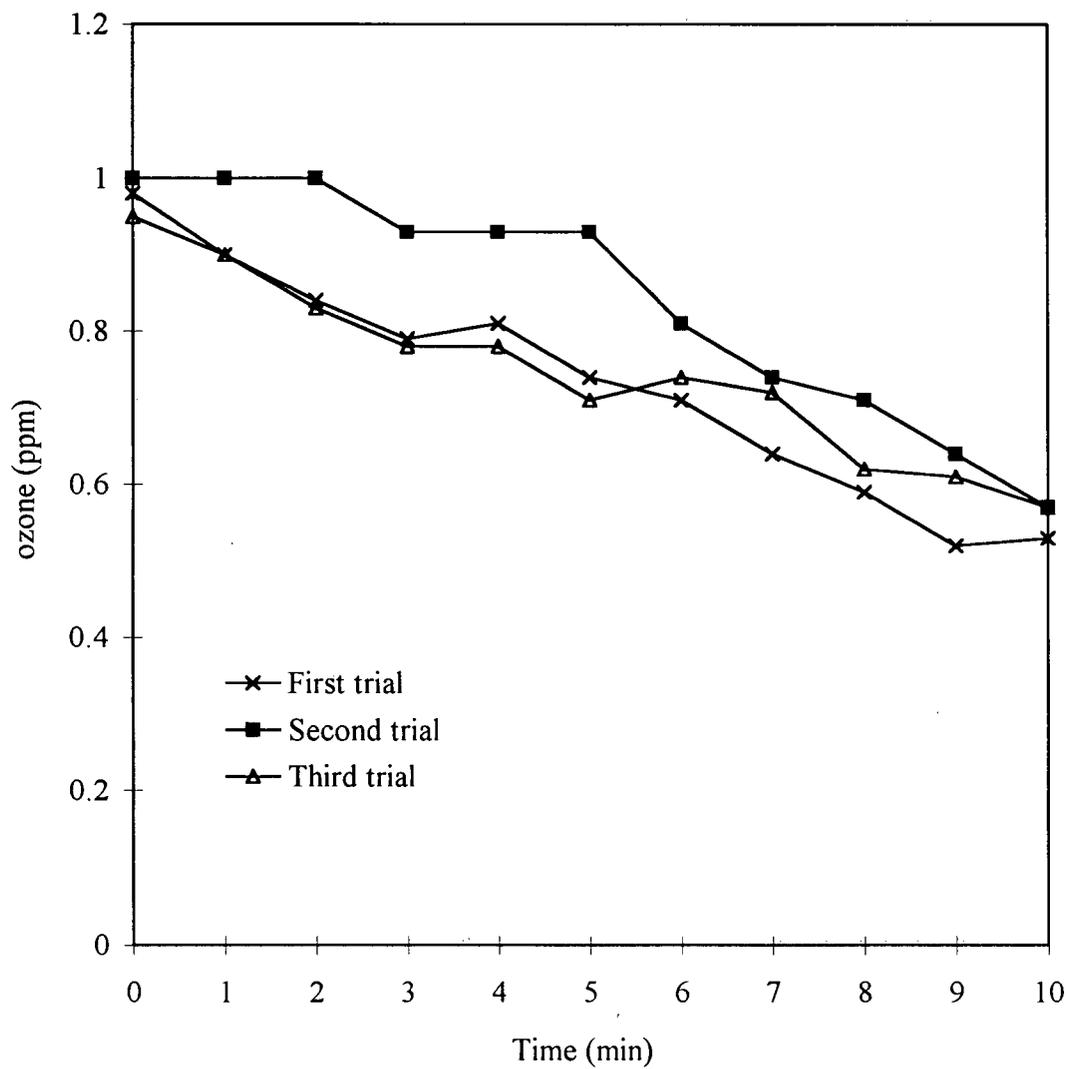


Figure 39. Ozone decay dissolved in distilled deionized water at a temperature of 25°C. Measurements were taken every 1 minute over a period of 10 min.

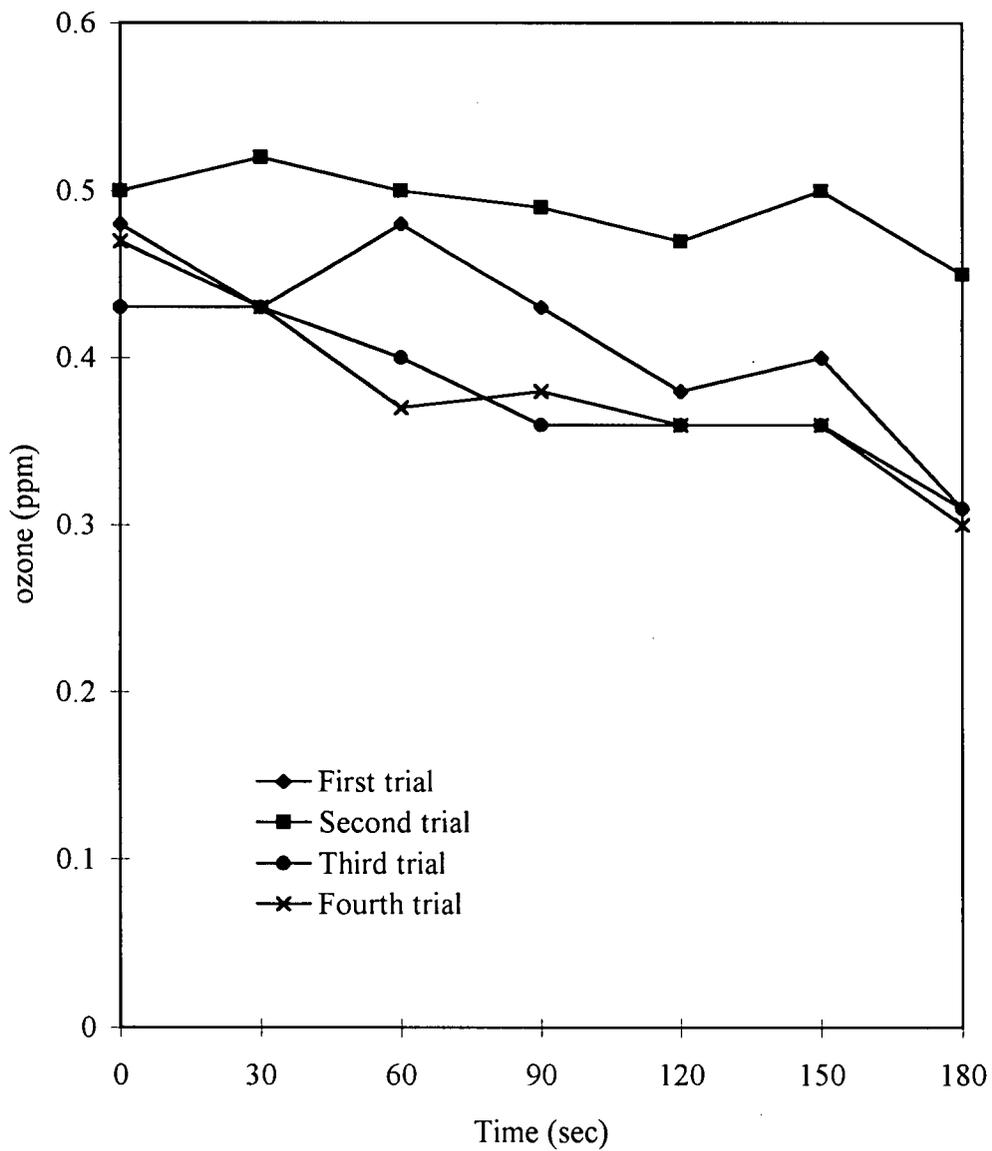


Figure 40. Ozone decay dissolved in distilled deionized water at a temperature of 25°C. Measurements were taken every 30 seconds over a period of 3 min.

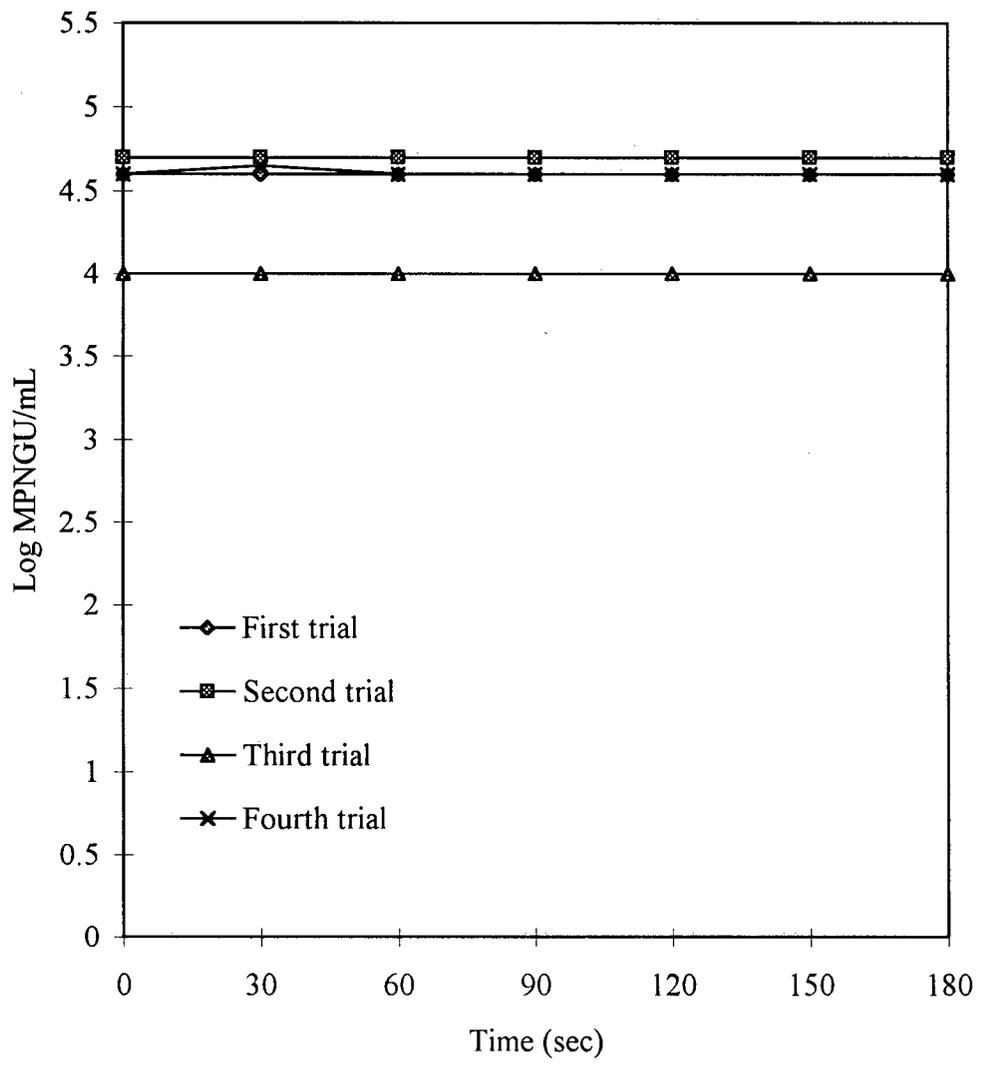


Figure 41. Effect of distilled-deionized water at 25°C on spores of *Penicillium* spp.

V. CONCLUSION AND RECOMMENDATIONS

The results of this study suggest the antimicrobial activity of the pepsin digest derived from bovine lactoferrin on spores of *Penicillium* spp. isolated from bottled water. Lactoferrin digest (LFD) at a concentration of 0.30 mg/mL in PYG medium inhibited spore germination and mycelial fusion for a period of 21 days. When the LFD concentration was brought down to 0.06 mg/mL, the inhibitory effect was also lowered, lasting only 9 days.

Ozone concentrations used for this study (0.1-0.4 ppm) were not sufficient to cause any decline in fungal population. The effect of ozone on fungal spores needs to be further studied in order to determine the exact time and concentration required to cause a significant decrease in microbial population. The growth stimulation effect imparted by low ozone concentrations should also be studied with more care in order to establish the mechanism(s) that may alter the spore and mycelium arising from the germinated spores, causing an accelerated or stimulated growth and sporulation.

Treatment of *Penicillium* spores with LFD and ozone led to slower development of mould colonies on the HGMF filters. Unfortunately, ozone concentration(s) could not be measured when working with LFD due to formation of a complex between the LFD and the indigo trisulfonate dye.

Finally, although LFD has shown effectiveness against fungal spores over a period of time, the feasibility of its addition prior to ozonation will have to be reviewed since ozonation using a diffusor caused the LFD to form a stable foam. Therefore, LFD would have to be aseptically added after the ozonation step.

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APPENDIX I

A. Key followed for the preliminary identification of fungi:

According to Pitt and Hocking (1985):

1. Growth on one or more standard media (CYA, MEA, G25N)
2. Growth filamentous, exceeding 10 mm diam on one or more standard media
3. Growth on CYA and/or MEA faster than on G25N
4. Hyphae frequently and conspicuously septate
5. Immature fruiting structures (or spores) of some kind present
6. Colonies and fruiting structures white or brightly coloured
7. Colonies or fruiting structures brightly coloured:

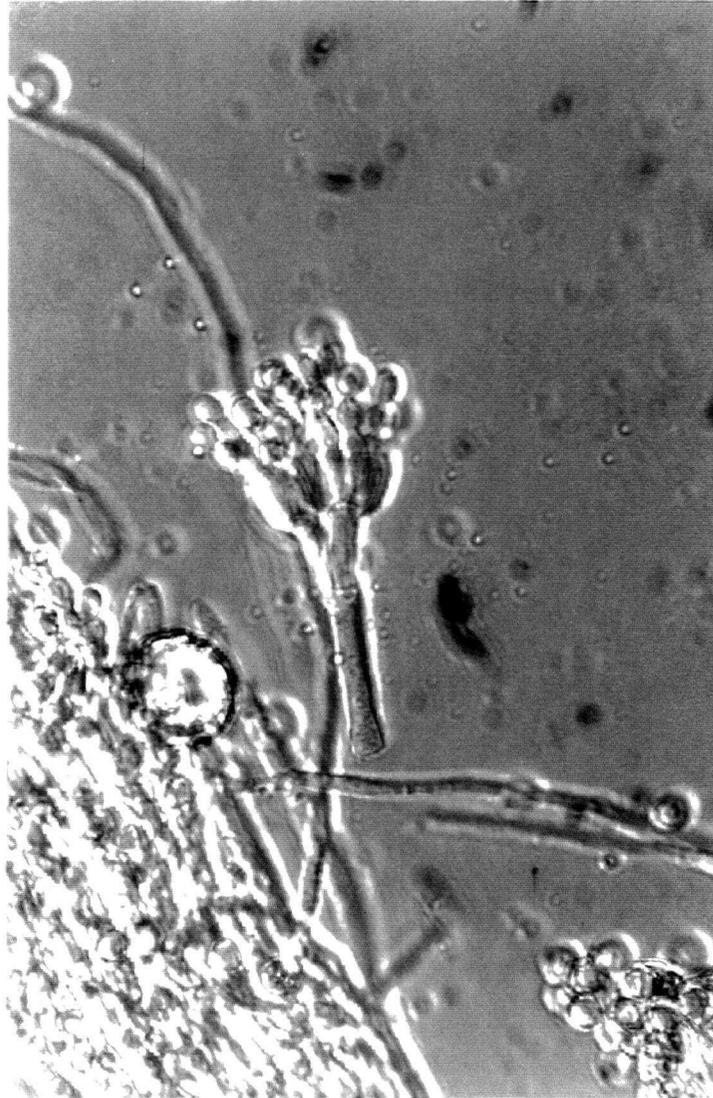
Refer to "*Penicillium* and related genera".

B. Key to genera producing penicilli

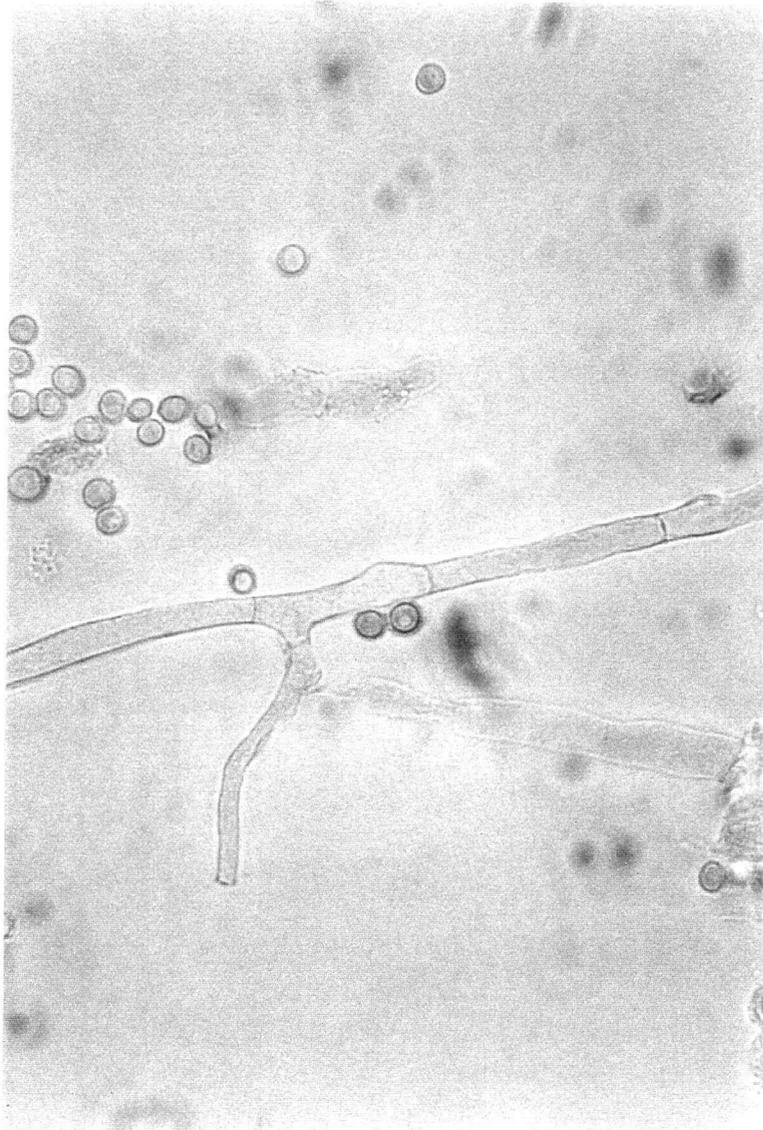
According to Pitt and Hocking (1985):

1. Conidia not truncate at the base, symmetrical from end to end; colony colour various
2. Mature conidia spherical to ellipsoidal, in shades of blue, green and/or grey

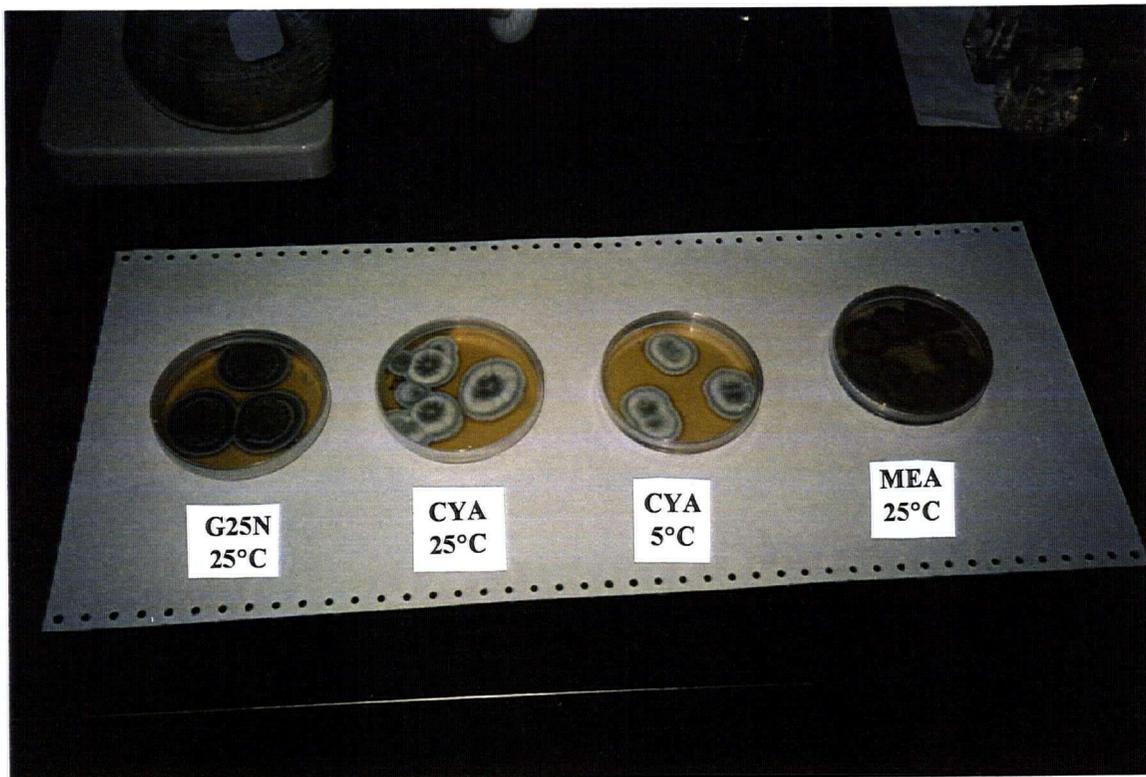
Penicillium



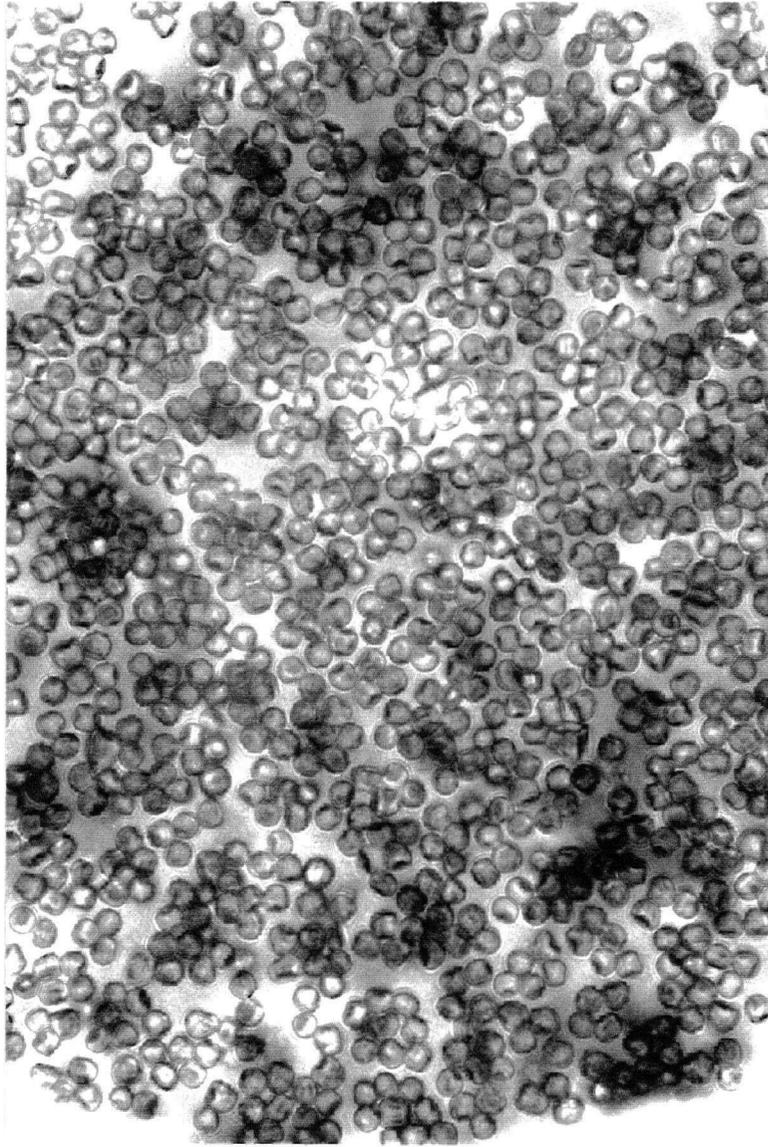
C. Microscopic observations of fungi isolated from bottled water (100x).



D. Microscopic observations of septate hyphae (100x).



E. Observations of *Penicillium* spp. on three standard media incubated at 25 and 5°C.



F. Microscopic observations from the spore stock suspension (100x).

APPENDIX II

A. Anova results of OD₅₉₅ for *Penicillium* spp. spores treated with LFD (0.06 mg/mL) in the antimicrobial assay.

Time (h)	LFD (0.06mg/mL) SAMPLE 1	BSAD (0.06 mg/mL) SAMPLE 2	Spore control SAMPLE 3
0	0.059 ^{xa}	0.047 ^{xa}	0.049 ^{xa}
12	0.067 ^{xa}	0.074 ^{xa}	0.080 ^{xa}
24	0.062 ^{xa}	0.105 ^{xa}	0.109 ^{xa}
36	0.065 ^{xa}	0.198 ^{yb}	0.243 ^{yb}
48	0.061 ^{xa}	0.220 ^{yb}	0.359 ^{yc}
70	0.061 ^{xa}	0.295 ^{ybc}	0.476 ^{yd}
118	0.064 ^{xa}	0.417 ^{ybc}	0.538 ^{yd}
168	0.075 ^{xa}	0.492 ^{yc}	0.664 ^{yd}
216	0.104 ^{xa}	0.566 ^{yc}	0.652 ^{yd}
336	0.162 ^{xa}	0.564 ^{xyc}	0.578 ^{yd}
504	0.214 ^{xa}	0.564 ^{xc}	0.578 ^{xd}

x,y rows not sharing the same letters are significantly different ($p < 0.05$) between samples at a given time.

a,b,c,d columns not sharing the same letters are significantly different ($p < 0.05$) within each sample at different times.

Note: Since the magnitude as well as standard deviation of the OD₅₉₅ values were much greater at the later times, corresponding to active mould growth in some wells, separate analysis of the data from two sets of statistical analysis were performed using the data set from 0 to 70 hours only for the first analysis and the entire data set from 0 to 504 hours for the second analysis. The shorter incubation times increased the power of the statistical analysis to distinguish smaller differences between the samples tested during the first 70 hours of the experiment.

B. General linear model example for LFD (0.06 mg/mL).

Analysis of Variance for OD 595, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
time	10	8.69473	8.69473	0.86947	30.57	0.000
sample	2	4.70338	4.70338	2.35169	82.67	0.000
time*sample	20	2.50596	2.50596	0.12530	4.40	0.000
Error	264	7.50984	7.50984	0.02845		
Total	296	23.41391				

Tukey Simultaneous Tests

Response Variable OD 595

All Pairwise Comparisons among Levels of **time*sample**

time = 0 h

sample = 1(LFD 0.06 mg/mL))subtracted from:

Level		Difference	SE of		Adjusted
time*sample		of Means	Difference	T-Value	P-Value
0	2	-0.01189	0.02860	-0.4156	1.0000
0	3	-0.00978	0.02860	-0.3418	1.0000
12	1	0.00900	0.02860	0.3146	1.0000
12	2	0.01500	0.02860	0.5244	1.0000
12	3	0.02122	0.02860	0.7419	1.0000
24	1	0.00311	0.02860	0.1088	1.0000
24	2	0.04644	0.02860	1.6237	0.9764
24	3	0.04989	0.02860	1.7441	0.9543
36	1	0.00667	0.02860	0.2331	1.0000
36	2	0.13978	0.02860	4.8865	0.0004
36	3	0.19089	0.02860	6.6734	0.0001
48	1	0.00267	0.02860	0.0932	1.0000
48	2	0.16100	0.02860	5.6285	0.0001
48	3	0.32200	0.02860	11.2569	0.0001

time = 336 h

sample = 1 subtracted from:

Level		Difference	SE of		Adjusted
time*sample		of Means	Difference	T-Value	P-Value
336	2	0.27600	0.07951	3.4714	0.1390
336	3	0.33056	0.07951	4.1575	0.0131
504	1	0.06822	0.07951	0.8581	1.0000
504	2	0.27600	0.07951	3.4714	0.1390
504	3	0.33056	0.07951	4.1575	0.0131

C. Anova results of OD₅₉₅ for *Penicillium* spp. spores treated with LFD (0.30 mg/mL) in the antimicrobial assay.

Time (h)	LFD (0.30mg/mL) SAMPLE 1	BSAD (0.30 mg/mL) SAMPLE 2	Spore control SAMPLE 3
0	0.061 ^{xa}	0.047 ^{xa}	0.050 ^{xa}
12	0.073 ^{xa}	0.073 ^{xa}	0.083 ^{xa}
24	0.068 ^{xa}	0.110 ^{xa}	0.111 ^{xa}
36	0.070 ^{xa}	0.201 ^{yb}	0.219 ^{yb}
48	0.067 ^{xa}	0.242 ^{yb}	0.292 ^{yc}
70	0.066 ^{xa}	0.251 ^{yb}	0.362 ^{yc}
118	0.067 ^{xa}	0.394 ^{ybc}	0.439 ^{yc}
168	0.067 ^{xa}	0.522 ^{ybc}	0.580 ^{yd}
216	0.068 ^{xa}	0.574 ^{yc}	0.566 ^{yd}
336	0.065 ^{xa}	0.514 ^{ybc}	0.534 ^{yd}
504	0.074 ^{xa}	0.514 ^{ybc}	0.536 ^{yd}

x,y rows not sharing the same letters are significant different ($p < 0.05$) between samples at a given time.

a,b,c,d columns not sharing the same letters are significant different ($p < 0.05$) within each sample at different times.

Note: Since the magnitude as well as standard deviation of the OD₅₉₅ values were much greater at the later times, corresponding to active mould growth in some wells, separate analysis of the data from two sets of statistical analysis were performed using the data set from 0 to 70 hours only for the first analysis and the entire data set from 0 to 504 hours for the second analysis. The shorter incubation times increased the power of the statistical analysis to distinguish smaller differences between the samples tested during the first 70 hours of the experiment.

D. General linear model example for LFD (0.30 mg/mL).

Analysis of Variance for OD 595, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
time (h)	10	5.08818	5.08818	0.50882	40.82	0.000
sample	2	5.23769	5.23769	2.61885	210.08	0.000
time*sample	20	2.55938	2.55938	0.12797	10.27	0.000
Error	264	3.29106	3.29106	0.01247		
Total	296	16.17630				

Tukey Simultaneous Tests

Response Variable OD 595

All Pairwise Comparisons among Levels of **time*sample**

time = 0

sample = 1 (LFD 0.30 mg/mL) subtracted from:

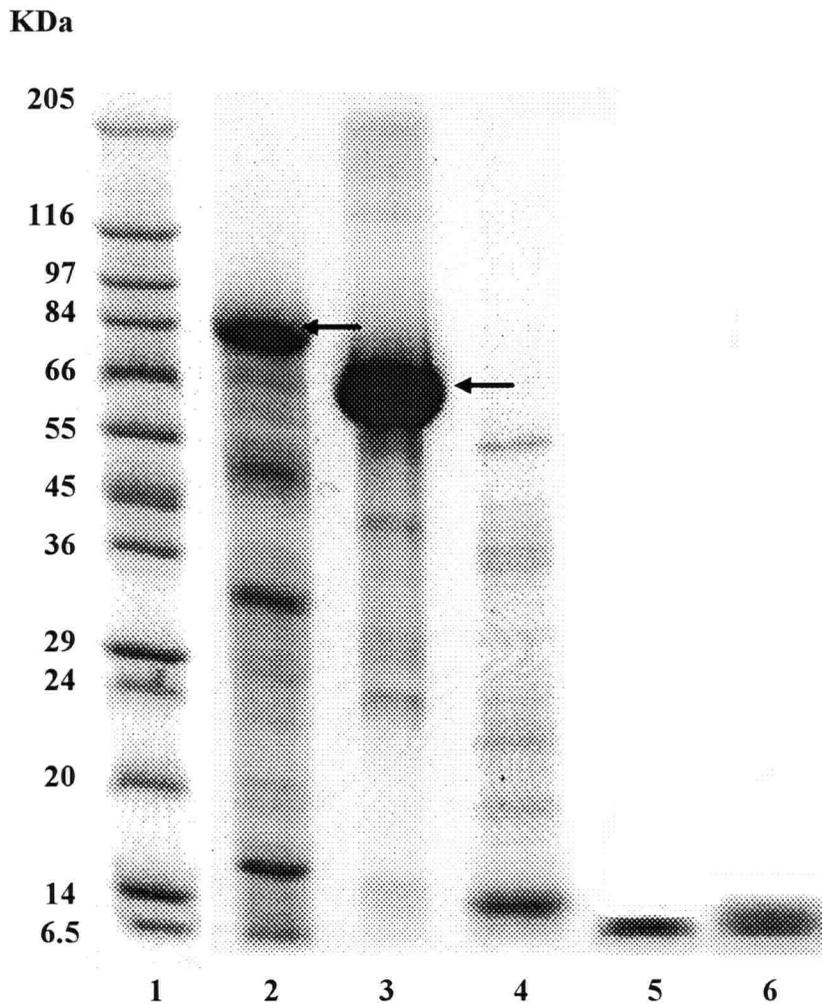
Level		Difference of Means	SE of Difference	T-Value	Adjusted P-Value
time*sample					
0	2	-0.01378	0.01428	-0.9646	0.9997
0	3	-0.01078	0.01428	-0.7546	1.0000
12	1	0.01222	0.01428	0.8557	0.9999
12	2	0.01211	0.01428	0.8479	0.9999
12	3	0.02144	0.01428	1.5014	0.9738
24	1	0.00633	0.01428	0.4434	1.0000
24	2	0.04944	0.01428	3.4617	0.0503
24	3	0.05000	0.01428	3.5006	0.0449
36	1	0.00844	0.01428	0.5912	1.0000
36	2	0.14111	0.01428	9.8794	0.0000
36	3	0.16033	0.01428	11.2252	0.0000
48	1	0.00578	0.01428	0.4045	1.0000
48	2	0.18789	0.01428	13.1544	0.0000
48	3	0.22944	0.01428	16.0638	0.0000

time = 336 h

sample = 1 subtracted from:

Level		Difference of Means	SE of Difference	T-Value	Adjusted P-Value
time*sample					
336	2	0.46656	0.05263	8.8643	0.0001
336	3	0.49467	0.05263	9.3984	0.0001
504	1	0.01133	0.05263	0.2153	1.0000
504	2	0.46656	0.05263	8.8643	0.0001
504	3	0.49467	0.05263	9.3984	0.0001

APPENDIX III



A. SDS-PAGE on a 10-15% gradient Phastgel. The profiles are lactoferrin (lane 2), BSA (lane 3), LFD (lane 5) and BSAD (lane 6). Lane 1 contains wide range molecular weight markers (Sigma Chemicals). Lane 4 contains fish mince (not used in this study). Arrows indicate band identified as lactoferrin (molecular weight of approximately 83,000 Daltons) and BSA (molecular weight of 66,000 Daltons).