ABSTRACTS & EXTENDED ABSTRACTS

Symposium Series on Food Microbiology

Sponsored by the
ILSI North America Technical Committee on Food Microbiology

in conjunction with the
International Association for Food Protection 87th Annual Meeting

August 6–9, 2000
Hilton Atlanta
Atlanta, Georgia, USA

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The Symposium Series on Food Microbiology was sponsored by the ILSI North America Technical Committee on Food Microbiology in conjunction with the IAFP 87th Annual Meeting, held in Atlanta, Georgia, USA, August 6–9, 2000, and made possible in part by an unrestricted educational grant from the National Food Processors Association.

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*Speaker's name is capitalized.

Preface

The *Symposium Series on Food Microbiology* consisted of two international symposia sponsored by the North American Branch of the International Life Sciences Institute (ILSI N.A.) Technical Committee on Food Microbiology at the International Association for Food Protection (IAFP) 87th Annual Meeting, held August 6–9, 2000, in Atlanta, Georgia, USA.

Listeria monocytogenes continues to be a serious concern to the food industry and to regulatory agencies. Foodborne outbreaks caused by this pathogen have had severe public health and economic consequences. The infective dose in humans is believed to be high but is still unknown. Evidence suggests that not all *L. monocytogenes* strains are equally likely to be implicated in human illness. The wide distribution and unique ecology of this pathogen in the food chain create challenges for intervention strategies and control. The day-long *Symposium on* L. monocytogenes*: Current Issues and Concerns* presented an update on current progress and developments to address these formidable issues.

Presentations in the first half of the symposium covered the application of animal models to investigate the virulence, and to determine the infective dose, of *L. monocytogenes*. The relationship between *L. monocytogenes* genotypes and virulence characteristics was also examined. Risk assessments of *L. monocytogenes* in the commercial food supply and in home-prepared foods were presented along with an update on the status of the Food and Drug administration's *Listeria* risk assessment. The second half of the symposium dealt with detection, enumeration, and intervention strategies for *listeria monocytogenes*, and featured presentations on gene-based detection methods, *L. monocytogenes* ecology, intervention strategies, and control measures in production environments.

The second committee-sponsored symposium focused on mycotoxins in human food and animal feeds. Efforts to limit exposure to mycotoxins are based on concerns about the adverse effects of direct exposure to contaminated food and feed on human or animal health, and potential mycotoxin residues in foods of animal origin. Food producers and processors are increasingly challenged to understand mechanisms of mycotoxin formation and to develop control and prevention measures. The *Symposium on the Significance of Mycotoxins in the Global Food Supply* provided an overview of the most important mycotoxins worldwide, including evidence for a relationship between mycotoxins and human disease. Speakers addressed factors influencing the occurrence of aflatoxins, fumonisins, and deoxynivalenol, their toxicology, and related human and animal health issues. Analytical detection methods and international food safety standards and industry efforts to control the occurrence of mycotoxins in the food supply were also discussed.

ILSI N.A. is a public, nonprofit foundation that advances the understanding of scientific issues related to the nutritional quality and safety of the food supply. By bringing together scientists from academia, government, industry, and the public sector, ILSI N.A. seeks a balanced approach to solving problems with broad implications for the well being of the general public. The ILSI N.A. Technical Committee on Food Microbiology was formed in 1987 to address issues related to microbial food safety hazards. The committee has funded over $2 million of research on several important foodborne pathogens and and has sponsored numerous scientific meetings in the area of microbial food safety. Since 1993, the committee has partnered with IAFP to sponsor an annual international symposium series on food microbiology. ILSI N.A. and the Technical Committee on Food Microbiology hope that making the abstracts and extended abstracts of the presentations in these symposia available to the public will provide important information to a worldwide audience and help stimulate initiatives to improve the safety of our global food supply.

LISTERIA MONOCYTOGENES: CURRENT ISSUES AND CONCERNS

Pathology, Virulence, and Risk Assessment of *Listeria monocytogenes*

Relevance of Animal Models to Study Virulence of *Listeria monocytogenes*

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Three-quarters of a century have passed since the initial description of *Bacterium monocytogenes* by Murray et al. (1926) in infected laboratory rabbits and guinea pigs. Since then, the organism has gone through a number of name changes and has emerged as an important foodborne pathogen for both humans and animals. Despite the decades of research that followed its initial isolation, a definitive animal model that mimics all aspects of human listeriosis remains elusive. Preliminary virulence studies focused on models using ruminants because of the high incidence of disease in these populations. However, as *Listeria monocytogenes* became more recognized as a human pathogen, rabbit, mouse, rat, and guinea pig models became more prevalent to mimic the disease observed in humans.

Human disease caused by *Listeria* infection was first described by Nyfeldt (1929) as an infectious mononucleosis, and is now known to cause a number of clinical manifestations, including sepsis, meningitis, meningoencephalitis, noninvasive gastrointestinal illness, and spontaneous abortion (Farber and Peterkin 2000).

Animal models of disease have been invaluable in attaining the current level of knowledge of the virulence of *L. monocytogenes* in human hosts. In the past few decades, increasing scrutiny and controversy surrounding the use of animals in research have led to the development of alternative in vitro models. Although in vitro systems have paved the way for the study of the biology of intracellular growth and bacterial determinants of pathogenicity, animal models are still very important. Traditionally, animal models have been used in four basic areas of *L. monocytogenes* research: (1) in studies of lethal dose/minimum infectious dose, (2) as indicators of invasiveness or infectivity, (3) to determine the mechanisms of pathogenesis, and (4) to understand the modes of infectivity. The following discussion will highlight the relevance of animal models in the study of the virulence of *L. monocytogenes*.

Determination of Lethal Dose (LD_{50}) or Minimum Infectious Dose (MID)

The LD_{50} is defined as the dose of a pathogen sufficient to kill half of the experimental animals, and the MID is defined as the minimum dose/numbers of the pathogen required to cause infection. Because of the foodborne nature of listeriosis, these measurements have received much attention in an effort to determine what level of *L. monocytogenes* may be safe in a food. Actual determination of MID values for humans has been extrapolated from outbreak data. However, animal models have provided invaluable information on differences in LD_{50} and MID with regard to the route of entry into the host, strain of *L. monocytogenes*, the effect of culture growth conditions on the pathogenicity of *L. monocytogenes*, and predisposition of the host to disease. The LD_{50} of *L. monocytogenes* can vary with each animal host and even within the same host. The strain used, growth conditions of the strain prior to inoculation, and mode of infection can also influence the outcome of infection (Gray and Killinger 1966, Kautter et al. 1963). Therefore, with respect to the dose and mode of infection, the extrapolation of animal LD_{50} values to human disease is difficult, although it is useful for demonstrating virulence differences among strains of *L. monocytogenes* and the importance of environmental and host factors in infection.

LD_{50} studies have consistently shown that serotypes 1/2a, 1/2b, and 4b are more virulent than other serovars for animals (Bracegirdle et al. 1994, Wirsing von Koenig et al. 1983, Osebold and Inouye 1954), and this is consistent with trends observed for human illness (Farber and Peterkin 2000). However, animal studies have also shown that some strains of these serotypes can be avirulent when lacking the hemolysin gene (Nishibori et al. 1995, Bracegirdle et al. 1994), indicating that serotype alone cannot reveal strain virulence. In addition, a number of researchers have used

LD_{50} models with virulence mutants in an effort to better understand virulence factors (Ermolaeva et al. 1997, Sheehan et al. 1996). Pine et al. (1990) demonstrated the importance of using LD_{50} values to directly compare the effect of intraperitoneal (i.p.) and intragastric routes of inoculation on the timing of death. At present, in vivo animal models are the only reliable means of determining the virulence of foodborne isolates.

Indicators of Virulence/Level of Infectivity

Using animal models, researchers have determined the invasiveness of virulent and avirulent strains of *L. monocytogenes.* Similar to the findings of Marco et al. (1997), Lammerding et al. (1992), and Rácz et al. (1972), work by MacDonald and Carter (1980) showed that following oral infection of mice, the organisms can spread from the Peyer's patches and the mononuclear leukocytes to reside in the spleen and liver by 4 days postinfection. Furthermore, Lammerding et al. (1992) observed that germ-free mice were more consistently colonized by *L. monocytogenes,* suggesting that the normal microflora of the mouse gastrointestinal tract interfered with colonization. The same group also found that a rough mutant of *L. monocytogenes* Scott A (SAR) was unable to infect placental tissues, whereas the wild-type strain was highly invasive. The wild-type Scott A (SA) strain migrated to the placental tissues by 3 days postinfection and infected the fetuses by days 4 and 5, whereas the rough mutant, although invasive to the maternal organs and placental tissues, was rapidly cleared. Interestingly, *L. monocytogenes* strain ATCC 19113 (serotype 3a) was avirulent, did not colonize the gastrointestinal tract, and was not invasive, possibly accounting for the low prevalence of this serotype in human illness. However, as recently observed in an outbreak in Finland linked to butter, serotype 3a can cause foodborne outbreaks (Lyytikainen et al. 2000). Thus, other factors involved in human infection that are not reproducible in the mouse model exist.

Variations in degrees of virulence among different strains of *L. monocytogenes* have frequently been reported (Bracegirdle et al. 1994, Stelma et al. 1987, Wirsing von Koenig et al. 1983). Lammerding et al. (1992) used i.p. inoculation of mice with *L. monocytogenes*, as well as peroral inoculation, and noted that SAR was as virulent as the wild-type strain, indicating that i.p. and other artificial inoculation methods may bypass the natural processes of immunity that decide the fate of foodborne pathogens (Pohjanvirta and Huttunen 1985). One component missing from the animal studies is the ability of any experimental model to mimic or explain the route of *L. monocytogenes* ascension to the brain and therefore its role in meningitis or meningoencephalitis. However, Altimira et al. (1999) recently showed that by repeatedly (7–10 days) dosing mice orally with a sublethal dose of a virulent *L. monocytogenes* strain, they could observe the development of severe central nervous system (CNS) lesions in 25% of experimental Swiss CD1 mice. It was speculated that the repeated oral dosing resulted in a persistent clinical bacteremia, owing possibly to the release of the organisms from the liver, which then led to the development of CNS listeriosis. This is consistent with the conclusions of Berche (1995), who found that the level and duration of bacteremia are directly associated with the development of CNS listeriosis in mice. The repeated oral dosing model may prove to be more useful in understanding human foodborne illness than other artificial inoculation models, i.e., middle ear inoculation of gerbils (Blanot et al. 1997) and intracerebral (Leist et al. 1988), intravenous (Berche 1995), or subcutaneous inoculation of mice (Prats et al. 1992). Although Altimira et al. (1999) clearly demonstrated the presence of *L. monocytogenes* in the brain of mice, histopathological analysis of the internal organs was done only upon euthanasia. Therefore, the stages of infection leading to CNS involvement were not clearly demonstrated and should be the focus of future studies.

Mechanism of Pathogenesis/Virulence Determinants

Many of the underlying signals and factors that determine the extent of invasiveness of *L. monocytogenes* have been elucidated through the use of animal models. Listeriolysin O (LLO) was first shown by a combination of genetic manipulation and animal model studies to be essential for virulence (Michel et al. 1990, Cossart et al. 1989). More recently, the presence of additional virulence-related genes have been discovered, and our understanding of their role is dependent on both genetic manipulation of strains and in vitro cell line models of virulence, as well as on animal model studies. Similarly, alteration of the expression of virulence genes on the outcome of disease has been monitored using animal models (Schluter et al. 1998, Buncic et al. 1996, Nishibori et al. 1995) as have the effects of immunosuppression (Okamoto et al. 1994, Pohjanvirta and Huttunen 1985), pregnancy (Lammerding et al. 1992, Gray 1958), and chemoprophylaxis (Walencka et al. 1994, Nakane et al. 1990).

Notwithstanding the knowledge gained using experimental animals, the need for a model that most closely mimics humans is clearly still required. Recently, while studying the host cell–*L. monocytogenes* interaction, Lecuit et al. (1999) discovered that mouse E-cadherin does not allow internalin-dependent entry into mammalian cells, and therefore the mouse may not be an appropriate model to study all aspects of human listeriosis. Recalling that Rácz et al. (1972) reported the detection of *Listeria* in enterocytes of experimentally infected guinea pigs, Lecuit et al. (1999) correctly anticipated that guinea pig epithelial cells expressing E-cadherin should permit infection by internalin-expressing bacteria. Sequence analysis work determined that, similar to human E-cadherin, both rabbit and guinea pig E-

cadherins share an essential proline at position 16. Because the fetoplacental unit and the CNS are bordered by E-cadherin–expressing cells, the guinea pig model may be extremely useful in studying these aspects of human listeriosis.

Mode of Inoculation

The majority of human listeriosis cases can be attributed to the consumption of contaminated food sources. However, many other modes of infection have been observed both experimentally and naturally in animals: respiratory, contact, cutaneous, intravaginal, middle ear, submucosal, intrauterine, subcutaneous, intravenous, intraperitoneal, intracerebral, intragastric, intranasal, and conjunctival. Kautter et al. (1963) determined that the intracerebral route is most lethal for mice, followed by the intraperitoneal, intravenous, and respiratory routes. However, for guinea pigs, although i.p. inoculation was ineffective, the respiratory route was much more lethal.

Pine et al. (1990) and Golnazarian et al. (1989) further demonstrated that for mice, respiratory inoculation is comparable to i.p. inoculation and that the intragastric route is similar to the i.p. route. Pohjanvirta and Huttunen (1985) examined various natural routes of listeriosis infection and discovered a link between wounds and CNS infection. In a group of subcutaneously inoculated mice, the purest form of ruminant-type cuffing encephalitis was encountered without any other pathological signs, suggesting that contaminated wounds in the head or neck area could allow seeding of the trigeminal nerve and infection of the brain. This idea was previously put forth as a mode of infection via ingestion of contaminated food that contacts wounds within the oral cavity (Barlow and McGorum 1985). However, it seems more likely that CNS listeriosis as the result of a wound infection is more common in ruminant populations.

The aerosol route of infection has consistently been used experimentally as an effective and reproducible mode of infection. The occurrence of an outbreak of neonatal listeriosis associated with aspiration of mineral oil underlies the fact that this mode of infection is not purely artificial, but may account for a small number of both animal and human forms of listeriosis (Schuchat et al. 1991). As previously discussed, repeated oral dosing of mice has reproduced CNS involvement seen in human listeriosis; however, the gastrointestinal symptoms usually seen in human infection were not observed. Perhaps the use of the repeated oral dosing method with a guinea pig model of infection may provide a more accurate model of human infection.

Many advances in our knowledge of *L. monocytogenes* and its role as a human pathogen have resulted from studies using animals as experimental models of infection. However, at present, no one animal model can be used effectively to mimic all aspects of human foodborne listeriosis. In the future, one can expect to see the emergence of transgenic animals as possible animal models of choice. In this regard, transgenic mice expressing E-cadherin may be a good choice for future studies involving animal models.

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Primates as a Model for *Listeria monocytogenes* Infective Dose: A Progress Report

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In the United States, an estimated 400 deaths per year result from listeriosis. About one-fourth of these are stillbirths, spontaneous abortions, or neonatal deaths. Despite the known susceptibility of many animals to listeriosis, no animal model has been developed to study human listeriosis because of differences in reproductive and immune systems. Rhesus monkeys have been used as a model for human reproduction because they share many similar characteristics, such as estrous cycle, ovulation, internal structure of the placenta, and placental membranes. The purpose of this study was to establish that the pregnant rhesus monkey can be used as an animal surrogate for human exposure and to develop a methodology for determining dose information to be used in a risk assessment for *L. monocytogenes*.

A six-strain mixture of *L. monocytogenes* serotypes most often associated with human or primate outbreaks was combined and used in the first challenge experiments. The origins of the six strains were a human clinical strain (4b), Vacherin Mont d'Or cheese (4b), Mexican-style cheese (4b), hot dog (4b), and two monkey clinical isolates (1/2a and 4b). The experimental design was patterned after phase I clinical trials for human testing of cancer therapeutic drugs. Pregnant rhesus monkeys from the Yerkes Regional Primate Research Center's timed breeding colony were identified at gestation day 30 and assigned to our study. Two animals were treated at any one concentration. If an adverse pregnancy outcome was observed from either pregnancy, the next dose was decreased by approximately 100-fold. This provided the largest number of animals in the lowest-dose groups. The treatments were administered by nasogastric intubation of the pregnant animals at approximately gestation day 112. The animals were monitored for the remainder of the pregnancies, and any changes in behavior or health status of the pregnant animal were noted by animal care technicians. Blood and fecal samples were collected immediately before treatment. Fecal samples were collected every day for 7 days, followed by every other day for 7 days, and at periodic intervals thereafter. *Listeria monocytogenes* was determined in the fecal samples and blood samples taken at 4 days posttreatment. Blood chemistry and serum antibody titer was determined for each animal.

Six of 15 pregnant animals treated with any one of the six strains or the mixture of *L. monocytogenes* strains had stillbirths. None of the animals presented outward signs of illness whether the pregnancy resulted in a stillbirth or a normal birth. Although *L. monocytogenes* was not detected in blood samples obtained 4 days posttreatment, *L. monocytogenes* was detected in fecal samples from all animals whose pregnancy resulted in a stillbirth. Although the fecal count varied on an individual basis, the animals whose pregnancy ended in stillbirth tended to shed *L. monocytogenes* over a longer time period and tended to have higher average fecal counts. For fecal samples, the animals with normal births had feces positive for *Listeria* 12% of the days collected, whereas animals with stillbirths had feces positive for *Listeria* 52% of the days collected.

Antibody titers have been determined for 10 of the treated animals to date. Of those, preinfection antibody titers ranged from 800 to 1600, with no change in titers at 4 days postinfection. However, at the time of delivery, all animals with normal deliveries had antibody titers no more than fourfold greater than preinfection titers. Animals with stillbirths had antibody titers between eight- to 32-fold greater than preinfection titers. Peripheral lymphocytes and plasma were subjected to analysis by several immunologic parameters to assess potential biomarkers for exposure. Mitogen-induced lymphocyte proliferation decreased between 75% and 95% at the time of stillbirth, but returned to preinfection levels by 30 days postdelivery. Levels of CD3+ cells remained consistent throughout the study. *Listeria*-specific proliferative responses and antibody titers were elevated at the time of stillbirth and remained elevated.

In conclusion, pregnant rhesus monkeys are susceptible to *L. monocytogenes* with the same disease outcome and pathology as humans. The lack of a good animal model for human pregnancy-related listeriosis has hampered re-

search in identifying susceptible pregnancies, predicting which exposures will end in stillbirth, and developing intervention and treatment strategies. Continued research using pregnant rhesus monkeys will help identify endpoints of infection and hopefully lead to biomarkers that could predict which pregnant women were at risk for having a stillbirth after exposure to *L. monocytogenes*.

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Relationships Between Virulence and *Listeria monocytogenes* Genotypes

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Listeria monocytogenes is a foodborne pathogen capable of causing serious invasive disease in humans and animals, including abortion, septicemia, meningitis, and meningoencephalitis. In humans, immunocompromised persons, pregnant women, the elderly, and neonates are particularly at risk of listerial infections. The frequency of clinical human listeriosis in most developed countries is estimated at between 2 and 15 cases per 1 million population, with case mortality rates between 13% and 34% (Farber and Peterkin 1991). Mead et al. (1999) estimated that 2500 cases of clinical listeriosis occur annually in the United States, resulting in a total of about 500 deaths.

Current regulations specifying a zero tolerance for the presence of any *L. monocytogenes* subtypes in ready-to-eat foods are based on historical taxonomic classification schemes. These classical taxonomic definitions of bacterial species often do not correlate with the ability of a group of bacteria to cause human disease. Rather, related bacteria that differ in their abilities to cause human and/or animal disease may be grouped together into the same species. Thus, a critical need exists for the development of better scientific definitions of bacteria with the ability to cause human disease independent of historical taxonomic species definitions.

Listeria monocytogenes as an Animal and Human Foodborne Pathogen

Although the majority of human clinical infections occur in sporadic cases, human listeriosis can also occur in large epidemics. Most sporadic human listeriosis cases and large human foodborne listeriosis epidemics have reportedly been caused by *L. monocytogenes* serotype 4b (Farber and Peterkin 1991, Rocourt 1988), including the most recent human listeriosis outbreak in the United States (Centers for Disease Control and Prevention 1998). The 4b strains isolated from most epidemic outbreaks form two closely related homogeneous groups (so-called epidemic clones) (Wiedmann et al. 1997, Piffaretti et al. 1989). Serotypes 1/2a and 1/2b are also responsible for significant numbers of sporadic cases of human illness. To illustrate, of 144 human isolates from sporadic cases serotyped by the CDC in 1986, 30% were 1/2a, 32% were 1/2b, and 34% were 4b (Schwartz et al. 1989). Of 1363 human isolates collected in the United Kingdom, 15% were 1/2a, 10% were 1/2b, and 64% were 4b (McLauchlin 1990). The remaining 10 currently recognized *L. monocytogenes* serotypes have been only rarely linked to human disease. This apparent association between a few specific *L. monocytogenes* strains and most cases of human listeriosis raises the intriguing challenge of identifying unique characteristics that enable these strains to be more effective than others in causing human disease.

Two hypotheses could explain the apparent predominance of serotype 4b strains in human epidemic listeriosis and of 4b, 1/2a, and 1/2b strains in sporadic human cases: (1) humans are more commonly exposed to these subtypes compared with other *L. monocytogenes* serotypes (i.e., these strains are found in foods more frequently than are other serotypes), and/or (2) these subtypes have a unique pathogenic potential for humans. If hypothesis 2 is true, one would expect a significant discrepancy between *L. monocytogenes* subtypes found in foods and those types that cause human disease. Specifically, one would expect that certain subtypes with limited or no human virulence are significantly less common in human isolates compared with their frequency in animal and food isolates.

Pathogenesis of Listeriosis

Listeria monocytogenes is a well-characterized facultative intracellular pathogen. A key group of virulence genes and their specific functions in the intracellular infection process have been identified and characterized. In tissue culture models of infection, the following stages of infection can be defined: (1) internalization of *L. monocytogenes* within the host cell; (2) bacterial escape from the host vacuole; (3) multiplication of the parasite within the host cell cytoplasm and movement through the cytoplasm by virtue of bacterially directed nucleation of host actin filaments; (4) bacterial movement to the host cell surface and extrusion of bacterial cells in pseudopod-like structures; and (5)

phagocytosis of these pseudopod-like structures by neighboring cells, followed by escape of the bacterium from the resulting double-membrane vacuole, thus allowing the cycle to repeat. Gene products essential for each step of the infection process have been identified. Six *L. monocytogenes* virulence genes (*prfA*, *plcA*, *hlyA*, *mpl*, *actA*, and *plcB*) are located together in one virulence gene cluster. Additional virulence-associated genes (e.g., *inlA*) are not linked to this virulence island.

L. monocytogenes Subtyping Methods

A variety of molecular typing (fingerprinting) methods that provide sensitive strain differentiation of *L. monocytogenes* have been described. These genetically based methods are often superior to classical methods (e.g., serotyping) because they generally provide more sensitive strain discrimination and a higher level of standardization and reproducibility. Serotyping, the classical strain differentiation method for *L. monocytogenes*, only provides discrimination of the species into 13 types. The most commonly used molecular methods that provide accurate and discriminatory typing results for *L. monocytogenes* include ribotyping (e.g., Bruce et al. 1995) and pulsed-field gel electrophoresis (e.g., Brosch et al. 1994).

Development of a *L. monocytogenes* Strain Collection and Database

Our research group has assembled a collection of human clinical and animal *L. monocytogenes* isolates to allow the development of a phylogenetic framework for *L. monocytogenes*. This collection now contains more than 400 human and more than 200 animal isolates as well as a limited number (approximately 200) of food isolates from selected food products (predominantly smoked fish and dairy products). The majority of *L. monocytogenes* isolates in our collection have been characterized by different DNA fingerprinting and phenotypic methods, including automated ribotyping and allelic analysis of the virulence genes *hly*, *actA*, and *inlA* using PCR-RFLP methods (Wiedmann et al 1997). Ribotype analysis identified more than 50 *L. monocytogenes* ribotypes that group into 11 ribotype subsets. PCR-RFLP analysis revealed 8 *hlyA,* 11 *inl*A, and 2 *actA* alleles. Selected isolates from our collection have also been characterized for virulence potential using a tissue culture plaque assay and by DNA sequencing of the virulence gene *actA* and of the *16S* rRNA gene (Wiedmann et al. 1997).

L. monocytogenes Phylogenetic Characterization

A combination of virulence gene alleles and ribotype patterns allowed us to separate *L. monocytogenes* strains into three distinct lineages designated I, II, and III (Wiedmann et al. 1997). These lineages may indeed represent different *L. monocytogenes* subspecies. Each lineage can be separated into three or four ribotype subsets, each of which contains between 5 and 20 genetic subtypes (ribotypes) with no detectable horizontal gene transfer among them. This work presents a unique phylogenetic framework for probing virulence differences among *L. monocytogenes* subtypes. For example, using a significant number of human and animal isolates, we assessed whether specific subsets of *L. monocytogenes* as defined by lineage, ribotype subset, and ribotype differed in their likelihood to cause human sporadic or epidemic listeriosis cases or animal listeriosis. Specifically, we examined linkages between independent phylogenetic and virulence markers in *L. monocytogenes* isolates (Jeffers et al. 2000).

The clinical histories of the *L. monocytogenes* strains suggested differences in pathogenic potentials among the three lineages. Lineage I contains 14 of 15 strains isolated during human epidemic outbreaks, whereas only 2% of human isolate was found in lineage III, suggesting the possibility that strains in this lineage may have reduced virulence for humans. Animal isolates were found in all three lineages. Specifically, we found a statistically significant predominance of human isolates compared with animal isolates in lineage I strains ($p = 0.0008$) as well as a significant predominance of animal isolates compared with human isolates in lineage II and III strains ($p = 0.041$ and $p = 0.002$, respectively) (Jeffers et al. 2000). These findings led us to hypothesize that lineage I strains or a subset of lineage I strains have a greater pathogenic potential for humans compared with strains in the other two lineages. This hypothesis is supported by the findings of Vines et al. (1992), who reported that perinatal listeriosis is caused more frequently by strains equivalent to those in our lineage I than by strains in lineage II. McLauchlin (1990) also found that serotypes 1/2b and 4b (grouped in lineage I) represent the majority of human isolates.

Additional evidence for virulence differences among *L. monocytogenes* strains was recently obtained from the investigation of a human listeriosis outbreak linked to the consumption of contaminated hot dogs. In this outbreak, a serotype 4b (lineage I) strain was responsible for more than 100 human listeriosis cases (even though it was present at <5 cells/gram), whereas a second strain (serotype 1/2a, lineage II) was present in many food packages from the same plant at higher levels (up to 3000 cells/gram) but was not linked with any human cases. These observations fit with other data from our laboratory that indicate that lineage I strains may be characterized by an increased human pathogenic potential compared with lineage II strains.

A *L. monocytogenes* Subset That Appears to Be Characterized by Reduced Human Virulence

Lineage III represents a unique subset of *L. monocytogenes* strains that show phenotypic and genetic features atypical for this species. Strains within lineage III have a unique

16S rRNA signature that differs by at least two nucleotides from strains in lineages I and II or from *L. innocua* strains (Wiedmann et al. 1997). Furthermore, by DNA-DNA homology, one representative of lineage III (ATCC 19114) is 72% related to the type strain of *L. monocytogenes* and 54% related to the type strain of *L. innocua* (Bruce et al. 1995), suggesting that these strains are not closely related. We also found that 70% of lineage III strains lack the putative virulence gene *lmaA*, which was found in all tested lineage I and II strains. *lmaA* encodes a listerial antigen capable of eliciting a delayed-type hypersensitivity reaction in mice (Schaferkordt and Chakraborty 1997). We found that only 2% of human isolates tested, but 11% of our animal isolates, were grouped in lineage III, a statistically significant difference in frequency of occurrence ($P = 0.002$). These findings indicate that strains in this lineage may be characterized by reduced virulence for humans and that this lineage might show specificity toward animal hosts (Wiedmann et al. 1997).

Lineage III can be further divided into two groups: lineages IIIA and IIIB. All isolates grouped in lineage IIIB are rhamnose-negative, although *L. monocytogenes* is characteristically rhamnose-positive. Although these strains appear closely related to *L. innocua*, the presence of the *L. monocytogenes* virulence genes *actA*, *hlyA,* and *inlA* strongly suggests that these strains should not be classified as *L. innocua* (Wiedmann et al. 1997, Bruce et al. 1995). No human isolates in our collection have been characterized as lineage IIIB, although a strain responsible for an outbreak of listeriosis in goats has been classified in this lineage (Wiedmann et al. 1999).

Detection of Unique *L. monocytogenes* Virulence Gene Alleles That May Serve as Markers for Virulence-attenuated and Human Pathogenic Subtypes

Two basic mechanisms may be responsible for differences in the pathogenic potential of *L. monocytogenes* subtypes: (1) the presence of different alleles of virulence-associated genes found in all *L. monocytogenes* and/or (2) the presence or absence of virulence genes in different *L. monocytogenes* strains. A variety of studies indicate that all known *L. monocytogenes* virulence genes appear to be present in the majority of *L. monocytogenes* isolates. Thus, we hypothesize that specific virulence gene alleles may contribute to differences in the pathogenic potential of different *L. monocytogenes* subtypes. For example, we have characterized three strains that showed attenuated virulence in a mouse model of listeriosis and attenuated tissue culture pathogenicity in a plaque assay (Wiedmann et al. 1997). Genetic characterization of these strains showed that they carry a unique *actA* allele encoding ActA proteins that is characterized by a unique combination of only three proline-rich repeats and a glutamic acid residue at aa 375 (Wiedmann et al. 1997). Thus, we have identified a unique *actA* allele that may represent a marker for a subset of *L. monocytogenes* with attenuated human virulence.

In a second set of experiments, we are currently exploring molecular markers for the major human epidemic clones of *L. monocytogenes*. Partial DNA sequencing of the *actA* gene from four isolates of the major epidemic clone (ribotype DUP-1038) revealed a unique, conserved amino acid sequence in the predicted ActA protein compared with 17 other isolates. This represents the first identification of a linkage between a specific virulence gene allele and this epidemic clone, which has caused the majority of human outbreaks. Using molecular genetic approaches (i.e., construction of isogenic mutants), we are in the process of determining whether these unique *actA* alleles are responsible for distinct virulence phenotypes.

Conclusions

Our results show that *L. monocytogenes* can be separated into three lineages that may be associated with human pathogenic potential. At least some *L. monocytogenes* strains found in ready-to-eat foods likely have limited or no human pathogenic potential. As an increasing amount of evidence is gathered in support of this hypothesis, a reevaluation of the zero tolerance ruling for all strains of *L. monocytogenes* may be warranted. Although zero tolerance appears to be justifiable for strains frequently associated with human disease (e.g., serotypes 1/2b and 4b or lineage I strains), the establishment of tolerance levels may be appropriate for lineages II and III or virulence-attenuated strains.

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Risk Assessment of *Listeria monocytogenes*: Prevalence in the Food Supply

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Listeria monocytogenes is widely distributed in nature, whence it easily gains access to food products. Occasionally it causes serious diseases like meningitis and sepsis in immunodeficient individuals as well as abortion in pregnant women. The annual incidence of listeriosis is about seven cases per million, with a fatality rate of 20–40% (Schuchat et al. 1991, Gellin and Broome 1989). The diseases might result from consumption of various food items contaminated with the bacterium. Most cases of listeriosis occur sporadically rather than as outbreaks, and are caused by the same kinds of foods as cause the epidemic cases (Schuchat et al. 1992, Pinner et al. 1992). Until now it has not been feasible to point out which strains cause listeriosis. These could be strains that are more virulent than others or strains that succeed in establishing themselves in the production environment and therefore occur more often in food items.

The purpose of this study was to identify and characterize *L. monocytogenes* in various food products and to compare them with those obtained from human cases of listeriosis to see whether matches exist among the strains.

Materials and Methods

The prevalence of *L. monocytogenes* on the processing line of broilers, turkeys, cold-smoked salmon, and related finished products as well as in various ready-to-eat products such as sandwiches and salads was determined. The samples collected were incubated for 2 days in Palcam broth (van Netten et al. 1989), and suspect colonies on Palcam agar plates were picked and identified as Gram positive, catalase positive, â-hemolytic, rhamnose positive, mannitol negative, and xylose negative.

Molecular characterization was performed by pulsed-field gel electrophoresis (PFGE) using the restriction enzymes Apa1 and Sma1 (Ojeniyi et al. 1996). The PFGE types of *L. monocytogenes* from food items were compared with the PFGE types of isolates from human cases of listeriosis.

Results

L. monocytogenes *in Broiler Production and in Final Products*

Samples from seven broiler abattoirs, including poultry processing lines, and final products were examined for *L. monocytogenes* (Ojeniyi et al. 1996). In the ready-to-cook poultry the occurrence of *L. monocytogenes* averaged 9.1% (range 0–27.5%). In sausages and similar products ready for consumption, 8.8% (range 0–50%) of the samples were positive for *L. monocytogenes*. This organism was isolated in 0.3–18.7% (average 8.0%) of the total of 385 samples examined from the processing line from each abattoir. In the cleaned and disinfected abattoirs, the contamination averaged 3.3% (range 0–10.0%). In the production line the highest number of positive samples was found on neck skin samples after the spin chiller (41.9%), followed by neck skin samples before the spin chiller (22.5%). In the spin chiller itself, *L. monocytogenes* was found in 21.3% of the samples. The evisceration machines and pluck sorter showed an average of 8.8% and 7.5% positive samples, respectively. In the cutting-up department, the number of positive samples was 13.1%. Cecal samples from 2078 broilers representing 90 randomly selected broiler flocks were negative for *L. monocytogenes*.

PFGE typing of 247 *L. monocytogenes* isolates revealed that the number of PFGE types from each abattoir ranged from 1 to 12. In each abattoir, identical PFGE types were found on the processing line before slaughter, in the evisceration machine, in neck skin before and after the spin chiller, in the cutting-up department, and in the ready-to-cook and ready-to-eat products. Nine of the PFGE types occurred in two or more of the abattoirs. In each abattoir where several *L. monocytogenes* strains were isolated, three to four of the PFGE types dominated. One of the PFGE types was identical to one of the types obtained from human cases of listeriosis.

L. monocytogenes *in Turkey Production and in Final Products*

An average of 10 turkey products, including ready-to-eat products as well as ready-to-cook products, were examined for *L. monocytogenes* on a weekly basis over a 9-week period. The prevalence of *L. monocytogenes* in ready-to-eat and ready-to cook products was 7.3% and 17.4%, respectively.

Two visits to the abattoir showed that the prevalence of *L. monocytogenes* in the processing line ranged from 25.9% to 41.4%. A total of 99 and 105 samples from the two visits were examined for *L. monocytogenes*. Cleaning and disinfection decreased the prevalence to 6.4%.

Twenty-two critical control points were selected in the abattoir. Gauze tampon samples from each of these control points were taken during processing as well as after cleaning and disinfection. This procedure was repeated seven times at 3-week intervals over a period of 18 weeks. Recovery rates before processing and after cleaning and disinfection ranged between 0% and 13.6% (average 5.8%). During processing the prevalence ranged from 4.5% to 45.5% (average 24.0%).

Two PFGE types dominated among the *L. monocytogenes* strains isolated, and they persisted throughout the 8 months of the study, whereas four other types of *L. monocytogenes* were detected only during shorter intervals. On the first visit, 27 of 41 (65.9%) isolates were of PFGE type B, 11 (26.8%) were of type D, and three were of types E, F, and G. Of the seven isolates from the second visit, two and four were of types B and D, respectively, and one was type C. After disinfection, three of five isolates were of type B and two were of type A. On both visits to the abattoir, PFGE types B and D were detected in all parts of the processing line, including crates and the slaughtering, evisceration, and cutting-up departments. Both PFGE types were also detected in the finished products. Two identical PFGE types were found in turkey products and in human cases of listeriosis.

L. monocytogenes *in Cold-smoked Salmon Production and in Final Products*

During two visits to plant 1 that took place 1 month apart, a total of 869 samples were collected; two similar visits at plant 2 yielded 962 samples. Samples were taken from the fresh salmon at the entrance to the plant, from critical control points during processing, and from the final products. The prevalence of *L. monocytogenes* ranged from 2% to 10% in plant 1 and from 13% to 31% in plant 2. Each plant was dominated by *L. monocytogenes* of a single PFGE type that was found both on the processing line and in the finished products, whereas only one sample from fresh salmon was positive for *L. monocytogenes*. In plant 1, 46 of 52 strains (88.4%) belonged to PFGE type K, which was also represented by a few strains in plant 2. In plant 2, 115 of 233 strains (49.3%) belonged to PFGE type L. The prevalence of *L. monocytogenes* in vacuum-packed cold-smoked salmon from plants 1 and 2 was 9% and 47%, respectively. A total of 16 PFGE types were identified. Types L and M dominated, representing 74 and 37 strains, respectively. Six other PFGE types were detected with a frequency of two to seven strains. Eight isolates represented eight different PFGE types. Four PFGE types were found in both salmon and in human cases of listeriosis.

L. monocytogenes *from Various Ready-to-Eat Products*

One hundred twenty-three *L. monocytogenes* strains were isolated from ready-to-eat foods sent to Danish municipal food and environmental laboratories. The strains originated from many different kinds of ready-to-eat products such as sandwiches, salads, cheeses, and ready-to-eat dishes. Forty-one *L. monocytogenes* strains were isolated from human cases of listeriosis in Denmark during the same period. All of the isolates were characterized by PFGE.

Nine PFGE types were detected in both human and food isolates. These constituted 25 of 41 (60.9%) of the human isolates and 94 of 123 (76.4%) of the food isolates. The number of human strains in each of these PFGE types ranged from one to five strains, whereas the number of food strains ranged from 1 to 44 strains. Ten PFGE types were detected only from humans, whereas 20 PFGE types were obtained only from foods.

On the basis of the results of this study, we could not point out specific food items as responsible for human listeriosis, since no specific food item or food category could be connected to a specific PFGE type. However, all of the food items may represent a reservoir of *L. monocytogenes* and thus constitute a potential source of human infection.

Conclusions

Poultry, turkey, and salmon products from Danish abattoirs might represent a reservoir of *L. monocytogenes* and a source for human infections. However, investigations of other ready-to-eat products in Denmark showed that a wide range of products could constitute a potential source for human listeriosis. The results of this study also indicate that the main source of contamination of poultry, turkey, and salmon products probably originates from improper hygiene along the processing line and, to a lesser degree, from the raw materials themselves. Therefore, improvement in cleaning and disinfection processes is recommended to reduce the occurrence of *L. monocytogenes* and, hence, to minimize the risk of human infection.

The finding of a few dominant and persistent clones in different types of plants may indicate that these strains are more resistant to disinfectants or that they may possess a special ability to colonize the processing environment. Isolation of identical strains in human cases of listeriosis and in different food products might also indicate that some of these strains are more virulent for human beings.

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Risk Assessment of *Listeria monocytogenes:* Impact of Cooking and Food Handling Procedures in the Home

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Consumer concern about the risk of harmful bacteria in food has increased. When volunteering comments on areas of serious threats to food safety, more consumers mention germs and bacteria-related factors than pesticides, chemicals, or any other risk category (Abt Associates Inc. 1997). Many consumers, however, have not adopted food selection and handling behaviors that protect against foodborne illness. This paper reviews consumer attitudes and practices that increase exposure to *Listeria monocytogenes*.

Listeria has been isolated from a variety of foods, including fresh produce, luncheon meats, beef jerky, and soft high-moisture cheeses (Beuchat 1996, Knabel 1995). Competitive pressures at retail may compel some manufacturers of fresh foods to place a long shelf life date on their products, in some instances longer than warranted to maintain optimum quality and safety. If the perishable food is temperature abused during storage, marketing, or consumer use, *Listeria* can grow and reach a population number of health significance.

Consumer handling recommendations to protect against *Listeria* may conflict with common consumer practices. Some foods that may contain *Listeria* are often eaten without further heating. Because this organism can survive and slowly grow under refrigeration temperatures, advice to keep food cold is not always effective.

Although recent outbreaks may have increased awareness of *L. monocytogenes*, consumer awareness is not high. More consumers were aware of *Salmonella* than of any other pathogen (Altekruse 1996). Only 10% of consumers were aware of *L. monocytogenes,* and 1% were able to name a food source of this pathogen, compared with 80% who were aware of *Salmonella,* with 54% able to identify a food source.

Consumers believe that food safety problems are most likely to occur at food processing or manufacturing facilities and at restaurants (Research International 2000). Only 14% believe that problems occur in the home. Fein and colleagues (Fein et al. 1995) theorize that when consumers are not aware of the source and consequence of foodborne illness, they will not be aware of preventive practices or be motivated to change behavior.

Consumer Practices

Consumers indicate that they are following several practices to ensure that the food they prepare is safe. Most consumers (60%) volunteer that they frequently wash their hands, and 36% indicate that they wash vegetables, 17% believe that they cook properly, and 13% mention that they refrigerate promptly (Research International 2000). Only 6% mention that they keep food separate, and 6% say that they use fresh foods before the expiration date.

Kitchen sanitation may be inadequate to prevent cross-contamination between foods that contain *Listeria* and other foods. Failing to wash cutting boards with soap and water between cutting meat or poultry and vegetables has been noted in 31–46% of consumers (Altekruse 1996, Audits International 1999, Bruhn and Schutz 1999, Williamson et al. 1992). Findings from a current study indicate that as many as 40% of consumers do not always wash the sink before preparing fruits and vegetables, half do not wash the sink after preparing produce, and some do not wash fruit at all, including at least 30% who do not wash melons before slicing (Bruhn, unpublished research, 2000).

People think that they keep food cold when they put it in the refrigerator, but some consumers set the refrigerator too warm. An Audits International survey of 121 households (Audits International 1999) found that 9% of households refrigerated ingredients above 45 °F and an additional 23% refrigerated ingredients between 42 and 45 °F. Participants in this study were not randomly selected, and included 70% of participants with a college degree, and may therefore not necessarily represent behavior typical of U.S. consumers.

Although heating can destroy *Listeria*, cooking practices did not meet recommended temperatures of 140–165 °F in 19% of households (Audits International 1999). The author has observed that some parents heat hot dogs only to warm them, so that the child can eat them immediately without getting burned. This is a reasonable practice from

the parents' perspective, but is a risky practice should the hot dog be contaminated with *L. monocytogenes*. Furthermore, many consumers judge thoroughness of cooking by appearance and do not use a thermometer.

Poor consumer inventory control of refrigerated products and failure to discard products when the package date has passed are potentially hazardous practices. The Audits International survey found that 46% of consumers had items in the refrigerator that were past the use-by date. In a California study, only 48% of consumers indicated that they discard luncheon meat or other products after the date on the package (Bruhn and Schutz 1999). Furthermore, up to 50% of consumers report always or sometimes relying on taste to determine if leftovers are safe. Consumers expect food to be usable as long as there are not visible signs of spoilage. To guide consumers, manufacturers should place the sell or use-by date in a more visible location. Storage temperature and the length of time consumption is expected to be safe should be indicated. This information could be coupled with point-of-sale material.

Infrequent refrigerator cleaning may increase risk by providing an opportunity for *Listeria* cross-contamination. Cleaning the refrigerator is a sporadic practice. Consumers indicate that they clean up after spills, but about 40% acknowledge that they clean the refrigerator only one to three times a year (Bruhn, unpublished data, 2000). Consumers use a variety of washing solutions, with dishwashing liquid the most frequent. Some use water only for cleaning. If refrigerators can be a source of *Listeria*, consumers need information on the effectiveness of different cleaning solutions and recommended cleaning frequencies.

Safe handling by some consumers has increased in recent years. Audits International found that in about 100 households, the average number of critical food safety violations decreased from 2.8 in 1997 to 1.7 in 1999 (Audits International 1999). A critical violation was defined as one that could potentially lead to foodborne illness. The incidence of cross-contamination decreased from 73% in 1997 to 31% in 1999. Hand washing frequency increased and violations decreased from 57% in 1997 to 29% in 1999. Less dramatic changes occurred in proper cooking and refrigeration, but the direction was toward increased safety.

These changes are likely the result of increases in knowledge; however, consumers who are knowledgeable about safe handling do not follow all safe handling practices (Altekruse 1996). Some know recommended practices but still follow less safe behavior for convenience, habit, or taste preference. For example, 75% of consumers with more than 12 years of schooling knew that cooking ground meat well done decreased the risk of food poisoning; however, only 63% actually served hamburgers medium or well done.

Both lack of knowledge and lack of motivation were responsible for food handling errors in the Audits International survey (Audits International 1999). In the area of cross-contamination, 65% of consumers were not aware that bacteria could transfer from cutting board or knife to food, and 16% did not think contamination was important. Of those who did not wash their hands, 59% did not realize that bacteria could be on visually clean hands and 19% did not think hand washing was important. Of those who had an insufficiently cold refrigerator, 70% indicated that they were not aware of the appropriate temperature and others either did not think it was important or were confused by multiple recommendations.

New food safety technologies such as food irradiation can reduce the incidence of *Listeria* in luncheon meats and soft cheeses. Consumer surveys indicate that people will purchase foods irradiated to destroy harmful bacteria. However, interest in purchasing irradiated food as recorded by the Food Marketing Institute annual nationwide survey decreased from 56% likely to buy in 1999 to 38% in 2000. Even fewer consumers reported interest in purchasing food that was processed to destroy harmful bacteria than in purchasing irradiated food.

In contrast, a nationwide survey in 1998 conducted after Food and Drug Administration approval of irradiation found that more than 80% of consumers considered irradiation an appropriate treatment for poultry, pork, and red meat (Food 1998). Recent marketing of frozen irradiated ground beef first introduced in Minnesota confirms consumer interest in this product. Frozen irradiated ground beef is now available in more than 15 states. Fresh irradiated beef sold in Florida is reported to sell well. When approved by the FDA, irradiation of luncheon meats, hot dogs, and other products could help reduce consumer exposure to *Listeria*.

Consumer Education

To protect themselves against listeriosis, consumers need information on high-risk foods, the seriousness of listeriosis, populations at greatest risk, and food purchase and handling practices to reduce risk. Kitchen sanitation and thorough heating of high-risk foods should be stressed. Tasting to determine safety should be targeted as an unsafe practice. Many consumers have not yet realized that pathogens can exist in foods that look and taste good. Consumers need to hear the recommended refrigerated storage temperature, and they need to understand that some pathogenic bacteria can grow under refrigerated conditions. The importance of inventory control of all foods including leftovers should be explained. The educational process should include both what to do and why so that knowledge and motivations work together to change behavior.

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Update on FDA's Risk Assessment of *Listeria monocytogenes*

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The U.S. Food and Drug Administration conducted a risk assessment based on current scientific knowledge as an initial step in reviewing its approach to maximizing public protection from foodborne *Listeria monocytogenes.* The risk assessment evaluated the presence and quantitative levels of *L. monocytogenes* in 21 groups of ready-to-eat foods. The potential growth of *L. monocytogenes* between retail, where contamination data originated, and consumption was modeled. The frequency and amount of consumption of these foods completed the data for the exposure assessment. For the hazard characterization or dose-response part of the risk assessment, data from animal studies, virulence assays, and epidemiological investigations were used to estimate the likelihood of illness for different human groups from consuming different numbers of *L. monocytogenes*. The quantitative modeling provides greater insight than does a qualitative review and also indicates the uncertainty of our knowledge. The risk assessment does not attempt to define an acceptable or tolerable level of *L. monocytogenes* consumption or to propose changes in regulations.

Note

The risk assessment for *L. monocytogenes* (Draft Assessment of the Relative Risk to Public Health from Foodborne *Listeria monocytogenes* Among Selected Categories of Ready-to-Eat Foods) and risk management action plan (Joint Response to the President: Reducing the Risk of *Listeria monocytogenes*) is available on the web at www.foodsafety.gov.

LISTERIA MONOCYTOGENES: CURRENT ISSUES AND CONCERNS

Detection , Enumeration, and Intervention Strategies for *Listeria monocytogenes*

Rapid Genetic Methods for the Detection of *Listeria monocytogenes*

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The history of *Listeria monocytogenes* goes back to 1918, when French workers isolated an unnamed organism from infected cerebrospinal fluid and lodged the culture in a collection at the Institut Pasteur. This organism was later identified as *L. monocytogenes*. The first description of the "*Bacterium monocytogenes*" was made by Murray and coworkers in 1926, and the name *Listeria monocytogenes* was given in the 1940s. From that point until the early 1980s, *L. monocytogenes* tended to be considered an organism that could be found fairly easily in the general environment but that could be a pathogen in a range of animals, including cattle, sheep, rodents, birds, and fish.

Contaminated animal feed was recognized as a cause of animal listeriosis, but the problem of human infection was not widely understood until the early 1980s, when the organism was found to be responsible for some large outbreaks of human food poisoning.

Test Methods

Listeria are slow growing and are poor competitors compared with other groups of interest to food microbiologists (e.g., members of the Enterobacteriaceae). This, together with a lack of recognition of *Listeria* as pathogenic to humans, meant that method development was not well advanced when the first recognized foodborne outbreaks occurred. At this point, the best method was a long enrichment (more than 40 days) at cold incubation conditions (*Listeria* is a psychrotroph; therefore, cold incubation acts selectively in enrichment) followed by isolation on selective agar. This method was far from ideal for use with foods, where more rapid isolation methods were required.

A large effort by many workers around the world led to the development of a number of analytical methods that allowed the isolation of *Listeria* from foods within 3 to 4 days. Although these methods were great improvements on cold enrichment, they were still far from rapid, and in a food industry in which fast results are of the greatest importance, there was a need for more rapid methods.

Rapid Methods

The long incubation times required by classical methods have resulted in a large effort to develop more rapid methods for the detection of *Listeria* from foods. Much of this work has led to the development of commercial products that are commonly used in food testing laboratories today. The general types of rapid tests are:

Microplate-based enzyme-linked immunosorbant assay (ELISA). A number of commercial products are available, all based on the same basic principles. Food or environmental samples are enriched and added to a microtitre plate containing "capture" antibodies. *Listeria* are immobilized in the wells of the plate and treated with a second antibody, which is labeled with an enzyme. The enzyme converts a substrate into a product that can be measured. The presence of a level of product above a predetermined threshold is taken to indicate that the organism was present in the sample under test. The main problems with this approach are that a 48-hour enrichment is still required before the ELISA test is applied. Additionally, because most tests are directed at *Listeria* species (a small number of *L. monocytogenes* ELISA tests are available), a series of confirmatory tests must be done to identify the presence of *L. monocytogenes*.

"Dipstick" immunoassay. A number of these kinds of commercial products are available, and the principles for all are similar. Food or environmental samples must initially be enriched before being added to the dipstick module, which is composed of a strip of absorbent material inside a plastic cover. The absorbent strip has colored latex particles linked to specific antibodies within it, and these move as the sample moves up the stick by capillary action. At the top of the stick, the absorbent material has a line of specific *Listeria* antibodies attached to it. These bind to the *Listeria* attached to the latex to form an immobilized line of colored latex, which can be seen by the eye.

As with ELISA tests, the negative side to these tests is the requirement for enrichments and the need to identify

isolates to confirm the presence of *L. monocytogenes*.

Immunomagnetic separation (IMS). These tests rely on small magnetic particles onto which are attached specific antibodies. The particles are mixed with an enriched sample and then separated from it by the use of a magnetic field. The separated particles will be linked to the target microorganism and will be more concentrated than they were in the enrichment and free from interfering food debris. The final detection test can be by plating the particles onto a suitable agar or by using them in an ELISA test. IMS is a fairly simple test that requires no equipment. Its main role is to shorten enrichment time and to separate microorganisms from the enrichment broth and associated food debris.

Genetic Methods

Genetic methods are based on an analysis of the genetic material within the cell. Tests of this type will thus be directed toward DNA or RNA targets. Because these materials carry the genetic identity of the microorganism, they can be engineered to detect whatever the method designer wishes. Thus, a test could be designed to be genus specific, species specific, and strain specific, or even to detect a particular characteristic of a strain, such as the presence of a known pathogenicity factor. Genetic methods can thus be uniquely useful to microbiologists.

The development of test methods based on genetic analysis has been going on for at least 20 years. The main problem has been that the skills, techniques, and equipment required have been more in keeping with a research laboratory than a food quality control laboratory. Since method protocols became more user friendly and were designed for use in food microbiology laboratories, these techniques have seen more widespread use.

Use of Genetic Methods

In food testing, genetic methods can be used to achieve the following: detection, confirmation/identification, amplification, speciation, and subtyping. Each of these can be achieved in a number of ways using the range of techniques available to the molecular biologist. In the rest of this review, only commercially available techniques to detect *Listeria* will be covered because only these can be used immediately in a wide range of food microbiology laboratories around the world.

Hybridization Probes

The first and currently the only nucleic acid hybridization probe kit marketed for the detection of *Listeria* from food products was produced by Gene Trak in the 1980s. The kit targets two probes to genus-specific regions of 16S rRNA. The two probes have different roles: one has a label that allows the bound probe to be visualized by the operator, and the other has a sequence that allows the probe to bind to a plastic dipstick. The bridge between the two probes is the target rRNA sequence from *Listeria*. Thus, only if the organism is present will the label be attached to the dipstick so that a positive reaction can be visualized.

This kit has been widely validated by organizations such as the Association of Official Analytical Chemists (AOAC) and the Association Français de Normalisation (AFNOR) and has given results that are comparable to classical methods. Gene Trak has also developed a similar kit based on a *L. monocytogenes*–specific capture probe, thus allowing the specific detection of this species.

Probes can also be used for the identification of *L. monocytogenes* through the use of the commercially available Gen Probe kit. This is a culture confirmation test that uses a species-specific DNA probe to rRNA (16S). The probe is prelabeled with a luminescent acridinium ester that is resistant to deesterification only when hybridized; the deesterified label is nonluminescent. An unidentified *Listeria* (usually from an isolation plate swab or colony pick) is added to a lysis reagent and the probe. If the isolate(s) are *L. monocytogenes*, the probe will bind to rRNA; the acridinium is protected and will luminesce. This will be detected in a luminometer and indicates the presence of *L. monocytogenes*. If the probe cannot bind (i.e., if *L. monocytogenes* is absent), the label will be deesterified and will be rendered nonluminescent. The whole test is very rapid, giving a result in approximately 30 minutes.

Polymerase Chain Reaction (PCR)

The sensitivity of nucleic acid hybridization probes is such that a 2-day enrichment is still required to increase to detectable levels the number of *Listeria* cells potentially present in the sample. A way to overcome this problem became available with the development of kits based on the PCR amplification reaction. PCR is a biochemical in vitro reaction that uses the enzyme DNA polymerase together with a cyclic change in reaction temperature to amplify a DNA sequence. PCR is capable of giving a 10^9-fold amplification of a DNA sequence in approximately 3 hours. This level of rapid amplification has the capacity to reduce the requirement for enrichment.

Currently at least six commercial kits produced by four companies (Qualicon Bax, BioRad Probelia, PE Applied Biosystems TaqMan, and Biotecon Food Proof) are targeted at the detection of *Listeria* in food and environmental samples. Four specifically detect *L. monocytogenes* and two are aimed at the *Listeria* genus.

The different methods vary in their requirement for enrichment, but the minimum is 24 hours, with some kits requiring 48 hours before the PCR test can be done. All methods also require specialist PCR equipment for thermocycling and other equipment for running electrophoresis gels or reading microtitre plates.

The sensitivity of these methods is lower than that for hybridization probes, with approximately 10^3 to 10^4 cells being required to give a positive result. The other advantage of these methods is that they are totally specific, so

that no confirmatory testing is required after a positive result is obtained.

Nucleic Acid Sequence–based Amplification (NASBA)

NASBA is another nucleic acid amplification test, which differs significantly from other such tests. NASBA uses three different enzymes to achieve amplification, and the reaction is isothermal, requiring no temperature cycling. Research papers report the use of NASBA to detect *L. monocytogenes* from foods. In these cases the target for the amplification reaction was either the 16S rRNA or hemolysin A mRNA of *L. monocytogenes*.

Because the NASBA protocol described in the published reports is too complex to be routinely used in food testing laboratories, simplification is required if this technique is to be for quality control laboratory use. The technique is reported to require 10^2–10^3 organisms to achieve a positive reaction, and the protocols used have required a 48-hour enrichment to achieve this.

Subtyping

The ability to identify an isolated microorganism below the species level has a number of advantages for the food microbiologist. The genetic fingerprint produced can be used to uniquely identify an isolate and will help in tracing the origin of an organism, in showing routes of contamination or cross-contamination, and in furthering understanding of the microbial ecology of foods and the food production environment.

A number of techniques have been used to subtype *Listeria* and *L. monocytogenes,* e.g., pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and ribotyping. Although most of these methods require specialized equipment and training to use, the automation and standardization of such methods are broadening their usefulness. Automated ribotyping has been available for a number of years with the use of the Qualicon RiboPrinter. This can uniquely identify more than 40 different types of *L. monocytogenes* and can be a valuable tool in investigations of food-related contamination.

The ability to subtype *L. monocytogenes* requires us to ask if pathogenic strains are to be specifically highlighted. Research suggests that many strains of *L. monocytogenes* are not pathogenic to humans in low-risk groups and that only certain identifiable strains are responsible for the major outbreaks of illness that have been seen around the world. In the future, it may be possible to use typing techniques to test directly for pathogenic strains of concern.

Conclusions

In the relatively short time that *L. monocytogenes* has been a known food pathogen, several methods to detect it have been developed. The genetic methods for its detection have moved from research laboratory–based techniques to relatively easy-to-use kits that are available commercially for use in routine food testing laboratories.

One of the major problems with *Listeria* remains its relatively slow growth rate and poor competition with other food-related organisms, such as members of the Enterobacteriaceae. Thus, enrichments tend to be selective and have to be for longer time periods. The selective nature of the enrichment also brings into question the ability of such procedures to recover injured or stressed cells. Another issue that must be raised is the "acceptable" levels of *L. monocytogenes* in foods. The United States requires zero tolerance in ready-to-eat foods, whereas other countries (e.g., European countries and Canada) permit countable levels of *L. monocytogenes* in certain foods under certain circumstances. Therefore, when considering detection methods, we should also look at enumeration procedures. Rapid enumeration methods are not now available but may be required in the future.

The genetic test methods themselves can offer great advantages over classical microbiology: shorter test times, greater specificity, and subtyping to trace contamination routes and even to allow direct testing for the pathogenic, epidemic strains of *L. monocytogenes*. These methods advance the science of food microbiology and give the microbiologist advanced tools to help produce safe and stable foods.

Ecology of *Listeria monocytogenes*: Studies on Incidence, Growth, and Microbial Competition in Primary Production

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Listeria monocytogenes is commonly found in soil and water and on plant material, particularly that undergoing decay, and these environments are regarded as the natural habitat of the organism (Rocourt and Seeliger 1985). *Listeria monocytogenes* is able to survive longer under adverse environmental conditions than many other nonsporing bacteria of importance in foodborne disease. This attribute, together with its ability to colonize, multiply, and persist on processing equipment, makes it a particular threat to the food industry and explains why the natural environment is considered a potential source of contamination of processed food products. Only relatively recently, with the introduction of improved molecular typing methods, has the full story of listeriosis epidemiology been emerging.

Soil and Vegetation

Soil is often referred to as the source of *Listeria* contamination, particularly contamination of silage (Fenlon 1988). Fertile agricultural soil is the recipient of decaying plant material, animal wastes, and sewage sludges, all of which are well-documented sources of *L. monocytogenes*. Weis and Seeliger (1975) showed that soil samples taken at a depth of 10 cm gave significantly fewer positive samples than surface soils, indicating that vegetation is a principal component in *Listeria* contamination of soil. The survival of *L. monocytogenes* in soil depends on the soil type and its moisture content (Welshimer 1960). From the evidence of the literature to date, it does not appear that soil is a natural reservoir in which *L. monocytogenes* multiplies. The presence of the organism in the soil is probably due to contamination by decaying plant and fecal material, which, together with damp surface soil providing a cool, moist protective environment, enables *L. monocytogenes* to survive from season to season.

A study by Fenlon et al. (1996) found that vegetable crops, such as lettuce and carrots, immediately postharvest did not carry *Listeria* to any significant extent. Confirming the findings of Faber et al. (1989), who found little *Listeria* contamination of unprocessed vegetables, a number of workers (Beuchat et al.1990, Gras et al. 1994, Carlin et al. 1995) reported that the degree of processing and packaging can significantly increase *Listeria* levels in processed vegetables. Similarly, this higher incidence of *L. monocytogenes* is particularly apparent in harvested (processed) grass prior to ensiling.

Animal Feeds

Most formulated animal feeds have low levels of available water, which restricts the multiplication of *L. monocytogenes*. The same is true of hay and cereal grains. Thus, although *L. monocytogenes* has been reported in such materials (Garcia et al. 1996), the numbers are unlikely to reach levels that present a serious risk to livestock. Many formulated feeds are sold in pellet form and have received a degree of heat treatment capable of killing a high proportion, if not all, *Listeria* present. It is well documented that the animal feed most closely linked with animal listeriosis is silage (Gitter 1986, Gitter et al., 1986, Grøntsøl 1979). Much of this association can be attributed to the high numbers of *L. monocytogenes* present in contaminated silage (Fenlon 1986a, 1986b). Good-quality silage prepared from grass, maize, whole-crop cereals, or leguminous plants rapidly undergoes a lactic fermentation, causing a rapid fall in pH, with well-preserved silages generally having a pH $\leq$ 4.5. These acidic conditions inhibit the growth of both spoilage microorganisms and *Listeria,* as long as anaerobic conditions are maintained. Grass silages in cooler, wetter climates tend to have lower sugar levels and higher moisture contents, resulting in poorer, slower fermentation, and so are more susceptible to *L. monocytogenes* contamination than grass and maize silages produced in countries with warmer climates.

Listeria monocytogenes contamination is most frequently associated with poor-quality silage, particularly silage that has been exposed to aerobic spoilage. Gitter (1986) noted that from 1975 to 1985 the incidence of listeriosis in sheep in Great Britain increased from less than 50 incidents per year to more than 250. He also noted that the pattern changed from isolated single incidents to much

larger flock outbreaks, which he attributed to a change in the method of conserving forage used to feed sheep. During the same period the rise in cattle listeriosis was much lower.

In the United Kingdom sheep are kept mainly on upland areas and were traditionally fed hay. The wet climate of these hill farms is not conducive to making good-quality hay, but silage was not an economic option prior to the mid 1970s, because it required expensive capital outlays in silos. The introduction of the half-ton round bale made silage feeding feasible by baling grass and sealing it in large plastic bags to make silage. Unfortunately, some of the early attempts to make silage in this way were not very successful, and baled silage was of poorer quality than was clamp silage (Fenlon 1985). Because *L. monocytogenes* is a surface problem and big bales have a much greater surface area for a given mass of silage than silage from clamps or silos, the potential for serious contamination to develop is much greater in bale silage, particularly if it is not sealed from air entry and aerobic deterioration, often associated with visible molding, takes place. Fortunately, much of the *L. monocytogenes* contamination in silage occurs in visibly moldy areas, so if these are removed and discarded prior to feeding, the challenge to the animal is considerably reduced.

Fecal Material

Given the ubiquitous nature of the organism, and given that most reports on enteric carriage have involved the testing of relatively large quantities of fecal material for the presence or absence of *L. monocytogenes* by enrichment techniques, with little quantitative data, it is not suprising that the feces of many animals have proved positive at some stage. Gray and Killinger (1966) listed 37 mammals from whose feces the organism had been isolated, and the list is now much longer. However, the gut is not a favorable environment for the survival and growth of *L. monocytogenes*. In the rumen, numbers of *L. monocytogenes* declined following ingestion, with significant protozoal predation (Shepherd et al. 1997). There was a further significant decline in the abomasum because of the acidic conditions, followed by a slight rise in *Listeria* numbers in the small intestine (Shepherd 2000). Thus, numbers in feces are lower than those in the diet. There is not much evidence of long-term colonization of the ruminant gut, although Fenlon et al. (1996) demonstrated shedding of a *L. monocytogenes* serotype 4 for at least 8 weeks after silage feeding had ceased.

The influence of diet on excretion of *L. monocytogenes* has more recently been studied, particularly in ruminants. Low et al. (1995) reported a low incidence of excretion in a flock of 100 grazing sheep; the incidence increased significantly to between 10% and 33% once silage feeding commenced, confirming the finding of Husu (1990) that the tendency is for excretion rates to be lower in grazing animals. In a study of diet and excretion, Fenlon et al. (1996) monitored two groups of cattle. While grazing, none of the tested cattle excreted *L. monocytogenes;* when retested after silage feeding commenced, 28.6% of one group and 30.8% of another excreted *Listeria*. Formulated diets for intensively reared pigs and poultry were free of the organism, which probably accounted for the fact that of nine samples of broiler poultry litter tested, only one was positive and all 10 swabs of feces taken at the processing plant from crates used to transport the birds were negative. Similarly, only one of 47 fecal samples from pigs and piglets was positive. Genigeorgis et al. (1989) in a study at a poultry processing plant found no *L. monocytogenes* in feather composite samples from live hanging birds or their hind gut contents, yet the incidence of *Listeria* on carcasses increased as processing progressed.

Water

The ubiquitous nature of *L. monocytogenes* and the discharge of sewage effluents and runoff of polluted water from agricultural land to surface water courses inevitably result in the presence of the organism in lakes, rivers, and streams. Dijkstra (1982) found the organism in 21% of surface water samples in the northern Netherlands and noted that even though the lakes were used by swimmers, no human cases were linked to this activity. A study of the course of the River Don in northeastern Scotland (Fenlon et al. 1996) found that 42% of 19 100-mL samples were positive for *L. monocytogenes* in May, and 53% 6 months later. Despite the high frequency of positive samples, *L. monocytogenes* numbers were low, ranging from 10 to 350 cfu per liter. The highest numbers were found at a sampling point below a sewage works, but this finding was not consistent for both sampling occasions. No factor, seasonal or otherwise, could be related to the presence or numbers of *L. monocytogenes,* which occurred over the whole course of the river. The main risk from water-courses appears to be the contamination of food products such as marine and freshwater fish with polluted water (Motes 1991, Jinneman et al. 1999).

Transmission

A noticeable feature of the primary production environment is the low incidence of *L. monocytogenes* found on plants and in animals and, when positive, the low numbers present. Once processing is initiated, however, *Listeria* numbers and the percentage of samples found to be positive increase significantly. Another feature is the diversity of types of *L. monocytogenes* present in the environment (Baxter et al. 1993, Boerlin and Piffaretti1991). In some of these instances in the Scottish study (Fenlon et al. 1996) it had been shown that the same types were found at various stages of the food chain, e.g., grass, silage, and forage-fed animals. However, in other cases, e.g., pigs and poultry, there appeared to be little contamination by *Listeria* at the

primary production level, yet much of the final product was found to have *L. monocytogenes* contamination. There is also evidence that although there is a wide variety of *L. monocytogenes* strains initially present on plant and animal raw materials or in the environment in which they are grown, the number of strains on processed products is much more limited; i.e., processing may select the relatively few that are capable of colonizing the processing system. Thus, a product that is sporadically contaminated with a variety of strains of *L. monocytogenes* prior to processing may be consistently contaminated with a single strain postprocessing (Boerlin and Piffaretti 1991). These strains may be process adapted, but they also appear to originate in the environment. However, the selective conditions enabling them to become established in particular processing environments remain unknown and are open to further investigation.

Suspicion must therefore lie with processing as a major hazard for cross-contamination and amplification of the level of *L. monocytogenes* contamination. This has important implications for hygiene management in the food industry, particularly with food testing procedures geared to zero tolerance in 25-g samples. As can be seen in silage, the time span between a product showing a nondetectable level and widespread low-level contamination can be very short when favorable conditions for growth occur.

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Production Intervention Strategies to Control *Listeria monocytogenes:* Prospects for the Use of Irradiation (or Pasteurization) for Packaged Ready-to-Eat Meats

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Listeria monocytogenes is viewed as a postprocessing contaminant in ready-to-eat (RTE) processed meats. *Listeria monocytogenes* is typically destroyed by normal thermal process and becomes an issue only when it contaminates the product after processing and prior to packaging. A typical thermal process for a processed RTE food may heat the interior of the product to a final temperature as high as 76 °C (170 °F), and the combined lethality of the thermal process may be the equivalent of a 3800 $\log_{10}$ reduction in the population. The bacteria are therefore surface contaminants on the product, and are not part of the microflora of the interior of RTE products. Current intervention strategies are therefore designed to eliminate the bacteria from the surface of RTE products, and not necessarily from the interior of the product.

The basic concepts of intervention strategies involve pasteurization of the exposed surfaces of the products either before or after packaging. Many of these methods involve thermal processes, such as steam and hot water. An example of a pasteurization technology applied prior to packaging is the method developed in the U.S. Department of Agriculture's Agricultural Research Service laboratories in Wyndmoor, Pennsylvania. This method involves applying steam in a vacuum chamber to sanitize individual hot dogs. As described in the literature, this process first applies a vacuum to remove the air and water on the surface of the hot dog, followed by a quick application of steam. The steam condenses on the product and transfers the thermal energy rapidly to the surface. A second vacuum is then applied to cool the product and remove the residual steam. Using multiple cycles (two and three) of this process, researchers (Cygnarowicz-Provost et al., 1994) reported greater than 4 $\log_{10}$ reductions of *Listeria* spp. that were inoculated on the surface of the product, with a total process time of less than 2 seconds.

The second approach to intervention strategies involves treatment after packaging. These interventions can be separated into thermal and nonthermal processes. An example of a typical thermal process for postpackaging pasteurization is water immersion. The processed RTE meats are packaged and then submerged in hot water, in either a batch or a continuous process. The hot water raises the surface temperature of the product to a point that eliminates the bacteria. A problem for RTE processed meats is that they are often packaged with multiple units in a single package, and these individual units typically touch or overlap. The surface area where this overlap occurs is somewhat insulated from the heat, and takes longer to reach a sufficiently high temperature to destroy microorganisms.

This type of thermal process has been investigated. It typically uses water temperatures that range from 70 to 96 °C (158–205 °F) and dwell times from 30 seconds to 10 minutes. One report from an equipment manufacturer indicated that with a water temperature of 90 °C (194 °F), the minimum surface temperature of a single layer of packaged hot dogs reached 72 °C (161 °F) in 9 minutes, and then required 16 minutes of chilling. A double layer of packaged hot dogs reached the same minimum temperature in 25 minutes and required 40 minutes of chilling. Other industry data suggest that *L. monocytogenes* inoculated onto the surface of packaged hot dogs could not be recovered when the surface temperature reached 66 °C (150 °F). As with any thermal process, the recovery of bacteria after any process is in part dependent on the initial inoculum. As indicated earlier, the random contamination that would occur after cooking but prior to processing would be expected to occur only at low levels.

The drawback of this process is the amount of space required to process typical production quantities of a product. In an effort to reduce this, a similar process is being investigated that uses steam in place of hot water. The steam would transfer the thermal energy faster than hot water, and therefore the equipment needed to perform this process would require less space to process the same volume of product.

Two nonthermal processes have been studied to reduce *L. monocytogenes* on the surface of RTE processed meats, although both are still considered experimental at

this time. The first is high hydrostatic pressure, which involves processing packaged RTE products at very high pressures (up to 900 MPa). This method inactivates microorganisms by disruption of the cell membrane and by the coagulation of proteins. Currently available data suggest that the decimal reduction (D_{10}) values for *L. monocytogenes* at a pressure of 414 MPa and either 2 or 25 °C are 1.28 or 1.48 minutes, respectively (Ananth et al., 1998). High hydrostatic pressure is currently an allowable process for foods, but its use is limited because the technology makes it either a batch process or at best a semicontinuous process. Although this works very well for experimental purposes, it limits applications in the industry because of production volumes.

The other experimental nonthermal process is ionizing radiation. At the present time, ionizing radiation is not allowed for processing RTE processed meats. However, this process is very effective in destroying microorganisms, and has potential applications in the future. The D_{10} values for *L. monocytogenes* have been reported to be between 0.18 and 2.0 kGy. Recent data with experimentally inoculated hot dogs suggest that the D_{10} value on RTE processed meats may in fact be between 0.45 and 0.7 kGy. This would make radiation processing, if it were approved for use, a practical process for large volumes of processed meats.

In summary, *L. monocytogenes* in processed RTE meats is a postprocessing surface contaminant. Intervention strategies must address all of the surfaces of the product, including those protected by the overlap of product in a package. Postpackaging pasteurization is an appealing intervention because the packaged product is protected from recontamination as long as the original package remains intact.

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Production Intervention Strategies to Control *Listeria monocytogenes*: Barrier Technology and High-Risk Production Area Control

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In terms of chilled food product safety, the major contamination risk is *Listeria monocytogenes* because of its ability to grow at low temperatures. For many products, *L. monocytogenes* could be associated with the raw materials used and thus may well be found in the low-risk preparation area. After the product has been heat processed or decontaminated (e.g., by washing) and has entered the high-risk postprocess area, it is essential that all measures be taken to protect the product from cross-contamination from external sources. A threefold philosophy has been developed in the United Kingdom to help reduce the incidence of *L. monocytogenes* in finished product (Holah and Thorpe 2000) and, at the same time, minimize other contamination sources:

1. Provide as many barriers as possible to prevent the entry of *Listeria* into the high-risk area.
2. Prevent the growth and spread of any *Listeria* penetrating these barriers during production.
3. After production, employ a suitable sanitation system to ensure that all *Listeria* are removed from the high-risk area prior to recommencing production.

Other information was recently published on *Listeria* control (Anonymous 1999, Tompkin et al. 1999).

High-Risk Barrier Technology

The building structure, facilities, and entrance practices associated with high-risk areas provide barriers for protecting manufacturing operations from contamination. These barriers are described below.

Heat-treated Product

Where a product heat treatment forms the barrier between low and high risk (e.g., an oven, fryer, or microwave tunnel):

- All product passing through the heat barrier must receive its desired cooking time/temperature combination.
- The heating device must be designed such that it forms a solid, physical barrier between low and high risk. Where this is not physically possible, air spaces around the heating equipment should be minimized and the low/high-risk floor junction should be fully sealed to the highest possible height.

Product Decontamination

Fresh produce should enter high risk via a decontamination operation, usually involving a washing process with the wash water incorporating a biocide. Similarly, the outer packaging of various ingredients on entry into high risk (e.g., some overwrapped cooked and processed intermediate products and canned foods) should also be decontaminated. If the outer packaging is clean, the use of ultraviolet light is preferred over biocides because it is dry and thus limits potential environmental microbial growth.

- Decontamination systems need to be installed such that all ingredients in high risk must have passed through the decontamination system.
- The established decontamination process should always be undertaken by controlling application temperature, concentration, and contact time.
- Periodic checking for critical parameters—e.g., blocked spray nozzles or UV lamp intensity and, from the low risk side, the loading of the transfer conveyor to ensure that product is physically separated such that all product surfaces are exposed—should be undertaken.

Other Product Transfer

All ingredients must be deboxed before transfer into high risk. Some ingredients, such as bulk liquids that have been heat treated or are inherently stable (e.g., oils or pasteurized dairy products), are best handled by being pumped across the low/high-risk barrier directly to the point of use. Dry, stable bulk ingredients (e.g., sugar) can also be transferred into high risk via sealed conveyors.

For nonbulk quantities, it is possible to open ingredients at the low/high-risk barrier and decant them through into high risk via a suitable transfer system (e.g., a simple funnel set into the wall) into a receiving container.

Packaging

Packaging materials (film reels, cartons, containers, trays, etc.) are best supplied to site "double bagged" (i.e., cardboard outer followed by two plastic bag layers surrounding the packaging). Once on site, the packaging is deboxed and stored double bagged until required. The packaging material is then brought to the low/high-risk barrier, the outer plastic bag is removed, and the inner bag and packaging enters high risk through a suitable hatch. The second plastic bag protects the packaging until time of use.

Liquid and Solid Wastes

Low-risk liquid or solid wastes should not be removed from the factory via high risk. Drains should flow from high- to low-risk areas, and backflow from low into high risk should be avoided.

Surfaces

In this context, surfaces are associated with sealed low/high-risk physical junctions to prevent microbiological penetration and are concerned with floors, walls, doors, and suspended ceilings. The materials themselves should also be impermeable, assessed for their cleanability, and laid and constructed to prevent microbial harborage points (Taylor and Holah 1996, Holah and Thorpe 2000).

Personnel

Best practice with respect to personnel hygiene is continually developing and has been recently reviewed by Guzewich and Ross (1999) and Taylor and Holah (2000). The high-risk changing room provides the only entry and exit point for personnel working in or visiting high risk. Additional clothing may be worn in high risk to further protect the food being processed from contamination arising from the operative's body (e.g., gloves, sleeves, masks, whole-head coveralls, coats with hoods, boiler suits). Clothing is color coded and is laundered at a temperature sufficient to significantly reduce microbiological levels. High-risk footwear should be captive to high risk; i.e., it should remain within high risk, with operatives changing into and out of footwear at the low/high-risk boundary.

The following high-risk entry hand hygiene maximizes hand cleanliness, minimizes hand transient microbiological levels, maximizes hand dryness, yet reduces excessive contact with water and chemicals that may lead to dermatitis issues and reduces the potential for water transfer into high risk (Taylor and Holah 2000):

- Remove low-risk/outside clothing.
- Remove low-risk/outside footwear.
- Cross over low/high-risk dividing barrier.
- WASH HANDS.
- Put on in the following order:
  —high-risk captive footwear,
  —hair net, beard snood, and hat (if appropriate), and
  —overall.
- Enter into high-risk production area and apply an alcohol-based sanitizer.
- Put on disposable gloves, sleeves, and apron, if appropriate.

Air

- For high-risk areas, the goal of the air handling system is to supply suitably filtered fresh air at the correct temperature and humidity and at a slight overpressure to prevent the ingress of external air sources (Brown 1996). The design of the air handling system should consider the following issues:
- The choice of filter will be dictated by the degree of microbial and particle removal required and is usually made up of a G4/F5 panel or pocket filter followed by an F9 rigid cell filter.
- The pressure differential between low and high risk should be 5–15 pascals, depending on the number and the size of openings and the temperature differentials between low and high risk (low-risk hot air will rise through the opening, whereas high-risk cold air will sink, causing two-way flow). The velocity of air through the opening from high risk may need to be 1.5 m/second or greater to ensure that one-way flow is maintained.
- To remove the heat load imposed by the processing environment and provide operatives with fresh air, generally five to 25 air changes per hour are adequate. The maximum air speed close to workers to minimize discomfort through "wind chill" is 0.3 m/second.
- Directional air that moves particles away from potential sources of contamination in a direction that does not compromise product safety should be provided.
- High-risk areas are usually operated at 10–12 °C to restrict the general growth of microorganisms in the environment, to prevent the growth of some (e.g., *Salmonella*) but not all (e.g., *Listeria*) food pathogens, and to reduce the heat uptake by the product. Alternatively, it is possible to provide filtered chilled air directly over or surrounding product both to cool the product and to produce a barrier that resists the penetration of microbial aerosols.
- A target humidity of 60–70% is recommended as a compromise between reducing microbial growth, avoiding condensation, and accelerating drying after cleaning (low humidity) and maintaining product quality and operative comfort (high humidity).

Utensils

Equipment, utensils, tools, etc., should be dedicated to use within high risk only.

- Ingredient or product transfer containers (trays, bins, etc.), utensils (e.g., stirrers, spoons, ladles), and other nonfixed equipment (e.g., depositors or hoppers) should be minimized and be cleaned and disinfected in a separate washroom area.

- All cleaning equipment, including hand tools (brushes, squeegees, shovels, etc.) and larger equipment (pressure washers, floor scrubbers/automats, etc.), should be color coded.
- Cleaning chemicals should be piped into high risk via a separate (from low risk) ring main.
- The most commonly used equipment service items and spares, etc., together with the necessary hand tools to undertake the service, should be stored in high risk.
- Occasionally used or new pieces of equipment entering high risk should be decontaminated prior to entry.

Production High-Risk Control

Control of *Listeria* during the production period is related to preventing its growth and spread. Both of these requirements are dependent on the absence of water, because of all the microorganism growth requirements (e.g., nutrients, temperature, pH, oxygen levels, available water), water is the only one that food processors can readily control. Water is also a requirement for translocation of microorganisms from one surface to another (e.g., hands to product or utensil to product) and, via aerosolization, the major route of aerial cross-contamination.

All cleaning equipment disperses microbial contamination by the formation of aerosols (Holah 2000). Two systems, high-pressure/low-volume spray (HPLV) lances and low-pressure/high-volume (LPHV) hoses, have the ability to disperse aerosols to a height greater than a typical processing height of 1 m (HPLV, 3 m height x 7 m distance; LPHV, 2 m x 4 m) and should not be used during production. Other techniques, however, are acceptable for use in clean-as-you-go operations, particularly floor scrubbers/driers (automats) that clean and remove excess water.

All sources of water during production should therefore be eliminated by design or controlled in practice. For example, footbaths should not be used and any equipment that has a water service should be isolated as much as possible.

To restrict the use of water for mid-shift cleaning during production, alcohol-based cleaning products are commonly used on a local scale. Ethyl alcohol and isopropyl alcohol are also bactericidal in the 60–70% range, and can be formulated into wipe and spray-based products.

Postproduction Sanitation Procedure

Sanitation programs have often been characterized as simply removing microorganisms from one part of the production area and redepositing them in another. This must not happen in high risk, and the sanitation procedure must be engineered to remove *Listeria* from surfaces, destroy any remaining surface-bound *Listeria,* and ensure that removed *Listeria* are directed out of the processing area.

Fundamental to this is that food production and service equipment should be designed hygienically such that any area of the equipment that could have been contaminated during production is accessible, or can be made easily accessible, to cleaning fluids. Suitable guidance is available from various organizations; see the Note toward the end of this abstract.

Once accessible, microbiological removal and destruction follow standard cleaning and disinfection practices, which can be conveniently described as preparation of work area, gross soil removal, prerinse, cleaning, interrinse, disinfection, and postrinse (as required).

The critical issue is the sequence in which the sanitation practices are undertaken such that all environmental surfaces and equipment in the area are cleaned at the same time. It is not acceptable to clean and disinfect one line and then move onto the next and start the sequence again, because this merely spreads contamination around the room. The following sanitation sequence has been demonstrated to minimize the proliferation of *Listeria* on food contact surfaces:

- Remove gross soil from production equipment.
- Remove gross soil from environmental surfaces.
- Rinse down environmental surfaces from "top to bottom."
- Rinse down equipment and flush to drain.
- Clean environmental surfaces, usually in the order of drains, walls, and then floors.
- Rinse environmental surfaces.
- Clean equipment.
- Rinse equipment.
- Disinfect equipment and rinse if required.
- Fog (if required).

Cleaning equipment is prone to contamination with *Listeria* and, by the nature of its use, provides an excellent way in which contamination can be transferred from area to area. Cleaning equipment should therefore be thoroughly cleaned, disinfected, and dried after use.

Sanitation equipment should be constructed of smooth, nonporous, easily cleanable materials such as stainless steel or plastic. Frameworks should be constructed of tubular or box section material, be closed at either end, be properly jointed, and contain no metal-to-metal joints. Crevices and ledges where soil could collect should be avoided, and exposed threads should be covered or dome nuts used. Tanks for holding cleaning chemicals or recovered liquids should be self-draining, have rounded corners, and be easily cleaned. Shrouds around rotating brush heads should be easily detachable to facilitate cleaning. Brushes should have bristles of colored, impervious material embedded in the head with resin, or alternatively, the head and bristles should be molded as one unit.

Note

Information on publications that discuss the hygienic design of equipment and food production facilities from the 3-A, National Sanitation Foundation (NSF), European Hygienic Design of Equipment Group (EHEDG), and Campden

& Chorleywood Food Research Association (CCFRA) can be found on the following websites: www.3-A.org, www.nsf.org, www.ehedg.org, and www.campden.co.uk.

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SIGNIFICANCE OF MYCOTOXINS IN THE GLOBAL FOOD SUPPLY

Mycotoxins in the World Food Supply

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Mycotoxins have affected human populations since the beginning of organized crop production. Ergotism is discussed in the Old Testament of the Bible. Some claim that the ancient Chinese used ergot for obstetrical purposes 5000 years ago. Many epidemics of ergotism were reported in western Europe from about AD 800. The screams of the victims, the stench of rotting flesh, extremities falling off, and death all feature in the descriptions of the disease. Large-scale mortalities persisted into the 18th century, when governments and the church promoted methods for the removal of sclerotia.

During the 13th century, rye was replaced by wheat in western Europe. The former is resistant to *Fusarium* diseases whereas the latter is typically susceptible, leading to the accumulation of trichothecene mycotoxins. These compounds are prevalent in small grains in western Europe today. *Poisons of the Past* (Matossian 1989) contains an analysis of weather and food consumption patterns during the plague epidemics in Europe. Plague epidemics took place when there were surpluses of small grains, the favorite food of rats. The occurrence of plague in the Middle Ages is very strongly associated with rainy and humid crop years.

Although hundreds of fungal metabolites are toxic in experimental systems, only five are of major agricultural importance: deoxynivalenol, aflatoxin, fumonisin, zearalenone, and ochratoxin (Miller 1995, 2000). In small grains, the toxins are concentrated into bran fractions during milling. All of these toxins are stable in the processes typical of food and feed processing. Animal products can be a minor dietary source of ochratoxin and fumonisin; for the remaining three toxins, animal sources are not important under normal circumstances.

A recent report by the U.S. National Academy of Sciences notes that even with the high-quality food system in the United States, the carcinogenic mycotoxins in U.S. diets may increase cancer rates. This is absolutely the case in many developing countries where mycotoxins are a major population health problem (National Academy of Sciences 1996, Miller 1998).

Deoxynivalenol and Zearalenone

These toxins occur when wheat, barley, corn, and sometimes oats and rye are infected by *Fusarium graminearum* and *F. culmorum*. These species cause *Fusarium* head blight in small grains, a major agricultural problem worldwide. These species cause a similar disease in corn called Giberella ear rot. Disease incidence most affected by moisture at flowering and most cultivars and hybrids used today lack genetic resistance to the disease. Because wheat, corn, and barley constitute two-thirds of the world production of cereals, deoxynivalenol is the mycotoxin to which the greatest number of humans are exposed.

"Red mold poisoning" was reported in rural Japan throughout the 1950s. Eventually, deoxynivalenol was discovered by Japanese researches from grain that had made humans ill. The same chemical was subsequently re-reported as "vomitoxin" from *F. graminearum*–contaminated corn in 1973 in the United States. Deoxynivalenol was a widespread contaminant of wheat in the northeastern United States and in eastern Canada in 1979–1981 and again in 1993–1996 and in 2000 in the Great Lakes area and the Red River Valley. Large-scale acute human toxicoses from deoxynivalenol have occurred in modern times in India, China, and Korea, among other countries.

Swine are the most sensitive domestic animal species to the effects of trichothecenes. Experiments feeding pure deoxynivalenol to swine suggested that diets containing < 2 mg/kg deoxynivalenol would have little impact on growth. However, many experiments using naturally contaminated grains often demonstrated that such grain was more toxic than indicated from the deoxynivalenol content (Prelusky et al. 1994). These co-occurring toxins were shown to increase the toxicity of deoxynivalenol fed in combination to insects, and might be also true in swine. The basis for the feed refusal in swine is the impressive neurotoxicity of deoxynivalenol. Experiments involving the dosing of the toxin by a continuous-exposure osmotic pump implanted intraperitoneally resolved that the effects could not be due to taste or learned responses (Prelusky 1997).

Deoxynivalenol exposure produces prolonged elevations in serum IgA and mesangial IgA, leading to hematuria. Human IgA disregulation (Berger's disease) is common, and the only agents so far demonstrated to reproduce this condition in experimental animals are trichothecenes (Bondy and Pestka 2000).

Crops that are contaminated by deoxynivalenol often contain zearalenone, albeit at a lower frequency. Zeara-

lenone is more common in maize than in small grains. Zearalenone is an estrogen analogue that causes hyperestrocism in female pigs at low levels; the dietary no-effect level is less than 1 mg/kg. Zearalenone has been implicated in several incidents of precocious pubertal changes in girls in Europe and South America.

Aflatoxin

Aflatoxin is a material problem in maize and groundnuts. Rice is an important dietary source of aflatoxin in circumstances of poor storage in tropical and subtropical areas. Aflatoxin contamination is managed by the development of systems to detect and segregate contaminated kernels and better storage systems. For corn and peanuts, all kinds of efforts have been made to prevent aflatoxin contamination, including plant breeding and biological control, to little effect.

Aflatoxin B_1, the most toxic of the aflatoxins, causes a variety of adverse effects in different animal species, especially chickens. In poultry, these include liver damage, decreased egg production in hens, inferior egg shell quality, inferior carcass quality, and increased susceptibility to disease. Swine are somewhat less sensitive than poultry; the acute and chronic effects of ochratoxin in swine are largely attributable to liver damage. In cattle the primary symptom is reduced weight gain as well as liver and kidney damage, and milk production is reduced. Aflatoxin is also immunotoxic in domestic and laboratory animals at oral exposures in the ppm range. Cell-mediated immunity (lymphocytes, phagocytes, mast cells, and basophils) is more affected than is humoral immunity (Bondy and Pestka 2000).

Many people in developing countries are seropositive for hepatitis B and C, which are also liver carcinogens. Although aflatoxin is a potent chemical carcinogen, its ability to alter response to the hepatocarcinogenic viruses is perhaps of greater importance. The relative rates of liver cancer in hepatitis B–positive populations are materially greater when exposed to high levels of aflatoxin. This is because the toxin interferes with the processing of the virus.

The human immunotoxicity of aflatoxin is also being increasingly studied. In one study, serum aflatoxin-lysine adducts were higher in protein energy–malnourished (PEM) children compared with control children. Aflatoxin metabolism was affected with relatively higher serum concentrations in PEM children. A second study compared PEM children with high and low serum aflatoxin concentrations. The serum aflatoxin–positive group of PEM children showed a significantly lower hemoglobin level ($P = 0.02$), longer duration of edema ($P = 0.05$), an increased number of infections ($P = 0.03$), and a longer duration of hospital stay ($P = 0.008$). This finding was echoed in another study that suggested that malaria infections increased in children exposed to aflatoxin (Adhikari et al. 1994; see also Miller 1998).

Fumonisins

Fumonisins are produced by *F. verticilloides* (formerly *moniliforme*), *F. proliferatum,* and several uncommon fusaria. Fumonisins were discovered in 1988 by two groups working independently. One was investigating the cause of human esophageal cancer in parts of southern Africa. The other was attempting to find the cause of a disease of horses known since 1850, equine leucoencephalomalacia (ELEM), a massive liquefactive necrosis of the brain. There are at least three naturally occurring fumonisins: B_1, B_2, and B_3. Fumonisin B_1 occurs at the highest concentration, followed by B_2 and B_3. Fumonisins have been found as a very common contaminant of corn-based food and feed in the United States, China, Europe, southern Africa, South America, and Southeast Asia (World Health Organization 2000).

Fusarium verticilloides and *F. proliferatum* can be recovered from virtually all corn kernels, including those that are healthy, which suggests that this may be an endopyte, i.e., in a mutualistic relationship. *Fusarium verticilloides* and *F. proliferatum* cause a "disease" called fusarium kernel rot, which is favored by temperature, drought, insect herbivory, and stresses caused by other corn disease (Miller 2000).

Fumonisins are toxic in all types of cells (yeast, plant, animal, human) because of their effects on sphingolipid synthesis. Alteration in sphingolipid base ratios occurs almost immediately after exposure because fumonisin inhibits ceramide synthetase. Many changes also occur in the amounts and ratios of complex ceramides. Additionally, fumonisins induce apoptosis leading to cell proliferation, which may explain their carcinogenic properties. Fumonisin causes an array of diseases of domestic animals. As noted above, ELEM was perhaps the first noted because horses are the most sensitive domestic species. In swine, high exposure causes pulmonary edema, with liver and kidney damage seen at dietary concentrations more likely to occur in feeds in the United States. Poultry, cattle, and catfish are more tolerant (World Health Organization 2000).

Exposure to *F. verticilloides*–contaminated maize has been linked to the elevated rates of esophageal cancer in the Transkei for 25 years. Very pure fumonisin produced tumors in male and female mice and rats in the U.S. National Toxicology Program 2-year bioassay (www.ntp.gov). An enormous amount is known about the rodent toxicities of fumonisins from the NTP assay. An exhaustive treatment of the toxicology of fumonisins can be found in a World Health Organization publication (2000).

Ochratoxin

Ochratoxin is known to be produced by only one species of *Penicillium, verrucosum. Aspergillus ochraceous* and perhaps some related species produce ochratoxin on other commodities. This species and *A. carbonarious* pro-

duce ochratoxin on grapes and coffee.

Ochratoxin is a potent nephrotoxin in swine and causes kidney cancer in male Fischer 344 rats. Pigs are affected at low exposures in terms of kidney damage, but typically there are no overt signs or biochemical/hematological changes. Poultry are similarly affected, with reduced growth rate and egg production at low ochratoxin concentrations less than 2 μg/g. Higher dietary ochratoxin concentrations are often fatal.

Ochratoxin is suspected as the cause of urinary tract cancers and kidney damage in areas of chronic exposure in parts of eastern Europe. Human exposure to ochratoxin occurs primarily from whole-grain breads. Some exposure comes from the consumption of animal products, especially pork and pig blood–based products. There is a great deal known about human serum ochratoxin concentrations in Europe (Miller 1999).

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Toxicology of Aflatoxin B_1

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Aflatoxins are toxic metabolites produced by some strains of *Aspergillus* fungi that grow on agricultural products under appropriate conditions of heat and moisture. Although identified in the early 1960s as the agents responsible for outbreaks of fatal hepatic necrosis in poultry and liver tumors in fish, subsequent investigation revealed elevated liver cancer incidences in human populations living in regions with high endemic aflatoxin levels (Eaton and Groopman 1994). Subsequently, aflatoxins have been considered major contributors to human hepatotoxicity and hepatocarcinogenesis, particularly in some developing countries.

The most potently cytotoxic and carcinogenic of the aflatoxins is aflatoxin B_1 (AFB_1). Although the liver clearly is the principal target organ for AFB_1 following ingestion, other tissues can be affected as well, including the respiratory system (Massey et al. 1995, Massey 1996).

Mechanisms of AFB_1 Toxicity

AFB_1 Biotransformation

As with most carcinogens, the mutagenicity and carcinogenicity of AFB_1 are not attributable to the parent compound, but rather to the production of a reactive metabolite. The principal organ for AFB_1 biotransformation is the liver, where it is reduced to aflatoxicol and oxidized by members of the cytochrome P450 (CYP) superfamily to a number of hydroxylated metabolites. However, the critical metabolic pathway for mutagenicity and carcinogenicity is epoxidation, resulting in AFB_1-8,9-*exo*-epoxide (AFB_1-epoxide). In the liver, this reaction is catalysed by a number of different CYP isoforms. If not detoxified by glutathione S-transferase (GST)-catalyzed conjugation with cellular glutathione (GSH), the epoxide can bind with high affinity to the N^7 position of guanine in DNA, leading to mutations in critical cellular genes and potentially to tumorigenesis.

The ability of GSTs to conjugate AFB_1-epoxide with GSH is a critical determinant of susceptibility and the major basis for observed species differences in AFB_1 hepatocarcinogenicity (Massey et al. 1995). Of the different classes of GST enzymes, in humans the mu class has particularly high activity for AFB_1-epoxide detoxification.

The cytotoxicity of AFB_1 is likely due to the actions of a number of different metabolites, the relative contributions of which remain to be unequivocally established. In any case, cytotoxicity likely contributes to the characteristic of AFB_1 being a "complete carcinogen", since it provides a stimulus for proliferation of initiated cell foci.

DNA Target Sites

Human hepatocellular carcinomas from individuals living in high aflatoxin-endemic regions of Africa and China were found to have a high frequency of point mutations at the guanine residue in the 3rd position of codon 249 of the *p53* tumor suppressor gene, resulting in an arginine to serine amino acid substitution (Bressac et al. 1991, Hsu et al. 1991). The gene most frequently altered in human cancers, *p53* codes for a nuclear phosphoprotein with numerous critical functions in cell cycle control and apoptosis. The AFB_1-associated mutations of *p53* were consistent with the DNA binding characteristics of AFB_1-epoxide, and suggested that AFB_1-mediated disruption of the gene is a critical step in hepatocarcinogenicity.

There is also evidence that factors other than AFB_1 contribute to hepatocarcinogenicity with human aflatoxin exposure. For example, products produced by hepatitis viruses may increase the frequency of AFB_1-associated *p53* mutations at codon 249 (Denissenko et al. 1998) and interact directly with the p53 protein (Greenblatt et al. 1997).

Experiments in rodents suggested that one of the *ras* genes, which code for proteins involved in GTP-associated cell signaling, was frequently mutated in AFB_1-induced liver tumors (Sinha et al. 1988, McMahon et al. 1990). However, this effect was not observed in human AFB_1-associated liver tumors (Chao et al. 1999).

Chemoprevention of AFB_1 Carcinogenicity

Numerous agents have been investigated for the potential to decrease AFB_1 hepatocarcinogenicity by altering the mycotoxin's bioactivation or detoxification. Experimentally, dietary constituents including ellagic acid, indole-3-carbinol, and green tea have been investigated, but the compound that has been developed the furthest is the dithiolthione oltipraz. Originally marketed as an antischistosomal agent, oltipraz appears to decrease the epoxidation of AFB_1 by inhibiting CYP1A2 and CYP3A4,

but its principal mechanism of action is probably induction of GSTs, thereby increasing conjugation of AFB_1-epoxide with GSH, and decreasing its availability for binding to DNA (Wang et al. 1999). An ongoing clinical trial in Qidong, China is determining the ability of oltipraz to reduce the incidence of AFB_1-associated liver cancer (Wang et al. 1999).

The Respiratory System as a Target

The lung can be exposed to AFB_1 due to occupational exposure to contaminated grain dusts, and there is evidence that human lung DNA can be a target of AFB_1 following ingestion (Harrison and Garner 1991). In isolated lung cells from rabbits and mice, we found AFB_1 to be bioactivated by CYP enzymes, primarily in nonciliated bronchiolar epithelial (Clara) cells. However, mutagenesis experiments suggested that the DNA-binding AFB_1-epoxide metabolite can leave the cells of origin, and potentially interact with other cell types (Daniels et al. 1993).

Consistent with DNA adduct studies, AFB_1-induced lung tumors in AC3F1 mice (a hybrid of highly lung tumor susceptible A/J and resistant C3H/HeJ parental strains) contained point mutations at guanine residues in K-*ras*, with the anticipated bias for the A/J allele (Donnelly et al. 1996a). Furthermore, following AFB_1 treatment but prior to tumor development, K-*ras* mutations occurred preferentially in mouse Clara cells (Donnelly and Massey 1999), which indicates that lung cells with the highest activity for AFB_1 bioactivation are targets of genotoxicity. However, in contrast to findings in mouse lung tumors induced by other carcinogens, AFB_1-induced mouse lung tumors demonstrated frequent, but heterogeneously distributed, overexpression of p53 protein as well as *p53* point mutations, suggesting a carcinogen-specific response (Tam et al. 1999).

Unlike lung tissue from mice and rabbits, and liver tissue from numerous species, human peripheral lung bioactivated AFB_1 primarily by prostaglandin H synthase and/or lipoxygenase-catalyzed cooxidation, and not by cytochromes P450, with activity concentrated in macrophages (Donnelly et al. 1996b).

In addition, although the mu-class glutathione S-transferase M1-1 had previously been shown to have high specific activity for AFB_1-epoxide conjugation, lung tissues from *GSTM1* null humans (approximately 50% of most human populations are homozygous null) did not demonstrate diminished rates of conjugation, compared to tissues from *GSTM1* positive individuals (Stewart et al. 1999). Thus, genetically determined differences in *GSTM1*-1 activity are not likely to play a role in susceptibility of the human lung to AFB_1 carcinogenicity.

In summary, AFB_1 tumorigenesis in mice demonstrates unique properties compared to effects of other carcinogens, and processes of biotransformation show significant species and tissue differences.

Conclusion

The adverse effects of AFB_1 on human and animal health remain a major concern, particularly in some developing countries. Experimentally, the molecular basis of interactions between infectious liver disease and AFB_1 toxicity is an area of intense research interest, and the mycotoxin serves as a valuable model chemical for studying the molecular processes involved in chemical carcinogenesis. On a positive note, the development of chemopreventive strategies based on detailed knowledge of the mechanisms of AFB_1 biotransformation and toxicity, holds promise.

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Significance of Fumonisins in the Global Food Supply

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Many fungi have evolved biochemical pathways that produce toxic secondary metabolites, which enhance their ability to survive and compete in a hostile environment. These compounds, known as mycotoxins, are remarkable for their diversity of chemical structures and for the myriad biochemical and physiological processes they are capable of affecting. Unfortunately for many organisms, including domestic animals and man, exposure to mycotoxins through consumption of fungally contaminated feed or food can have disastrous consequences. As expected based on the dissimilarities in properties of mycotoxins, these consequences of exposure encompass a multitude of expressed toxicological symptoms. Examples of symptoms encountered can be as subtle as reduced feed consumption or skin rashes and as severe as gangrene, brain hemorrhage, and other potentially fatal lesions.

In 1988 the structures of a unique family of mycotoxins were published (Bezuidenhout et al. 1988). Because they were isolated from a common endophyte of corn, *Fusarium verticillioides* (syn. *F. moniliforme* teleomorph *Giberella fujikuroi*, mating population A), they were given the trivial name fumonisins. For many years *F. verticillioides* was known for its ability to cause toxicity in farm animals, particularly equids, and was suspected as a causative factor in human esophageal cancer in people who consume large quantities of corn that is frequently of poor quality and moldy. The agent or agents produced by the fungus that caused the observed symptoms was unknown until Gelderblom et al. (1988), using a short-term cancer promotion bioassay to monitor fractions, were able to extract and purify fumonisins from corn cultured with a known toxic isolate, *F. verticillioides* MRC 826. Shortly thereafter, purified fumonisins were experimentally shown to duplicate the highly species-specific toxicological signs known to be caused by *F. verticillioides*—contaminated corn. Thus, fatal brain lesions in horses (equine leucoencephalomalacia, ELEM), as well as fatal hydrothorax in swine (porcine pulmonary edema, PPE), were demonstrated in fumonisin feeding studies (Kellerman et al. 1990, Haschek et al. 1992). The ability of purified fumonisin to initiate as well as promote liver cancer in rats was also reported by Gelderblom et al. (1991, 1992).

Mechanism of Fumonisin Toxicity

Fumonisin B_1, the predominantly occurring fumonisin analog, is 1,2,3-propanetricarboxylic acid, 1,1′-[1-(12-amino-4,9,11-trihydroxy-2-methyltridecyl)-2-(1-methylpentyl)-1,2-ethanediyl]ester. Examination of the structure does not reveal the presence of functional groups normally considered to be reactive with biological sites (macromolecules, receptors, or enzymes) that could result in toxicity. However, Wang et al. (1991) noticed that the 20-carbon chain with the associated amino group at the terminal end of the fumonisin backbone bears a resemblance to the sphingoid bases, sphingosine and sphinganine. Fumonisin was subsequently found to be a potent, specific inhibitor of ceramide synthase, a key enzyme in the biosynthesis and turnover of sphingolipids. The significance of this finding is that both sphingoid bases and the complex sphingolipids are known to modulate the activity of many regulatory proteins and to control other processes critical for cell growth, differentiation, and apoptosis. Examples of parameters affected by fumonisin-induced disruption of sphingolipid biosynthesis include Na^+/K^+ ATPase, protein kinase C, retinoblastoma protein, mitogen-activated kinases, transcription factor AP-1, Ca^{+2} homeostasis, and growth factor receptors. For a review of the consequences of these multifaceted effects, see Merrill et al. (1997).

As further evidence that the specific inhibition of ceramide synthase by fumonisin is the underlying cause of fumonisin toxicity, a number of studies have been conducted that demonstrate high correlation between the degree of disruption of sphingolipid metabolism and the development of overt toxicity, including ELEM, PPE, hepatotoxicity, and nephrotoxicity (reviewed by Riley et al. 2000). An important consideration about which there is little information is the effect of chronic disruption of sphingolipid metabolism that might result from the daily consumption of fumonisin-contaminated corn products. Limited surveys have indicated the presence of fumonisins in corn intended for human consumption in most if not all countries studied. Whether there is a relationship between diseases such as cancer or arteriosclerosis and fumonisin intake remains to be determined, but recently completed long-term feeding studies in rodents have stimulated much discussion

and concern among scientists, regulatory officials, corn producers, and consumers.

National Toxicology Program Study of Fumonisin B_1

Because of (1) the extent of corn consumption by man and animals throughout the world, (2) the widespread occurrence of *F. moniliforme* and fumonisins no matter where corn is grown (Shephard et al. 1996), (3) the demonstrated ability of fumonisins to alter cellular and biochemical processes through specific inhibition of ceramide synthase, and (4) the hepato- and nephrotoxicity and apparent carcinogenicity of fumonisin B_1, the U.S. National Toxicology Program (NTP) selected fumonisin B_1 for inclusion in its 2-year testing protocol. A draft report of the completed study was released in June 1999 (available as NIH Publication 99-3955 from NTP Central Data Management, National Institute of Environmental Health Sciences, P.O. Box 12233, MD E1-02, Research Triangle Park, NC 27709). Very briefly, after being fed fumonisin-containing diets for 2 years, male rats, but not female rats, developed kidney tumors. Female mice, but not male mice, developed liver tumors. The results of the study provided "clear evidence" of carcinogenic activity of fumonisin B_1. "Clear evidence" means "a dose-related increase of malignant neoplasms, increase of a combination of malignant and benign neoplasms, or marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy."

The results of the NTP study have stimulated much activity, concern, and discussions regarding what should be done to protect consumers while preventing needless harm to corn growers, producers, and exporters that could result from setting unjustifiably low action limits. Most recently, the U.S. Food and Drug Administration has issued draft guidelines for fumonisins that recommend not exceeding levels from 2 to 4 ppm of total fumonisins in corn products for human consumption, and maximum levels from 5 to 100 ppm in feed for animals, depending on the species and age of the animals (Federal Register 2000).

Worldwide Significance of Fumonisins and Future Considerations

Even before the completion of the NTP study, the International Agency for Research on Cancer designated toxins derived from *F. moniliforme* as group 2B, which means a substance is possibly carcinogenic to humans. In light of the results of the NTP investigation, and as more data become available, there will be increased scrutiny of these mycotoxins by scientists and government officials.

In the brief period of 12 years since the discovery of the fumonisins, a remarkable amount of research has provided important information regarding these unique mycotoxins, including occurrence, toxicological effects, mechanism of action, and fate and distribution. The problem that now faces countries worldwide that grow or import corn for both animal and human consumption is how to deal with the problem. Should regulations be established limiting fumonisin content in corn or corn products, or will advisory guidelines suffice? Imposing action levels of less than 1 ppm could have disastrous consequences for corn growers, but the overriding concern must be for the health of consumers. As yet, only Switzerland has established any official guidelines for fumonisins. The Swiss Federal Office of Public Health issued a "tolerance value" of 1 ppm total fumonisins B_1 and B_2 in August 1997. Other countries must now begin to assess the extent to which fumonisins may affect their human and animal populations, and decide if and at what level guidelines or restrictions need to be implemented. Because there are still many gaps in our knowledge base on fumonisins, caused primarily by the incomplete picture of the ramifications underlying sphingolipid metabolism disruption, any limits established for fumonisins should be flexible and subject to change, either up or down, as new information becomes available.

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Deoxynivalenol

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The trichothecenes are a group of more than 180 sesquiterpenoid metabolites produced by *Fusarium* and other fungi that can be found as food or environmental contaminants worldwide. Trichothecenes bind readily to ribosomes and are extremely potent inhibitors of translation (Ueno 1987). These mycotoxins are capable of causing a variety of toxicoses in animals and humans. Acute exposure of experimental animals to high doses of trichothecenes can cause "radiomimetic" effects that include diarrhea, vomiting, leukocytosis, and gastrointestinal hemorrhage. At very high doses, these effects are accompanied by circulatory shock, reduced cardiac output, and ultimately death. Trichothecenes found in moldy grains have been retrospectively associated with a human illness known as "taumalgetriede" (staggering grains) in Siberia in the 1890s that included in its symptoms vomiting, headache, and vertigo (Pestka and Casale 1990). T-2 toxin and other related trichothecenes are believed to be responsible for "alimentary toxic aleukia" (ATA) in the Orenburg region of the USSR from the 1930s to the late 1940s. ATA was associated with overwintered wheat, barley, and millet, and had as its symptoms vomiting, diarrhea, leukopenia, hemorrhage, and shock, with mortality in some years reaching 60%. Of the trichothecenes, deoxynivalenol (DON) is the most commonly encountered in wheat, corn, and barley throughout the world (Rotter et al. 1996). It is thus pertinent to highlight key features of DON relevant to its occurrence, toxicologic effects in experimental animals, and potential impact on human health.

DON Occurrence and Control

Morooka et al. (1972) first characterized and named DON following its isolation from *Fusarium*-infected barley in Japan. Nearly concurrently, Vesonder et al. (1973) isolated the same compound from *Fusarium*-infected corn and named it "vomitoxin" because of its capacity to induce emesis in swine. DON is produced in grains infected with *F. graminearum* and *F. culmorum*. DON is primarily a preharvest problem in developed countries where grains are dried to = 13% moisture content but can be produced in storage in areas of the world where the moisture content of stored grains is less rigorously controlled. Concurrent infection and DON elaboration in the field are largely dependent on weather and are favored by low temperatures and high humidity (Rotter et al. 1996). Thus, DON levels in corn, wheat, and barley can vary widely from year to year and from region to region. DON at low levels (<1 ppm) is frequently encountered but can sporadically occur at levels as high as 5–20 ppm (Rotter et al. 1996) even in human foods such as corn meal and granola (Abouzied et al. 1991). Its presence in finished foods is not surprising because it is heat resistant and can survive processing.

In North America, DON is associated with the plant diseases ear rot in corn and head blight ("scab") in wheat and barley. During the last decade, the latter has occurred with greatly increased frequency in the Midwestern United States (McMullen et al. 1997). Economic losses caused by scab from 1991 to1996 in barley, winter wheat, and spring wheat have been estimated to exceed $1 billion because of both DON occurrence and decreased grain quality. Increased scab has been attributed to recurrent cool rainy weather during wheat and barley flowering. Another causative factor is the increase in no-till farming, which facilitates the retention of high levels of infective fusaria propagules in the field from one season to the next.

To combat and control *Fusarium* head blight in a coordinated manner, the U.S. Department of Agriculture initiated the National Wheat and Barley Scab National Initiative (see http://www.scabusa.org), which is currently coordinated at Michigan State University. This effort involves researchers from major land grant universities, industry, and government, and receives input from more than 40 national wheat- and barley-associated organizations. The effort is multidisciplinary in that it includes plant pathologists, plant breeders, food scientists, agronomists, and toxicologists. Preharvest and postharvest strategies undertaken by the U.S. Wheat and Barley Scab Initiative are outlined in Table 1.

Table 1. Strategies for U.S. Wheat and Barley Scab Initiative Research Projects

Preharvest	Postharvest
Crop epidemiology and management	Monitoring DON occurrence
Fungicide application	Analytical methods development
Conventional plant breeding	Sampling
Molecular variety development	Regional labs/reporting network
Novel germ plasms	Processing methods to reduce DON
Regionwide evaluation	Human risk assessment

Toxic Effects of DON in Experimental Animals

Numerous studies have been conducted to assess DON toxicity in a variety of animal species (Rotter et al. 1996). Although DON is less toxic than other trichothecenes such as T-2 toxin, acute exposure to high doses can cause vomiting as well as lesions in lymphoid tissue, bone marrow, and intestine. At extremely high doses that are unlikely to be encountered in food, it can cause shock and death. Lethal doses (LD_{50}) for mice of 49 mg/kg body weight administered intraperitoneally and 78 mg/kg body weight administered orally have been reported (Forsell et al. 1987). Chronic effects of DON ingestion include anorexia, reduced weight gain, and immunotoxicity, but not neoplasia.

DON susceptibility in different species follows the rank order pig > mouse > rat > poultry > ruminant (Rotter et al. 1996). The susceptibility of pigs apparently relates to more rapid and extensive DON absorption, wide tissue distribution, slower clearance rate, and minimal/no de-epoxidation capacity in this species. In pigs, DON levels of 1–5 ppm cause partial feed refusal, whereas 12 ppm causes complete refusal; DON at 20 ppm can initiate vomiting. The anorectic and emetic responses are believed to be mediated by the serotoninergic system based on increased levels of serotonin or its metabolites in DON-treated animals (Fitzpatrick et al. 1988, Prelusky 1993) as well as the capacity of serotonin receptor antagonists to prevent DON-induced emesis (Prelusky and Trenholm 1993).

In addition to its neuroendocrine effects, DON and other trichothecenes can affect leukocyte function and be immunostimulatory and immunosuppressive (reviewed in Bondy and Pestka 2000). At low levels, DON can have hormetic effects and actually stimulate immune functions such as resistance to pathogens, cytokine expression, and immunoglobulin A production. Our laboratory has studied DON-induced IgA hyperelevation and found it to mimic the common human glomerulonephritis, IgA nephropathy relative to IgA immune complex formation, and kidney mesangial deposition and hematuria (Dong et al. 1991). At the mechanistic level, we found that DON enhances cytokine gene expression in vitro and in vivo, most notably IL-6, which appears to drive the polyclonal activation and differentiation of IgA-committed B cells to IgA-secreting plasma cells. Increased cytokine expression appears to involve both transcriptional and posttranscriptional mechanisms (Bondy and Pestka 2000). In contrast to these findings, high doses of DON and other trichothecenes are immunosuppressive and cause leukopenia, decreased host resistance, decreased hypersensitivity, and reduced immunoglobulin production. Immunosuppression is likely to occur because trichothecene-induced apoptosis in leukocyte populations is critical in regulatory and effector arms of the immune response (Islam et al. 1998, Yang et al. 2000).

Potential Effects of DON on Humans

From the above-described animal studies it seems possible that DON could initiate acute toxic effects such as gastroenteritis in humans. There were 35 outbreaks of food poisoning associated with ingestion of scabby wheat or moldy corn in China from 1961 to 1985, and these affected 7818 persons (Kuiper-Goodman 1994). The onset time was 5–30 minutes, and hallmark symptoms included nausea, vomiting, diarrhea, headache, dizziness, and fever. In one gastroenteritis outbreak in Xingtai County in 1984, 362 persons who ate moldy corn containing 0.34–92.8 ppm DON developed gastroenteritis. Another outbreak of gastroenteritis was reported that affected several thousand individuals consuming products made from rain-damaged moldy wheat in the Kashmir Valley of India (Bhat et al. 1989). In this study, 11/24 samples taken contained DON in the range of 0.34–8.4 ppm.

No recorded outbreaks of gastroenteritis in the United States have been etiologically linked to DON. However, the U.S. Centers for Disease Control and Prevention reported 16 gastroenteritis outbreaks in schoolchildren from six states who consumed burritos from two manufacturing plants (Anonymous 2000). Symptoms included abdominal cramps, vomiting, diarrhea, headache, and dizziness. In a Georgia outbreak, the median onset and duration times were 15 min and 4.5 hours, respectively. The rapid onset times suggested that a toxin was causing the illness. *Bacillus cereus* and *Staphylococcus aureus* toxin were not detectable in associated samples. Other tests for putative toxic agents, such as metals, alkaloids, biogenic amines, and pesticides, were negative. Interestingly, DON was detectable in some of these samples; however, the levels were within the U.S. Food and Drug Administration's advisory guideline of 1 ppm. Although the investigators concluded that the etiology of these outbreaks remains unknown, the results suggest that the possible link between DON and human gastroenteritis outbreaks requires further scrutiny.

The potential for DON to cause chronic effects such as anorexia, reduced weight gain, or immunotoxicity in humans also is of obvious concern. No epidemiological reports exist that support this possibility. However, animal studies using the most sensitive endpoints (i.e., food re-

fusal, reduced weight gain) have been used to establish Tolerable Daily Intakes (TDIs) for DON. Kuiper-Goodman (1994) proposed TDIs of 1.5 and 3.0 ìg/kg body weight per day for children and adults, respectively, whereas Pieters et al. (1999) used more recent animal studies to generate a TDI of 1.1 ìg/mg body weight per day.

The United States has established tolerance limits of 1 ppm DON for bran, flour, and germ targeted for human consumption (Pieters et al. 1999). Canada set guidelines of 2 ppm DON in uncleaned soft wheat used for nonstaple foods except for infant foods, where the guideline is 1 ppm (Kuiper-Goodman 1994). Currently, DON screening is conducted by mills and processors in the United States and other countries to divert grains exceeding established limits from entering the human food supply. This level of surveillance has increased sharply in recent years because of the availability of rapid ELISA tests and because of increased awareness of DON resulting from the *Fusarium* head blight epidemic. Thus, the likelihood of high levels of DON to enter humans has greatly decreased.

Future Research Needs

Further research in several areas is needed to improve our capacity to conduct risk assessments for DON and to validate current tolerance limits/guidelines. Such studies might include (1) routine surveys of DON in raw and finished foods over several years, (2) mechanistic studies in human leukocytes and comparisons with other species, (3) epidemiological studies of acute (e.g., gastroenteritis) and chronic diseases (e.g., IgA nephropathy) in geographical areas of high and low DON exposure, (4) testing for DON in outbreaks of foodborne gastroenteritis, and (5) human clinical trials.

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Methods of Analysis of Mycotoxins

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Some hundreds of bioactive metabolites have been isolated from fungal cultures, including mycotoxins, which have quite different chemical structures and physicochemical characteristics, thus requiring different approaches to their analytical determination. The five major groups of mycotoxins—aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, and zearalenone—are naturally occurring in different agricultural commodities at levels that may be of concern for human and animal health. They require separate analytical protocols because they have completely different structures.

Adequate analytical methods for these mycotoxins are essential if we are to obtain reliable data on human exposure through the diet and to support the implementation of international food standards and trading agreements. Sensitive, precise, and accurate methods are required that have been validated by interlaboratory studies for different commodities and that can be applied horizontally to several matrices susceptible to contamination. In this respect the European Committee for Standardization (CEN) has established criteria for the acceptance of mycotoxin analytical methods when official standards are not available (CEN 1999).

Many methods have been developed for mycotoxin analysis in a variety of foodstuffs, with high-performance liquid chromatography (HPLC) now routine for most mycotoxins and with the increasing use of immunoaffinity clean-up in internationally validated methods. Our laboratory recently developed and validated two major methods that can be applied horizontally to the analysis of fumonisins in corn-based foodstuffs (sponsored by the Standards, Measurements and Testing [SMT] Programme) and of ochratoxin A in wine and beer (Solfrizzo et al. 2000a, Visconti et al. 1999, 2000).

Analysis of Fumonisins in Corn-based Foods

Several methods are available to determine fumonisin levels in corn, but they are often used improperly for the analysis of corn-based products and can lead to underestimation of the true levels of contamination. They involve in general an extraction step (mainly shaking or blending with different mixtures of water, methanol, and/or acetonitrile); a clean-up step (using strong anion exchange [SAX], immunoaffinity [IMA], or reversed-phase C_{18} columns); and an end-determination step using a variety of chromatographic (e.g., TLC, HPLC, GC/MS, LC/MS) or immunological (ELISA) techniques (Shephard 1998). The most widely used method, adopted as standard by AOAC International, foresees the use of methanol-water extraction, SAX clean-up, and HPLC determination after reaction with o-phthaldialdehyde/2-mercaptoethanol (OPA) to form fluorescent derivatives (Sydenham et al. 1996). This method performs well with corn flour, but it is not suitable for the determination of fumonisins in most corn-based food products.

After testing for several factors (type and volume of extraction solvent, sample intake, extraction mode, and clean-up) that may affect analytical performance, our laboratory developed an accurate and horizontal method for the determination of fumonisin B_1 (FB_1) and B_2 (FB_2) in corn flour, corn flakes, extruded corn, muffins, and infant formula that is based on immunoaffinity clean-up and HPLC analysis (Solfrizzo et al. 2000b). We extracted samples twice with acetonitrile-methanol-water (25:25:50) and applied the combined extracts to a Fumoni test immunoaffinity column after dilution with phosphate-buffered saline solution. We eluted fumonisins from the column with methanol and analyzed them by HPLC after OPA derivation (Solfrizzo et al. 2000a, 2000b).

The use of immunoaffinity columns for clean-up was necessary to obtain clean extracts, particularly for corn flakes, corn muffins, and infant formula. The average recoveries of $FB_1 + FB_2$ from corn flour, corn flakes, extruded corn, corn muffins, and infant formula (spiked at levels of 0.5, 1.5, and 4.2 µg/g) were 85%, 100%, 93%, 84%, and 78%, respectively. Higher fumonisin levels were found in naturally contaminated samples with the new method compared with other methods (Solfrizzo et al. 2000a). The AOAC official method was not applicable as such to the analysis of extruded corn and infant formula, because most of the extraction solvent was absorbed by the matrix and because it provided much lower fumonisin recoveries, particularly for corn flakes (Solfrizzo et al. 2001, DeGirolamo et al. 2001).

The new method has been validated for corn flour and corn flakes by an international collaborative study supported by the European Union SMT Programme, and it is

currently under consideration as a standard method by AOAC International and CEN.

Analysis of Ochratoxin A in Wine and Beer

Immunoaffinity columns have been used successfully for the analysis of ochratoxin A (OTA) in various agricultural and food matrices, and there is increasing interest in their use for the analysis of wine and beer. A rapid, sensitive, and accurate method has been developed in our laboratory for the analysis of OTA in wine and beer using commercial immunoaffinity column for clean-up and HPLC for quantification (Visconti et al. 1999, 2000). The method avoids the use of high volumes of dangerous extraction solvents (aromatic or chlorinated solvents) that are required by other OTA methods.

We diluted wine or beer samples with a solution containing 1% polyethylene glycol and 5% sodium bicarbonate, which we then filtered and cleaned up with an Ochra test immunoaffinity column. After washing the column with a solution containing sodium chloride (2.5%) and sodium bicarbonate (0.5%) followed by water, we eluted OTA with methanol and quantified it by reversed-phase HPLC with a fluorometric detector (I_{ex} = 333 nm, I_{em} = 460 nm) using acetonitrile:water:acetic acid (99:99:2) as the mobile phase. Average recoveries of OTA from white, rosé, and red wine and beer samples spiked at levels from 0.04 to 10 ng/mL and ranged from 88% to 103% with 0.01 ng/mL detection limit and coefficients of variation generally lower than 5%. The range of applicability of the method was from 0.01 to 30.0 ng/mL OTA in wine or beer. The method was applied in a survey of 56 samples of red, rosé, and white wines (bottled as well as home-made wines) from Italy and 35 samples of pale beer (<6% alcohol) and 26 of strong beer (≥6% alcohol) from several countries. The levels of OTA in wine ranged from <0.01 to 7.6 ng/mL, with red wines more contaminated than rosé and white wines (Visconti et al. 1999). The levels of OTA in beer ranged from <0.01 to 0.14 ng/mL, with no significant difference between OTA contamination levels in strong and pale beers (Visconti et al. 2000).

The method has been validated for wine and beer by interlaboratory studies and is under consideration as a standard method by AOAC International, CEN, and the Office International de la Vigne et du Vin.

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Control of Mycotoxins in the Food Supply: A Food Industry Perspective

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Mycotoxins can have serious health effects on populations that consume large amounts of moldy food. Mycotoxins can also have a devastating economic impact on farmers of grain, nut, and spice crops globally. Major international food companies employ a wide variety of strategies to ensure the safety of their raw materials. The impact of mycotoxins can be minimized using an integrated strategic approach involving seed companies, farmers, grain millers, universities, government agencies, consumers, the media, and food companies. The basic strategies used by the food industry to control mycotoxins are discussed herein.

Seed companies are the first line of defense for mold and mycotoxin prevention. However, the seed companies' customer is the farmer, who has a primary concern with the cost of seed and whether it is offset with higher yields. This is especially true when farmers sell the majority of their crop for feed. Food companies need to be more proactive in educating the seed companies as to their needs with respect to mycotoxins. For example, one traditionally bred corn variety has a much higher yield with a larger ear. However, the ear can be so large that it often protrudes from the protective husk and invites insects and mold. Complete husk coverage lowers the risk of infestation and subsequent mold damage. Some seed companies are proactive and have used biotechnology to develop varieties that are more resistant to mold and varieties with enzymes to break down mycotoxins. The U.S. Department of Agriculture and Iowa State University conducted a survey of corn enhanced with *Bacillus thuringiensis* and found a 96% reduction in corn mold and a 90% reduction in fumonisin (Munkvold et al. 1999).

Farming has progressed over recent years with the use of "precision farming" techniques. Many farmers have equipment that uses a Global Positioning Satellite system to measure the moisture in grain correlated with the location of a tractor. This field information is transmitted via satellite to a computer to plot moisture, soil type, and location on a map. The U.S. Weather Service and the local extension office often can assist the farmer to determine the appropriate harvest time. Food companies also should work with farmers to educate them on the proper storage of grain to prevent further mycotoxin damage, with emphasis on temperature and moisture control.

Most large food companies do not actually purchase grain from individual farmers. The farmer sells his crop to a grain miller, who processes it and sells it to the food company. Millers who have worked with farmers should know what to expect in terms of overall grain quality. As added insurance, millers check the quality of each incoming load. Some millers may have 300 truckloads of grain a day, which makes it difficult to use traditional analytical methods for mycotoxin testing. If a truck is approved, the grain is dumped into a clean, sealed storage bin. Millers monitor the grain throughout its storage life by controlling temperature, moisture, and insect damage. Additionally, the transportation of grain can be a critically important step, especially when it is shipped overseas.

Most raw materials for food are milled fractions of the grains. Removing fines, or small particles of the grain, before milling can reduce mycotoxin levels. Another method used in the peanut and rice industry to reduce aflatoxins is color sorting, which is used to remove molded material. Millers have conducted simulated studies to determine the fate of mycotoxins. For example, when corn highly infected with *Fusarium* mold was milled, most of the *Fusarium* remained in the germ and bran fractions, with small amounts in the flour and none in the flaking grits (Bullerman and Tsai 1994). These processing studies are useful in identifying potential issues with milled fractions.

The food processing plant should work with millers and farmers to prevent "surprises" from occurring. Plants set specifications for raw materials, including limits for moisture and mycotoxins. Most multinational companies use the same standards internationally in countries that do not regulate mycotoxins. The plant inspects the incoming grain to make sure it meets required specifications.

Corporate food safety groups in food processing companies play a significant role in strategic partnerships to control mycotoxins. A group may manage a mycotoxin audit testing program. In a year where a mycotoxin occurs at relatively high levels in a given grain, the group may work with suppliers to reemphasize the importance of the issue. It may alert food processing plants to increase mycotoxin

testing and may also work with purchasing departments to seek alternative supplies from a different region with no mycotoxin problems. For example, in 1996, the Midwestern wheat crop was severely damaged with deoxynivalenol, and food companies paid enormous fees to have wheat shipped in from the Pacific Northwest that did not have the same mycotoxin problem.

The corporate food safety group can also participate in proactive initiatives to lessen the impact of mycotoxins. It may sponsor research with universities to determine the fate of mycotoxins in processing. This might involve, for example, spiking a mycotoxin onto a grain and sending it through simulated food equipment at a university laboratory (Bullerman 1996, Katta et al. 1999). Some mycotoxins are more heat stable than others, but overall food processing cannot be relied on as a complete kill step.

The corporate food safety group may monitor global regulations in mycotoxins and participate in international conferences such as the Codex Food Additive and Contaminants Meeting. It may voluntarily submit data to the U.S. Food and Drug Administration or other government agencies to assist them in making valid risk assessments.

All parties should make use of recognized mycotoxin experts. Many universities have extension offices that focus on farmer education and early warning systems for mold problems. Some universities conduct research on processing and milling methods, methodology development, plant pathology, and toxicology. The U.S. Weather Service is also useful in determining when conditions are favorable for heavy mold proliferation.

Government agencies should work with industry to share data on the prevalence of mycotoxins in grains and other components of the food supply. They should review the toxicology literature and perform assays needed to fill data gaps. A good example is the 2-year rodent bioassay on fumonisin conducted by the National Toxicology Program. Using all of this information, government agencies should conduct a scientific quantitative risk assessment and set limits based on sound science. They should not set limits lower than justified by science, because such limits can have an unnecessary negative impact on farmers and others. Government agencies should also work to harmonize mycotoxin regulations globally based on science.

The consumer and media also play a role in food safety. Consumers have a right to expect high-quality, safe foods, but they should understand the risks associated with eating food with some molds. For example, in South Africa some people use very moldy corn to make alcohol, and this alcohol still contains very high levels of mycotoxins (Marassas, 1995). Consumers and the media also need to understand that government limits for mycotoxins are already precautionary and have built-in safety factors.

Overall, the food industry uses a comprehensive strategic approach to control mycotoxins to protect the consumer and to protect food companies' reputations.

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