REFERENCE 1

2002F.0316

BKG 1

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration

Memorandum

Date: June 3, 2005

From: Division of Petition Review, Toxicology Group I

Subject: **Safety Review of LMP-102™ as an antimicrobial agent in ready-to-eat-foods, fresh meat, meat products, fresh poultry, and poultry products**

To: Raphael Davy
CSO, DPR

Through: Catherine Whiteside, Ph.D. Catherine Whiteside
Toxicology Group Supervisor, DPR, HFS-265

FAP 2A4738

Intralytix, Inc
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Intralytix, Inc. is proposing the use of LMP-102™, a phage preparation effective against *Listeria monocytogenes* (*L. monocytogenes*), as an additive to be applied to the surface of ready-to-eat meat and poultry products to reduce pathogen contamination. The petitioner proposes to use LMP-102™ at levels of approximately 1mL per 500 cm^2 food article surface area.

Identity:

LMP-102™ (Trade Name) is a mixture of equal proportions of six unique clonal bacteriophages (referred to as "component phage(s)" or "component monophage(s)") contained in phosphate-buffered saline solution in water that are effective against *L. monocytogenes* (DPR Chemistry Review Group, 6/16/04 H. Lee memo). Table 1 shows a listing of the components in the LMP-102™ solution. Intralytix, Inc. has indicated that the LMP-102™ mixture will be purified using a series of membrane filtration steps (involving the use of .022 μM, filtration steps and a 100 kDa filtration step) in the manufacturing process of LMP-102™ that are expected to effectively remove virulence factors of a size up to 100 kDa (see Table 2). Based on these procedures, LLO and most of the other smaller virulence factors (<100 kDa) are unlikely to be present in the final phage preparation in significant levels. The virulence factors larger than 100 kDa, autolysin Ami (102 kDa) and Protein p104 (104 kDa), may be present in the final phage preparation. However, these factors are not classified as toxins, and thus are of no toxicological concern.

Using the proposed purification process, the petitioner states that LLO is undetectable in the final product based upon method analytical detection limits of approximately 5 hemolytic units (HU/ml). Therefore, because of its known toxicity, we are evaluating the potential toxicity of LLO that would be expected when present at the low level of 5 HU/ml as a residue of LMP-102™.

TABLE 1. Chemical Components of LMP-102™

Chemical Component	Concentration
Active Phages	0.1-0.3 mg/kg
Total Dissolved Solids	1%
Ash	<0.95%
Phosphorus	300-370 mg/kg
Potasium	150-157 mg/kg
Sodium	3600-3900 mg/kg
Chloride	4800-5100 mg/kg
Heavy Metals	<0.5 mg/kg
Total Organic Carbon	<40 mg/kg
Total Protein	<20 mg/kg
Total Nitrogen	<7 mg/kg
Total kjeldahl nitrogen	<7 mg/kg
Mono/polysaccharide	<80 mg/kg

TABLE 2. Virulence Factors Described for *Listeria monocytogenes*

Virulence Factor	KiloDaltons (kDa)
Phospholipases	29 – 33
Metalloprotease	35
Protein p60	60
Listeriolysin O (LLO)	55 – 60
Internalin B	71
Internalin A	80
ActA protein	90
Autolysin Ami	102
Protein p104	104

Estimated Dietary Intakes of residual components of LMP-102™:

Dietary Exposure of Phage: Our DPR Chemists have estimated the eaters-only dietary intake of phage from LMP-102™ at the 90th percentile would be 1.2 μg/p/d for the 2+ years age group (all ages) and 1.4 μg/p/d for the 20+ years age group for males (DPR Chemistry Review Group, 4/11/05 H. Lee memo).

Exposure of LLO: The petitioner has determined that LLO was undetectable as residues in LMP-102™ based upon a detection method having detection limits at 5 hemolytic units. One hemolytic unit (HU) is defined as the amount of toxin needed to release half the hemoglobin (50% lysis) of the erythrocytes in a standard assay. The detection method, analytical calculations, and limits of detection for LLO have been evaluated and accepted by our Microbiology Reviewer and Chemistry reviewer and have been determined as appropriate and acceptable (N. Belay, Memo, 06/01/05, H. Lee, Memo, 04/11/05). DPR Chemists have estimated the eaters-only dietary intake of LLO from

LMP-102™ at the 90th percentile would be 78 HU/p/d for the 2+ years age group (all ages), and 104 HU/p/d for the 20+ years age group for males (DPR Chemistry Review Group, 4/11/05 H. Lee memo).

Antifoaming Agent Dimethylpolysiloxane (DMPS): The petitioner did not provide an intake estimate for the antifoam agent, dimethylpolysiloxane; however, DRP Chemists have estimated the eaters-only intake of DMPS from LMP-102™ to be 0.16 mg/p/d for the 2+ years age group (all ages) and 0.21 mg/p/d for the 20+ years age group for males (DPR Chemistry Review Group, 4/11/05 H. Lee memo).

Total Organic Solids (TOS): Our DPR Chemists have estimated the eaters-only dietary intake of TOS from LMP-102™ at the 90th percentile would be 0.41 mg/p/d for the 2+ years age group (all ages) and 0.54 mg/p/d for the 20+ years age group for males (DPR Chemistry Review Group, 4/11/05 H. Lee memo).

Inorganic substances: Our DPR Chemists have estimated the eaters-only dietary intake of inorganic substances from LMP-102™ at the 90th percentile would be 157 mg/p/d for the 2+ years age group (all ages) and 209 mg/p/d for the 20+ years age group for males (DPR Chemistry Review Group, 4/11/05 H. Lee memo).

Our DPR Chemists have indicated that these exposure estimates represent either worst-case scenario estimates or conservative estimates based upon maximum use levels of LMP-102™ or maximum levels of the components of LMP-102™.

Safety Evaluation of LMP-102™ and its Components

The petitioner has provided an addendum petition (Volumes 11 – 14) to the original petition, (Volumes 1 – 4 and supplemental volumes 5-10) because numerous deficiencies were noted in the safety/toxicology information presented in the original petition (see previous Toxicology memoranda, T. Turner, 9/23/02; and T. Walker, 6/14/04 and 8/20/04). We note that Volumes 11 - 14 now contain a complete safety narrative (Volume 11, Section 5) that discusses all relevant studies regarding LMP-102™ from previously submitted literature searches (Appendices B43, H17, J/S) and include hard copies of all relevant studies (Appendix K). Specifically, the petitioner has submitted toxicology information on LLO, and a review of the immunologic effects and potential allergenicity of phages.

Safety Information Submitted on the *Listeria* Monoclonal Bacteriophages: The petitioner has estimated that the number of *L. monocytogenes* specific phages released into the environment is approximately 6 X 10^7 to 1 X 10^8 PFU/person/day (Volume 5, Section 8.2, p. 000081 – 000082). This estimate was based on (1) a comparison to a known concentration of F-specific phages for Salmonella serotype Typhimurium WG49 in sewage in Europe (Volume 9, Appendix I14, pp. 001484 – 001488),[1] and (2) an estimate of the sewage generated per day for the average person in the United States (Volume 9, Appendix I18, pp. 001525 – 001527). Thus, humans are exposed to high background levels of *L. monocytogenes* in the environment on a daily basis with no apparent harm to public health. Additionally, a significant portion of humans (2 – 10%) harbor *L.*

monocytogenes in their gastrointestional tract without showing any adverse effects or signs of illness.[2,3] Also, a human oral inoculation study using attenuated *L. monocytogenes* strains (*actA / plcB* deleted) showed no serious side effects when administered to healthy study participants in an 8 week study.[4]

We note that *L. monocytogenes* minus virulence factors is similar to the nonpathogenic species of *Listeria, Listeria innocua* (*L. innocua*). *L. monocytogenes* is a pathogenic species of *Listeria* due to its virulence factors, including the LLO toxin. However, *L. innocua* is a nonpathogenic species of *Listeria* that does not contain the virulence factors associated with *L. monocytogenes*, therefore will not cause harm if consumed with food.[5] Both species are similar in structure and composition minus the virulence factors. We have concluded that due to the petitioner's purification process, which reduces LLO and most of the virulence factors to nondetectable/trace levels, the final product will be toxicologically comparable to the nonpathogenic *Listeria* species, *L. innocua.*

Together, these points provide support for concluding that the phage formulation which may contain carry-over *L. monocytogenes* components do not present a toxicological concern.

Safety Information Submitted on LLO: Information regarding the identity and toxicity of LLO, the stability of LLO, the concentration of LLO in LMP-102™, and the safety margin for LLO was discussed by the petitioner in the safety narrative sections in Volume 5, (Section 5.1.2.3, pp. 000063 - 000065) and Volume 11, (pp. 001684 – 001685 and 001695 - 001696). In these sections the petitioner discussed their conclusion that "LLO is expected to be rapidly and irreversibly degraded by proteolytic enzymes and stomach acid when administered orally and thus, LLO would not be expected to pose a toxic threat to humans". The petitioner stated that "variants of LLO…irreversibly degrade at low pH", and supported this claim with data from Matar et al., 1992 (Volume 9, Appendix H29, pp. 001187 – 001196),[6] which shows that LLO activity was lost or significantly decreased at pH 5 and not regained upon serial dilution and incubation in 0.01 M PBS at pH 6.0 (an optimal pH for hemolytic activity as reported by this study).

Additionally, a detailed review of LLO-related scientific literature was performed by the petitioner (Volume 11, pp. 001685 - 001695) and hardcopies were submitted in Appendix G12 (Volume 8), and Appendices K22 – K77 (Volumes 12 - 14) in the petition. The review of this literature was summarized and categorized according to: studies on Listeriolysin O, genetic studies of *Listeria* virulence, studies and reviews on the pathogenesis of Listeriosis, immunological studies, and other studies. The literature publications of the general studies on Listeriolysin O discussed basic background information on LLO, its detection and characterization, its mechanism of action, and the similarity of LLO to other toxins (supporting references were submitted in Appendices G12, K22 – K43, K45 – K50, and K68). The genetic studies of *Listeria* virulence discussed the hly gene that encodes LLO, and how that gene is regulated (supporting references were submitted in Appendices K51 – K54). The studies and reviews on the pathogenesis of Listeriosis discussed how LLO is specialized to function within the intracellular milieu to facilitate the escape of *L. monocytogenes* from the phagosome, and subsequently lead to pathogenicity (supporting references were submitted in Appendices K44, and K55 – K65). The immunological studies described the cellular, rather than humoral, immune response to *L. monocytogenes* (supporting references were

submitted in Appendices K66 – K72), and the final literature section summarized other miscellaneous studies on LLO that did not fall under any of the previously mentioned categories (supporting references were submitted in Appendices K73 – K77).

One notable review article submitted in the section "Studies and Reviews on the Pathogenesis of Listeriosis" discussed *L. monocytogenes* and food safety (Volume 14, Appendix K62, pp. 002460 – 002478).[7] With regards to LLO, this review focused on the control of LLO expression and its role in the virulence of *L. monocytogenes*. Also, the use of oral and intragastric infection models to better simulate aspects of foodborne infection was discussed. The petitioner noted that this review yielded "...no mention whatsoever of foodborne illness caused by elaboration of listeriolysin O directly onto food. Disease occurs only in the presence of an active intracellular infection with *L. monocytogenes*". He concluded that the information gathered from this entire literature review provided "strong evidence that LLO is the only toxin produced by *L. monocytogenes*, that the toxin is produced in low levels, and that there are no reports of oral toxicity of the LLO toxin by itself or as a contaminant on food" (Volume 11, p. 001654).

Based upon our review of available published information on LLO, we have determined that the residual levels of LLO found in the phage preparation do not present a toxicological concern based on the following:

1) **Residues of LLO in LMP-102™ are undetectable with a method sensitive to 5 HU/ml:**
   The petitioner stated that any residues of LLO in LMP-102™ are undetectable with a method sensitive to 5 HU/ml; therefore, the concentration in each batch will be limited to no more than 5 HU/ml. A quantitative amount for 1 HU of LLO can be calculated from the specific activity to be 10^{-6} mg of protein, or 1 nanogram (ng) of protein[8] (specific activity of LLO = 10^6 HU/1 mg of protein, or 10^6 HU = 1 mg of protein, or 1 HU = 10^{-6} mg of protein X 1 g / 10^3 mg X 1 ng / 10^{-9} g = 1 ng of protein). Therefore, the amount of toxin present in the final product, based on the detection limit, is 5 HU/ml or 5 ng/ml. Additionally, Geoffroy et al., 1987 (Volume 9, Appendix H28, pp. 001180 – 001185),[8] determined the LD_{50} value of purified LLO in mice to be 0.8 μg per mouse, or 0.8 μg/0.03 kg bw/d = 26.7 μg/kg bw/d (Volume 11, p. 001684). Since the specific activity of LLO is 10^6 HU/mg, or 1 HU /10^{-6} mg, the LD_{50} value of LLO is approximately 26,700 HU/kg bw/d (26.7 μg/kg bw/d, or 0.0267 mg/kg bw/d X 1 HU/10^{-6} mg = 26700 HU/kg bw/d) (Volume 11, p. 001685). DPR Chemists have estimated the exposure of LLO to be 104 HU/p/d for males, age 20+, or 1.73 HU/kg bw/d (104 HU/p/d ÷ 60 kg bw = 1.73 HU/kg bw/d). The margin of safety between the LD_{50} value of LLO and the exposure level in humans is 26,700 HU /kg bw/d ÷ 1.73 HU/kg bw/d = 15433, and therefore would not be a cause for toxicological concern. This potential exposure of LLO represents a worst-case concentration and is not realistically expected to be present as a residue component of LMP-102™.

2) **Inactivation of LLO by Cholesterol:**
   Listeriolysin O belongs to a superfamily of pore-forming toxins known as cholesterol-dependent cytolysins (CDCs) that function exclusively on cholesterol containing membranes (Volume 12, Appendix K26, pp. 002075 – 002083; Volume 13, Appendix K35,

pp. 002241 – 002246).[2,9,10] In fact, the presence of membrane cholesterol is required in order for the CDC cytolytic mechanism to occur.[11] It has been suggested that toxins in this family, including LLO, act by binding to the surface of cholesterol-containing membranes, in which they oligomerize to form pores; thus, damaging the membranes of target cells resulting in lysis of the cell.[2,12] The effect of cholesterol on LLO and other CDCs has been examined for each step in this process.[9,11,13] Studies show that depletion of membrane cholesterol stalls CDCs (perfringolysin, streptolysin O, and intermedilysin) in their prepore complex; thus, preventing pore formation.[11] Additionally, cytolytic activity has been shown to be significantly reduced following preincubation of LLO with exogenous cholesterol *in vitro* [8] by as much as 200- to 2000-fold.[13] While the cytolytic activity of LLO and other CDCs is significantly reduced by the loss of membrane cholesterol and in the presence of exogenous cholesterol, membrane binding appears to be largely unaffected;[11,13] this reportedly occurs due to evidence which suggests that two different domains of the LLO toxin are involved in cytolytic activity and cholesterol binding (Volume 14, Appendix K68, pp. 002547 – 002551).[14] However, preincubation of LLO with cholesterol has been shown to interfere with the subsequent oligomerization step in this process, which is thought to be responsible for inhibition of cytolytic activity.

Since the petitioner has proposed the use of LMP-102™ on meat/animal products, any minimal amount of LLO that may be present in the final product is likely to be inactivated by the cholesterol normally present on the surface of meat/animal products.

3. pH and LLO activity:

The direct relationship between pH and LLO activity inside *L. monocytogenes*-containing vacuoles has been studied as well. Some studies show that LLO activity is lost or significantly decreased in acidic environments.[6,15] Variants of LLO have been shown to be maximally stable at pH 8.0, while they irreversibly degrade at pH 5.0.[6] However, other studies show that the lytic action of LLO may be triggered by vacuolar acidification (Volume 12, Appendix K23, pp.002055 – 002059).[8,16] Perforation of vacuoles by LLO occurred over a narrow range of acidic pH values from 4.9 to 6.7 with a mean of 5.94;[16] and an increase in pH by the addition of NH_4Cl (a weak base) or bafilomycin A_1 (an inhibitor of vacuolar proton ATPases) resulted in reduced or complete prevention of vacuolar perforation. This study reported the lowest pH achieved inside the *L. monocytogenes*-containing vacuole, and the results of an increase in pH; however, no studies were performed at lower pH levels that are more consistent with that of the gastric environment. Similar results were reported by Geoffroy et al., 1987,[8] where optimal LLO activity was observed at pH 5.5 and hemolysis was undetectable at pH 7.0; pH levels below 4.5 were not studied.

We conclude that if LMP-102™ treated foods are uncooked before consumption, then any residual carry-over components from *L. monocytogenes,* e.g. LLO, are likely to be inactivated by host defense mechanisms (low pH gastric secretions, the normal intestinal microflora, and the activity of macrophages and neutrophils), and therefore would not be a cause for toxicological concern.

4. Inactivation of orally consumed *Listeria* and LLO by Host Defense Mechanisms:

Host defenses against orally consumed LLO and *Listeria* include stomach acidity, the normal gut microflora, and cell mediated immunity.[3,5] Studies show that an increase in the pH of the stomach results in increased gastric transit of *Listeria*.[17] Also, antacids and H2 blocking agents have been reported to be a risk factor for listeriosis.[18,19] This indicates that the gastric acid in the stomach may destroy a significant number of the *Listeria* organisms ingested with contaminated food; thus, the stomach may serve as a primary barrier against listeriosis.

The normal intestinal flora, macrophages, and neutrophils offer the next level of protection against orally consumed *Listeria*. *L. monocytogenes* must first traverse the intestinal wall before it can cause severe systemic illness characteristic of listeriosis. *In vivo* experiments demonstrate that both the normal intestinal flora and the activity of macrophages and neutrophils in the intestines inhibit listeriosis infection.[20]

DPR Toxicology was asked by DPR Chemistry (H. Lee June 16, 2004 memo) to comment on the appropriateness of the petitioner's claim that:

> **"LLO is expected to be rapidly and irreversibly degraded by proteolytic enzymes and stomach acid when administered orally and thus, LLO would not be expected to pose a toxic threat to humans" (Section 5.1.2.3 pp. 000063 – 000065).**

Therefore, based upon the points discussed above, Toxicology agrees with the petitioner's statement that "LLO is expected to be rapidly and irreversibly degraded by proteolytic enzymes and stomach acid when administered orally and thus, LLO would not be expected to pose a toxic threat to humans". We conclude that potential negligible residues of LLO found in the phage formulation do not pose a safety concern for the proposed use of LMP-102™ as an antimicrobial agent. DPR Toxicology agrees with our DPR Chemists that a specification limit of 5 HU/ml for LLO should be included in the proposed regulation of LMP-102™.

Evaluation of the Potential Allergenicity and Immunotoxicity of Bacteriophages: An evaluation of potential immunological and allergenicity of bacteriophages was performed by the petitioner (Volume 11, Section 5, pp. 001667–001679 in the petition). Several studies were submitted that evaluated the use of ΦX174, a small bacteriophage specific for *E. coli*, in human and animal studies in both immunocompromised patients and healthy volunteer controls (hardcopies were submitted in Appendices H03 – H09 and K02 – K05). These studies examined treatment with bacteriophages in a range of diseases and complications, such as Down Syndrome, Wiskott-Aldrich Syndrome, steroid-mediated immunosuppression, severe dermatoses and skin infections, HIV/AIDS, and other immunodeficient diseases. These studies were performed in patients from a range of age groups spanning from infants to adults, including an immunocompromised infant as young as 8 months old (Volume 12, Appendix K02, pp. 001853 – 001858),[21] and children with Down Syndrome ranging from 12 – 18 years of age (Volume 8, Appendix H03, pp. 000997 -

001001).[22] No complications or adverse effects were noted in the submitted studies that could be directly attributed to the use of the ΦX174 bacteriophage.

The petitioner submitted additional studies which evaluated the treatment of patients with therapeutic bacteriophages in the United States and Europe (hardcopies were submitted in Appendices K06 – K07 (U.S. studies) and K08 – K21 (European studies) in the petition). The submitted studies performed in the U.S. examined the use of bacteriophage therapy in complications such as furunculosis, chronic and pustular acne, pyoderma, excema, bronchial asthma, respiratory infections, and intestinal disorders. The submitted European studies focused on using bacteriophage therapies to treat dysentery, leukemia, suppurative infections, skin infections, chronic open wounds/ulcers, burn patients, lung abscesses, osteomyelitis, colitis, allergic rhinopharyngitis, allergic dermatitis and conjunctivitis, staphylococcal pneumonia, and sepsis in newborns and infants. Notably, in the study that evaluated bacteriophage therapy in immunocompromised newborns and infants, the author reports that "no patients revealed toxic, allergic, or pyrogenic reactions or accessory phenomena caused by the treatment carried out by the offered scheme" (Volume 12, Appendix K21, pp. 002047).[23] Additionally, some studies in Appendices K06 - K21 reported occasional side effects when crude, unpurified phage preparations were used in immunocompromised, ill patients. However, none of the studies reported observations of allergic or immunological responses that could be directly attributed to phage exposure when purified phage preparations were used in humans or animals. The petitioner has indicated that several filtration steps will be used in the manufacturing of LMP-102™; thus, the final phage product is a purified phage product.

Antifoam Agent: Dimethylpolysiloxane (DMPS) will be used as an antifoam agent in the manufacturing process of LMP-102™ at a maximum use level of 10 mg/kg. In the original petition, Intralytix proposed to use the antifoaming agent, Antifoam 204, in the manufacturing process of LMP-102™; however, this compound was not approved for use as proposed by the petitioner. Subsequently, the petitioner elected to use an antifoam agent previously approved by the Agency. The Agency was notified by the Petitioner in an email response (dated 02/18/05) that they are no longer considering the use of Antifoam 204 in the manufacturing process of LMP-102™ and instead will use DMPS as the antifoam agent.

DMPS is currently regulated for use as a defoaming agent in food processing at levels up to 10 mg/kg under 21 CFR 173.340. DPR Chemists have accepted the petitioner's use of DMPS at a maximum level of 10 mg/kg (DPR Chemistry Review Group, 4/11/05 H. Lee memo). Therefore, the use of DMPS at the proposed level does not present a toxicological concern.

Other Chemical Components of LMP 102™:
DPR Toxicology has no concerns regarding the estimated dietary intake levels for other chemical components of LMP-102™ (e.g. total organic solids, heavy metals, and inorganic substances).

Overall Safety Conclusion For FAP 2A4738:
DPR Toxicology has evaluated the safety of LMP-102™, a bacteriophage formulation that will be used as an antimicrobial agent effective against *L. monocytogenes*, on ready-to-eat meat and poultry

products. Based upon our review of the available data we conclude that the proposed phage formulation is safe.

Tina W. Walker, PhD

Tina W. Walker, PhD

ATTACHMENT 1: References for the Toxicology Record for FAP 2A4738 (June 3, 2005)
ATTACHMENT 2: Additional References Found by the DPR Toxicology Reviewer (June 3, 2005)

CC: HFS-265 (Biddle, Varner, Whiteside, Walker)

ATTACHMENT 1: References for the Toxicology Record for FAP 2A4738 (June 3, 2005)

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ATTACHMENT 2: Additional References Found by the DPR Toxicology Reviewer (June 3, 2005)

Reference #2 Swaminathan B. 2001. *Listeria monocytogenes*, p 383 – 409. *In* M P Doyle et al. (ed.), *Food microbiology: Fundamentals and Frontiers,* 2nd ed. ASM Press, Washington, D.C.

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