

**Evaluation of Micronutrients
Supplementation Effects on a Novel
Infection Model with *Listeria
monocytogenes*.**

**(新規リステリア・モノサイトゲネス感染モデルを
用いた微量栄養素投与効果の検討)**

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ABSTRACT

Listeriosis, caused by the gram-positive, facultative intracellular bacterium *Listeria monocytogenes*, is one of the leading causes of death due to foodborne illness in the industrialized world. *Listeria monocytogenes* had been isolated in humans as early as the 1920. Nevertheless, it was not identified as an important cause of neonatal infection until after World War II in Germany. Over the past 50 years, the human immunodeficiency virus (HIV) epidemic has significantly enlarged the immunodeficient population, with a relative risk of developing listeriosis 500 times higher in acquired immunodeficiency syndrome (AIDS) patients as compared to the general population. However, globally, human listeriosis remains a rare disease, and its prevalence is declining in industrialized countries in which food control measures have been implemented.

L. monocytogenes infects humans through the ingestion of contaminated food. It is able to cross the intestinal barrier and is thought to disseminate from the mesenteric lymph nodes to the spleen and the liver. *Listeria* has been isolated in a variety of raw foods, such as uncooked meats and vegetables, or food items that become contaminated during their processing, such as soft cheeses and cold cuts at deli counters. Unpasteurized milk or foods made from unpasteurized milk may contain the bacterium. *L. monocytogenes* is killed by pasteurization and cooking. However, in certain ready-to-eat foods such as hot dogs and deli meats, contamination may occur after cooking, before packaging.

There is growing interest in the role of micronutrients (essential trace elements and vitamins) in optimizing health, and in the prevention or treatment of disease, thus, because the nutritional status of an individual is profoundly affected by most disease states (usually by a combination of increasing demand at a time when there is reduced intake). This stems partly from the increase in knowledge and understanding of the biochemical functions of these nutrients, but also from the extensive threat that the growing population of antibiotic resistant organisms pose to public health.

There are many lines of in vitro evidence and human trials that have shown the essential effect of trace elements and vitamins on all aspects of immune function. In a study of tuberculosis in Indonesia, supplementation with zinc and vitamin A led to much earlier resolution of radiological changes and time to sputum negativity. Zinc (Zn) is the second most abundant transition metal after iron. It plays a vital role in living organisms and affects multiple aspects of the immune system. Vitamin A and related retinoids influence many aspects of immunity. Combined zinc and vitamin A synergistically reduced the prevalence of persistent diarrhoea and dysentery.

In this study I challenged the efficacy of this combination against *Listeria monocytogenes* in a mouse model. I demonstrated that the supplementation with Zn and atRA enhanced resistance against *L. monocytogenes* infection in mice and treatment with both Zn and atRA showed a higher protective effect than treatment with either alone. Supplementation with Zn, atRA or their combination decreased the number of *L. monocytogenes* present in target organs. In vitro, its combination increased the bacterial uptake by macrophage cells and reduced the replication of *L. monocytogenes*.

Animal models have played fundamental roles in elucidating the pathophysiology and immunology of listeriosis, and will almost certainly continue to be integral components of the research on listeriosis. Data derived from animal studies helped for example characterize the importance of cell-mediated immunity in controlling infection, allowed evaluation of chemotherapeutic treatments for listeriosis, and contributed to quantitative assessments of the public health risk associated with *L. monocytogenes* contaminated food commodities. Newly emerging data about species-specific differences have recently raised concern about the validity of most traditional animal models of listeriosis.

Insect infection models have been used increasingly to study various pathogenic agents, including *L. monocytogenes*, in evaluations of pathogenicity and drug efficacy. Although, insect models have been criticized for being “too distant” from human disease processes, silkworms may provide a useful model for analyzing innate immunity because insects and mammals share common innate immune mechanisms.

In this study, I also demonstrated that larvae of the silkworm *Bombyx mori* are useful for studying *Listeria monocytogenes* infections in insects. Infection with the *L. monocytogenes* wild-type strain induced silkworm death. Infection by a Listeriolysin O (LLO) deletion mutant also induced silkworm death, but the bacterial numbers in silkworms were lower than those of the wild-type strain. Intracellular growth was observed when the silkworm ovary-derived cell line BmN4 was infected with the wild-type strain. Pretreatment with vitamin A did not affect silkworm mortality after bacterial infection, but the efficiency of infecting the hemocytes and BmN4 cells was decreased

with vitamin A treatment. Our results indicate those silkworm larvae are a useful insect infection model for *L. monocytogenes* and that vitamin A has protective effects against bacterial infection in silkworms.

Non-mammalian models have logistical, budgetary, and ethical advantages over mammals. In particular, the ease and low costs required to obtain large numbers of larvae facilitate large-scale screening, which would be prohibitive in mammals. The relevance of *in vivo* studies in so-called animal models has always to be revisited in the light of medical observations and *in vitro* findings. The “model” status of a given animal has constantly to be challenged, in order to uncover its limitation and ultimately propose ways to replace it, or “fix” it by the use of transgenesis or KI approaches.

DEDICATION

To all the Dominican women:

The highway that takes you from daydreaming to realization does exist and have a name: courage. Someone said, “Women are the largest untapped reservoir of talent in the world [...]”*, so allow yourself to brainstorm your future, be brave and daring and never give up until you conquer your dreams. Never allow anyone, or yourself, to tell you that you are incapable of anything: be your own backbreaking judge. Don’t be afraid of your dreams.

Personally I dedicate this work to my grandparents Francisco and Aida, my uncle Martin and Ms. Melania: they who believed in me... my heavenly cheerleading crew.

To my niño Anibal: I love you with all my heart...

*Hillary Clinton, *Elle*, 2012.

PREFACE

The Gram-positive bacterium *Listeria monocytogenes* is a ubiquitous pathogen that thrives in diverse environments such as soil, water, various food products, humans and animals. Discovered in 1926, it is responsible for a severe food-borne disease characterized by meningitis, meningo-encephalitis, materno-fetal and perinatal infections and is also responsible for febrile gastroenteritis (Cossart, 2011).

L. monocytogenes is a facultative intracellular bacterium considered an opportunistic psychrophile organism whose life cycle reflects its remarkable adaptation to intracellular survival and multiplication in macrophages and other cell types. The disease caused by this bacterium, listeriosis, is acquired by ingesting contaminated food products and mainly affects immunocompromised individuals, pregnant women and newborns, resulting in death in 25–30% of cases (Seveau et al., 2007)

The diverse clinical manifestations of infection with *L. monocytogenes* reflect its ability to cross three tight barriers in the human host due to the capacity of *Listeria* to escape intracellular killing when phagocytosed by macrophages. Following ingestion, it is generally considered that bacteria after crossing the intestinal barrier, reach via the lymph and the blood, the liver—in which they replicate in hepatocytes—and the spleen. Then bacteria via hematogenous dissemination can reach the brain and the placenta (Hamon et al., 2006; Cossart and Toledo-Arana, 2008).

There is growing interest in the role of micronutrients (essential trace elements and vitamins) in optimizing health, and in the prevention or treatment of disease. This stems partly from the increase in knowledge and understanding of the biochemical functions of these nutrients, but also from the extensive threat that the growing population of antibiotic resistant organisms pose to public health.

There are many lines of in vitro evidence and human trials that have shown the essential effect of trace elements and vitamins on all aspects of immune function with vitamin A and zinc at the central stage. Both, vitamin A and zinc deficiency reportedly increase the susceptibility to pneumonia and alimentary tract diseases. Inversely, supplements in populations with poor vitamin A and zinc status, have been shown to reduce mortality from diarrhoea in community studies, and deaths from pneumonia in measles studies (Villamor and Fawzi, 2005; Mora et al., 2008; Rahman et al., 2002). In a large study in India on residential schoolchildren with biochemical evidence of poor status for several micronutrients, a multi- micronutrient supplement did not reduce the incidence of common childhood infections, but did reduce the duration of such illnesses, therefore, supplementation effects extends to innate and adaptive immune responses (Sarma et al., 2006).

Being that an infection is a multistep process integrating a series of host and microbial variables, the comprehension of the pathophysiology of a human infection necessitates an animal model in which the infectious agent has as much as possible the same cell and tissue tropism as in humans. Animal species naturally infected by *L.*

monocytogenes should be logically used as firsthand models to study the pathophysiology of human listeriosis (Lecuit, 2007).

However, this apparently straightforward approach has obvious technical limitations, since the animals developing a disease most closely resembling human listeriosis are not classical laboratory animals such as the rat or the mouse, but rather farm animals such as sheep, cattle and goats. In this study I challenged the effect of micronutrients supplementation against infection with facultative intracellular pathogen *Listeria monocytogenes* in two different infection models:

1. Effect of the combination of all-trans Retinoic Acid (atRA) and Zinc on infection in mouse. (Chapter 1)
2. Effect of vitamin A on infection in silkworms. (Chapter 2)

CHAPTER 1

**Combination of zinc and all-trans retinoic acid
promotes protection against *Listeria monocytogenes*
infection.**

INTRODUCTION

One promising area of current public health research is the possibility that micronutrient supplementation may decrease the incidence of infectious disease. With the development of modern medicine, the prevention, treatment, and cure of infectious diseases have become progressively dependent on vaccines and refined antimicrobial drugs. For decades, we have focused on the production of newer and stronger medicines and have failed to remember that simple compounds can effectively contribute to the treatment or prevention of infectious diseases. Among the micronutrients, zinc (Zn) and vitamin A are thought to have the largest impact on the prevention of diseases. Their deficiencies have been known to increase susceptibility to infection and to raise the incidence of infectious respiratory and alimentary tract diseases (Semba, 1994; West et al., 1989).

Zn is well known to be both an essential and toxic micronutrient for development of all organisms, including bacteria (Christian and West, 1998; Rahman et al., 2002). Zn is the second most abundant transition metal in the human body and has crucial roles in many facets influencing growth and affecting the development and integrity of the immune system. It is important for enzymes of all six classes, as well as transcription and replication factors (Smith, 1980). Studies reveal that supplementation and optimal intake of Zn restore impaired immune responses and decrease the incidence of infection *in vivo*. T-cells levels increase significantly after Zn supplementation and cell-mediated immune response are improved (Todd et al., 1934; Rink, 2001). The *in vivo* and *in vitro* effects of

Zn on immune cells depend mainly on the Zn concentration, considering the fact that Zn has significant toxicity at high concentrations. However, the molecular basis of Zn toxicity remains poorly defined (Fischer and Black, 2004; Dardenne, 2002).

All-trans retinoic acid (atRA), also known as tretinoin, is the acid form of vitamin A. This appears to be its active form in all tissues except retina (Napoli, 2012). Supplementation with vitamin A and its metabolite, atRA, has been reported to decrease the incidence and severity of infectious diseases, although the regulation of immune function by vitamin A may also vary widely depending on the type of infection and the immune responses involved (Villamor and Fawzi, 2005). Certain aspects of a functional synergy between Zn and vitamin A are well defined. Zn status influences several aspects of vitamin A metabolism, including: 1) absorption—Zn is essential for the lymphatic absorption of retinol; 2) transport—Zn is fundamental for the synthesis of retinol-binding protein; and 3) utilization—Zn is required for the conversion of retinol to retinal for dark adaptation. There is also evidence that vitamin A affects Zn absorption and utilization (Stephensen, 2001; Yang et al., 2011).

Listeriosis is caused by the Gram-positive bacterium *Listeria monocytogenes*. In humans, this pathogen has the ability to cross the intestinal, placental, and blood-brain barriers, leading to gastroenteritis, materno-fetal infections, and meningoenzephalitis, respectively. A key feature of the virulence of *L. monocytogenes* is its ability to avoid the killing mechanisms of professional and non-professional phagocytic host cells (Portnoy et al., 1992; Cossart and Lebreton, 2014). *L. monocytogenes* infections in humans are

caused mainly by the ingestion of contaminated foods, such as dairy products, raw vegetables, fish, poultry, processed chicken, and beef (Lorber, 1997).

Worldwide, few trials of dietary supplements have examined the practical importance of the Zn-vitamin A interaction for human respiratory tract affections and diarrhea (Kartasurya et al., 2012; Rahman et al., 2002; Long et al., 2006). Those that have been conducted have suggested that Zn and vitamin A statuses directly affect the morbidity of these infections in their trial populations. Since both Zn and vitamin A primarily affect aspects of innate immunity, the effect of supplementation against infection with facultative intracellular pathogen *L. monocytogenes* was examined in mice.

MATERIALS AND METHODS

Bacterial strain

L. monocytogenes EGD was maintained as a frozen glycerol stock and cultured in brain heart infusion (BHI) broth (Becton Dickinson, Franklin Lakes, NJ, USA) or on BHI broth containing 1.5% agar.

Zn or atRA treatment in mice

Six to 10-week-old BALB/c male mice were obtained from Kyudo Co., Ltd. (Saga, Japan). All mice were maintained under specific pathogen-free conditions in sterile cages, which were put into a ventilated isolator. Fluorescent lights were cycled 12 hours on/12 hours off, and ambient temperature ($23\pm 1^\circ\text{C}$) and relative humidity (40–60%) were regulated. Groups of four mice per assay were used. Mice were intraperitoneally administered 150 μg of ZnSO_4 (Wako, Osaka, Japan) in 0.2 ml PBS or 400 μg of atRA (Wako) dissolved in 0.2 ml corn oil. PBS or corn oil was injected, as a control. ZnSO_4 was administered six times over 1 week; atRA was administered seven times over 2 weeks (Fig. 1A). Pre-treated mice were infected intravenously via the tail vein with approximately 10^5 cells of *L. monocytogenes* in 0.1 ml saline and survival was observed on day 5 of infection. Depending on the progress of the disease, animals were monitored every 3–4 hours during the day-phase (7:00 am to 7:00 pm) by veterinarian. Mice were determined endpoints when mice showed loss of ability to ambulate and inability to access food or water, and mice were sacrificed by isoflurane euthanasia. Humane endpoint by isoflurane euthanasia was conducted if death of the animals during the

following hours was to be expected. For counting the number of bacteria in the liver and spleen, pre-treated mice were infected intravenously with approximately 10^4 cells of *L. monocytogenes* in 0.1 ml saline. Two days after infection, their liver and spleen were removed and homogenized in saline. The tissue homogenates were serially diluted with PBS and plated on BHI agar plates to estimate the number of colony-forming units (CFU). All the protocols for animal experiments have been approved in the Animal Research Committee of Yamaguchi University (Permit Number: 141). Animal studies were performed in compliance with the Yamaguchi University Animal Care and Use guidelines. The mice were sacrificed by cardiac puncture under isoflurane anesthesia and overdose of isoflurane, and all efforts were made to minimize suffering by using isoflurane anesthesia.

Cytokine measurement.

The serum levels of IFN- γ , TNF- α , IL-6, and IL-1 β were measured for infected and uninfected mice. Pre-treated mice were infected intravenously with approximately 10^4 cells of *L. monocytogenes* in 0.1 ml saline and blood was collected at 2 days after infection. Blood was collected at the same times for uninfected mice. Serum levels of cytokines were measured with an enzyme linked immunosorbent assay (ELISA) kit (Bio legends, San Diego, CA, USA), according to the manufacturer's instructions.

Cell culture

J774 cells were cultured in RPMI 1640 (Sigma, St. Louis, MO, USA) containing 10% FBS. J774 cells were seeded ($1-2 \times 10^5$ per well) in 12-well tissue culture plates.

Efficiency of phagocytosis and bacterial survival

J774 cells were grown for 3-4 days in 12-well tissue culture plates to confluency. ZnSO₄ (40 μM) or atRA (20 μM) was added to the J774 cells 48 h before infection. Bacterial strains were deposited onto J774 cells at a multiplicity of infection of 0.1 by centrifugation at 150 × g for 10 min at room temperature. To measure phagocytosis efficiency, after 30 min of incubation at 37°C, the cells were washed once with medium and then incubated in a medium containing gentamicin (50 μg/ml, Sigma) for 30 min. The cells were then washed three times with PBS and lysed with cold distilled water. CFU values were determined by serial dilution on BHI plates. To measure bacterial survival efficiency, the infected macrophages were incubated at 37°C for 30 min, washed once with RPMI 1640, and incubated with RPMI 1640 containing gentamicin (50 μg/ml) for 24 h. Cell washing, lysis, and plating procedures were the same as for the bacterial invasion efficiency assay. Cell viability analysis was the use of trypan blue dye exclusion staining.

In vitro bacterial growth assay

Bacterial strains were cultured in BHI broth containing ZnSO₄ (200 or 1,000 μM) or atRA (10 or 50 μM) at 37°C for 24 h. CFU values were determined by serial dilution on BHI plates.

Statistical analyses

One-way ANOVA was used to make statistical comparisons between the groups. Results with $p < 0.05$ were considered significantly different and are indicated by

asterisks. Data are expressed as the mean of triplicate samples from three identical experiments and the error bars represent the standard deviations (SD).

RESULTS

Pretreatment with Zn and atRA enhances host resistance against *L. monocytogenes* infection.

To examine the effect of Zn and atRA on *L. monocytogenes* infection, Zn- or/and atRA-pretreated mice were infected intravenously with *L. monocytogenes*. All the control mice died within 4 days (Fig. 1B). In contrast, 66.6% of Zn- or atRA-pretreated mice survived at least 5 days (Fig. 1B). All of the mice pretreated with both Zn and atRA survived for 5 days (Fig. 1B). Next, to measure bacterial number in the liver and spleen, the mice were infected with *L. monocytogenes* and the bacterial number were determined 2 days after infection. The number of bacteria observed in these organs was significantly lower in pretreated mice than in control mice (Fig. 2). Treatment with the combination of Zn and atRA showed a higher protective effect than treatment with either alone (Fig. 2).

Pretreatment with Zn and atRA affect cytokine production.

Multiple components of the cellular compartment of both the innate and the adaptive immune systems are simultaneously required for the resistance to infection by *L. monocytogenes*. To investigate the effect of Zn and atRA pretreatment on cytokine production, pretreated mice were infected with *L. monocytogenes* and cytokine production was determined 2 days after infection using ELISA kits. *L. monocytogenes* infection induces the production of IFN- γ , TNF- α , IL-1 β , and IL-6 (Fig. 3). Pretreatment with atRA or a combination of Zn and atRA decreased IFN- γ , TNF- α , and IL-1 β

production (Fig. 3A-C). Pretreatment with Zn, atRA, or a combination of Zn and atRA decreased IL-6 production (Fig. 3D).

Zn and atRA treatment affect bacterial infection in J774 cells.

To examine the effect of Zn and atRA on phagocytosis and bacterial survival in J774 cells, J774 cells pretreated with Zn and atRA were infected with bacteria. Since J774 cells were infected with bacteria at low multiplicity of infection, J774 cells still were intact as seen by light microscopy at 24 h after infection. Cell viability was also confirmed by the trypan blue dye exclusion staining. Pretreatment with Zn, atRA, or a combination of Zn and atRA significantly increased the relative phagocytosis efficiencies of *L. monocytogenes* in J774 cells (Fig. 4A). Pretreatment with these reagents inhibited growth of *L. monocytogenes* in J774 cells (Fig. 4B and C).

Zn has bacteriostatic effect against *L. monocytogenes* in vitro.

To investigate the bacteriostatic abilities of Zn and atRA, bacteria were cultured in BHI broth with or without Zn and/or atRA. Although the addition of atRA to the growth medium did not affect the growth of *L. monocytogenes* (atRA⁺, 89.4%; atRA⁺⁺, 80.3%), addition of Zn significantly reduced the growth of *L. monocytogenes* in a dose-dependent manner (Zn⁺, 53.7%; Zn⁺⁺, 4.8%; Zn and atRA⁺, 34.5%, Zn and atRA⁺⁺, 5.9%) (Fig. 5A and B).

DISCUSSION

Many biochemical and physiological roles of Zn have been indicated, reinforcing the importance of Zn in various biological phenomena, such as the immune response (Bonaventura et al., 2015). It has been reported that the influence of cadmium (Cd) and Zn on mice improves resistance to *L. monocytogenes* infection. Cd-exposed mice are more susceptible to *L. monocytogenes* infection, while Zn significantly reduces the negative effect of Cd on the antimicrobial defense of mice (Simonyte et al., 2003). Because the effect of Zn treatment alone against *L. monocytogenes* infection remained unknown, we investigated the role of Zn in *L. monocytogenes* infection in mice in this study. Our results showed that Zn contributes to the defense against *L. monocytogenes* infection in mice. The protective ability of Zn appears to be due to a bacteriostatic effect on *L. monocytogenes*. Although Zn is an essential micronutrient for bacteria, it is toxic at high concentrations (Corbin et al., 2008). Zn plays a protective role against infection by other pathogens, such as shiga-toxigenic *Escherichia coli* (Crane et al., 2014) and *Trypanosoma evansi* (Bottari et al., 2014). A bacteriostatic effect of Zn was demonstrated using in vitro bacterial growth assays. These results suggest that Zn acts on bacteria directly.

Vitamin A is a fat-soluble essential nutrient that is acquired from the diet as atRA, retinyl esters, or β -carotene. Retinoic acid (RA) can be generated in multiple isoforms although the all-trans isoform predominates in most tissues (Harrison, 2012). Vitamin A or RA is important for an extensive range of biological processes, including

immunomodulatory functions (Hall et al., 2011). Vitamin A deficiency can lead to an increased susceptibility to infectious diseases (West et al., 1989). During infection with *Toxoplasma gondii*, an intracellular replicating pathogen controlled by IFN- γ (Suzuki et al., 1988), the acute Th1 response, and parasite clearance are significantly impaired in vitamin A-deficient mice (Hall et al., 2011). RA has been shown to enhance macrophage activation in response to *Mycobacterium tuberculosis* infection in vitro (Yamada et al., 2007). Our results also suggest that pretreatment with atRA also enhances host resistance against *L. monocytogenes* infection.

Although Zn and atRA have been shown to protect mice from infection by intracellular pathogens in previous studies and in our study, the mechanism is still unclear. In our study, pretreatment with Zn or atRA inhibited bacterial growth in the liver, spleen, and macrophages; and these protective effects were enhanced by pretreatment with a combination of Zn and atRA. These results suggest that the bacteriostatic effect is increased by a combination of Zn and atRA. Experimental and human studies have demonstrated that type 1 cytokines, including IL-1 β , IL-6, IFN- γ , and TNF- α , play an important role in the development of cell-mediated immune responses to intracellular infections.

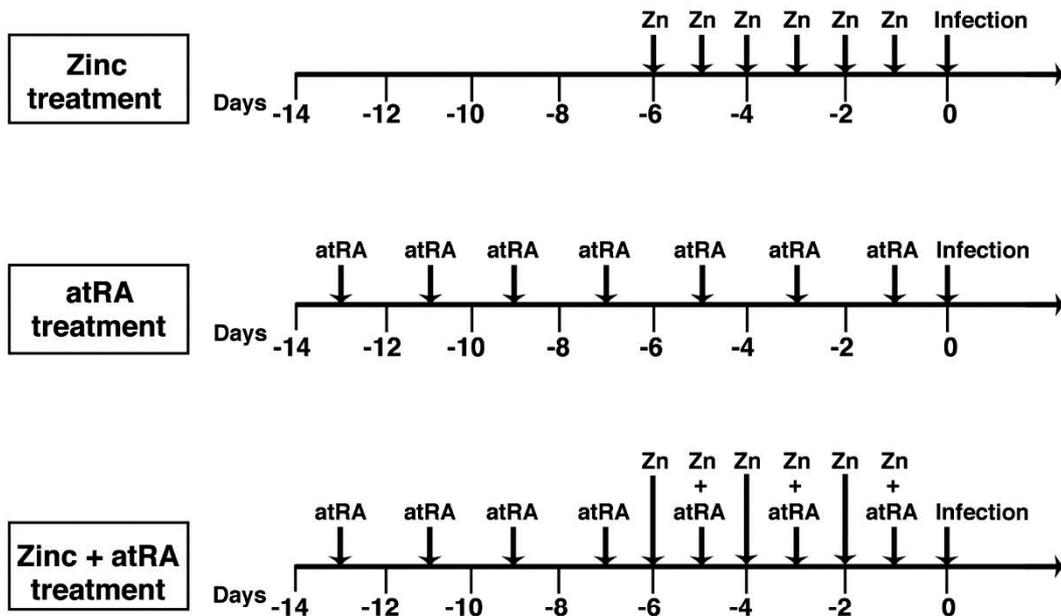
Taking this into consideration, we speculated that the bacteriostatic effect increased by the combination of Zn and atRA, is caused by the induction of cytokine production. The production of cytokines in mouse serum after *L. monocytogenes* infection was measured using ELISAs. Unexpectedly, Zn and atRA treatment decreased the production of cytokines. The decrease in cytokine production may be caused by the

decrease in bacterial numbers in mice due to Zn and atRA treatment and its bacteriostatic effect. Although vitamin A and Zinc supplementation, individually, reduces morbidity and mortality from diarrheal and respiratory disease in general, it is unclear whether these compounds enhance immunity against all pathogens or have specific effects for certain organisms. The modulation of immune function by supplementation of combined atRA and Zn appeared to be complex and may involve many arms of the immune system since it is unclear how these compounds combine to enhance immunity.

Because the bacteriostatic activity of J774 macrophages was increased by Zn and atRA treatment, zinc and atRA may affect phagocytosis by macrophages. Indeed, it has been reported that the supplementation of Zn or atRA enhances bacterial clearance by macrophages and phagocytosis (Lahiri, 2014; Lo et al., 2014). Although no enhancement of the bacterial clearance activity of macrophages by treatment with a combination of Zn and atRA was observed *in vitro*, the combination treatment may be effective *in vivo* or in primary macrophages.

FIGURES

A



B

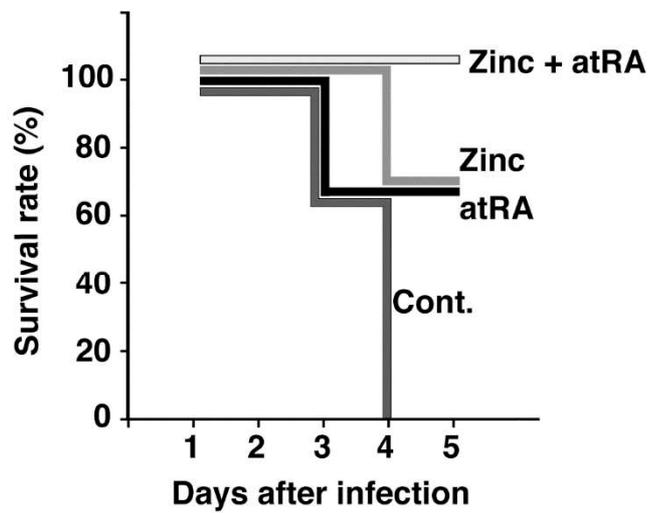


Fig. 1. Survival of lethally infected mice supplemented with Zn and atRA. Panel A shows the administration procedure. At the end of atRA and Zn administration, mice were

inoculated intravenously with $10^5/100 \mu\text{l}$ *L. monocytogenes* per animal. Groups of four mice per compound were checked daily for survival. Survival was observed until day 5 post-infection.

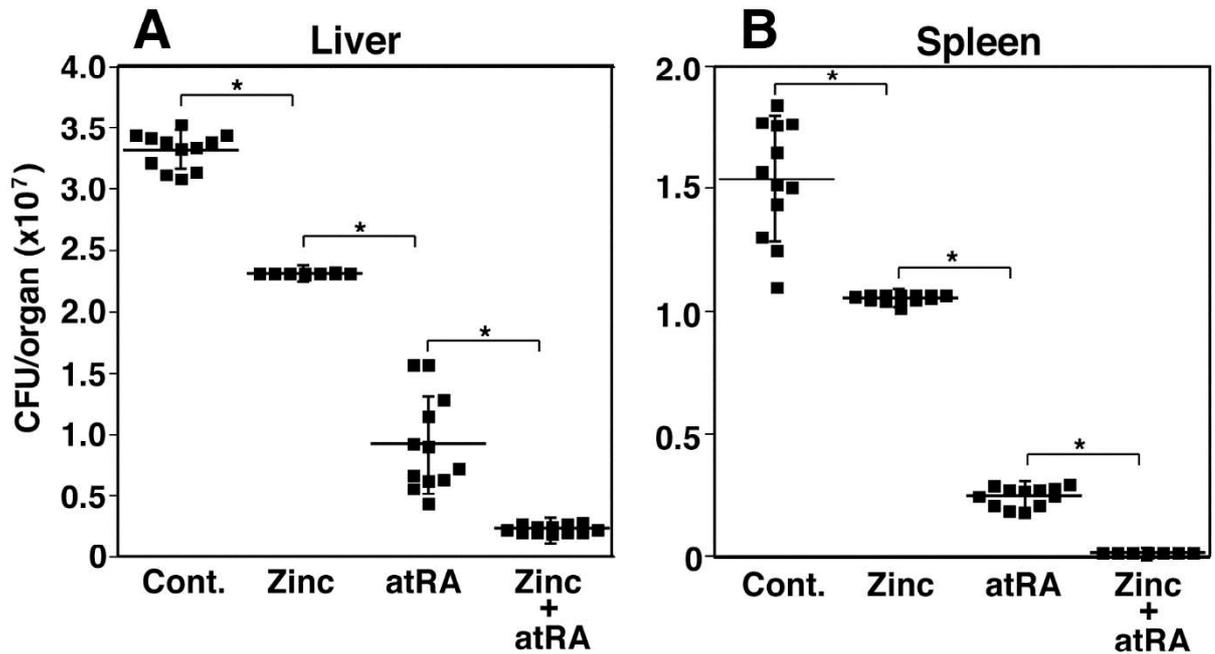


Fig. 2. Number of *L. monocytogenes* in the target organs of Zn- and atRA-supplemented mice. Mice were injected intraperitoneally with Zn, atRA or a combination of both compounds. Twenty-four hours after the last dose, the mice were infected intravenously with 10^4 cells/100 μ l per mouse. CFU was measured in the liver and spleen 48 h after infection. The experiment was performed three times with four mice per group each time. Control mice received corn oil alone. Data are mean \pm SD values of 12 mice per group. Asterisks indicate statistically significant differences, compared with each group.

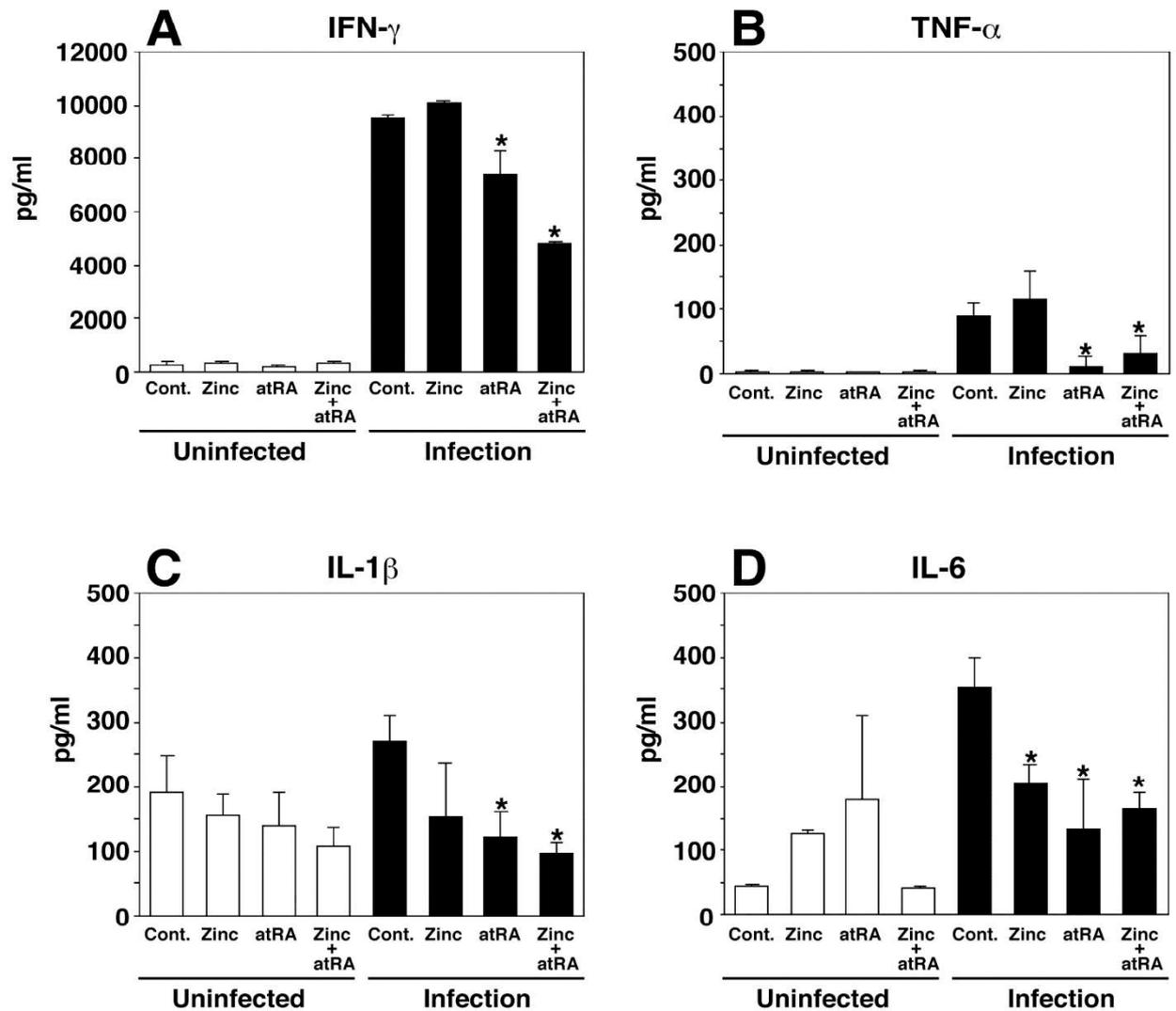


Fig. 3. Cytokine levels in normal and *L. monocytogenes*-infected mice supplemented with Zn, atRA, or a combination of both. Doses were given intraperitoneally for 7 intraday and 6 consecutive days, respectively (Fig. 1A). Twenty-four hours after the last dose, the mice were infected intravenously with $10^4/100 \mu\text{l}$ *L. monocytogenes* per mouse. IFN- γ , TNF- α , IL-1 β , and IL-6 were measured using an ELISA 48 h post-infection. Experiment was performed three times with four mice per group each time. Control mice

received corn oil (atRA) or PBS (Zn) alone. Data are mean \pm SD values of 12 mice per group. Asterisks indicate statistically significant differences, compared with the control.

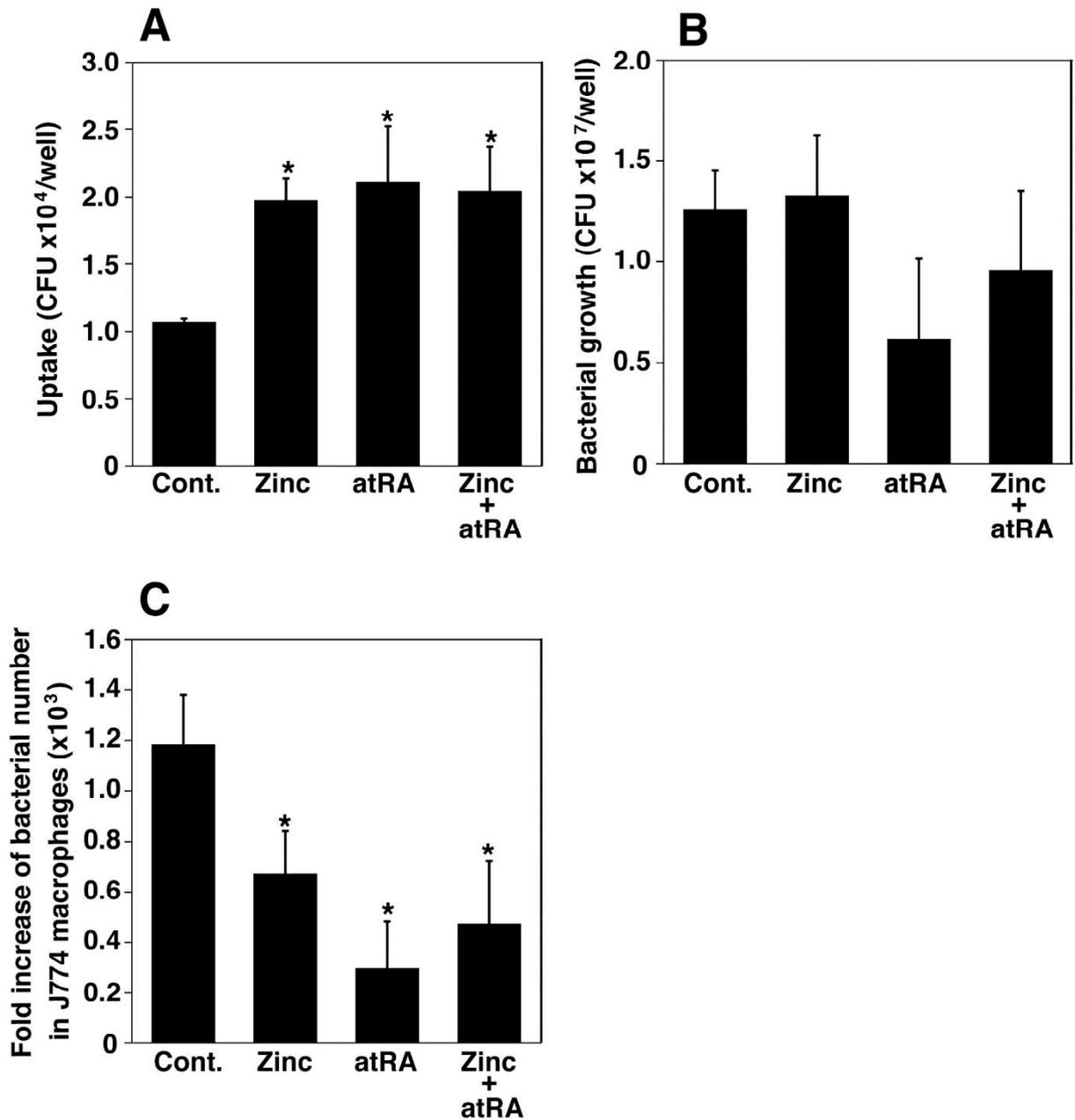


Fig. 4. *L. monocytogenes* macrophage uptake and bacterial survival after Zn and atRA supplementation. J774 macrophage cells were infected with *L. monocytogenes* by centrifugation at $150 \times g$ for 10 min at room temperature at a multiplicity of infection of 0.1. Bacterial uptake (A) was measured after 30 min of incubation at 37°C and bacterial

survival (B) was measured after 24 h. ZnSO₄ (40 μM) or atRA (20 μM) were added 48 h before infection. Panel C shows fold increase of bacterial number in macrophages. Results are presented as fold increase of bacterial number relative to the number of phagocytosed bacteria. Data are the averages of triplicate samples from three identical experiments and error bars represent SD. Statistically significant asterisks indicate differences, compared with the control.

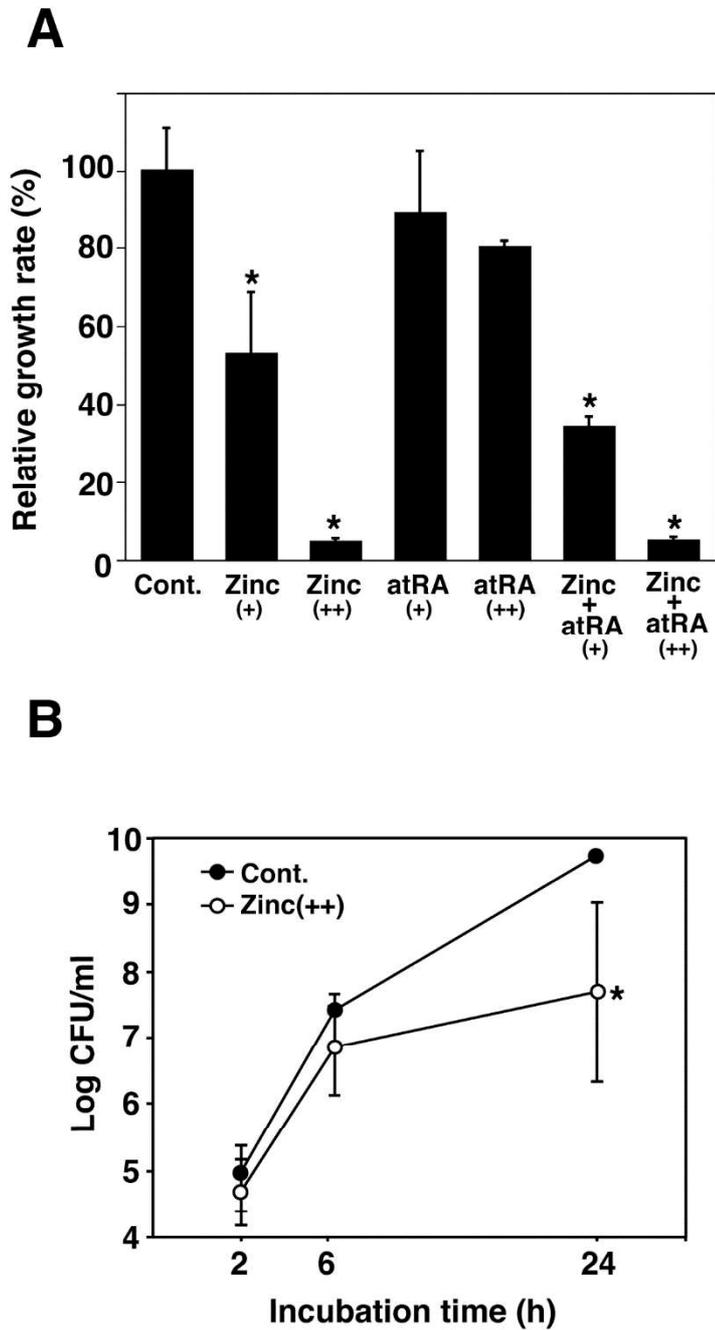


Fig. 5. *L. monocytogenes* growth in vitro in the presence of Zn, atRA or their combination. *L. monocytogenes* was cultured in BHI broth for 24 h in tubes containing ZnSO₄ (+; 200 or ++; 1,000 μM) or atRA (+; 10 or ++; 50 μM) at 37°C. CFU values were

determined by serial dilution on BHI plates. For each sample, CFU values of *L. monocytogenes* observed in the absence of Zn and atRA was set at 100% and CFU values in the presence of Zn and atRA was showed relative to this value. Data are the averages of triplicate samples from three identical experiments and error bars represent SD. Statistically significant asterisks indicate differences, compared with the control. Panel B shows growth curves of bacteria in BHI broth with or without Zn.

CHAPTER 2

Effect of vitamin A on *Listeria monocytogenes* infection in a Silkworm model.

INTRODUCTION

Studying host-pathogen interactions and their related factors is of major importance for understanding the molecular mechanisms of human infectious diseases. Model systems are essential for investigating the complex processes of infectious diseases in humans. *Listeria monocytogenes* is a major food-borne pathogen that causes listeriosis, which is an invasive disease that can lead to meningitis, meningoencephalitis, septicemia, and abortion in its severest form (Midelet-Bourdin et al., 2006).

Listeriosis occurs primarily in pregnant women, newborn infants, and the elderly as well as in immunocompromised patients, with a mortality rate of about 30%. The pathogen has a facultative intracellular life cycle, with the capacity for cellular invasion, intracellular replication and movement from cell to cell without an extracellular phase (Hamon et al., 2012). LLO is an exotoxin of 58 kDa pore-forming cytolysin secreted by *L. monocytogenes* and is required for its intracellular survival, allowing bacterial escape from the phagosomal compartment and intracytoplasmic growth (Hernandez-Flores and Vivanco-Cid, 2015). Rodents have been established as a useful model for analyzing the systemic phase of *L. monocytogenes* infections (Lecuit, 2007), but costs and ethical issues are associated with the use of mammalian hosts, and thus non-mammalian models have proved useful surrogate hosts (Disson et al., 2009).

Some non-mammalian studies have contributed to our understanding of the infectious process of *Listeria* species. The nematode *Caenorhabditis elegans* was the first

invertebrate model to be employed (Thomsen et al., 2006), followed by the larva and adults of the fruit fly *Drosophila melanogaster* (Cheng and Portnoy, 2003; Mansfield et al., 2003), and the larvae of the wax moth (*Galleria mellonella*), which has been validated as an alternative host model for analyzing *L. monocytogenes* (Mukherjee et al., 2010; Joyce et al., 2010). The larvae of *G. mellonella* have a significant advantage compared with previous models because they can be incubated at human body temperature (37 °C).

In recent years, the larvae of *Bombyx mori*, commonly known as the silkworm, and its ovary-derived cultured cell line BmN4 have been employed as an infection model for a large variety of agents including bacteria, viruses, and fungi (Jiang et al., 2016; Kaito, 2016; Uchida et al., 2016; Kaito et al., 2002). The silkworm has been utilized to produce silk for more than 4,000 years, so there is a large volume of accumulated technical information about this insect. Therefore, the silkworm has various advantages compared with other insects, such as its easy handling, low cost, and ready availability.

Vitamins are one of the various organic compounds used by organisms and limited amounts of them are essential for natural performance. Vitamins are essential for silkworm and its direct supplementation to the insect body have been reported to affect the larvae metabolism (Mora et al., 2008).

The pathogenesis of *L. monocytogenes* has been studied using insects, but a comparative analysis of the pathogenic potential of a listeriolysin O (LLO) mutant has not been addressed previously in silkworms. Thus, in the present study, we explored the possibility of using silkworms as an infection model for *L. monocytogenes*. We examined

whether the mutants could kill silkworms in an effective manner at room temperature. In addition, we examined whether vitamin A, an essential nutritional requirement with major effects on the immune system by extending both innate and adaptive immune responses (Mora, 2008), can offer protection to silkworms during the infection process.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Listeria monocytogenes strain EGD, an LLO deletion mutant (*Δhly*), and an LLO-complemented strain (*Δhly::hly*) were used in this study (Hara et al., 2007). The *L. monocytogenes* strains were maintained in frozen glycerol stocks and cultured overnight in brain-heart infusion (BHI) broth (Becton Dickinson, Franklin Lakes, NJ, USA) at 37 °C with shaking, or on BHI broth containing 1.5% agar.

Vitamin A supplementation in silkworms

Fourth instar *Bombyx mori* larvae (Hu·Yo × Tukuba·Ne) were obtained from Ehime-Sanshu (Ehime, Japan). The larvae were raised by feeding them with Silkmate (Nosan Corporation, Kanagawa, Japan) at room temperature. Fifth instar larvae weighing 3.2-3.8 g were injected into the dorsolateral hemocoel with a single dose of 0.1 mL vitamin A acetate (MP Biomedicals, LLC) at 2.25 mg/mL (1,250 IU/mL) dissolved in phosphate-buffered saline (PBS). Pressure was applied immediately at the injection site by using a finger for 10 s to stop the bleeding. Syringes (1 mL) and needles (27G × 3/4") were obtained from Terumo Inc., Tokyo, Japan.

Infection of silkworms with *L. monocytogenes*

Bacterial inoculum were injected dorsolaterally into the hemocoel of pretreated and control (3.2-3.8 g body weight) last-instar larvae using 1 mL disposable syringes. Fifth instar larvae were injected with 0.05 mL of a bacterial suspension containing

approximately 10^4 cells of *L. monocytogenes* EGD, the LLO deletion mutant, or the LLO-complemented strain in PBS. After injection, the larvae were incubated with food at 25 °C. Caterpillars were considered dead when they exhibited no movement in response to touch. The day of infection was considered to be day 0 of infection. To obtain colony-forming unit (CFU) counts at 1 h, 24 h, and 48 h post infection, the infected silkworm larvae were weighed and placed in a disposable 15 mL centrifuge tube, chopped up with scissors, homogenized with a disposable homogenizer (Biomasher SP (EOG-sterellied), Funakoshi Co. Ltd, Tokyo, Japan) and suspended in 3 mL of PBS. Subsequently, the suspension was centrifuged at $300 \times g$ for 30 s and solid tissues were separated from the concentrated suspension. After appropriate dilutions, the sample suspensions were spread on BHI agar plates, incubated overnight, and the numbers of colonies were counted. To calculate the CFU count, the summed volumes of hemolymph and tissues were estimated together (1 g = 1 mL). The number of hemocytes was counted in six independent fields of view by confocal laser scanning microscopy, and the relative hemocyte number was calculated as the ratio of each group relative to that of the control. The larvae were maintained at room temperature with food post infection. All of the experiments were replicated at least three times independently and examples of representative results are reported.

Cell culture

The *Bombyx mori* ovary-derived cultured cell line BmN4 was a gift from Dr. Kusakabe (Mon et al., 2003). BmN4 cells were cultured in IPL-41 insect medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) at 25 °C in the atmosphere. BmN4 cells were seeded (1×10^5 per well) in 48-well tissue culture plates

coated with concanavalin A at 48 h prior to use. Plate coating was performed according to the following steps: the bottoms of the wells were covered with 100 mM concanavalin A and incubated for 2 h at room temperature, after removing the concanavalin A solution, air drying completely, and washing once with PBS.

Efficiency of bacterial invasion and intracellular replication

BmN4 cells were grown in 24-well tissue culture plates for 24 h to reach confluence. For the supplemented treatment, IPL-41 medium containing vitamin A (0.45, 2.25, or 11.25 mg/ml) was added to the BmN4 cells at 24 h before infection. Bacterial strains were deposited onto BmN4 cells at a multiplicity of infection (MOI) of 10 by centrifugation at $150 \times g$ for 10 min at room temperature. To measure the invasion rate, after incubating for 2 h at 25 °C, the cells were washed twice with PBS, and then incubated in PBS containing gentamicin (100 µg/mL, Sigma) for 30 min. The cells were then washed two times with PBS and incubated for 30 min in IPL-41 medium containing vitamin A (when supplementation was performed) and gentamicin (25 µg/mL). Next, the cells were washed three times with PBS and lysed with cold distilled water. The CFU counts were determined on BHI plates by serial dilution. To measure the intracellular replication efficiency, the infected cells were incubated at 25 °C for 2 h, washed twice, and incubated again in PBS containing gentamicin (100 µg/mL) for 30 min. The cells were then washed and incubated for 24 h in IPL-41 medium containing vitamin A and gentamicin (25 µg/mL). The cell washing, lysis, and plating procedures were the same as those used in the bacterial invasion efficiency assay.

Immunoblotting

Each strain of *L. monocytogenes* was cultured in BHI broth supplemented with PBS and 0.45, 2.25, or 11.25 mg/ml of vitamin A acetate for 8 h or 12 h at 25 °C or 37 °C. Each supernatant was collected after centrifugation ($16,000 \times g$, 4 °C) for 30 min. After separating each protein from 5 μ L of culture supernatant by SDS-PAGE with 10% polyacrylamide gel, the proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). After blocking with 5% nonfat dry milk Tris-buffered saline (TBS) at room temperature for 2 h, the membranes were incubated overnight with anti-LLO antibody (1:1,000; ab43018; Abcam plc, Cambridge, UK) at 4 °C. After washing with TBS containing 0.02% (v/v) Tween 20, the membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (0.01 μ g/mL) at room temperature and, immunoreactions were visualized using the enhanced chemiluminescence detection system (GE Healthcare Life Science, Little Chalfont, UK).

Fluorescence microscopy

Aliquots of hemolymph (200 μ L) were harvested from supplemented and non-supplemented infected larvae under sterile conditions and deposited onto a 24-well tissue culture plate on sterile coverslips, which contained 500 μ L IPL-41 insect medium supplemented with 10% FBS, and were incubated at 25 °C in the atmosphere. Hemocytes were promptly centrifuged at $150 \times g$ for 5 min, washed twice with PBS and incubated at room temperature with FBS-supplemented IPL-41 medium for 15 min. The hemocytes were then washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 30 min at room temperature, and washed twice with PBS. The samples were permeabilized with 0.2% Triton X-100, washed twice with PBS, and then treated with 1 μ M rhodamine

phalloidin and 5 µg/mL anti-*L. monocytogenes* rabbit antibody (Viro Stat, Portland, ME) diluted in blocking buffer (5% bovine serum albumin in PBS) to identify intracellular bacteria. Fluorescent images were obtained using a FluoView FV100 confocal laser-scanning microscope (Olympus Tokyo, Japan).

Statistical analysis

Statistical analyses were performed using the Student's t-test or one-way ANOVA with the post hoc Tukey–Kramer test. Statistically significant differences between groups were accepted at $P < 0.05$ in three identical experiments. The survival curves were estimated with the Kaplan-Meier method and the log-rank test was used to determine significant differences ($P < 0.05$).

RESULTS

Infection of silkworm larvae with human pathogenic *L. monocytogenes*

In order to determine whether the silkworm *B. mori* can be used as a model system for *L. monocytogenes* infection, we first examined the infectivity of wild-type *L. monocytogenes* EGD, an LLO deletion mutant, and an LLO-complemented strain at room temperature. *L. monocytogenes* was serially diluted and then injected into 15 individual silkworms per group, before monitoring the time required to kill 50% of the silkworms (LT50). The PBS-injected larvae survived for at least six days and subsequently matured into pupae (data not shown). The LT50 was determined when approximately 10^4 cells of the wild-type strain, LLO deletion mutant, or LLO-complemented strain were injected at room temperature. The wild-type strain and LLO-complemented strain obtained LT50 values of 120 h and 96 h (five and four days), respectively (Fig. 1A). A delay in the LT50 (approximately six days) was observed for the LLO deletion mutant. All of the infected larvae looked normal for at least two days and the activity then decreased, before they stopped eating and finally died. Their skin color became dark only after death.

Growth of *L. monocytogenes* in silkworms

To determine whether *L. monocytogenes* could proliferate in infected larvae, we analyzed the hemolymph and tissues isolated from the larvae injected with the wild-type strain, LLO deletion mutant, or LLO-complemented strain. To determine the CFU counts per larva, bacteria were harvested from infected fifth instar larvae and the bacterial colony counts were quantified. The CFU counts showed that the bacterial numbers of the three

strains were at the same level 1 h after the initial inoculation (Fig. 1B). However, at 24 h post injection, there was a rapid increase in the bacterial number of the LLO-complemented strain compared with the wild-type strain. The proliferation of the *L. monocytogenes* LLO mutant decreased following the initial inoculation but recovered to the same levels as the wild-type strain and the LLO-complemented strain at 48 h after inoculation (Fig. 1B).

To further understand the host-pathogen interactions in this silkworm model, we examined hemocyte isolated from infected larvae using confocal microscopy. Intracellular bacteria belonging to the three strains were observed at 1 h and 24 h after infection (Fig. 1C). Infection with the LLO-complemented strain decreased the number of hemocytes by $55.3\% \pm 9.7\%$ compared with those injected with the wild-type strain and LLO deletion mutant at 24 h after infection. These results demonstrate that the LLO produced by *L. monocytogenes* contributes to bacterial proliferation in the silkworm. We also confirmed the *in vitro* expression of LLO at room temperature by immunoblotting. High levels of LLO expression were observed with the wild-type strain and LLO-complemented strain at 37 °C and slightly lower LLO expression at 25 °C (Fig. 1D). LLO expression was not detected in the LLO deletion mutant at 37 °C and 25 °C.

Intracellular replication of *L. monocytogenes* in BmN4 cells

It is well known that *L. monocytogenes* is an intracellular pathogen. To examine the intracellular replication kinetics of *L. monocytogenes*, we infected the silkworm ovary-derived cell line BmN4 with the wild-type strain, LLO deletion mutant, and LLO-complemented strain at an MOI of 10. We measured the bacterial invasion and

intracellular replication of each strain at 3 h and 24 h after infection. The bacterial count of the LLO deletion mutant was significantly lower in BmN4 cells compared with those of the wild-type strain and LLO-complemented strain at 3 h and 24 h post infection (Fig. 2A). Intracellular replication by all three strains was observed at 24 h post infection.

Next, we analyzed intracellular replication by the bacteria using confocal microscopy, where we observed the wild-type strain, LLO deletion mutant, and LLO-complemented strain in BmN4 cells at 3 h post infection (Fig. 2B). Explosive replication was observed in BmN4 cells infected with the wild-type strain and LLO-complemented strain, but low level replication was detected in BmN4 cells infected with the LLO deletion mutant at 24 h post infection (Fig. 2B).

Effects of vitamin A pretreatment on *L. monocytogenes* infections in silkworms

The innate immune system of the silkworm is considered to function at both the cellular and humoral levels. The role of vitamin A in the immune system extends to both innate and adaptive immune responses in vertebrates (Mora et al., 2008). At first, we check the effects of vitamin A treatment in non-infected silkworms. No significant differences were observed in the health conditions, such as body weight, feeding activity and time to pupation, between the silkworms pretreated with vitamin A and control (data not shown). To examine the effects of vitamin A supplementation on *L. monocytogenes* infections in silkworms, we infected vitamin A-pretreated larvae with approximately 10^4 cells of the wild-type strain at 12 h after supplementation, and we then measured the survival rates of the supplemented silkworms and their CFU counts. There was no significant difference between the vitamin A-supplemented silkworms and the control

(Fig. 3A). There was a one-day delay in mortality between the two groups. Next, we determined the bacterial numbers in infected silkworms based on the CFU counts.

To measure the bacterial numbers in infected silkworms, the larvae were infected with the wild-type strain and the bacterial counts were determined at 1 h, 24 h, and 48 h after infection. The number of bacteria was significantly lower in vitamin A-supplemented silkworms compared with the non-supplemented silkworms (Fig. 3B). This effect was maintained throughout the course of infection. To investigate the effect of vitamin A supplementation on bacterial invasion into the hemocyte of silkworms, we examined hemocyte isolated from infected larvae using confocal microscopy. Intracellular bacteria were observed at 1 h and 24 h after infection, and there were no differences between vitamin A-supplemented and non-supplemented silkworms (Fig. 3C).

Effects of vitamin A pretreatment on *L. monocytogenes* infection in BmN4 cells

To determine the effects of vitamin A on non-hemocyte silkworm cells, we infected BmN4 cells with the wild-type strain at an MOI of 10 and we measured bacterial invasion and intracellular replication at 3 h and 24 h after infection. We found that the number of intracellular bacteria decreased at 3 h and 24 h after infection in a dose-dependent manner (Fig. 4A). Pretreatment with vitamin A significantly reduced bacterial invasion at concentrations of 2.25 mg/ml and 11.25 mg/ml.

Next, we analyzed intracellular replication by the bacteria using confocal microscopy. The wild-type strain was observed in BmN4 cells treated with or without vitamin A at 3 h post infection, but the number of intracellular bacteria was lower in BmN4 cells treated with vitamin A (Fig. 4B, arrowheads). Explosive replication was observed in BmN4 cells

treated with or without vitamin A at 24 h post infection (Fig. 4B, open arrowheads), thereby suggesting that vitamin A treatment did not inhibit intracellular replication by the bacteria.

Vitamin A is known for its cytotoxic effects on vertebrate cells (Penninston and Tanumihardjo, 2016) but there were no effects on the viability of BmN4 cells at the vitamin A concentration used in this study (11.25 mg/ml). Confocal microscopy demonstrated that the shape of the BmN4 cells was changed by vitamin A treatment and filopodia-like structures were observed in BmN4 cells treated with vitamin A (Fig. 4B arrows).

DISCUSSION

Recently, insect infection models have been used to study various human infectious diseases including *L. monocytogenes* (Joyce et al., 2010). However, insect models have been criticized for being too “distant” from human disease processes. The strongest aspects of this argument are: (i) the temperature ranges employed, which means that certain pathogens cannot express virulence factors; and (ii) physiological problems, such as the absence of the same target organs (O’Callaghan and Vergunst, 2010). Bacterial infections can cause opportunistic diseases and innate immunity has a major role in combating these diseases. Silkworms may provide a useful model for analyzing innate immunity because insects and mammals share common innate immune mechanisms. Thus, silkworms provide a model for studying host-pathogen interactions and cellular infection in the presence of innate immunity.

Non-mammalian models have logistical, budgetary, and ethical advantages over mammals. In particular, the ease and low costs required to obtain large numbers of larvae facilitate large-scale screening, which would be prohibitive in mammals. In addition, the amounts of drugs needed to examine therapeutic effects are generally much smaller in invertebrates than in mammals due to their low body weight. Other significant benefits of this model are that *B. mori* can be incubated at room temperature (25 °C) and the specific virulence factors of *L. monocytogenes* that are active at this temperature can be found. In the present study, we found that silkworm larvae were killed by infections with the *L. monocytogenes* wild-type strain, LLO deletion mutant, and LLO-complemented strain

when infected at room temperature. LLO is a major virulence factor, which was originally shown to be crucial for bacterial escape from the internalization vacuole after entry into the host cell (Hamon et al., 2012). The delay until bacterial proliferation in silkworms infected with the LLO deletion mutant and the number of hemocytes were reduced dramatically when silkworms were infected with the LLO-complemented strain, which indicates that LLO may contribute to the early stage of infection in silkworms. Proliferation of the LLO deletion mutant and the wild-type strain in silkworms as well as the slight expression of LLO were shown in an *in vitro* experiment at 25 °C, and thus LLO may have a limited role in bacterial infection so mortality may require bacterial growth in silkworms.

Recently, an insect infection model using *Galleria mellonella* was employed to study *L. monocytogenes* pathogenesis (Mukherjee et al., 2010; Joyce et al., 2010), which showed that the production of LLO is necessary for toxicity and bacterial growth (Joyce et al., 2010). The virulence of strains with mutated LLO production was attenuated completely and these strains did not cause mortality in the insect. The results obtained using our silkworm infection model differed from those with the previous insect infection model because we maintained the silkworms at 25 °C. Silkworms appeared unhealthy when maintained at 37 °C so it would have been difficult to perform experiments at 37 °C under our test conditions. Therefore, this model can be useful for investigating virulence factors that are not regulated by temperature. Silkworms may also be suitable for analyzing environmental infection mechanisms. It is expected that novel virulence factors involved in insect infections will be identified using the silkworm model.

A visible phenotype associated with bacterial infection in insects is increased pigmentation in the larvae bodies, which usually indicates pro-phenoloxidase (PPO) induction to cause melanization (Bidla et al., 2009; Tanaka and Yamakawa, 2011). In silkworms, melanization has been documented in infections by both Gram-negative (*Pseudomonas aeruginosa*, *Escherichia coli*, and *Vibrio cholerae*) and Gram-positive bacteria (*Staphylococcus aureus*) (Kaito et al., 2002). In this silkworm model, *L. monocytogenes* killed silkworms without inducing melanization (data not shown). This may suggest that *L. monocytogenes* escape from immune responses of silkworm and proliferate rapidly. However, further study is needed to demonstrate if *L. monocytogenes* possess a mechanism for inhibiting the induction of melanization.

In recent years, the *B. mori* ovary-derived BmN4 cell line has been used to study viral replication and the development of antiviral compounds (Iwanaga et al., 2014; Orihara et al., 2008). To the best of our knowledge, this is the first study to use the BmN4 cell line for investigating intracellular replication by *L. monocytogenes in vitro*. We observed intracellular spreading in BmN4 cells infected with the wild-type strain and LLO-complemented strain, but not with the LLO deletion mutant. These results suggest that LLO has an important role in escaping from vacuoles in BmN4 cells. The intracellular replication phenotype differs between hemocytes and BmN4 cells. LLO may have strong cytotoxic effects on hemocytes, so intracellular spreading was not observed clearly. Intracellular replication in other types of cells and the extracellular replication of *L. monocytogenes* may be direct factors that lead to the death of silkworms rather than the intracellular replication of the bacterium.

Vitamins are essential dietary components and they influence the immune system (Hall et al., 2011). Especially, vitamin A shows immunomodulatory function (West et al., 1989) and enhances resistance to various infectious diseases (Suzuki et al., 1988). For example, vitamin A plays an important role in the decrease of *Toxoplasma gondii* or *Mycobacterium tuberculosis* (Yamada et al., 2007; Rosenberg, 2007). In addition, we previously demonstrated that *all-trans* retinoic acid, the active form of vitamin A, induces protection of the host against *L. monocytogenes* infection in a murine model and that vitamin A may have a bacteriostatic effect on *L. monocytogenes* (Castillo et al., 2015). Therefore, we hypothesized that vitamin A supplementation in silkworms might enhance the host immune system. Multi-vitamin supplementation with vitamins C, B12, D3, and A has been used in the silkworm industry to enhance biological and economical parameters (larval weight, female cocoon and pupal weight, and egg productivity) with good results (Etebari and Matindoost, 2005).

Due to the cytotoxic properties of vitamin A, our main concern when using insects as an infection model was a possible difference in drug toxicity and metabolism between invertebrates and mammals. However, some studies have reported that the LD50 values for cytotoxic substances in silkworms are consistent with those in mammals (Hamamoto et al., 2009). Our results showed that vitamin A only had limited protective effects during the early stage of infection in silkworms. Insects do not possess an acquired immune system where antibodies have a significant role (Tanaka and Yamakawa, 2011), which might influence the chemotherapeutic effects of vitamin A. In mammals, vitamin A affects adaptive immune-cell subsets and general lymphocyte functions. In this study, we observed that vitamin A treatment changed the shape of BmN4 cells; thereby suggesting

that vitamin A may induce cell activation and differentiation in BmN4 cells. However, our understanding of the metabolite conjugation pathways is quite limited in silkworms and further investigations are needed.

FIGURES

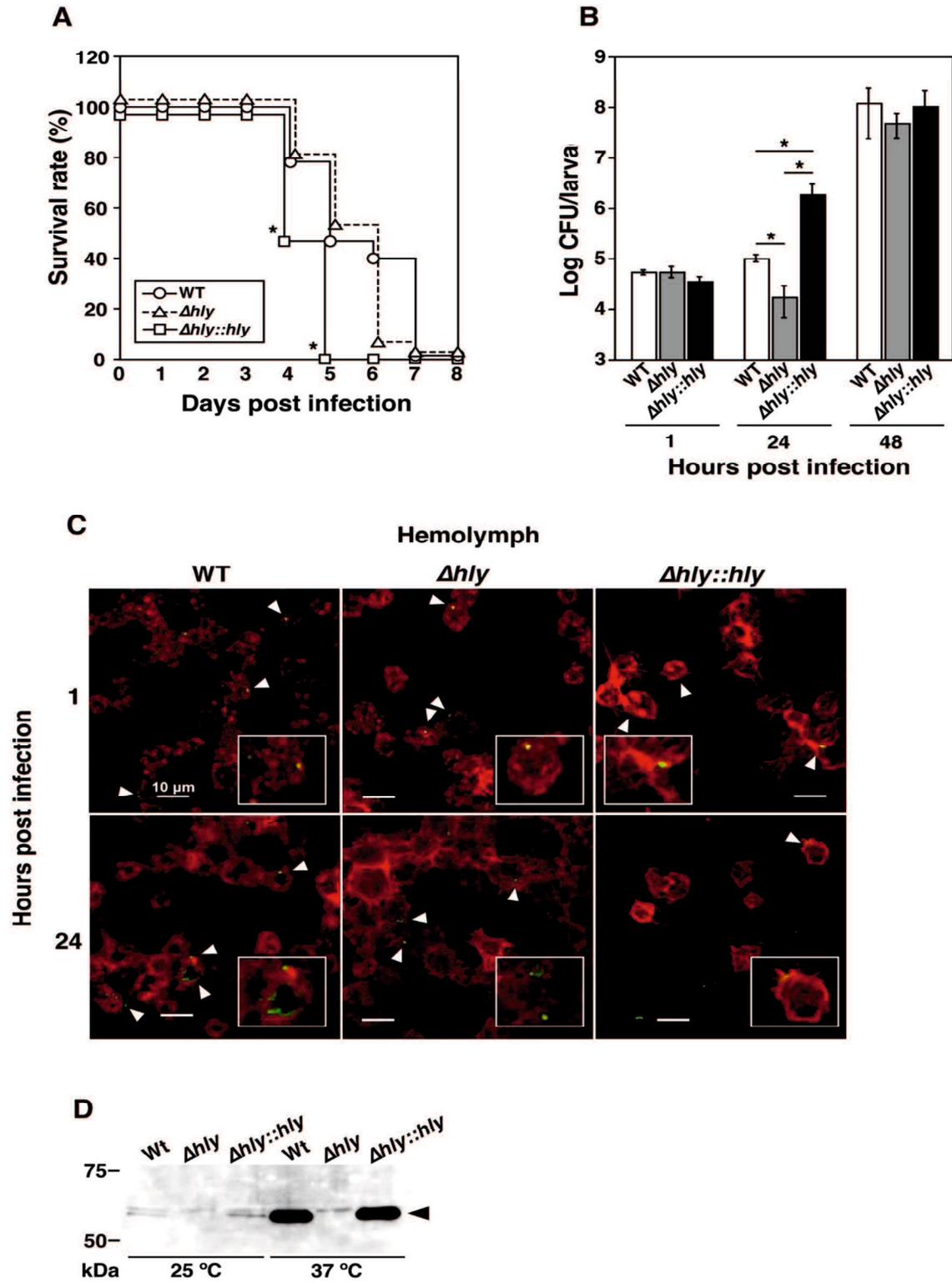


Figure 1. Infection of silkworms with *L. monocytogenes*. (A) Survival rate of silkworms. Fifteen insects per group were infected and monitored to determine the time required to kill 50% of the silkworms. Approximately 10^4 bacteria belonging to the wild-type strain (WT, circles), LLO deletion mutant (Δhly , triangles), and LLO-complemented strain ($\Delta hly::hly$, squares) were sufficient to kill 50% (LD50) of the silkworms within 4 and 5 days. Significant differences were accepted at $P < 0.05$ and they are indicated by asterisks (*). (B) The bacterial numbers of each strain detected in silkworms at 48 h post inoculation. The data represent averages based on triplicate samples from three identical experiments and the error bars represent the standard error of the mean (SEM) ($n = 9$). Significant differences were accepted at $P < 0.05$ and they are indicated by asterisks (*). (C) Hemocytes (red) containing *L. monocytogenes* strains (green, arrowheads) were observed by confocal laser scanning microscopy at 1 h and 24 h post inoculation. Scale bar represents 10 μm and zoomed areas represent 2x the original photo. (D) Immunoblot analysis of LLO expression. Samples were prepared from the wild-type strain, LLO deletion mutant, and LLO-complemented strain cultivated at 37 °C or 25 °C in BHI broth.

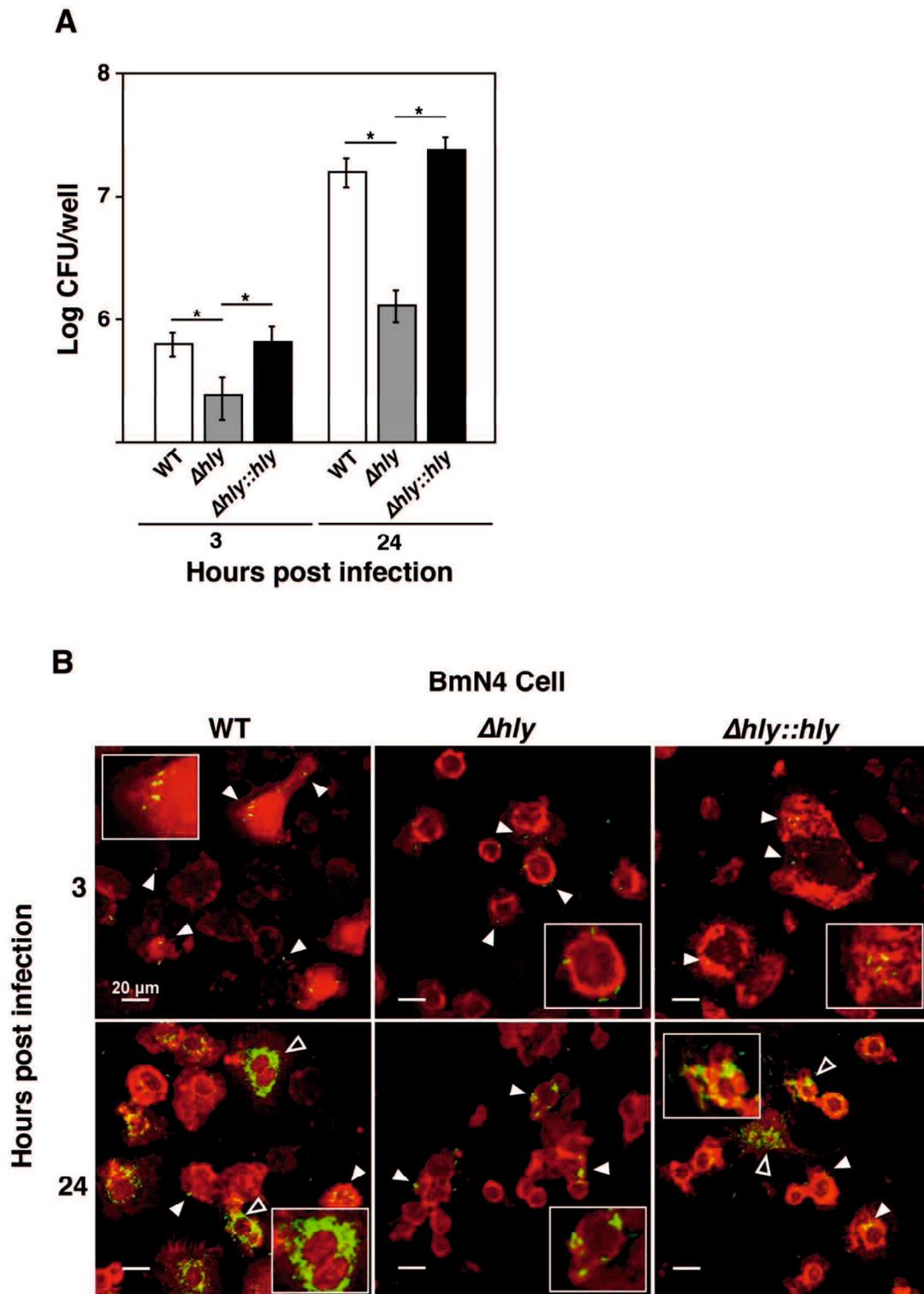


Figure 2. Intracellular replication of *L. monocytogenes* in BmN4 cells. (A) *L. monocytogenes* replicates intracellularly in BmN4 cells. The silkworm ovary-derived cell

line BmN4 was infected with the wild-type strain (WT), LLO deletion mutant (Δhly), and LLO-complemented strain ($\Delta hly::hly$) at an MOI of 10. Bacterial invasion and intracellular replication by each strain were measured at 3 h and 24 h after infection. The data represent averages based on triplicate samples from three identical experiments and the error bars represent the standard deviations. Significant differences were accepted at $P < 0.05$ and they are indicated by asterisks (*). **(B)** BmN4 cells (red) containing *L. monocytogenes* strains (green, white arrowheads) were observed by confocal laser scanning microscopy at 3 h and 24 h post inoculation. Scale bar represents 20 μm and zoomed areas represent 2x the original photo. Open arrowheads indicate explosive bacterial replication. The experiment was replicated three times independently.

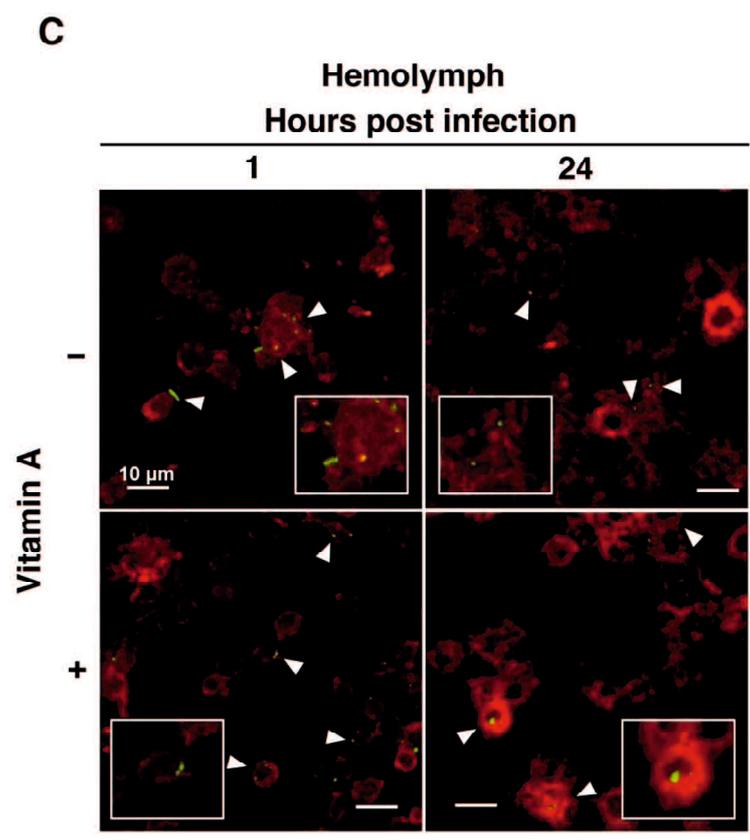
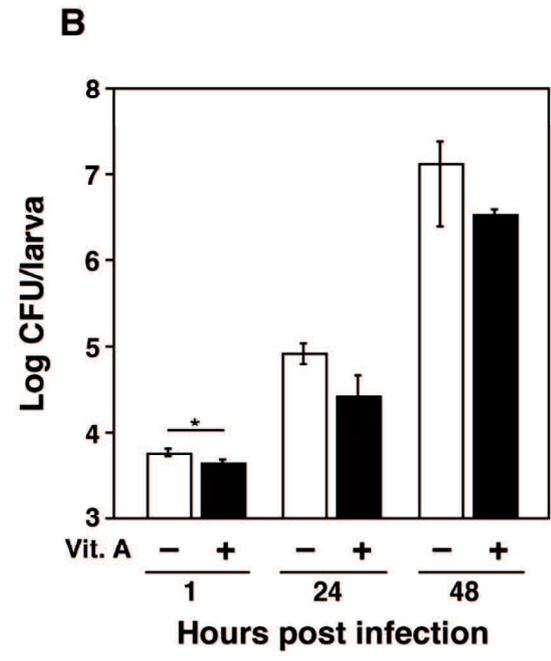
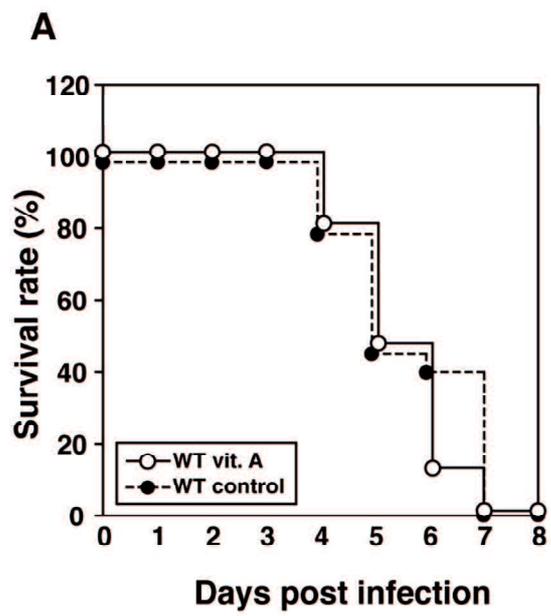


Figure 3. Infection of *L. monocytogenes* to silkworm pretreated with vitamin A. (A)

Survival of infected silkworms after previous supplementation with vitamin A.

Approximately 10^4 bacteria of the wild-type strain were used to infect silkworms pretreated with (open circles) or without vitamin A (black circles). Groups of five insects per group were checked daily for survival. Survival was observed until 8 days post infection.

(B) Bacterial numbers of the wild-type strain in silkworms pretreated with (black bars) or without vitamin A (open bars) until 48 h post inoculation. The data

represent averages based on triplicate samples from three identical experiments and the error bars represent the SEM ($n = 9$). Significant differences were accepted at $P < 0.05$

and they are indicated by asterisks (*). **(C)** Hemocytes (red) containing *L. monocytogenes*

strains (green, arrowheads) isolated from silkworm pretreated with or without vitamin A were observed by confocal laser scanning microscopy at 3 h and 24 h post inoculation.

Scale bar represents 10 μm and zoomed areas represent 2x the original photo.

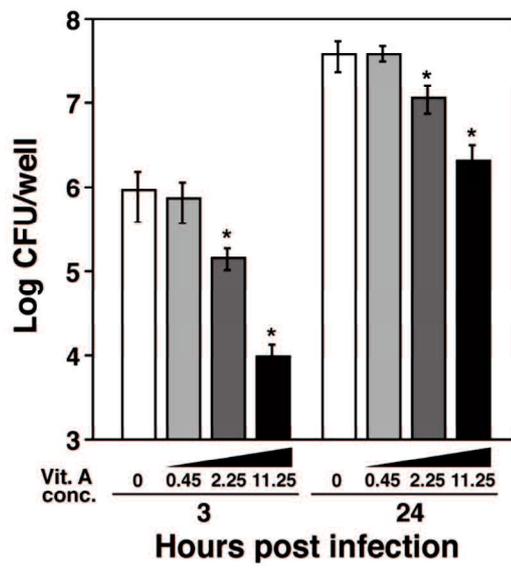
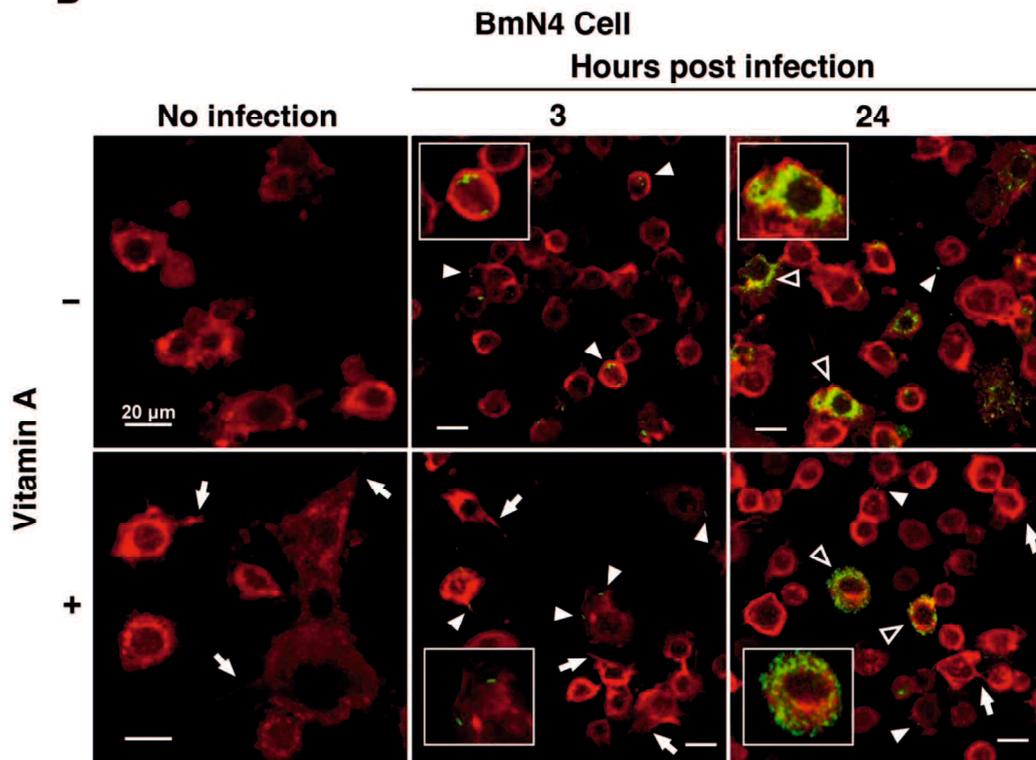
A**B**

Figure 4. *L. monocytogenes* infection of BmN4 cells after pretreatment with vitamin

A. (A) Pretreatment with vitamin A inhibited *L. monocytogenes* infection in BmN4 cells. BmN4 was infected with the wild-type strain at a multiplicity of infection of 10. Bacterial invasion and intracellular replication were measured at 3 h and 24 h after infection in BmN4 cells at the indicated vitamin A concentrations (mg/ml). The data represent averages based on triplicate samples from three identical experiments and the error bars represent the standard deviations. Significant differences were accepted at $P < 0.05$ and they are indicated by asterisks (*). **(B)** BmN4 cells (red) containing *L. monocytogenes* strains (green, white arrowheads) were observed by confocal laser scanning microscopy at 3 h and 24 h post inoculation. BmN4 cells were pretreated with (11.25 mg/ml) or without vitamin A. Scale bar represents 20 μm and zoomed areas represent 2x the original photo. Open arrowheads indicate explosive bacterial replication. Arrows indicate filopodia-like structures. The experiment was replicated three times independently.

CONCLUSION

It is a well-known fact that *Listeria monocytogenes* is potentially fatal and widespread in the environment. However, it has only recently emerged as a significant cause of human infection in industrialized countries, owing to appearance of a vulnerable population of immunocompromised individuals, and the concomitant development of large-scale agro-industrial plants and refrigerated food. Human listeriosis is a rare but potentially very severe infection associated with a mortality of up to 30%, even when an adequate antimicrobial treatment is administered. This accounts for the high economic cost associated with human listeriosis despite its relative low incidence. Furthermore, listeriosis remains under-diagnosed, particularly at its early stages, and this leads to delay in the administration of antimicrobial therapy, which is absolutely critical for a favorable outcome, here again in contrast to most other foodborne infections.

In Chapter 1, I demonstrated that pretreatment with Zn or atRA inhibited bacterial growth in the liver, spleen and macrophages; and that this bacteriostatic effects were increased with the combination of Zn with atRA. The detailed mechanism by which the combination of Zn and atRA enhances the bacteriostatic effect is still unclear; this is a task for future research. My results demonstrated that treatment with a combination of Zn and atRA may be useful against infection by intracellular pathogens and may provide a new therapeutic option for bacterial infection.

In Chapter 2, I developed a silkworm infection model with *L. monocytogenes*. Using this model, I demonstrated that *L. monocytogenes* is able to replicate intracellularly, and LLO is an important pathogenic factor in invertebrates. Moreover, vitamin A is an inhibitory agent against *L. monocytogenes* infection. Although, it is unclear invertebrates play an important role for survive of *L. monocytogenes* in environment, the results of this study may help to prevent the spread of *L. monocytogenes* in the environment.

En masse, there are many lines of in vitro evidence that have shown the essential effect of trace elements and vitamins on all aspects of immune function. It has therefore been assumed that patients (human and animals) who have subclinical deficiencies of trace elements and vitamins may be at risk of impaired immune function and hence an increased risk of infection. It is important that doctors and other health professionals are aware of the evidence for the nutritional essentiality of these substances, and for the situations where an increased intake may lead to clinical benefit.

Animal models have played fundamental roles in elucidating the pathophysiology and immunology of listeriosis, and will almost certainly continue to be integral components of the research on listeriosis. Data derived from animal studies helped for example characterize the importance of cell-mediated immunity in controlling infection, allowed evaluation of chemotherapeutic treatments for listeriosis, and contributed to quantitative assessments of the public health risk associated with *L. monocytogenes* contaminated food commodities. Nonetheless, a number of pivotal questions remain unresolved, including dose-response relationships, which represent essential components of risk assessments. Newly emerging data about species-specific differences have

recently raised concern about the validity of most traditional animal models of listeriosis.

However, considerable uncertainty about the best choice of animal model remains.

The relevance of *in vivo* studies in so-called animal models have always to be revisited in the light of medical observations and *in vitro* findings, as one would expect for a research field that is supposed to fill the gap between the bench and bedside. The “model” status of a given animal has constantly to be challenged, in order to uncover its limitation and ultimately propose ways to replace it, or “fix” it by the use of transgenesis or KI approaches.

This may lead to an infinite delay in performing definitive *in vivo* experiments, the never-ending quest of the ultimate animal models substituting the use of those readily available despite their known limitations. This postponing stance might impede the pace of discoveries, but might also help to avoid premature conclusions drawn from unsuitable models. Somewhere in between the ultimate multi-transgenic “humanized” model and a reductionist pure *in vitro* approach lie very efficient model organisms for high throughput screens, that have proven to be very successful tools in bacterial pathogenesis, such as *Galleria mellonella*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Danio rerio*, despite their noticeable differences with the human model. It is hoped that, altogether, these systems will complement each other and finally help us to fully understand the complexity of listeriosis pathophysiology.

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