Centro de Investigación en Alimentación y Desarrollo, A.C.

EFECTO DEL ZINC SOBRE *Giardia lamblia*, SU PATOGENICIDAD E INDUCCIÓN DE RESPUESTA INMUNE HUMORAL

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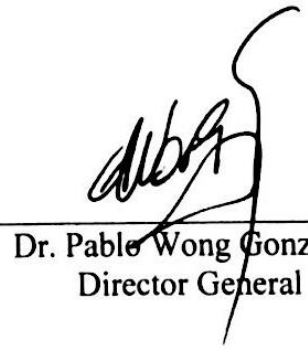

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RESUMEN

La giardiosis es una enfermedad gastrointestinal común y una causa importante de diarrea y malabsorción. La Organización Mundial de la Salud y el Fondo de las Naciones Unidas para la Infancia recomiendan la administración de suplementos de zinc como terapia auxiliar en niños con diarrea. Evidencia reciente indica que el efecto benéfico del zinc es patógeno-específico. Por lo anterior, es de interés investigar la relevancia del zinc en la patogénesis de la infección por *Giardia*. En éste trabajo, el gluconato de zinc (GZ) provocó alteraciones morfológicas e inhibición (>90%) en el crecimiento de trofozoítos *in vitro* ($P<0.001$). El análisis de trofozoítos por microscopía electrónica de barrido (MEB) reveló alteraciones topológicas severas; análisis por microscopía electrónica de transmisión (MET) mostratron cambios ultraestructurales: formación de áreas vacías en citoplasma, acumulación de material granular electrodenso, desplazamiento del disco adhesivo, alteración y pérdida de flagelos. En consecuencia, se consideró estudiar como la deficiencia o suplementación con zinc influyen en la capacidad de colonización y patogenicidad de *Giardia in vivo.* Jerbos (*Meriones unguiculatus*) fueron asignados a tres grupos: ZA (zinc- adecuado), ZD (zinc-deficiente) y ZS (zinc-suplemento) (ZA+50 mg GZ/kg peso/día). La infección se asoció con efectos adversos sobre la ganancia de peso del grupo ZA. La deficiencia de zinc (ZD) acentuó la severidad del efecto, incrementando significativamente la pérdida de peso. La administración de zinc (ZS) mejoró la ganancia de peso. Jerbos ZD desarrollaron altas cargas parasitarias, mientras que en ZS se observó una reducción significativa en la intensidad de la infección ($P<0.05$). MEB mostró alteraciones morfológicas en trofozoítos recuperados de jerbos ZS. Mientras que en ZA y ZS no se detectaron trofozoítos al día 30pi, en jerbos ZD la infección persistía con altas cargas parasitarias ($6.6 \log_{10}$). El título de anticuerpos (IgG) incrementó al avanzar la infección en todos los grupos. La administración de zinc potenció las respuesta inmune anti-*G. lamblia* (ZS 0.249 vs ZA 0.131, 5dpi). Jerbos ZD mostraron menores incrementos en IgG (ZD 0.249 vs ZA 0.525, 30dpi), lo que sugiere una respuesta inmune deteriorada. Estos datos

muestran un efecto dual del zinc al beneficiar la respuesta inmune del hospedero mientras de forma directa afecta la patogenicidad del parásito. Se demuestra aumento en la susceptibilidad del hospedero deficiente en zinc a desarrollar infecciones persistentes, potenciando la pérdida de peso inducida por la infección. Se concluye que la suplementación con zinc podría ejercer un papel protector y contribuir al control de la giardiosis.

Palabras clave: deficiencia de zinc, suplementación con zinc, giardiosis, *Giardia lamblia*

ABSTRACT

Giardiosis is a common gastrointestinal disease and an important cause of diarrhea and malabsorption. The World Health Organization and the United Nations Children´s Fund recommend zinc supplementation as adjunct therapy for childhood diarrhea. Recent evidence indicates that zinc can have pathogen-specific protective effects. Thus, it was of interest to investigate the relevance of zinc in the pathogenesis of *Giardia* infection. In this work, ZG induced morphological alterations and inhibited (>90%) the growth of *Giardia* trophozoites *in vitro* ($P<0.001$). Inspection of trophozoites by scanning electron microscopy (SEM) revealed severe topological alterations; transmission electron microscopy (TEM) analyses showed ultrastructural alterations: formation of empty areas in the cytoplasm, accumulations of electrodense granular material, displacement of the adhesive disk, flagella alteration and loss. On this basis, it was of interest to explore how zinc deficiency or supplementation influence *Giardia* colonization and pathogenicity *in vivo*. Gerbils (*Meriones unguiculatus*) were randomly assigned into three groups: ZA (zinc-adequate), ZD (zinc-deficient) and ZS (zinc-supplemented) (ZA+ 50 mg ZG/kg body weight/day). Infection was associated with significant adverse effects on body weight gain of ZA gerbils; ZD accentuated the severity of these growth decrements and significantly increased the amount of weight loss; ZS gerbils significantly improved body weight gain. In addition, ZD gerbils developed higher numbers of parasitosis earlier and exhibited prolonged infection. The administration of zinc was able to reduce significantly the intensity of infection at 15 dpi ($P<0.05$); MEB showed morphological alterations on trophozoites recovered from ZS gerbils. By day 30 pi parasites were usually undetectable in ZA-ZS gerbils, but persisted in the ZD group (6.6 $\log_{10}$). Antibody titers increased as the infection progressed; while ZS enhanced anti-*G. lamblia* immune response earlier in infection (ZS 0.249 vs ZA 0.131, 5 dpi), ZD gerbils showed much smaller increases in anti-parasite IgG (ZD 0.249 vs ZA 0.525, 30 dpi), suggesting an impaired immune response. These data show a dual effect of zinc in benefiting the host while impairing *Giardia* pathogenicity. In the current study, we were able to demonstrate increased susceptibility to develop persistent

infection and *G. lamblia*–potentiated growth decrements in ZD gerbils. In conclusion, ZS might exert a protective role in controlling *Giardia* infection.

Keywords: zinc deficiency, zinc supplementation, giardiosis, *Giardia lamblia*

SINOPSIS

La interacción entre nutrición e inmunidad es un fenómeno complejo. Todas las células de nuestro cuerpo necesitan de ciertos nutrientes para funcionar adecuadamente, y las células en constante proliferación involucradas en las defensas inmunitarias son particularmente vulnerables a deficiencias nutricionales (Chandra, 1997). Es un hecho bien conocido que un pobre estado nutricional conlleva un mayor riesgo de contraer infecciones, sin embargo, el conocimiento del papel que juegan los nutrientes en los mecanismos inmunológicos de defensa es mucho más reciente. Los micronutrientes –vitaminas, minerales y elementos traza- son componentes esenciales de la dieta y tienen un profundo impacto sobre la salud; ejercen un papel importante como cofactores de muchas vías metabólicas y se consideran esenciales para la integridad y el perfecto funcionamiento del sistema inmune.

La influencia que ejercen los micronutrientes sobre la inmunocompetencia ha cobrado importancia en los últimos años; aunque los estados de malnutrición en el humano normalmente son síndromes complejos en los que se observan deficiencias múltiples de nutrientes, las deficiencias de un solo micronutriente pueden aparecer en relación con el hierro, el zinc y la vitamina A.

El zinc es un buen ejemplo para ilustrar el concepto de cómo la deficiencia de un único nutriente puede afectar al sistema inmunitario. En este sentido, la literatura sobre la deficiencia de zinc es muy vasta (Prasad, 1998; Dardenne, 2002), hecho que no es de extrañar dada su participación en procesos de división celular, crecimiento y diferenciación, como componente estructural, catalítico y regulador en proteínas, como factores de transcripción, enzimas, transportadores y receptores (Stefanidou et al., 2006).

La deficiencia de zinc se reconoció como un problema de salud por vez primera en 1961 (Prasad, 2003); desde entonces, el zinc se convirtió en un foco de atención

importante. La deficiencia de zinc es especialmente frecuente en países de bajos ingresos debido al bajo consumo de alimentos ricos en zinc y a una absorción inadecuada; se calcula que una tercera parte de la población mundial habita en países identificados por tener un alto riesgo de deficiencia de zinc (IZiNCG, 2004). Este problema se asocia con mayor riesgo de infecciones gastrointestinales en niños debido al efecto adverso que tiene esta deficiencia sobre la función y estructura del tracto gastrointestinal (Fischer y Black, 2004). Es por lo tanto también un reconocido factor que contribuye a las muertes infantiles por diarrea. A pesar de esto, aun no tenemos un entendimiento claro acerca de los mecanismos que llevan a un aumento en la susceptibilidad a infecciones.

Las enfermedades gastrointestinales infecciosas son uno de los principales problemas de salud pública en México y otros países del mundo. Se transmiten, ya sea por vía fecal-oral, o bien por el consumo de agua y alimentos contaminados. Afectan principalmente a la población infantil, y tanto su incidencia como su prevalencia dependen de factores socioeconómicos y nutricionales.

La giardiosis, causada por *Giardia lamblia* (sinónimo: *Giardia intestinalis*, *Giardia duodenalis*), constituye una parasitosis de gran importancia epidemiológica y clínica por su alta prevalencia y patogenicidad, fundamentalmente entre la población infantil. *Giardia lamblia* se encuentra clasificado dentro de la clase Zoomastigophorea, es decir, tiene flagelos como medios de locomoción; incluida dentro del orden Diplomaida y familia Hexamitidae; rubro que caracteriza a protozoos que presentan axostilo, dos núcleos y simetría bilateral. En su ciclo biológico (Figura 2) (Adam, 2001) tiene la capacidad de adoptar dos formas: trofozoíto (forma móvil) y quiste (forma infectante) (Figura 1a y 1b, respectivamente). El parásito en su forma de trofozoíto se establece en su hábitat (el epitelio en cepillo de los dos tercios superiores del intestino delgado -duodeno y yeyuno) fijándose a la mucosa mediante su disco suctor; cada trofozoíto se multiplica de manera activa por medio de un proceso de división longitudinal surgiendo un gran número de elementos en poco tiempo. Los trofozoítos colonizan primariamente el yeyuno, lo que sugiere que requieren una alta concentración de nutrientes para su supervivencia y proliferación.

La infección por *Giardia* es generalmente un proceso autolimitado, lo que indica la presencia de una defensa eficaz por parte del hospedero. La presencia del trofozoíto

en la mucosa intestinal estimula la respuesta inmune local donde, tanto factores inmunológicos, como no-inmunológicos, tienen diferentes niveles de importancia durante el curso de la infección. Amplias revisiones se han enfocado a los mecanismos involucrados en la respuesta inmune contra *Giardia* (Faubert, 2000; Solaymani-Mohammadi y Singer, 2000; Eckman, 2003; Roxtrom-Lindquist et al., 2006; Heyworth, 2014; López-Romero et al., 2015), y en la Figura 3 se presentan de forma muy general algunos de estos mecanismos. Individuos inmunocompetentes infectados con *Giardia* producen anticuerpos séricos e intestinales contra trofozoítos del parásito, lo que indica que respuestas inmunológicas del hospedero limitan la intensidad y/o duración de esta infección. La literatura sugiere que alteraciones en la producción de anticuerpos anti-*G. lamblia* es una de las principales razones por la que estados de inmunodeficiencia predisponen a infecciones severas y prolongadas.

La giardiosis posee un cuadro clínico polimorfo que va desde las formas asintomáticas (aunque con la posibilidad de anormalidades funcionales y morfológicas en el tracto gastrointestinal), hasta las formas que cursan con diarrea crónica, síndrome de talla y peso bajo y malabsorción intestinal (Cañete et al., 2004; Luján, 2006; Ayala y Vega, 1961). Sus mecanismos patogénicos no están bien comprendidos, pero trabajos experimentales *in vivo*, *in vitro* y estudios sobre la infección en el humano coinciden en que se trata de un proceso multifactorial, en el que se encuentran involucrados aspectos inmunológicos y funcionales de hospedero y parásito (Ortega-Pierres et al., 2009; Cotton et al., 2011). Al respecto, Bartelt y Sartor (2015) realizan una interesante revisión acerca de las determinantes y los mecanismos implicados en el establecimiento crónico de la infección (Figuras 4 y 5), tratando de comprender cómo y cuándo este protozoario causa la enfermedad. De acuerdo a estos autores, en niños con giardiosis crónica, el estado nutricional podría determinar la severidad de la enfermedad.

El 60% de los niños infectados con *G. lamblia* desarrollan sintomatología asociada con diarrea crónica la cual puede durar varios meses y es devastadora en la población infantil; en esta fase, se ha observado que las cargas parasitarias altas interfieren con los mecanismos de absorción de algunos nutrientes, entre ellos el zinc (Cordingley y Crawford, 1986; Gillon, 1985; Becerril, 2008). En un estudio realizado en Perú, la presencia de *Giardia lamblia* en menores de 2 años llamó mucho la atención,

encontrándose riesgo de infección (4 a 8 episodios por año) en áreas endémicas, produciendo alteraciones en la absorción de metales, especialmente zinc (Berkman et al., 2003). En 1993 Jendryczko et al., reportan por primera vez a la giardiosis como un factor de riesgo para la malabsorción de zinc en niños; otros estudios también indican alteraciones en el estado de zinc de niños con giardiosis (Quihui et al., 2010, 2012; Abou-Shady et al., 2010; Karakas et al., 2001; Ertan et al., 2002).

En el capítulo I del presente trabajo se presenta un artículo de revisión (Astiazarán-García et al., 2015) donde se exploran algunos aspectos de esta compleja interacción, discutiendo información reciente sobre el estado de zinc y su posible contribución al encuentro entre hospedero y parásito; se discuten a fondo los estudios ya mencionados acerca de cómo la infección por *Giardia* lleva a alteraciones en el metabolismo de zinc, y a la vez se discuten posibles mecanismos implicados: el estado de zinc podría verse alterado por malabsorción intestinal, redistribución hacia distintos órganos o competencia hospedero-parásito; las potenciales propiedades de unión a metales que posee *Giardia* sugieren formas inusuales en las que el parásito interactúa con el hospedero.

La suplementación con zinc como estrategia preventiva para la diarrea ha recibido gran interés en la última década, observándose por lo general un efecto benéfico en los niños (Bhutta et al., 2000; Patel et al., 2010; Bajait and Thawani, 2011); sin embargo, en los últimos años ha aumentado la evidencia de que el efecto benéfico del zinc no es universal frente a los distintos agentes patógenos causales de diarrea (Patel et al., 2010, 2011), por lo que el efecto protector podría ser patógeno-específico.

Estudios de suplementación con zinc en niños reportan una disminución en la tasa de casos de diarrea asociada a *Giardia* (Long et al., 2007; Veenemans et al., 2012). Estos resultados indican que el zinc podría impactar significativamente la salud pública en áreas endémicas de infección por este parásito. Actualmente se buscan alternativas para la prevención y tratamiento de la giardiosis, ya que los casos de re-infección justo después del tratamiento son muy comunes (Gilman et al., 1988), por lo que resulta difícil el control de la enfermedad y el estado de los individuos como portadores del parásito puede ser prolongado, reflejándose en defectos en el desarrollo físico y cognitivo (Cotton et al., 2011; Faubert, 2000).

De acuerdo a lo reportado por Bartelt y Sartor (2015), la infección por *Giardia* puede reducir la absorción de zinc (Ertan et al., 2002; Rosado et al., 2009; Astiazarán-García et al., 2010; Abou-Shady et al., 2011) e influenciar la respuesta del hospedero a la dosis de fortificación o suplementación. La giardiosis y la deficiencia moderada de zinc presentan una distribución geográfica muy similar, por lo que las mismas personas podrían experimentar ambos problemas durante un importante periodo de sus vidas; sin embargo ningún estudio ha explorado el efecto de distintos niveles de zinc en la dieta en un hospedero infectado. En el capítulo II se presenta un artículo original (Iñigo-Figueroa et al., 2013) cuyos objetivos fueron precisamente dilucidar si la giardiosis continúa siendo un factor de riesgo para desarrollar deficiencia de zinc, independientemente del nivel de la ingesta alimentaria; cómo esto afectaría el rendimiento del crecimiento, y la forma en que el sistema inmune responde a este parásito de acuerdo con el nivel de zinc en la dieta.

Se utilizó un modelo experimental de giardiosis en ratones alimentados con 1 de 4 dietas experimentales: adecuada en zinc, bajo contenido de zinc, alta en zinc y zinc suplementado. *G. lamblia* provocó retraso en el crecimiento y niveles bajos de zinc sérico en ratones que consumieron dietas baja y adecuada en zinc con respecto a su control no infectado. Las dietas alta y suplemento evitaron esta pérdida de peso durante la infección, mejorando la tasa de crecimiento y los niveles de zinc. A los 10 días post-infección, se detectó la presencia de anticuerpos específicos en todos los grupos, sin embargo en los grupos alto y suplemento, se observó mayor producción de IgG anti-*G. lamblia*. Los hallazgos de este bioensayo sugieren que un período corto de suplementación con zinc dietario podría tener efectos benéficos sobre el crecimiento, los niveles séricos de zinc y los mecanismos de defensa del hospedero.

Hoy en día es evidente el estrés que la deficiencia de zinc genera sobre el sistema inmune y cómo la suplementación influye de manera positiva en el desarrollo y progresión de diversas enfermedades infecciosas, por lo que el zinc nos provee un modelo bien caracterizado para entender cómo el sistema inmune modifica y adapta sus repuestas a esta deficiencia tan frecuente en la población. Pocos estudios han examinado los efectos del nivel de zinc sobre la respuesta inmune en el hospedero infectado, y aún menos han considerado los eventos que ocurren a nivel intestinal, que es donde se lleva

a cabo la absorción de nutrientes, y a su vez el tejido linfoide asociado a mucosas juega un papel vital al dirigir las respuestas inmunológicas locales y sistémicas; debido a que la respuesta inmune desencadenada por un agente infeccioso normalmente incluye muchas redundancias, el efecto de la deficiencia o suplementación con zinc en el control de la infección debe estudiarse en un hospedero infectado. Con el auge de la biología y la medicina, los modelos animales se han utilizado con la finalidad de conocer aspectos relacionados con la infección que eventualmente puedan extrapolarse a humanos. Durante los últimos cuarenta años se han estudiado infecciones naturales y experimentales por *Giardia* en una gran variedad de animales como ratas, ratones, gatos, perros y gerbos, y estos últimos son considerados el modelo animal más adecuado para estudiar las infecciones con *Giardia* (Belosevic et al., 1983; Faubert y Belosevic, 1990).

Considerando lo anterior, se planteó la evaluación del efecto del zinc sobre *G. lamblia*, su patogenicidad e inducción de respuesta inmune humoral. (capítulos III y IV). La hipótesis primaria del estudio fue que en un modelo *in vivo* de giardiosis, la deficiencia de zinc dietario causa atrofia de la mucosa intestinal y del tejido linfoide asociado a mucosas, reflejándose en defectos funcionales durante la respuesta antígeno-específica y promoviendo la supervivencia de trofozoítos en duodeno. Por otro lado, la suplementación con zinc hace más eficiente la eliminación de la infección al modular la respuesta inmune del hospedero e inducir alteraciones sobre el trofozoíto, afectando de esta forma su patogenicidad y evitando el establecimiento del parásito en la mucosa.

Primero se evaluó la actividad biológica del zinc sobre el crecimiento de *G. lamblia* bajo condiciones axénicas *in vitro*, prestando especial atención a la presencia de alteraciones morfológicas y/o ultraestructurales en el trofozoíto (Capítulo III). A continuación, se evaluó la capacidad que tiene el zinc de alterar la patogenicidad de *Giardia in vivo* utilizando un modelo experimental de giardiosis en jerbos (*Meriones unguiculatus*) alimentados con distintos niveles de zinc (ZD- dieta deficiente en zinc; ZA- dieta adecuada en zinc; ZS- dieta adecuada en zinc + 50 mg gluconato de zinc/kg peso/día).

El gluconato de zinc, de forma dosis dependiente, indujo alteraciones morfológicas e inhibición en el crecimiento de trofozoítos en cultivo. Utilizando el modelo experimental de giardiosis en jerbos fue posible demostrar un aumento en la

susceptibilidad del hospedero deficiente en zinc a desarrollar infecciones persistentes, potenciando la pérdida de peso inducida por la infección; esto podría estar relacionado a una respuesta inmune deteriorada, como se demuestra por menores incrementos de IgG anti-*Giardia* con respecto a grupos control. En vista de que en el grupo ZS se observó una reducción significativa en la intensidad de la infección a los 15 dpi, así como potenciación de la respuesta inmune anti-*G. lamblia* de forma más temprana en la infección, la suplementación con zinc podría ejercer un papel protector y contribuir al control de enfermedad.

Es necesario generar más información para mejorar nuestra comprensión de esta infección parasitaria que recientemente fue incluida por la Organización Mundial de la Salud en la "Iniciativa de enfermedades olvidadas". Las parasitosis representan un grave problema de Salud Pública en todo el mundo, y actualmente existe una actitud de tolerancia e indiferencia hacia este problema. Las altas tasas de infección y sus consecuencias, especialmente durante la infancia, exigen la implementación de programas de control y prevención de enteroparasitosis a corto y largo plazo.

Antes de la era de los antibióticos, la dieta era una parte integral del manejo de las infecciones. Ahora, es necesario tomar un vistazo a esta interacción debido a que la comprensión sobre la respuesta inmune se ha expandido de manera considerable. En comparación, se ha llevado a cabo poca investigación acerca del impacto de las intervenciones nutricias en el manejo de las enfermedades infecciosas, siendo la mayoría de las observaciones de carácter epidemiológico. Hoy día se debate si componentes de la dieta pueden condicionar la respuesta metabólica e inflamatoria a las agresiones, de forma que incida en la evolución clínica del paciente, con el ideal de modular la dieta, diseñándola específicamente para cada proceso patológico. La comprensión de cómo la dieta y sus componentes modifican nuestra respuesta a la infección, representa un área de necesidad, oportunidad y desafío científico.

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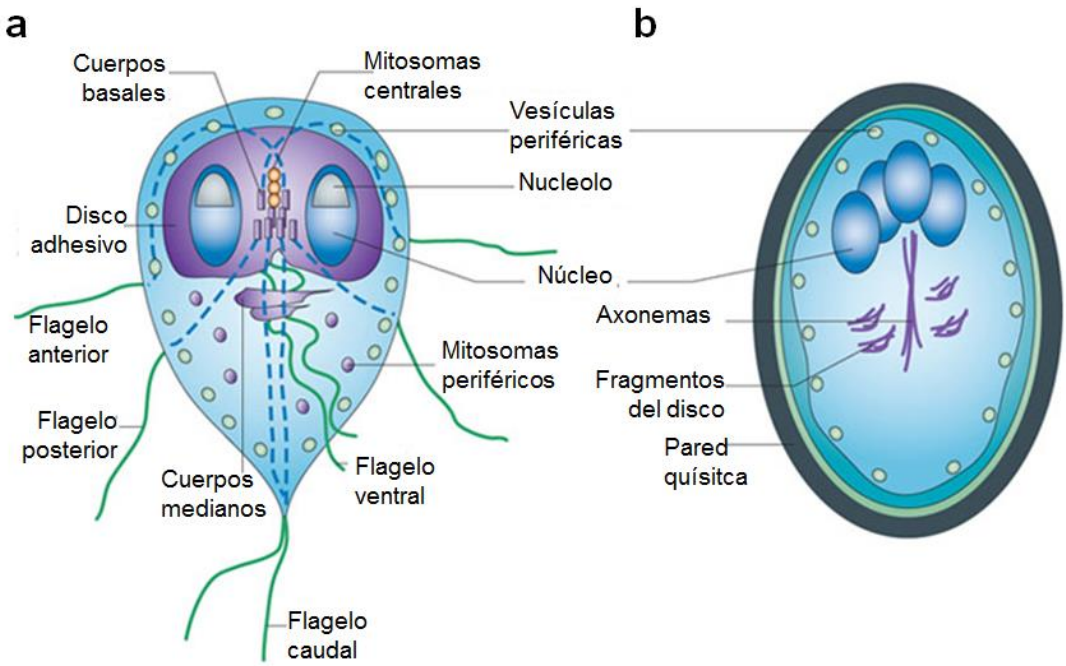

Figura 1. *Giardia* en su forma de trofozoíto y quiste. **a** | El trofozoíto de *Giardia* mide 12–15 μm de largo y 5–9 μm de ancho. Aquí se muestra el trofozoíto desde una vista dorsal. Presenta ocho flagelos organizados en cuatro pares: flagelos anteriores, flagelos ventrales, flagelos posteriores y flagelos caudales; las líneas punteadas indican estructuras internas. Los cuerpos basales son los sitios de donde se originan los flagelos. Los cuerpos medianos son un estructura microtubular cuya función se desconoce. El disco adhesivo es una estructura de adherencia, grande y rígida, compuesta de microtúbulos. Hay varios mitosomas centrales y periféricos en la célula. Las vesículas periféricas son vesículas tipo-lisosomas que se encuentran debajo de la membrana plasmáticas a través de toda la célula. **b** | En el quiste, la pared quística y la capa interna, consistente en dos membranas, protegen al parásito. Los quistes de *Giardia* son de forma ovalada, no móviles, y miden 8–12 μm de largo por 7–10 μm de ancho. La capa externa del quiste tiene un grosor de 0.3–0.5 μm, y se compone de una red de filamentos que van de 7 a 20 nm de diámetro. Esta pared se compone principalmente de N-acetilgalactosamina y tres proteínas de pared quística diferentes (CWP1, CWP2 y CWP3). El disco adhesivo y los flagelos se desmontan y almacenan en el parásito. El quiste tiene cuatro núcleos tetraploides. Ankarklev et al., 2010

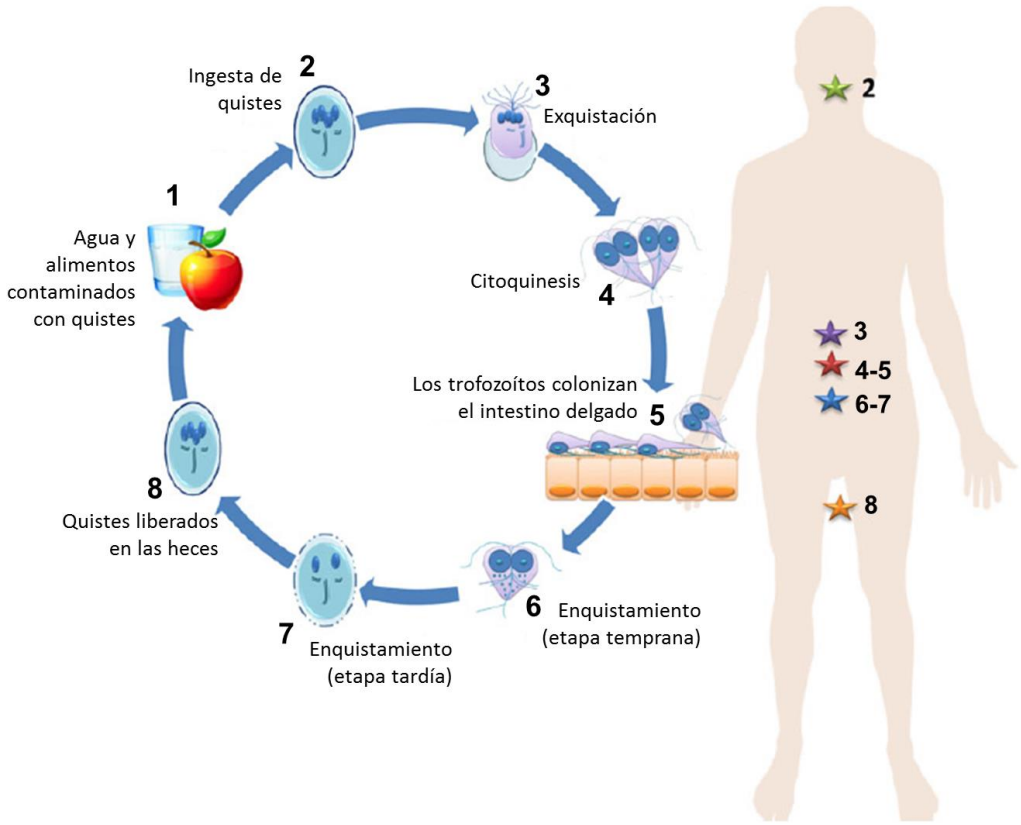

Figura 2. Ciclo de vida de *Giardia*. La ruta de transmisión de la giardiosis es la fecal-oral. La infección inicia con la ingesta de quistes en agua o alimentos contaminados (1 y 2). La exposición al ambiente ácido en el estómago induce el proceso de exquistación (3). Cada quiste produce dos trofozoítos (4). Los trofozoítos colonizan y se replican en el intestino delgado, pudiéndose adherir epitelio intestinal (5). A medida que los trofozoítos viajan a través del intestino, el ambiente bajo en colesterol, alto en bilis y ligeramente alcalino, puede inducir una etapa temprana de enquistamiento, en la cual los trofozoítos adquieren una forma redondeada y proteínas específicas de enquistamiento son transportadas hacia la superficie celular mediante vesículas para formar la pared del quiste (6). El disco adhesivo se desmonta y la célula se somete a replicación de ADN para obtener una célula que contiene dos núcleos (4N cada uno). Durante la etapa final de enquistamiento, el núcleo se divide, dando lugar a cuatro núcleos (2N cada uno), y el DNA se duplica de nuevo para generar un quiste maduro con cuatro núcleos (16N) (7). Los quistes son liberados en las heces permitiendo de esta forma la finalización del ciclo de transmisión al infectar un nuevo hospedero (8). Adam, 2001; López-Romero et al., 2015

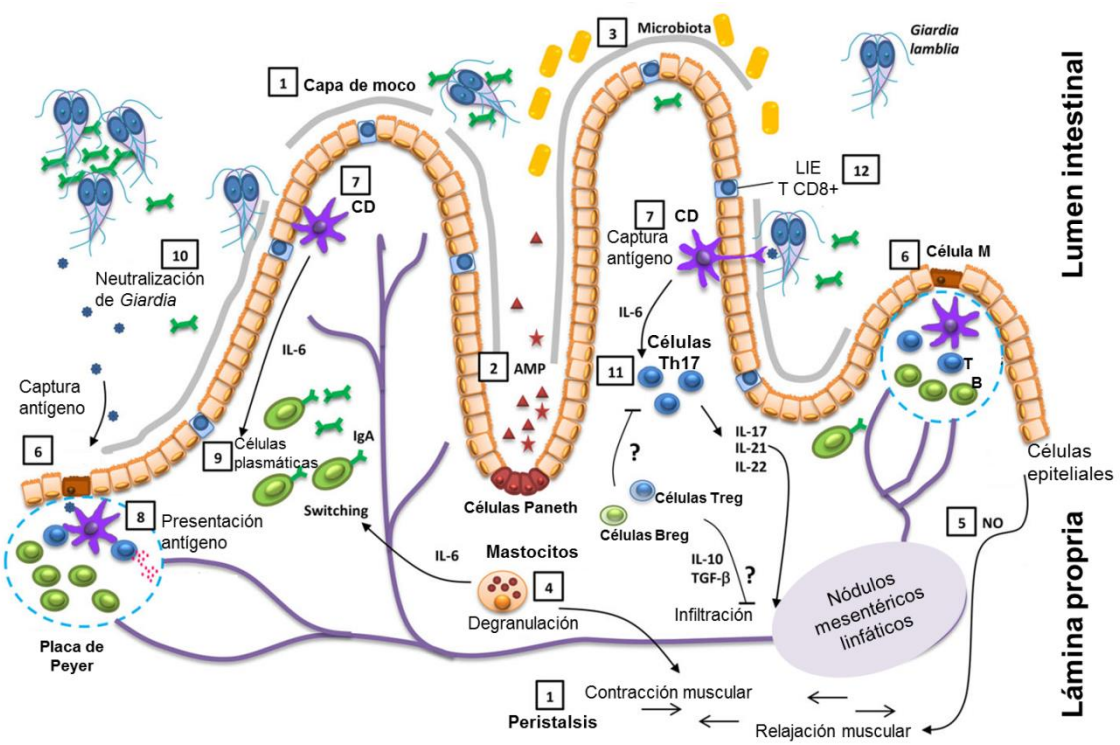

Figura 3. Mecanismos de defensa del hospedero contra *Giardia lamblia*. El sistema inmune innato y adaptativo actúan en sincronía para controlar la infección por *Giardia*. Los mecanismos de inmunidad innata son la primera línea de defensa ante la colonización por *Giardia*. La capa de moco en la superficie intestinal y los movimientos peristálticos constituyen barreras mecánicas para la adherencia de *Giardia* (1). Los péptidos antimicrobianos (AMP) liberados por las células de Paneth y otras células, pueden matar trofozoítos (2). La microbiota intestinal tiene un efecto anti-*Giardia* por competencia, toxicidad directa o modulación de la respuesta inmune. Adicionalmente, la microbiota contribuye a preservar la integridad intestinal (3). Los mastocitos liberan citocinas pro-inflamatorias, como IL-6; la degranulación de mastocitos promueve la peristalsis (4). Células epiteliales e inmunes producen óxido nítrico (NO), que tiene un efecto citoestático sobre trofozoítos de *Giardia*, inhibe procesos de desenquistamiento/enquistamiento y contribuye a la peristalsis (5). Mediante endocitosis las células M capturan antígenos del lumen intestinal hacia las placas de peyer (PP) para inducir respuestas inmunes (6). Las células dendríticas (DC) juegan un papel como "comunicador" entre la inmunidad innata y adaptativa; se localizan en la lámina propia y en las PP, donde reconocen antígenos; también pueden expandir sus dendritas hacia el lumen intestinal para capturar antígenos (7). Las CD ingieren y procesan antígenos de *Giardia* para posteriormente presentarlos a células T "naive" mediante moléculas de MHC II. Los linfocitos T activados liberan un panel de citocinas que modulan la respuesta anti-*Giardia* (8). La IL-6 liberada por los mastocitos, CD y células T, es un importante modulador de la maduración de células B, e induce cambio de isotipo de anticuerpos para producir IgA (9). Las células plasmáticas migran hacia la lámina propia para liberar IgA, que puede inhibir la adherencia de *Giardia* a las células epiteliales (10). Las células T CD4+ Th17 son activadas durante etapas tempranas de la inmunidad adaptativa contra *Giardia*, y liberan citocinas como IL-17, IL-21, IL-22, que juegan un papel pro-inflamatorio (11). Los linfocitos intraepiteliales (LIE), principalmente células T CD8+, juegan un papel en la patología observada en el intestino durante la giardiosis (12). Falta información importante en nuestro conocimiento de la interacción *Giardia*-hospedero: ¿Qué papel juegan las células Breg y Treg en la regulación del proceso inflamatorio durante la giardiosis? Los trofozoítos de *Giardia* no invaden la mucosa ¿Cómo pueden antígenos del parásito inducir respuestas inmunes tanto en mucosa como sistémicas? ¿Cómo rompe la tolerancia en mucosa? ¿Qué antígenos de *Giardia* pueden inducir una respuesta inmune protectora? ¿Modula *Giardia* la manipulación del antígeno por células presentadora de antígeno (CD, macrófagos)? López-Romero et al. 2015

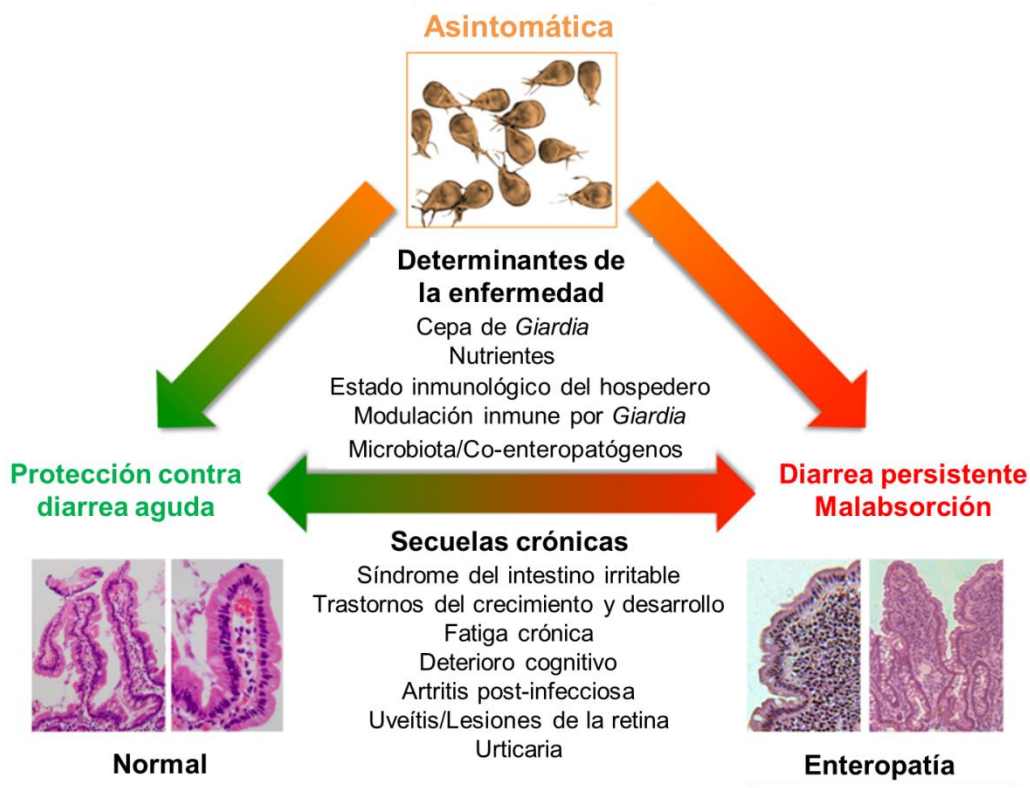

Figura 4. Espectro clínico de la infección por *Giardia lamblia*. La mayor parte de los individuos infectados con *Giardia* son asintomáticos. Se hipotetiza que algunas de las determinantes de la forma en que se manifiesta la enfermedad incluyen: la virulencia de la cepa infectante de *Giardia*, estado nutricional del hospedero, enteropatógenos co-infectantes, la composición y función de la microbiota residente, modulación inmune por *Giardia*, genética e inmunidad del hospedero. Cambios en estas variables dinámicas pueden modificar las manifestaciones de la enfermedad. Los cambios histológicos varían de la histopatología normal, a acortamiento de vellosidades e infiltrado inflamatorio crónico (enteropatía). Las secuelas crónicas asociadas a giardiosis incluyen: síndrome del intestino irritable, fatiga crónica, trastorno del crecimiento en niños, retraso en el desarrollo, deterioro cognitivo, y manifestaciones extra-intestinales que se presume están relacionadas a fenómenos inmunológicos (artritis reactiva, manifestaciones inflamatorias oculares y urticaria) y no son necesariamente dependientes de la severidad de las manifestaciones diarreicas, pudiendo persistir aún después de no detectar al parásito. Bartelt y Sartor 2015

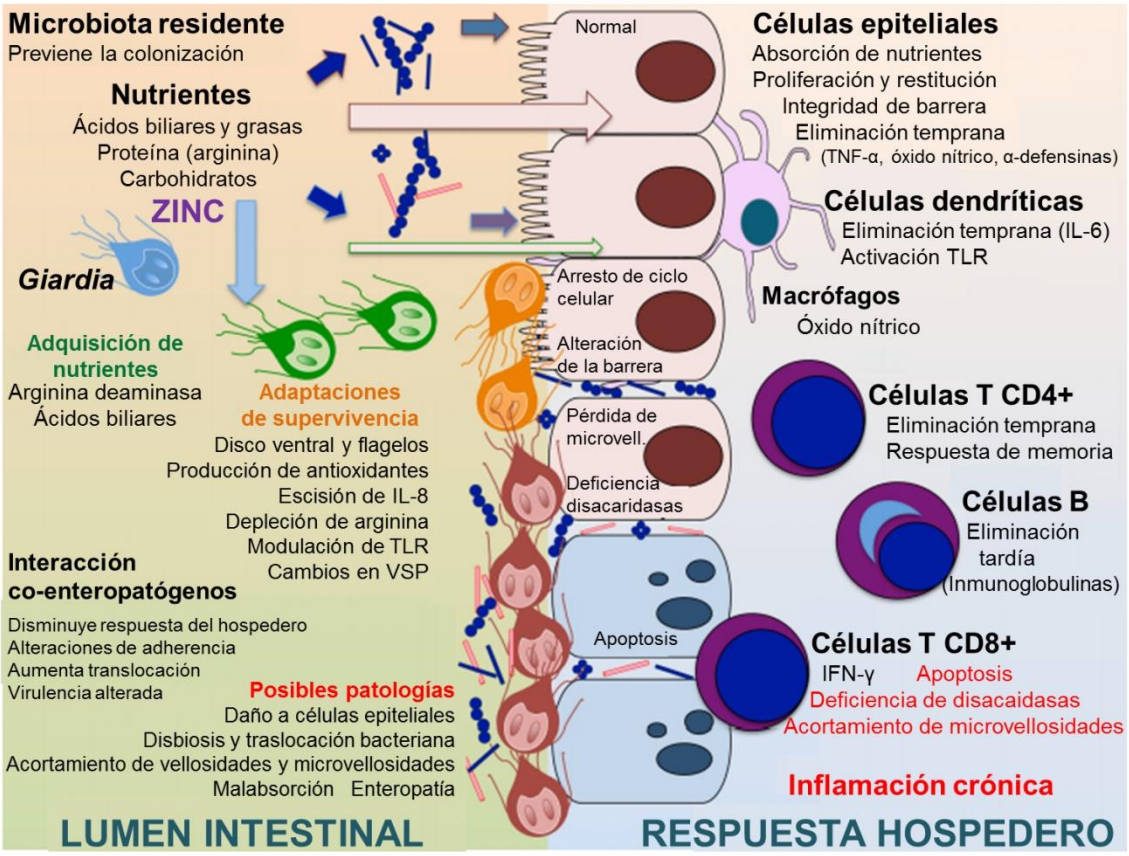

Figura 5. Determinantes y mecanismos implicados en el establecimiento crónico de la infección. Interacciones complejas entre la microbiota, nutrientes, cepa de *Giardia*, co-enteropatógenos, y respuestas moleculares del hospedero en el ambiente luminal y de la mucosa, muy probablemente influencian la infectividad de *Giardia* y la forma en que se establece la enfermedad. (Izquierda) La microbiota residente mantiene la resistencia a la colonización. *Giardia* utiliza, y potencialmente secuestra, nutrientes como la bilis, arginina y zinc para poder sobrevivir, replicarse, y evadir la microbiota y respuesta inmune del hospedero. Los flagelos y el disco ventral, son estructuras del trofozoíto que le ayudan en la unión y adherencia a células epiteliales. *Giardia* utiliza factores de virulencia para evadir las repuestas inflamatorias del hospedero a través de la producción de antioxidantes, escisión de interleucina 8 (IL-8), depleción de arginina mediada por arginina deaminasa (ADI), y cambios en la expresión de proteínas variables de superficie (VSP). Los efectos de *Giardia* en las células epiteliales (arresto del ciclo celular, alteración de la proliferación, ruptura de uniones estrechas, y apoptosis) pueden ser directos o indirectos, y dependientes de la cepa. Cambios subsecuentes en la disponibilidad de nutrientes, composición de la microbiota, defensas inflamatorias, y sitios de unión de patógenos a células epiteliales, podrían secundariamente alterar las manifestaciones de la enfermedad de enteropatógenos co-infectantes. (Derecha) Respuestas inmunes redundantes en la mucosa promueven la eliminación temprana de *Giardia* (absorción de nutrientes en células epiteliales para beneficio del hospedero, mantenimiento de la función de barrera, y moléculas pro-inflamatorias; IL-6 derivada de células dendríticas y mastocitos; y células T CD4+ y CD8+), y hacia el final de la enfermedad (células de memoria T CD4+ y células B). Células T CD4+ inducen respuestas de memoria, pero también contribuyen a inflamación crónica y pueden promover deficiencia de disacaridasas. Células T CD8+ son las encargadas de mediar la apoptosis, acortamiento de microvellosidades y deficiencia de disacaridasas. El daño a las células epiteliales puede persistir después de la eliminación del parásito, dando lugar a la traslocación sostenida de la microbiota y productos microbianos. La alteración de la homeostasis de la mucosa, la inflamación (enteropatía) y la composición de la microbiota, pueden obstaculizar aún más la absorción de nutrientes y contribuir a secuelas a largo plazo que incluyen alteraciones en el crecimiento y el desarrollo cognitivo. Bartelt y Sartor 2015

CAPÍTULO I

Artículo:

Crosstalk between zinc status and *Giardia* infection: a new approach

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Review

Crosstalk between Zinc Status and *Giardia* Infection: A New Approach

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Abstract: Zinc supplementation has been shown to reduce the incidence and prevalence of diarrhea; however, its anti-diarrheal effect remains only partially understood. There is now growing evidence that zinc can have pathogen-specific protective effects. Giardiasis is a common yet neglected cause of acute-chronic diarrheal illness worldwide which causes disturbances in zinc metabolism of infected children, representing a risk factor for zinc deficiency. How zinc metabolism is compromised by *Giardia* is not well understood; zinc status could be altered by intestinal malabsorption, organ redistribution or host-pathogen competition. The potential metal-binding properties of *Giardia* suggest unusual ways that the parasite may interact

with its host. Zinc supplementation was recently found to reduce the rate of diarrhea caused by *Giardia* in children and to upregulate humoral immune response in *Giardia*-infected mice; *in vitro* and *in vivo*, zinc-salts enhanced the activity of bacitracin in a zinc-dose-dependent way, and this was not due to zinc toxicity. These findings reflect biological effect of zinc that may impact significantly public health in endemic areas of infection. In this paper, we shall explore one direction of this complex interaction, discussing recent information regarding zinc status and its possible contribution to the outcome of the encounter between the host and *Giardia.*

Keywords: zinc supplementation; zinc deficiency; *Giardia lamblia*; giardiasis; parasite infection; micronutrient supplementation

1. Introduction

Nutrition plays a fundamental role in the maintenance of health and the treatment of disease. The ability of the immune system to prevent infection and disease is strongly influenced by the nutritional status of the host, and an inadequate intake of macronutrients or selected micronutrients may compromise its ability to resist infectious pathogens; however, nutrition does not influence all infections equally [1,2].

Nowadays, there is growing interest in dietary factors, in particular micronutrients, from the perspective of disease pathogenesis and potential for treatment. Results of field and laboratory studies provide convincing evidence that micronutrient deficiencies contribute to the mortality and morbidity of infectious diseases [1,2]. This awareness has led to the conduct of several micronutrient supplementation studies which have been successful in reducing illness and decreasing deaths. Micronutrients offer a potentially inexpensive feasible means of altering the outcome of infectious diseases in the developing world. The evidence is strongest for the essential mineral zinc, which has caught wide scientific attention for the conceptual promise it has to offer for prevention, control and treatment of childhood diarrhea.

Diarrhea is one of the most common health problems affecting children, it can lead to malnutrition, developmental disorders, and even death [3]. In 2004, the World Health Organization (WHO) and the United Nations International Children's Emergency Fund (UNICEF) issued a global recommendation for the daily supplementation with 20 mg of zinc in children 6 months of age and older, and 10 mg of zinc in infants younger than 6 months for 10–14 days on diarrheal onset [3]. This recommendation was based on several randomized controlled trials, meta-analyses and reviews [4] that have

demonstrated the utility of zinc supplementation to reduce the incidence and prevalence of diarrhea, as well as the severity of the current episode, prevent subsequent episodes and improve other diarrhea related outcomes. Nevertheless, the protective mechanism of zinc has remained elusive.

Zinc has been tested for its ability to treat and prevent diarrheal diseases in many large field trials over a period of over 4 decades and has generally been found effective. However, significant heterogeneity of zinc on diarrhea-related outcomes has been observed across trials [4–6]. Potential contributors to this heterogeneity are currently not fully understood. Patel *et al.* [7] conducted a systematic analysis of several large studies and showed that the influence of zinc supplementation in acute diarrhea differs by the isolated organism, and that the beneficial effect of zinc may not be equivalent against the common causative agents. At present, evidence has shown that zinc can have pathogen-specific protective effects [8–11]. These findings suggest that the current strategy of zinc supplementation may optimize the therapeutic benefit based on the causative organism, but further studies are required to support this. The interactions of nutrition and infection with regard to individual infections and defined nutrients are now better known. Giardiasis remains as a common yet neglected cause of acute and chronic diarrheal illness worldwide [12]. This infection has been related to disturbances in the zinc metabolism of infected children [13], and may represent a risk factor for zinc deficiency [14]. In this paper we shall explore one direction of this complex interaction, discussing recent information regarding zinc status and its possible contribution to the outcome of the encounter between the host and *Giardia*.

2. Zinc

2.1. Zinc Biology

Since its discovery as an important element for human health in the 1960s, zinc has been widely studied but many questions regarding its mechanism of action and utility still remain unanswered [15]. It is the second most abundant trace element in the human body and is required for its normal functioning [16]. It plays critical catalytic, structural, and regulatory roles [17]. Zinc enables hundreds of enzymes to function, facilitates protein synthesis and folding, and regulates processes such as gene expression and apoptosis [18]. Zinc is also important for DNA and RNA metabolism, as well as cellular replication, differentiation and growth [19]. Therefore, organs that are dependent on continuous cell division for proper function, such as the immune system and the gut, are particularly sensitive to zinc deficiency.

2.2. Zinc Deficiency

A regular intake of zinc is required as there are no large stores in the body from which it can be easily mobilized [20]. Zinc is widely distributed in foods. Meat, fish and poultry are the major contributors of zinc in the diet, although dairy products and cereals also contribute substantial amounts [21].

Zinc deficiency is largely related to inadequate intake or absorption of zinc from the diet. High levels of inhibitors in the diet, such as fibre and phytates (mainly found in plant-based diets), may result in low absorption of zinc, even though intake of zinc may be acceptable. In general, zinc absorption from a diet high in animal protein will be greater than from a diet rich in plant derived proteins [22].

Although severe zinc deficiency is rare nowadays, mild-to-moderate zinc deficiency is quite common throughout the world. It is estimated that 17% of the global population is at risk of inadequate zinc intake [23]. Regional estimated prevalence ranges from 7.5% to 30%, with specific countries in South and South-East Asia, Sub-Saharan Africa, and Central America having the greatest risk of inadequate zinc intake. Part of the problem is that many people do not eat enough zinc-rich foods, while the mineral is also not well absorbed.

Children are especially vulnerable to deficiency because their periods of rapid growth create increased zinc needs that may remain unmet [24]. Zinc is recognized as problem nutrient for the older breastfed infant because of the challenge of obtaining adequate intake from exclusive breastfeeding and the resultant dependence on complementary foods to meet dietary requirements [25,26]. Meat is an excellent source of zinc and increased use of animal products has been recognized as an option, but it has often been considered unrealistic [26,27]. The choices of complementary foods are affected by economic and socio-cultural factors like family dietary pattern, culture, customs, beliefs of food taboos, previous experience of feeding patterns, inadequate nutritional knowledge, among others [28,29]. The majority of culturally acceptable and affordable complementary foods are plant- and cereal-based with relatively high phytate content which decreases zinc bioavailability [29].

In the developing world there is not only a zinc shortage in food but intestinal parasites inhibit its absorption as well [30,31]. In resource-scarce settings, poor water and sanitation systems lead to frequent exposure to gastrointestinal pathogens and high rates of infectious disease and diarrhea [32,33]. For instance, diarrhea can compromise intestinal function and damage the gastrointestinal tract lining, thereby causing increased zinc intestinal excretion [34]. Furthermore, zinc deficiency has been shown to increase the susceptibility of infants and children to gastrointestinal infections due to its adverse

effects on gastrointestinal tract structure and function [34]; as a result, a cycle of zinc deficiency and infection may develop.

2.3. Zinc and Immune Function

Nutritional importance of zinc has been known for a long time, but in the last decades its importance in immune modulation has arisen. Although its function as a structural component of many enzymes has been known for many years, current experimental evidence points to an additional function of the concentration of free or loosely bound zinc ions as an intracellular signal [35].

Zinc functions as a modulator of the immune response through its availability [36], which is tightly regulated by a network based on ZnT-ZIP proteins and metallothionein for storage [37]. When this mechanism is disturbed, zinc availability is reduced, altering survival, proliferation and differentiation of the cells of different organs and systems [36]. The immune system is one of the most highly prolifarating organs [38,39], and the activity of cells involved in both innate and adaptive immunity is modulated by zinc. These cells include monocytes, polymorphonuclear-, natural killer-, T-, and B-cells. T cell functions and the balance between the different T helper cell subsets are particularly susceptible to changes in zinc status [36,37].

While acute zinc deficiency causes a decrease in innate and adaptive immunity, chronic deficiency increases the production of inflammatory cytokines, influencing the outcome of a large number of inflammatory diseases [36]. In 2008, Prasad [40] reported that in both young adults and elderly subjects, zinc supplementation decreased oxidative stress markers and generation of inflammatory cytokines.

Lymphopenia and thymic atrophy, which were the early hallmarks of zinc deficiency in humans and higher animals, are now known to be due to high losses of precursor T and B cells in the bone marrow [41]. Changes in gene expression for cytokines, DNA repair enzymes, zinc transporters, signaling molecules, *etc.*, suggest that cells of the immune system are attempting to adapt to the stress of suboptimal zinc [42]. That these effects can be functionally significant is demonstrated by the increased susceptibility of zinc-deficient animals to a number of bacterial, viral, and parasitic challenges [43]; zinc-deficient persons also experience increased susceptibility to a variety of pathogens [40].

Impaired immune functions due to zinc deficiency are shown to be reversed by an adequate zinc supplementation, which must be adapted to the actual requirement. Care must be taken on this matter as high dosages of zinc evoke negative effects on immune cells and show alterations that are similar to those observed with zinc deficiency [39].

It is clear that zinc affects multiple aspects of the immune system, from the barrier of the skin to gene regulation within lymphocytes [43]. Better understanding of the

molecular and cellular changes made in response to inadequate zinc should lead to the development of immunotherapeutic interventions [42].

2.4. Zinc and Intestinal Parasitic Infections

Micronutrient deficiencies and parasitic diseases have similar geographical distribution with the same people experiencing both health problems in their lives. Intestinal parasitic infections are especially problematic because they have negative lifelong health consequences [44]; these infections can contribute to malnutrition which in turn results in delayed physical development and cognitive growth. Infection generally causes sequestration of zinc in the liver, and conditions that affect intestinal function and integrity can influence zinc homeostasis [21]. Parasitic infections are thought to contribute to child malnutrition through subtle reduction in digestion and absorption, chronic inflammation and loss of nutrients. Parasites may affect the intake of food, its subsequent digestion and absorption, metabolism and the maintenance of nutrient pools [30]. The most important parasites related to nutritional status are soil transmitted helminthes,protozoa such as *Giardia lamblia* and *Entamoeba histolytica*, followed by other parasites such as coccidia, *Schistosoma* sp. and malarial parasites [30]. Management guidelines for treatment of undernutrition in children explicitly recognize that treatment of overt and occult infection is a first step in breaking the cycle of infection, undernutrition, and immune impairment [45].

3. Giardiasis and Zinc Status

3.1. Giardia lamblia Infection

Giardiasis is a major protozoan infection associated to diarrheal disease worldwide. The flagellate protozoan *Giardia lamblia* (*G. lamblia*) (synonym *Giardia intestinalis*, *Giardia duodenalis*), its causative agent, is the most commonly identified intestinal parasite in the United States and the most common protozoal intestinal parasite isolated worldwide [46–49]. In developing countries, the prevalence of giardiasis commonly ranges from 20% to 30%, with reports of 100% prevalence in some populations [50]. In some regions of the world, giardiasis is endemic and infection is practically universal by 2 years of age [51].

Giardia is transmitted through the ingestion of cysts in contaminated food or water, or directly via the fecal/oral route [51]. A striking feature of giardiasis is the spectrum of clinical symptoms that occur in infected individuals, from asymptomatic, to acute or chronic diarrheal disease associated with intestinal malabsorption, abdominal pain and nausea [12]. Multiple factors have been proposed to account for the disease variability,

including the state of the host immune system, host age and nutritional status, strain genotype, infectious dose and, possibly co-infections [52–54].

Immune responses to *Giardia* occur in the intestinal mucosa and a spectrum of inflammatory mechanisms company this infection [12,52]. Secretory antibodies of the IgA class are important candidate for immune defense against *Giardia*, because they are secreted in large quantities into the intestinal lumen and their actions are antigen-specific [53].

In recent years, this protozoan attracted and renewed scientific attention because of the recognition of pathologies beyond the regular symptoms of infection—reviewed by Halliez and Buret [50]. These included chronic fatigue [55], post-infectious irritable bowel syndrome [55], and particularly, in early childhood, poor cognitive function and failure to thrive [56]. In addition, the inclusion of *Giardia* in the WHO's Neglected Diseases Initiative in 2004 [57] and its re-emergence in industrialized countries, because of its recognized role in numerous outbreaks of diarrheal disease in day-care centers and in waterborne infections [58], led to a greater appreciation of the public health consequences of giardiasis.

Despite significant advances in the knowledge of the biochemistry and molecular biology of *Giardia*, little is known about its pathogenesis, where a combination of parasitic factors and host responses seems to be involved [59]. The pathophysiological consequences of *Giardia* infection include heightened rate of enterocytes apoptosis, shortening of brush border microvilli with villous atrophy, disaccharidase deficiencies, intestinal barrier dysfunction, activation of host lymphocytes, small intestinal malabsorption, anion hypersecretion and increased intestinal transit rates [12,60–67]. All these consequences are clearly multifactorial, and involve both host and parasite factors, as well as immunological and non-immunological mucosal processes [50].

3.2. Giardiasis and Zinc Deficiency

The interactions of nutrition and infection with regard to individual infections and defined nutrients are now better known. The association between zinc deficiency and giardiasis has scarcely been investigated although the association of giardiasis with undernutrition and malabsorption of micronutrients such as vitamin A [68–70] is well recognized.

Zinc is an element which cannot be stored in the body and therefore it can easily decline during the course of infective diseases [71]. In 1993 giardiasis was reported as a first-time risk factor for zinc malabsorption in children [13]. According to Jendryzcko *et al.* [13] disturbances were found in the zinc metabolism of *Giardia*-infected children. Elimination of zinc via urine, and serum-erythrocyte-zinc concentration were lower in

infected children compared to non-infected; concentration of serum zinc carriers, total protein, albumin fraction and transferrin were not differing between both studied groups. Authors concluded that children with giardiasis had lower zinc absorption from the gastrointestinal tract, which led to zinc deficiency.

Several studies conducted regarding trace elements in giardiasis have also shown a significant decrease in zinc levels. Yones *et al.* [72] studied the effect of enteric parasitic infections on serum trace elements and nutritional status in Egyptian children; stunting, wasting and coincident decrease in serum zinc levels were more prominent among *Giardia lamblia* patients. Another study in Egyptian children [73] also reports affection of weight, intermittent diarrhea and significantly decreased serum zinc levels in the infected group compared to control. Two studies from Turkey [74,75] showed that serum zinc levels were significantly lower among the children, 2 years to 14 years old, with chronic giardiasis compared to their matched *Giardia*-free group. Zarebavani *et al.* [76] compared mean serum levels of immunological and biochemical parameters between *Giardia*-positive and *Giardia*-negative Iranian children, founding that zinc level in *Giardia* patients was remarkably lower, with no significant difference in serum levels of vitamin B12 and folic acid. In a recent study by Lazarte *et al.* [31], trace elements status was evaluated and associated to the presence of intestinal parasites in a group of children from a rural area of Bolivia. A multiple regression model showed the significant effect of the presence of the parasite *Giardia lamblia* on the serum zinc levels.

Quihui *et al* [14] investigated the association between giardiasis and zinc deficiency in schoolchildren from northwestern Mexico. Longitudinal analysis demonstrated a significant increase of the serum zinc levels in the *Giardia*-infected group six months after treatment, even though no difference was observed in the socioeconomic characteristics and daily intakes of zinc between the groups. Another study from Turkey [77] found a significant increase in the serum zinc levels after treatment in 20 *Giardia*-infected children, 3 months to 14 years old.

How zinc metabolism is compromised by *Giardia* is not well known. During infection the mucosal epithelium has a high turnover rate and functional immaturity of enzyme and transport systems. Thus, it is hypothesized that the increased intestinal absorption of zinc associated with anti-*Giardia* treatment may be explained by the restoration of the impaired intestinal mucosa as a result of the infection [78]. Another hypothesis has suggested that zinc deficiency may result from organ redistribution of zinc, from plasma to the liver, as part of the acute phase response of the host; apparently, the immune response of the host leads to activation of the synthesis of metallotionein in the liver and other tissues, altering the hepatic uptake of zinc [79,80].

Competition between the host and the pathogen for an important nutritional resource may be another way by which *Giardia* alters zinc status; in this situation the parasite and the host make great efforts to control zinc's availability. Recent studies have shed some light on the role of zinc in gut infections. In order to survive within a host, some gut pathogens may evolve specialized systems to gain an advantage. Such is the case of *Salmonella thyphimurium*, a pathogen that thrives in the inflamed intestine by overcoming calprotectin-mediated zinc chelation through the expression of a high affinity zinc transporter. These findings highlight the importance of zinc acquisition in bacterial intestinal colonization [81].

The surface and flagella of the *Giardia* trophozoite are covered by a protein coat composed of a single variant-specific surface protein (VSP) [82,83]; these are metal-binding proteins and different cloned trophozoite lines all bound zinc [84]. The benefit of metal binding of VSPs to *Giardia* is unknown. Metal ions could maintain structure, bind neighboring VSPs or prevent oxidation; most interestingly, no other surface-residing Zn-finger protein exists in any other organism [85].

This group of proteins forms the major component of interface between *Giardia* and its host. VSPs can vary spontaneously or in response to antibody [86] or environmental selection [87], and loss of a VSP leads to replacement with another, which is usually immunologically distinct [86,88]. *Giardia* undergoes antigenic variation as a mechanism assumed to allow parasites to evade the host's immune response, producing chronic and/or recurrent infections [84]. Because *Giardia's* mechanism of protection may depend on switching expression among immunologically distinct VSPs, the host should be able to prevent infection by simultaneously developing specific immune responses to all variable surface molecules [85]. Despite the frequency of VSP switching, certain features are common to all known VSPs. An interesting idea at this point would be how the zinc status of the host could affect the mechanism controlling VSP switching during *Giardia* infection, and eventually its survival within the host.

As discussed by Nash *et al.* [84], in some infections the parasite burden is large, and the villi of the small intestine are covered with trophozoites. VSPs could bind zinc and inhibit the function of zinc or metal-requiring intestinal enzymes, or compete with the host for zinc and contribute to zinc malnutrition, which is increasingly recognized and occurs in populations and areas where *Giardia* is prevalent. In such scenario, only the presence of enough trophozoites, but not necessarily the presence of disease, would be needed to cause zinc malnutrition. The potential metal-binding properties of *G. lamblia* suggest unusual ways that the parasite may interact with its host, but whether this sequestration could affect the patient is not clear, especially in view of the non-specificity of metal binding by the VSPs [89].

3.3. Zinc Treatment and Giardiasis

Persistence of intestinal parasitic infections during de-worming campaigns in schoolchildren has been reported [90]. Rapid reinfection by *Giardia lamblia* after treatment is a common problem in endemic areas [91]. The front line treatment for giardiasis is antimicrobial therapy. Although the infection is usually self-limiting and recovery occurs in the majority of cases, there is a need for safe and effective ways of preventing and treating this disease. Several classes of antimicrobial drugs are available for the treatment of giardiasis. Among the most commonly used are members of the nitroimidazole family such as metronidazole and tinidazole [92]. However, first-line therapy fails in up to 20% of cases and cross-resistance between different agents can occur [93–95]. Alternative agents exist for treatment failures and for special circumstances (e.g., pregnancy), but these are generally less effective than nitroimidazole drugs [96].

Therefore, because of the prevalence of giardiasis and limited treatment options new interventions are required. Andrews and Mylvaganam [97] tested the sensitivity of *Giardia lamblia* to bacitracin and its zinc salt *in vitro*. The activity of bacitracin was enhanced 5–10 times by equimolar concentrations of zinc. This enhancement was not due to zinc toxicity and was zinc dose dependent. Significant *in vivo* activity was also demonstrated in a clinical trial in patients infected with the protozoa [98]. Nash and Rice [88] showed efficacy of zinc-finger-active drugs against *Giardia lamblia*. Zinc-finger-active compounds at 300 μM or less inhibited *Giardia lamblia* growth. In the adult mouse model, significant *in vivo* activity was demonstrated by increased cure rates and decreased parasite burdens. Because of high reinfection rate after traditional treatment and lack of effect on nutritional status or growth, anti-*Giardia* drug treatment of asymptomatic carriers generally is not recommended.

Zinc supplementation was recently found to reduce the rate of diarrhea caused by giardiasis [11,99]. A randomized, double-blind, placebo-controlled trial [11] was conducted among Mexican children who were assigned to receive either vitamin A, a daily zinc supplement, a combined vitamin A and zinc supplement, or a placebo; then children were followed for 1 year. *G. lamblia* infections were reduced among children in the combined vitamin A and zinc group or the zinc alone group. In a study by Veenemans [99], there was no evidence that the efficacy of zinc supplements in reducing diarrhea rates is enhanced by concurrent supplementation with other micronutrients. Two other trials that investigated the added benefit of multinutrients in addition to zinc in preventing episodes of diarrhea also failed to show an advantage of combined supplementation above supplementation with zinc alone [100–102]. Therapeutic application of zinc as a pharmacological agent during infection seems to be

an interesting idea, but all the different impacts of zinc treatment on the host must be considered in order to achieve a desired therapeutic effect [103].

Our group work using an experimental murine model of giardiasis [104] showed that *Giardia*-infected mice fed zinc-low or zinc-adequate diets had a significant growth retardation in comparison to non-infected controls. Supplementation of the diet with high levels of zinc improved growth performance, by increasing the body weight gain, and up-regulated the host's humoral immune response by improving specific antibodies production. Clinical outcomes of zinc supplementation during giardiasis included significant weight gain, earlier and higher *Giardia*-specific antibody response and improved serum zinc levels despite the ongoing infection. These findings probably reflect biological effect of zinc that could be of public health importance in endemic areas of infection. Therefore, safe and inexpensive measures such as supplementation with oral zinc are apparently an attractive management and treatment option.

4. Conclusions

Zinc supplementation has been shown to reduce the incidence and prevalence of diarrhea. There is growing evidence indicating that zinc can have pathogen-specific protective effects. There is a need for additional research to understand the effect of zinc supplementation on diarrhea associated to different pathogens in order to use zinc treatment depending on the relative prevalence of causative organisms among a specific population. Intestinal parasites remain an important worldwide public health problem. *Giardia lamblia* is one of the important intestinal parasites associated to acute and chronic diarrheal diseases in human. The nutritional problems associated with persistent diarrhea seem to be more severe and less easily managed than those accompanying acute diarrhea. Giardiasis appears to be an interesting disease model to study the mechanisms by which zinc deficiency might contribute to pathogenesis of diarrhea or the success achieved in diarrhea control and treatment by zinc supplementation. Further studies are in progress to confirm the benefit of zinc supplementation during the acute phase of the disease. Understanding the mechanisms by which zinc contributes to the outcome of the encounter between an individual and an infectious agent requires additional research. Such understanding could bring micronutrient research from broad supplementation programs to potentially targeted nutritional therapy in specific infectious diseases.

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Author Contributions

Humberto Astiazarán-García conceived the principal idea of the review; Gemma Iñigo-Figueroa linked the ideas and contributions from all of the authors and wrote the paper; Luis Quihui-Cota Contributed as reviewer with expertise ideas in parasitology and public health; Ivan Anduro-Corona contributed as basic research reviewer.

Conflicts of Interest

The authors declare no conflict of interest.

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CAPÍTULO II

Artículo:

Effects of dietary zinc manipulation on growth performance, zinc status and immune response during *Giardia lamblia* infection: a study in CD-1 mice

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Article

Effects of Dietary Zinc Manipulation on Growth Performance, Zinc Status and Immune Response during *Giardia lamblia* Infection: A Study in CD-1 Mice

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Abstract: Associations between *Giardia lamblia* infection and low serum concentrations of zinc have been reported in young children. Interestingly, relatively few studies have examined the effects of different dietary zinc levels on the parasite-infected host. The aims of this study were to compare the growth performance and zinc status in response to varying levels of dietary zinc and to measure the antibody-mediated response of mice during *G. lamblia* infection. Male CD-1 mice were fed using 1 of 4 experimental diets: adequate-zinc (ZnA), low-zinc (ZnL), high-zinc (ZnH) and supplemented-zinc (ZnS) diet containing 30, 10, 223 and 1383 mg Zn/kg respectively. After a 10 days feeding period, mice were inoculated orally with 5×10^6 *G. lamblia* trophozoites and were maintained on the assigned diet during the course of infection (30 days). *Giardia*-free mice fed ZnL diets were able to attain normal growth and antibody-mediated response. *Giardia*-infected mice fed ZnL and ZnA diets presented a significant growth retardation compared to non-infected controls. Zinc supplementation avoided this weight loss during *G. lamblia* infection and up-regulated the host's humoral immune response by improving the production of specific antibodies. Clinical outcomes of zinc supplementation during giardiasis included significant weight gain, higher anti-*G. lamblia* IgG antibodies and improved serum zinc levels despite the ongoing infection. A maximum growth rate and antibody-mediated response were attained in mice fed ZnH diet. No further increases in body weight, zinc status and humoral immune capacity were noted by feeding higher zinc levels (ZnS) than the ZnH diet. These findings probably reflect biological effect of zinc that could be of public health importance in endemic areas of infection.

Keywords: zinc; *Giardia lamblia*; giardiasis; micronutrients; immune response; IgG; mice

1. Introduction

Nutrition and infection have been linked for centuries and a considerable amount of research has recently been focused on specific nutritional deficiencies [1]. There are links between micronutrient deficiencies and immune impairment [2]. This evidence is strongest for the trace element Zinc (Zn). Zn deficiency could be important for susceptibility to infections, since it is essential for numerous immune functions (Reviewed in [3]). Both epidemiological and clinical experiences indicate an important role of zinc in immunologically mediated host defense [4]. Although nutritional deficiencies are often associated with inadequate food intake and poor dietary quality, many studies have shown that other factors such as intestinal parasites also play an important role as predictors of such deficiencies [5].

A consistent change in level of zinc in the blood of children infected with *G. lamblia* has been noted by some investigators [6–11]. A recent study in Peru showed high risk of *Giardia* infection in children aged 2, with 4–8 episodes per year in endemic areas which caused alterations in the absorption of metals, especially Zn [9]. This data has been supported by other authors who have also reported decreased serum Zn levels during giardiasis [6–11]. On the other hand, eradication of *G. lamblia* led to a significant improvement in the mean serum Zn levels six months after treatment in schoolchildren from northwestern Mexico [12]. The above-mentioned results show association between giardiasis and zinc levels in human hosts.

This intestinal parasite causes a generally self-limited clinical illness characterized by diarrhea, abdominal cramps, bloating, weight loss, and malabsorption. However, asymptomatic giardiasis with high reinfection rates occurs frequently, especially in developing countries for reasons that remain obscure [13, 14]. The study of recurring infectious diseases is a powerful investigative tool; as a rule, the occurrence of a recurrent intestinal infection by *Giardia lamblia*, is a condition that warrants consideration of humoral immune deficiency [15], and a failure to improve may reflect a failure to correct an undefined specific nutrient deficiency, for example, the need for adequate zinc repletion.

Mild to moderate Zn deficiency is now known to occur among children and adults of many countries and is thought to be an important public health problem; its global prevalence was estimated to be 31% in 2004, whereas rates ranged from 4% up to 73% in developing countries [16]. On the other side, giardiasis is endemic in many developing countries, where infection prevalence varies from 20% to 30% [13] and up to 90% of children between the ages 2–4 can become infected at least once.

Moderate zinc deficiency and giardiasis have a strikingly similar geographical distribution and the same people may be experiencing both insults together for a

considerable time of their lives. Interestingly, no studies have examined the effects of different dietary zinc levels on the parasite-infected host. Because the immune response elicited to infectious agents normally includes many redundancies, the ultimate consequence of zinc deficiency or supplementation in controlling infection needs to be established in an infected host. Based on all these considerations, the aims of this study were to elucidate whether giardiasis remains a risk factor for zinc deficiency regardless of the level of dietary intake, how this would affect growth performance, and the way the immune system responds to this parasite and shapes the eventual adaptive response according to the dietary zinc level.

2. Experimental Section

2.1. Mice, Diets and Study Design

An *in vivo* feeding trial was conducted accordingly to the protocol presented in Figure 1 in order to examine the effect of different dietary zinc levels on the growth performance, zinc status and immune response in mice during experimental *Giardia lamblia* infection. Young (6–8 week old) CD-1 male mice were obtained from a colony maintained by the Animal Resource Centre at Universidad de Sonora. Mice were housed in stainless steel cages at a temperature (25 ± 2 °C), humidity (50%–60%) and lightning (lights on from 8:00 to 20:00 h) controlled environment and randomly assigned to either a low-zinc (ZnL, n = 20), adequate-zinc (ZnA, n = 20), high-zinc (ZnH, n = 20) or supplemented-zinc (ZnS, n = 20) diet. All diets were prepared based on a modified AIN93G rodent diet [17] with additional zinc as zinc gluconate according to experiment needs (see diet formulations in Table 1).

Figure 1. Experimental protocol used in the present study.

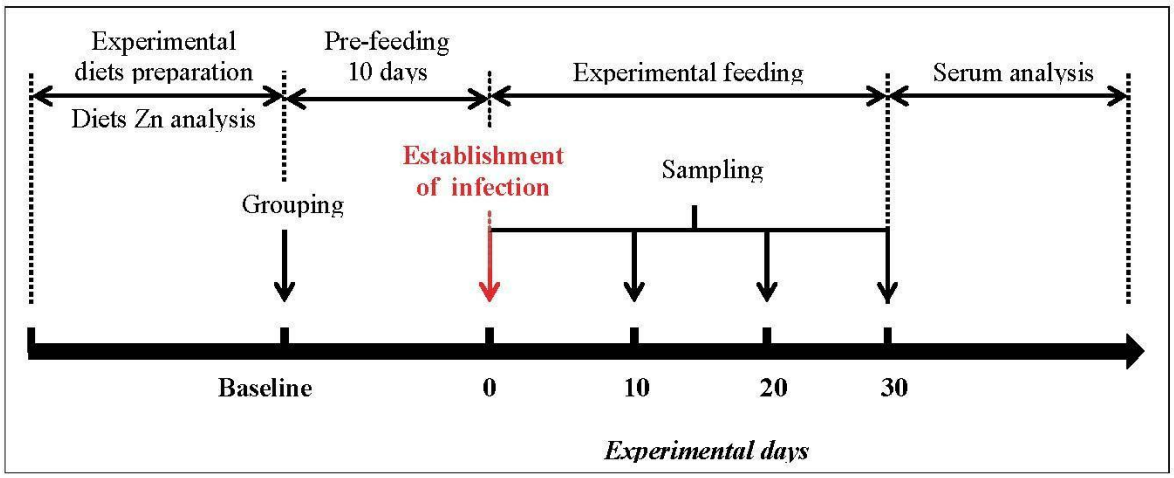


Table 1. Composition of experimental diets.

Ingredient	g/kg diet
Corn starch	653
Casein	200
Corn oil	50
Cellulose	50
Vitamin mix [1]	10
Mineral mix [2]	35
Zinc gluconate	*
Choline bitartrate	2

All ingredients purchased from Dyets Inc.; [1] AIN-76-A rodent vitamin mix; [2] AIN-93-G mineral mix without zinc carbonate; * 0, 0.03, 0.25 and 3.0 g/kg for the ZnL, ZnA, ZnH and ZnS diets respectively.

Mice were fed *ad libitum* the assigned diets for 10 days to accommodate the experimental feeding system. After this adjustment period, half of the mice in each diet group (n = 10) were exposed to peroral inoculation of *G. lamblia* trophozoites, while the remaining mice (n = 10) were mock infected. The assigned feeding program was followed for the next 30 days post-infection, paying special care with the feed and water to ensure no other sources of infection were introduced to these animals throughout the course of the study.

Body weight was recorded at baseline and then after once a week until the end of the experiment using a precision electronic balance (OHAUS 7124331499). Blood sampling of mice began prior to allocation of dietary treatment and *G. lamblia* infection (B = baseline), and was performed on day 0, 10, 20 and 30 post-infection (Figure 1). Mice were bled from the tail vein and serum was recovered and stored at −20 °C until analysis. The experiments were performed in compliance with the guidelines of the Institutional Animal Care and Use Committee [18].

2.2 *Establishment of Giardia lamblia Infection*

G. lamblia trophozoites (clone GS/M-83-H7) were obtained from the American Type Culture collection (ATCC 50581). Axenic *G. lamblia* cultures were maintained in TYI-S-33 medium supplemented with 7.0 mL of 10% bovine serum (Bovine Adult SERUM, SIGMA B2771, St. Louis, MO, USA) using a Purifier Class II Biosafety Cabinet (Delta Series, LABCONCO, Kansas City, MO, USA). For experimental inoculation, actively growing trophozoites (48–72 h old cultures) were harvested by being chilled in ice for

20 min. Trophozoites were washed with PBS at pH 7.2 (GIBCO) by 10 min centrifugation (800× *g*) at 4 °C and were resuspended in 500 µL of PBS. Dilutions were prepared with PBS and 0.4% trypan blue (Sigma, Co., St. Louis, MO, USA) to obtain a suspension of 5×10^6 trophozoites in 200 µL. Before inoculation, a 6–9 h fasting period with no water restraint was required to facilitate infection procedure. The trophozoites were inoculated directly into the mice's duodenum using a syringe fitted with a cannula needle to prevent tissue damage [19].

2.3 Diet and Serum Analysis for Zinc Content

All diets were analyzed for zinc content by atomic absorption spectrophotometry (Varian-Spectra AA-20) according to the AOAC 968.08 official method [20]. Before measurement, triplicate samples of each diet were digested with concentrated HNO_3 (Fisher Scientific, TM grade, Pittsburgh, PA, USA) in a microwave digestor (MDS 2000, CEM Corp., Matthews, NC, USA). The determination of the serum zinc levels was developed according to D'Haese *et al.* [21]. The concentration of the final solutions was measured at 213.9 nm using a hollow cathode zinc lamp. Quality control was monitored using bovine liver standard reference material 1577b (US Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD, USA) and NIST standard reference material 1577b (US Department of Commerce, National Institute, Gaithersburg, MD, USA). Zinc standards, prepared from a reference solution (Fisher Scientific, Pittsburgh, PA, USA) in 5% nitric acid, were used as internal control. All analyses were conducted in acid-washed glassware.

2.4. Immunoglobulin ELISA

To evaluate serum anti-*G. lamblia* IgG production of infected mice, an ELISA was carried out using standard techniques. Briefly, 96 well plates (Corning) were coated overnight with 50 µL (2.5 µg) of soluble *G. lamblia* antigen in 0.1 M sodium bicarbonate buffer pH 9.6. Soluble *G. lamblia* trophozoite antigens were obtained by using the method described by Gottstein *et al*. [22] with slight modifications [23]. Briefly, *G. lamblia* trophozoites from confluent cultures were harvested during log-phase by chilling on ice for 30 min. One hundred million trophozoites were washed three times with sterile phosphate buffer saline (PBS), resuspended in 1.5 mL of PBS, frozen (liquid nitrogen) and thawed (room temperature) three times, and then sonicated (30 cycles for 2 min (Brandon sonifier 250, Shelton, CT, USA) in the presence of protease inhibitor cocktail (23 mM/L 4(2-aminoethyl) benzenesulphonyl fluoride (AEBSF)), 0.3 mM/L pepstatin A, 0.3 mM/L E-64, 2 mM/L bestatin, and 100 mM/L sodium EDTA (Sigma, St. Louis, MO, USA). Cell debris was removed by

centrifugation (10,000 *g* for 30 min). The protein concentration of the soluble antigen preparation was determined by the Bradford method (Bio-Rad).

After overnight incubation with soluble *G. lamblia* antigen at 4 °C, plates were washed with PBS-0.05% Tween 20 (PBST), and blocked with PBS-1% BSA for 1 h at room temperature and washed. Mouse serum samples (diluted 1:10 in PBS 1% BSA) from both infected and non-infected mice were added to triplicate wells and incubated for 1 h at room temperature. After washing with PBST, antibody binding was detected with 50 µL of HRP-conjugated goat anti-mouse IgG (1:1000 diluted in PBS 1% BSA) (Sigma, St. Louis, MO, USA). After 1 h of incubation at room temperature, the plates were washed, and developed with 1 mL ABT-S in citrate buffer with 0.03% H_2O_2. Optical density was measured at 415 nm with an ELISA reader (Benchmark Microplate Reader, Bio-Rad, Hercules, CA, USA).

2.5. Statistical Analysis

All values are given as means ± S.D. Statistical analyses were performed in the statistical software NCSS 2000 (NCSS Statistical Software, Kaysville, UT, USA) either by paired or unpaired Student's t testing, as appropriate, for analyzing two sets of data and by ANOVA if multiple groups were compared. Differences among means were analyzed using Duncan's test, with $p < 0.05$ considered as significant.

3. Results

3.1. Zinc Content of Experimental Diets

All diets were analyzed for zinc content by atomic absorption flame spectrophotometry. The results of the zinc analyses of the four diets indicated the following levels, in mg/kg: 10, 30, 223 and 1383 for the ZnL, ZnA, ZnH and ZnS diets respectively.

3.2. Growth Performance as Affected by Dietary Zinc and Infection

Mice in each group were fed with 1 of the 4 experimental diets for 10 days before exposure to *G. lamblia* and during the course of the infection (30 days). The effect of dietary zinc level and infection on body weight is presented on Table 2 where ZnL, ZnA, ZnH and ZnS mice received diets containing 10, 30, 223 and 1383 mg of Zn/kg of diet respectively.

Table 2. The effect of dietary zinc and infection on body weight (BW) (g) at day 0 and 30 p.i.

	Giardia-free					*Giardia*-infected				
Diet	***N***	**Initial BW**	**Final BW**	**Gain**	***p* ***	***N***	**Initial BW**	**Final BW**	**Gain**	***p* ***
ZnL	9	34.9 ± 2.1	38.5 ± 1.9 b	3.6	< 0.001	10	35.0 ± 2.0	33.9 ± 1.6 a	−1.2	0.214
ZnA	10	35.8 ± 3.0	39.2 ± 2.8 b	3.4	< 0.001	10	34.2 ± 2.6	34.5 ± 2.9 a	0.3	0.182
ZnH	10	32.5 ± 1.5	43.6 ± 2.6 c	11.1	< 0.001	10	33.0 ± 2.1	42.8 ± 2.6 c	9.6	< 0.001
ZnS	8	33.3 ± 1.9	43.1 ± 2.9 c	9.8	0.008	8	34.0 ± 1.7	43.1 ± 2.2 c	9.1	0.002

ZnL = low-zinc, ZnA = adequate-zinc, ZnH = high-zinc, ZnS = supplemented-zinc; Values are expressed as mean ± S.D.; * Paired *t* test, Initial BW *vs*. Final BW, significance at $p < 0.05$; ^{a,b,c} Different superscript letters among initial or among final weights indicate significant difference between means, $p < 0.05$.

On the day of the establishment of infection no significant difference was found between the mean weights of the mice from the different groups ($p = 0.09$). At the end of the 30-day period continual weight gain was seen for *Giardia*-free mice in all four dietary groups; in addition, mice fed ZnH and ZnS diets weighed significantly ($p < 0.0001$) more than mice fed the ZnL or ZnA ones (43.6 ± 2.3 g and 43.1 ± 2.9 g *vs*. 38.5 ± 1.9 g and 39.2 ± 2.8 g). On the other hand, *Giardia*-infected mice consuming ZnL or ZnA diets essentially failed to grow as demonstrated by the fact that weights remained unchanged ($p = 0.214$ and $p = 0.182$ for the ZnL and ZnA diets, respectively) after the 30-day period, but there was a significant weight gain in infected mice which received ZnH or ZnS diets during the same period of time (33.0 ± 2.1 g *vs*. 42.8 ± 2.6 g and 34.0 ± 1.7 g *vs*. 43.1 ± 2.2 g for the ZnH and ZnS respectively). This weight improvement was similar to that of the mice not exposed to the infection (9.6 g *vs*. 11.1 g and 9.1 g *vs*. 9.8 g for the ZnH and ZnS infected and non-infected mice respectively).

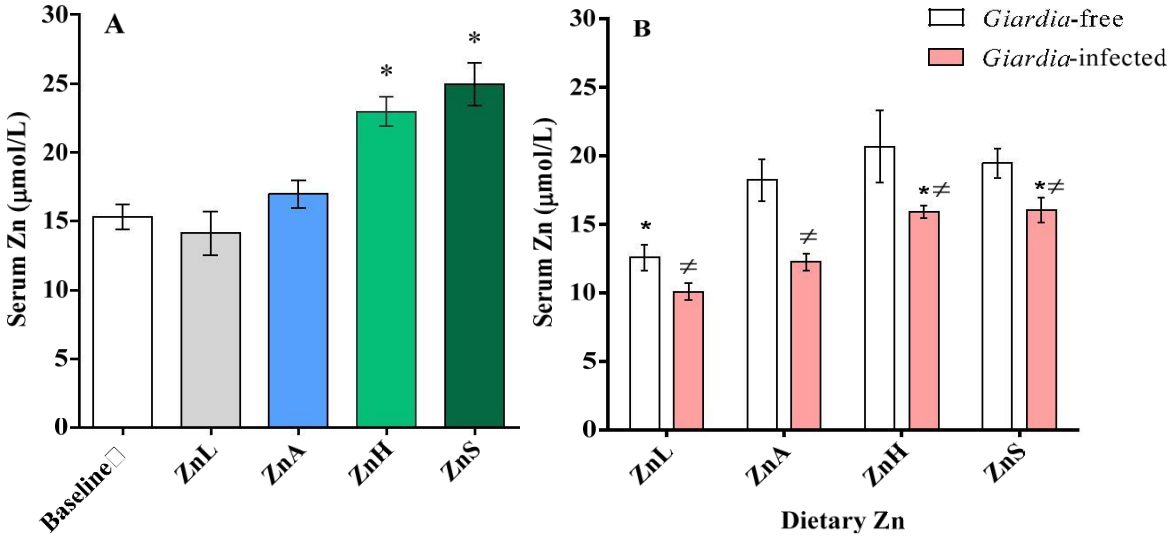
3.3. Serum Zinc Changes Associated with Dietary Zinc Level and Infection

Both dietary zinc and infection affected zinc status as assessed by the serum zinc concentration (Figure 2). After 10 day on the feeding regimen, serum zinc concentration was increased in mice fed ZnH and ZnS diets ($p < 0.05$), whereas for ZnL and ZnA diets serum zinc level maintained similar to the baseline values (14.1 ± 1.6 µmol/L and 16.8 ± 1.0 µmol/L *vs*. 15.3 ± 0.9 µmol/L, respectively, $p = 0.09$) (Figure 2A). Moreover, although a large increase in dietary zinc is observed from the ZnH (223 mg Zn/kg) to the ZnS (1383 mg Zn/kg) diet, serum zinc did not statistically differ between these groups (22.9 ± 0.6 µmol/L and 24.9 ± 1.5 µmol/L, respectively, $p = 0.13$). As expected, infected mice had lower serum zinc levels than non-infected mice in all dietary groups ($p = 0.01$) (Figure 2B). However, zinc concentration of *Giardia*-infected mice consuming ZnH or ZnS diets were significantly higher than infected mice fed ZnA diets (15.89 ± 0.46 µmol/L and 16.04 ± 0.92 µmol/L *vs*. 12.24 ± 0.61 µmol/L), and similar to the zinc level of control mice (*Giardia*-free ZnA, 18.21 ± 1.53 µmol/L).

3.4. Immune Response as Affected by Dietary Zinc

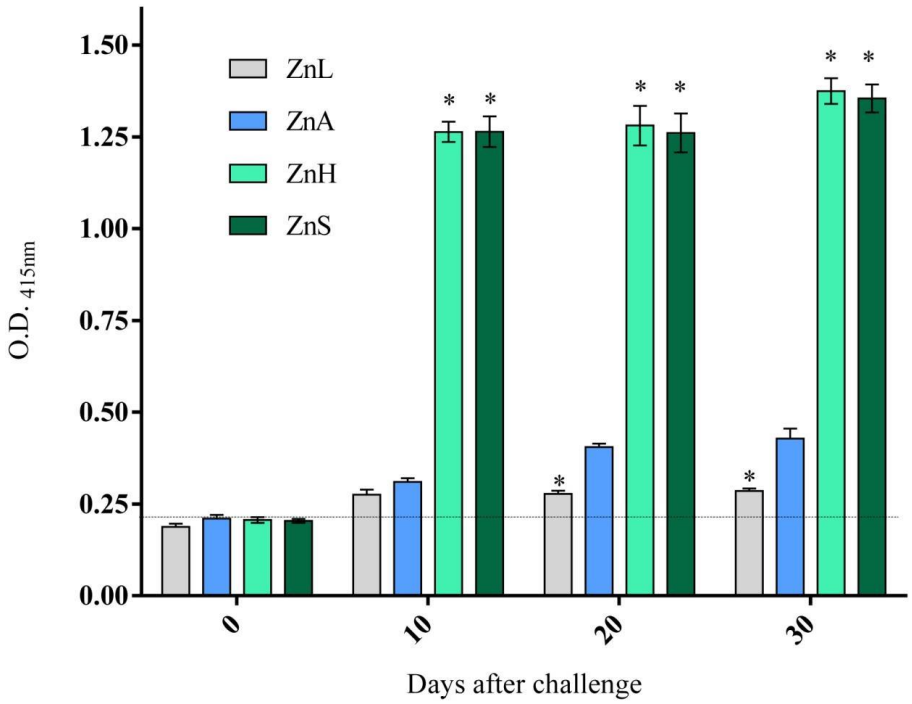
To determine the effect of dietary zinc level on immune function, we measured the anti-*G. lamblia* IgG response during the course of primary infection. Antibody response was evaluated at day 0, 10, 20 and 30 p.i. None of the serum samples obtained from uninfected animals had significant levels of antibodies against *G. lamblia* antigens (Data not shown). Figure 3 shows that systemic anti-*G. lamblia* antibody responses became evident at day 10 p.i. in all dietary groups. Nevertheless, ZnH and ZnS mice had notably

Figure 2. Effects of dietary zinc and infection on serum Zn concentration. Zinc levels in serum of mice following a 10-day feeding period (**A**) and of *Giardia*-free and *Giardia*-infected CD-1 mice following an additional 30-day feeding period p.i. (**B**) with diets containing 10 mg Zn/kg (ZnL), 30 mg Zn/kg (ZnA), 223 mg Zn/kg (ZnH) and 1383 mg Zn/kg (ZnS). Values are expressed as means ± S.D. * Significantly different from ZnA mice (2A) or significantly different from its respective ZnA-non-infected or ZnA-infected control mice (2B), $p < 0.05$. ≠ Significantly different from non-infected dietary control, $p < 0.05$.



higher mean levels of *G. lamblia*-specific IgG than ZnA mice (1.263 and 1.281 *vs*. 0.376 optical density units; $p = 0.004$). Notably, low zinc diet had little effect on IgG production, since the antibody production of this experimental group was no different from the production of the ZnA group at this time point. However, there was no further increase from day 10 p.i. and IgG levels maintained the same throughout the infection period for this dietary group. On the other hand, IgG values progressively increased throughout the 30-day period for the ZnA, ZnH and ZnS groups, even though differences did not reach statistical significance.

Figure 3. Time-course of parasite-specific IgG antibody response in CD-1 mice after oral immunization with 5×10^6 *G. lamblia* trophozoites. The horizontal dotted line indicates the mean optical density plus two fold standard deviation at 415 nm (O.D. 415 nm) for the negative control and the group of non-infected mice, which represents the cutoff point for positivity of the ELISA. Results are expressed as means ± S.D. from three measurements, each containing pooled sera from 8 to 10 mice.
* Significantly different from ZnA mice at each time-point, $p < 0.05$.



4. Discussion

The CD-1 mouse was used as a mode1 of giardiasis to investigate the effects of dietary zinc intake on growth performance, zinc status and immune response during infection. Zinc is a metal with great nutritional importance and is particularly necessary in cell replication and the development of the immune response [3]. Even though zinc deficiencies are characteristically known to initiate anorexia and delay growth in animal models and humans [24, 25], in our study body weight gain was not affected by low zinc diet; *Giardia*-free mice fed ZnL or ZnA diets presented a normal growth rate during the experimental 30 day period according to growth data from CD-1 mice suppliers (Charles River Laboratories, San Diego, CA, USA and Harlan Laboratories, Wilmington, MA, USA). On the other hand, mice fed ZnH and ZnS diets manifested rapid growth, with an approximate 30% increase in body weight compared to the 10% increase in mice fed ZnA diet. The explanation for the profound impact of zinc supplemented diets on mouse growth could be attributed to the fact that zinc participates in the regulation of cell proliferation; it is essential for enzyme systems that influence cell division and it also has a role in hormonal regulation of cell division [26]. Dietary zinc supplementation (ZnH and ZnS diets) was possibly effective in improving the body zinc status and the secretory levels/potentiation of growth hormones, thus increasing growth velocity.

Weight loss or reduced weight gain has been associated with giardiasis. In our study weight gain was significantly affected by the *G. lamblia* challenge in mice fed ZnL or ZnA diets, where *Giardia*-free mice had greater weight gain than *Giardia*-infected animals. This is consistent with data from Barthold [27], which reports that although mice infected with *Giardia* are usually asymptomatic, impairment of weight gain is the most common sign of infection. Even though the mice in our study only showed moderate growth retardation, severe weight loss has been reported in lambs infected with *G. lamblia* [28], with similar effects of human giardiasis on body weight [29]. There is increasing evidence to suggest that infection with *Giardia* leads to the development of chronic disorders in the gastrointestinal tract. This impairment of weight gain may be due to nutrient malabsorption, which has been reported in rodent models [19]. The pathophysiological mechanisms that occur during *G. lamblia* infection are not completely understood. Evidence indicates that, shortly after colonization of the small intestinal lumen, *Giardia* trophozoites heighten the rates of enterocyte apoptosis, decrease intestinal barrier function, and these changes lead to diffuse shortening of small intestinal brush border microvilli, maldigestion, and malabsorption, via the activation of CD8+ T-lymphocytes [30].

Animals fed the ZnH and ZnS diets had a higher weight gain than mice fed ZnL and ZnA diets, regardless of whether the animals were infected or not. In this experimental

model, dietary zinc level appeared to be more important in regard to growth performance than *Giardia*-infection. Therefore, the growth retardation observed in the ZnL and ZnA *Giardia*-infected groups may have resulted from an interaction of the dietary treatment and the infection, as infection did not significantly affected body weight gain in the ZnH and ZnS groups.

To establish the influence of our diets and *Giardia* infection on zinc status, serum zinc determinations were taken following a 10-day feeding period before the establishment of *Giardia* infection and then 30 day post-infection. The consumption of a ZnL diet for 10 days had no effect on serum zinc level, as mice fed this diet maintained their baseline values, which were similar to that of ZnA mice. Given that mice were at maintenance and that the level of Zn restriction was moderate for a relatively short period, this response is not surprising. However, the consumption of ZnH and ZnS diets resulted in a significant 50%–60% increase in serum zinc levels (Figure 2A). Despite a large increase in dietary zinc is observed from ZnH to ZnS diet, serum zinc did not statistically differ between these groups.

Adjustments in gastrointestinal zinc absorption and intestinal endogenous zinc excretion are the primary means by which the body maintains constant tissue levels of zinc with varying intakes [31]. Zinc transporter systems in enterocytes ZIP4 and ZnT1 [32] respond appropriately to dietary zinc availability and are responsible for a saturable, energy-dependent and regulated uptake of zinc [33]. Studies in experimental rats demonstrate a capacity to maintain a relatively constant content of zinc in the whole body while dietary zinc intakes vary by as much as 10-fold [34]. When the zinc intakes of weanling experimental animals ranged from 10 to 100 mg/kg, the zinc content on the whole remained constant. According to Kirchgessner [34], changes in the concentration of zinc in the whole body present only when very low (<10 mg/kg) or very high (>100 mg/kg) intakes were consumed.

Regarding the influence of *Giardia* infection on zinc status, a consistent change in the level of zinc in the blood of children infected with *G. lamblia* has been reported by some authors [6–11]. In our study, *Giardia*-infected mice in all dietary groups had lower serum zinc levels than non-infected mice after 30 days p.i. This is in agreement with other authors whose studies showed that giardiasis may be a risk factor for zinc deficiency in mice regardless of the dietary intake [35]. However, the “extra” zinc provided by the ZnH and ZnS diets helped maintaining the zinc levels near those of the ZnA *Giardia*-free mice. Interestingly, a study by Jendryczko *et al*. [6] shows that in children infested with *G. lamblia* occurs a decrease of zinc absorption in the gastrointestinal tract which causes zinc deficiencies in those children; when compared with non-infected children, mean concentrations of serum zinc carriers, total protein, albumin fraction, transferrin and picolinic acid—a zinc absorption factor in the

gastrointestinal tract—were not differing between both studied groups of children. These shows that disturbances might be occurring in the zinc metabolism of infected children and these modifications could be independent of the acute phase response. Therefore, interventions to improve children's zinc nurture should be considered in populations at risk of zinc deficiency, especially in endemic areas where high reinfection rates of giardiasis are present.

Most infections with *Giardia* are controlled by the host within a few weeks, suggesting the ability of the immune system to control the infection. The high rate of proliferation and differentiation of immune cells requires constant supply of sufficient amounts of zinc. We investigated whether dietary zinc levels adequate for growth would also produce normal response to an infection. Several studies suggest an important role for B cells in clearing *Giardia* infection [36]. B cells differentiate into immunoglobulin secreting plasma cells and hereby induce humoral immunity. For example, *Giardia* infection in humans and mice induce the production of antigiardial antibodies of the immunoglobulin A (IgA), IgM, and IgG isotypes in mucosal secretions and serum, and this specific antibody production correlates with the clearance of infection [37–41]. Such antibodies reach their targets *in vivo*, since antigiardial IgA and IgG antibodies coat trophozoites in infected mice [37]. It has been shown that zinc deficiency affect B cell lymphopoiesis and also leads to a reduction in antibody-mediated immune defense [42]. However, in our experiment, the consumption of a ZnL diet for a 30 day period had no effect on the capacity of the mice to respond to *G. lamblia* since anti-*G. lamblia* IgG production was comparable to that of the ZnA group. Thus, although these mice consumed a diet with only 30% the amount of zinc in the ZnA diet, it was not sufficient to interfere with their response to *G. lamblia*. This suggests that activated B-cells were able to proliferate and produce antibody in spite of the 30 day period of zinc restriction. These data supports the notion that the Zn deficiency these animals experienced was only moderate, and that the discussed alteration of gastrointestinal physiology giving improved recovery of zinc via transport mechanisms, may have been sufficient to support B-cells proliferation reasonably well in ZnL mice. An extension of the experimental time period might have resulted in a greater loss of body weight and a reduction in immunity in the ZnL mice, as suggested by results of King *et al*. [43], where the thymus of chronic zinc deficient mice were unchanged at day 34, but by day 45 an alteration in lymphopoiesis was observed. Under our experimental conditions, the consumption of a ZnL diet had no quantitative effect on the humoral immune capacity, whereas zinc supplementation led animals to an enhanced immune response, which can be seen through the higher levels of specific IgG antibodies in ZnH and ZnS mice. Our data points in the direction that the zinc concentration used in this experiment seem to have a profound positive effect on humoral immune response. Although zinc is generally

regarded as a non-toxic essential metal of particular importance to the immune system, overdosing zinc supplementation can also have a negative impact on immune efficiency [44, 45].

Zinc toxicity in rats or mice has not been clearly defined. Studies have indicated that under certain circumstances dietary zinc concentration in excess of 250 mg Zn/kg diet leads to symptoms of toxicity, whereas a more generally recognized toxic concentration is 5000 mg Zn/kg diet [46]. Rather than being a toxic metal ion, zinc is an essential trace element and the human body has efficient mechanisms, to maintain homeostasis over a broad exposure range; consequently, a severe impact on human health by intoxication with zinc is a relatively infrequent event [47].

Results of the present study demonstrate that zinc supplementation can influence the development of adaptive humoral immune response. However, before making recommendations for supplementation, issues of dose and safety need to be addressed. Zinc supplementation with high amounts of Zn (223 and 1383 mg Zn/kg of diet) did not seem to compromise growth performance or specific IgG production in mice. Contrary, there was a higher weight gain and a boosted immune response when compared to diets containing adequate amounts of zinc.

5. Conclusions

Taken together, our results showed that *Giardia*-free mice fed a ZnL diet during a 30-day experimental period were able to attain normal growth and antibody-mediated response. *Giardia*-infected mice fed ZnL and ZnA diets present a significant growth retardation compared to non-infected diet controls. Zinc supplementation can avoid this weight loss during *G. lamblia* infection and may be of considerable benefit in improving humoral specific immune response. Clinical outcomes of zinc supplementation during giardiasis include significant weight gain and improved serum Zn levels despite the ongoing of infection. Also, a maximum growth rate and antibody-mediated response were reached in mice fed ZnH diet. No further increases in body weight, zinc status and humoral immune capacity were noted by feeding higher zinc levels (ZnS diet). If the key mechanisms by which zinc exerts its regulatory effect on growth performance and immune response can be identified, successful strategies for preventing/treating this infection may be implemented in the future.

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Conflicts of Interest

The authors declare no conflicts of interest.

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CAPÍTULO III

Artículo:

Growth inhibition, morphological and ultrastructural alterations induced by zinc on *Giardia duodenalis* trophozoites

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Growth inhibition, morphological and ultrastructural alterations induced by zinc on *Giardia duodenalis* trophozoites

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Abstract Antimicrobial effects of zinc (Zn) have been reported for several pathogenic enteric bacterial common in childhood diarrhea. Since giardiosis is a common cause of acute-chronic diarrheal illness worldwide, this study investigated the *in vitro* effect of Zn on growth and morphology of the pathogen protozoa gastrointestinal parasite *Giardia duodenalis* (*G. duodenalis*). An inoculum of 10^4 *G. duodenalis* GS/M-83-H7 trophozoites was used. Zinc-gluconate (ZG) in increasing concentrations (0 – 7.0 mM) was added to TYI-S-33 growth medium and the remaining trophozoites after 48 h exposure were counted. Growth, viability and morphological changes of trophozoites after treatment were analyzed by light and electron microscopy. Our data showed cell growth inhibition of *Giardia* trophozoites *in vitro* by ZG in all tested concentrations. At 4.9 – 7.0 mM ZG concentrations reduced up to 90-95% the *G. duodenalis* growth when compared to controls ($P<0.001$). Inspection of trophozoites by scanning electron

microscopy revealed deformation of the typically pear-shaped body, with irregularly shaped rough-porous ventral and dorsal surface and content spill out. Transmission electron microscopy images showed formation of extensive empty areas in the cytoplasm and accumulations of electrodense granular material; the adhesive disk was frequently found displaced and flagella emergence was altered with extensive loss. In conclusion, increased ZG concentrations induced morphological alterations and inhibited the *G. duodenalis* trophozoites growth *in vitro*. Based on this, it is worthwhile to explore how zinc status can influence the colonization and pathogenicity of *Giardia in vivo*.

Keywords *Giardia duodenalis*, zinc gluconate, anti-*Giardia*, in vitro, *Giardia* SEM, *Giardia* TEM

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Introduction

Diarrhea caused by enteric infections is a serious threat all over the world with great economic implications especially in developing countries (Black et al. 2003). Infectious diarrhea may be associated with bacteria, viruses and parasites (Hodges and Gill 2010). These organisms are easily transmitted through food or water or from person to person, being foodborne pathogen invasion a recurrent health problem. Some of these agents are of health concern especially in individuals with compromised immune systems or structural abnormalities of the gastrointestinal tract (Guerrant et al. 2001). Although oral rehydration solutions save lives of children with diarrhea, current rates of morbidity by infectious diarrhea remain a major public health problem (Wardlaw et al. 2010).

Currently, different studies support the effectiveness of zinc (Zn^{2+}) in the treatment of acute and persistent diarrhea and its prophylaxis. Zinc is an essential trace element for all forms of life (Kaur et al. 2014) and plays fundamental roles in different physiological processes; in vital cellular reactions, acting as a cofactor during catalytic and structural activities. Its significance to health is increasingly appreciated as its deficiency may play an important role in the appearance of diseases (Black 2003; Chasapis et al. 2012). Zinc supplementation has been associated with clinical reduction in duration and severity of diarrheal episodes and likelihood of subsequent infections for 2-3 months in infants and young children (Bhutta et al. 2000; Lukacik et al. 2008); probable mechanisms for its antidiarrheal effect include improved absorption through better intestinal transport of water and electrolytes, faster regeneration of gut epithelium and restoration of its function, increased levels of enterocyte brush border enzymes, and improved immune response which helps in clearing the pathogens from gut during diarrhea (Jones and Peters 1981; Gebhard et al. 1983; Ghishan 1984; Moran and Lewis 1985; Fenwick et al. 1990; Roy et al. 1992; Shankar and Prasad 1998; Cario et al. 2000; Iñigo et al. 2013). However, evidence suggests that the beneficial effect of zinc may not be equivalent against the common causative organisms (Patel et al. 2010). Consequently it is possible that the influence of zinc supplementation on diarrhea may be dependent on the organisms present in the gut. However, the anti-diarrheal mechanism of zinc remains poorly understood.

Zn salts have shown antibacterial activity against both Gram-positive and Gram-negative bacteria involved in childhood diarrhea such as *Escherichia coli* (EPEC), *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonellae*, *Shigellae* and *Vibrio cholera* (Atmaca et al. 1998; Surjawidjaja et al. 2004; Jones et al. 2008; Faiz et al. 2011; Pasquet et al. 2014); anyhow, information about the antiparasitic activity of zinc is not available.

According to the Center for Disease Control and Prevention (CDC), parasites are the pathogens most commonly isolated from patients with persistent diarrhea (Connor 2013). For example, *Giardia duodenalis* (*G. duodenalis*) has a slow onset of diarrhea

and can persist for months, while most bacterial and viral infections are limited from 1 to 2 weeks (Hodges and Gill 2010). This parasite is a common parasitic infection in children and common cause of diarrhea in travelers presenting to clinics (Muhsen and Levine 2012; Ross et al. 2013; Bartelt and Sartor 2015). Trophozoites are the active form of this parasite and they colonize the upper small intestine by adhering to the apical surface of the epithelium (Muller and von Allmen 2005).

Treatment of giardiosis is still a challenge since most of the anti-*Giardia* drugs show a high incidence of undesirable side-effects and treatment failure rates up to 20% (Gardner and Hill 2001; Lalle 2010). Limitations of current drugs emphasize the requirement of alternative, efficient and well-tolerated therapeutics. Long et al. (2007) investigated the effect of micronutrient supplementation on gastrointestinal parasitic infections among Mexican children.

Zn supplementation was associated with distinct parasite-specific outcomes, including reduced both incidence and duration of *Giardia* infections. Zn is being efficiently used in various forms like Zn-sulphate (ZS), Zn-gluconate (ZG) and Zn-acetate among supplementation trials for the treatment of diarrhea (WHO 2006; Scrimgeour and Lukaski 2008). Nevertheless, supplementation trials have shown a significant homogeneous reduction in diarrheal duration using ZG compared to other Zn-salts (Dutta et al. 2000; Bahl et al. 2002; Patel et al. 2010). Therefore, the current study was designed to investigate the inhibitory effect of ZG on *G. duodenalis* growth *in vitro*, and the morphological and structural changes of *Giardia* trophozoites associated to this effect.

Materials and Methods

Culture of G. duodenalis Trophozoites

G. duodenalis (GS/M-83-H7) was obtained from the American Type Collection (ATCC 50581). Trophozoites were maintained in axenic culture at 37°C in 10 ml TYI-S-33 medium supplemented with 10% newborn calf serum (NBCS; Gibco) with antibiotics (ceftriaxone) as modified by Keister (1983), in screw-cap cell culture vials. Log-phase cultures (48–72 h) were harvested by cooling culture vials (4°C/15 min) and centrifuged (1,500×g/5 min). *G. duodenalis* trophozoites were washed and then counted in a haemocytometer (Neubaeur cell-counter chamber). These cells were used as inoculum to study the effects of zinc on its growth, viability and morphology.

Growth Inhibition Assay

To investigate the effects of Zn^{2+} on *G. duodenalis* development, growth inhibition assays were performed in the presence or absence of Zn. Susceptibility of *G. duodenalis* growth to Zn *in vitro* was determined. Stock solution of Zn-gluconate (ZG)

was prepared by dissolving $C_{12}H_{22}O_{14}Zn$ in distilled water. The solution was sterilized by filtration, using a millipore filter. To obtain a ZG-working solution [20 mg/ml], a further dilution of the stock solution was made in TYI-S-33 medium. ZG-working solution was diluted in 2.0 ml screw-cap cell culture vials containing TYI-S-33 medium in order to get a range of concentrations from 0 to 7.0 mM (Table 1). Control tubes with no Zn were also prepared. Cultures of log-phase trophozoites (5×10^4) were exposed to increasing Zn concentrations in the culture medium and the remaining trophozoites after an incubation period of 48h/37°C were counted. The detachment of trophozoites was carried out at 4°C/15 min, and the suspension centrifuged at 1200 ×g /10 min. Trophozoites were washed three times in cold PBS and the cell pellets were re-suspended. The total number of cells was determined by using a hemocytometer, counting under light microscope (VE-B6 Velab). These results were expressed as the percentage of proliferation of the control (not treated).

Electron Microscopy

Cultures of treated and untreated *Giardia* trophozoites were prepared for scanning (SEM) and transmission (TEM) electron microscopy to reveal the overall morphology of the trophozoites and ultrastructural elements.

Transmission Electron Microscopy After the incubation period with ZG, cultured trophozoites were chilled on ice for 15 min, centrifuged (200×g/10 min), washed three times with PBS, and fixed with 2.5 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 1 h. Then, trophozoites were post-fixed with 1 % osmium tetroxide solution for 1 h, and dehydrated through a graded ethanol series (50–100 %). Samples were embedded in polybed epoxy resins and polymerized at 60°C for 24 h. Thin sections (60nm) were contrasted with uranyl acetate and lead citrate, and examined in a JEOL JEM-1011 transmission electron microscope.

Scanning Electron Microscopy For SEM, trophozoites previously fixed in 2.5 % (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 1 h, were placed on cover glass covered with 1.5 % gelatin before CO2 critical point dried using a Tousimis Samdri 780 device. Samples were covered with gold in a JEOL JFC-1100 ionsputtering, and examined in a JSM-7100F field emission scanning electron microscope.

Data Analyses

All experiments were carried out in triplicate and in at least two independent assays. Values were expressed as mean ± standard deviation (SD) and compared by ANOVA, followed by Tukey-Kramer´s multiple comparisons. P value ≤ 0.05 was considered statistically significant.

Results

ZG inhibits the growth of Giardia trophozoites in vitro

The effects of different concentrations of Zn^{2+} were evaluated on the cell growth of log phase trophozoites after 48 h incubation. Treatment of *Giardia* trophozoites with ZG revealed a dose-dependent inhibition of parasite growth compared to the control ($P<0.001$) (Fig 1). While the parasites in the control group showed a typical growth rate, a significant reduction on parasite proliferation was observed in the ZG-treated groups at all the concentrations tested (0.7 – 7.0 mM). Briefly, 0.7 mM ZG inhibited *Giardia* growth by 51.65% ± 26.33; 1.4 mM, 64.16 ± 10.77 %; 2.1 mM, 70.97 ± 3.99 %; 2.8 mM, 78.18 ± 7.84 %; 3.5 mM, 72.47 ± 4.39 % and 4.2 mM, 86.79 ± 3.82 %. Concentrations from 4.9 to 7.0 mM, produced a >90% growth inhibition on *Giardia* when compared to non-treated controls. The highest growth inhibition was observed at 5.6 mM, with 5.0 ± 1.70 % of growth. The trophozoite counts included all cells identified as trophozoites, irrespective of viability. Therefore, the results suggested that ZG inhibited *Giardia* proliferation in vitro. Light microscopic observations of ZG-treated trophozoites after 48 h exposure revealed that ZG induced loss of viability with morphological alterations. Non-treated trophozoites showed the typical pear-shaped structure, flagellar motility and structural integrity (Fig 2a). As ZG concentration increased, the cells appeared slightly condensed in shape, acquired a rounded morphology with abundant cellular debris as well as abnormal motion, slower flagella beating and loss of motility (Fig 2b-d).

ZG leads to altered morphology of Giardia trophozoites in vitro

To evaluate the effect of Zn^{2+} on trophozoites morphology, cells were treated with increasing concentrations of ZG (0 – 7.0 mM) for 48 h. Microscopic inspection revealed diverse morphological alterations. While untreated trophozoites presented the typically pear-shaped body, ventral disk, four pairs of flagella and lateral flange (Fig 3a-c); the cell pear-shape was lost in the trophozoites exposed at increased ZG concentration (Fig 3d-o). After treatment, the surface of trophozoites was rough and some cells showed pores on the dorsal (Fig 3f, 3i, 3m) and ventral faces (Fig 3k-l); other cells showed flange membrane bending and a concave depression of the ventral region (Fig 3h); most trophozoites had an irregularly shaped ventral (Fig 3e, 3h) and dorsal (Fig 3g) surface. Incubation in 4.9 mM ZG produced evident deformation of trophozoites (Fig 3n-o), identified as rounding with severe surface damage and content spilled out (Fig 3k, 3m).

The effects induced by ZG were further analyzed by TEM (Fig 4). In the control trophozoites (Fig 4a-b), the two nuclei (n) were evident, the ventral disk formed by

microtubules associated to thin proteic bands was observed close to the plasma membrane and protruding laterally of the cell body (fl); small peripheral vesicles (pv) aligned in the cytoplasm down the plasma membrane, and the axonemes of flagella were also visible between the two nuclei and located to left and right of the cell body. After treatment with 1.4 mM ZG (Fig 4d-e) parasites exhibited cytoplasmic differences in respect of the control such as: formation of extensive empty areas in the cytoplasm (Fig 4c, arrow) and accumulations of electrodense granular material. The adhesive disk was frequently found displaced and contacting the nuclei (Fig 4d). The flagella emergence was altered and loss of them has been suggested.

Discussion

Results of the present investigation indicated that ZG inhibits the growth of *Giardia* trophozoites *in vitro*. Interestingly, growth percentage is different at different ZG concentrations. The more ZG concentration the lesser proliferation of trophozoites. At concentrations 4.9 – 7.0 mM, Zn2+ produced an inhibition of > 90% on *Giardia* growth when compared to non-treated controls. Analysis of variance (ANOVA) showed that the ZG compound has a significant effect on the growth of *Giardia* trophozoites ($P<0.001$). At present information about the effect of Zn^{2+} on the growth of gastrointestinal parasites is limited. Vega Robledo et al. (1999), observed that zinc sulfate (ZS) at 1.0 mM concentration reduced amebic replication *in vitro*. Other studies published the antibacterial effect of some salts of Zn^{2+} on enteric pathogens. Surjawidjaja et al. (2004) showed that ZS concentrations between 1.2 and 1.8 mg/ml (4.2 – 6.3 mM) inhibited the growth of enteric pathogens associated with diarrheal disease (*Salmonella typhi, Salmonella* groups A, B, C, D and E, *Escherichia coli, Enterobacter, Shigella* and *Vibrio cholera*). These concentrations agreed with those obtained by other authors for enteric bacteria. Faiz et al. (2011) also showed that ZS (Zn^{2+} 0.06 - 1.0 mg/ml, 0.9 – 7.7 mM) inhibited the growth of enteric bacterial pathogens (*Salmonellae, enteropathogenic Escherichia coli, Shigellae* and *Vibrio cholerae*), and determined the inactivity of the sulfate part in ZS, confirming that antibacterial activity against microorganisms was only due to Zn^{2+} and that the salt part was totally inactive. Other studies (Iwalokun and Bakare 2008; Crane et al. 2007) determined the in vitro antibacterial activity of ZS against certain common pathogens in childhood diarrhea and found comparable results. In this study, ZG showed the same inhibitory pattern as ZS (Data not shown).

In addition, Zn prompted modifications on the cell shape of the parasite in this study. The overall morphology and ultrastructure of *Giardia* trophozoites by SEM and TEM after ZG exposition were observed. Most trophozoites had an irregularly shaped ventral and dorsal surface; some cells became completely deformed presenting membrane undulations and holes, with severe surface damage and the content spilled out, possibly indicating loss of membrane function. TEM studies also evidenced

important ultrastructural morphological changes induced by ZG; mainly distorted cell shape, formation of extensive empty areas in the cytoplasm and altered integrity of the adhesive disk.

Trophozoites are the disease causing stage and colonize the upper small intestine. Flagella and the ventral disc are structures of trophozoites that aid attachment and adherence to intestinal epithelial cells where the parasite uses, and potentially sequesters, nutrients such as zinc in order to survive, replicate, and evade microbiota and host defenses (Bartelt and Santor 2015). Thus, motility and attachment to host cells are essential in the pathogenesis of giardiasis and alterations induced by ZG in both form and movement of trophozoites may have an impact in the establishment of the infection. Studies are needed to determine if these morphological and ultrastructural changes are related to disruption of the cell membrane activity or metabolic stress of the trophozoites, which are essential for the infectivity and outcome of giardiosis.

Zinc is an essential trace element for growth in organisms (Gielda and DiRita, 2012). Luján et al. (1995) determined that the major metals present in *Giardia* trophozoites were zinc (0.43 mM) and iron (0.80 mM). It has been described that the surface of *Giardia* trophozoites, including the adhesive disk and flagella, is covered by a protein coat composed of a single variant specific surface protein (VSP) that binds zinc *in vitro* (Nash et al. 1990, 1993; Pimenta et al. 1991; Zhang et al. 1993). The benefit of metal binding of VSPs to *Giardia* remains unknown. VSPs may function in the binding-transport-storage of zinc or other metal ions. In addition, Nash (1992) and other authors (Nash and Mowatt, 1993; Aley and Gillin, 1993; Zhang et al. 1993; Luján et al. 1995) proposed that metal coordination stabilizes VSPs, providing them resistance to proteolytic attack in the upper small intestine. Most interestingly, no other surface residing Zn-finger protein exists in other organism (Nash et al. 1991). On the basis of abundance and location of zinc binding motifs in VSPs, it is suggested that trophozoites may require large amounts of zinc for survival and colonization (Zhang et al. 1993). In such a way, the *Giardia*'s ability to bind zinc ions may play an important role in malabsorption and nutritional deficiency in heavily infected persons (Luján et al. 1995). These findings have suggested unusual ways in which the parasite may interact with its host, but whether the availability of Zn within the host could affect the parasite VSP's mechanism is not clear, especially in view of the non-specificity of metal binding by the VSPs (Zhang et al. 1993).

Levels of zinc must be tightly regulated, as too little zinc does not support cellular growth, while too much zinc exerts toxic effects on the cell, possibly through inhibition of key enzymes and competition with other metal ions (Costello et al. 1997; Dineley et al. 2003; reviewed in Plum et al. 2010). Thus, the uptake of zinc into cells and its transport into and out of intracellular organelles requires transporter proteins that span these membranes to facilitate the movement of zinc (Eide, 2006). Zn concentration is regulated under physiological conditions by several transporters so that Zn ions are essentially nontoxic to higher organisms (Liuzzi and Cousins 2004). Mechanisms to

evade zinc toxicity are also widespread in the microbial world (Rouch et al. 1995; Nies, 1999; Chodhury and Srivastava 2001). Most microbes have evolved high-affinity binding and transport systems (Gielda and DiRita, 2012).

Despite the biological importance of zinc to *Giardia* and its potential toxic effects, no information exists regarding the processes underlying zinc homeostasis in this parasite. The genome of *G. duodenalis* possesses genes that encode zinc transporter domain proteins (Morrison et al., 2007). Members of this family are integral membrane components considered in the cation efflux family protein that participate in the regulation of zinc ion sequestration. These components of membrane might increase tolerance or mediate toxicity to Zn by cation efflux that removes these ions from the cell. Zinc efflux is a critical component of zinc homeostasis. The role of zinc efflux transporters in cellular zinc homeostasis may be particularly important during gastrointestinal disease, where high levels of unabsorbed zinc might accumulate after supplementation.

Although homeostasis regulates Zn uptake by cells, it does not control zinc adsorption to cell membranes. As discussed by Atmaca et al. (1998) and Faiz et al. (2011), it has been suggested that Zn binds to surface membranes of microorganisms prolonging the lag phase of the growth cycle and increasing the generation time of the organisms, so that it takes each organism more time to complete cell division (Selahattin et al. 1998). Among other mechanisms proposed for the antimicrobial effect of Zn, a direct interaction with microbial membranes leading to membrane destabilization and enhanced permeability has been discussed. Cellular internalization of Zn and the production of active oxygen species have been proposed in earlier studies. Increase of Zn concentrations above optimal levels perturbs its homeostasis and allows its entry inside cells, so that Zn starts being cytotoxic.

It can be assumed that the *in vitro* changes in growth and morphology observed on the ZG-treated trophozoites may represent changes really observed *in vivo*. The World Health Organization (WHO) and the United Nations International Children's Emergency Fund (UNICEF) recommends short term zinc supplementation (20 mg Zn/day for children 6-59 months of age or 10 mg Zn/day for children less than 6 months, from 10 to14 days) for the management of childhood diarrhea (WHO-UNICEF 2004; WHO 2006). Different forms of zinc contain different amounts of elemental zinc; the WHO recommends the use of water-soluble compounds, zinc sulfate (23% Zn), zinc acetate (30% Zn), or zinc gluconate (14% Zn). So, for a dose of 10 – 20 mg of elemental Zn, 71 – 143 mg of ZG must be taken. Notably, Zn has poor intestinal absorption rate and an important amount of the initial dose given may remain in the intestinal lumen unabsorbed until its fecal excretion (Krebs 2000).

Our *in vitro* study showed that zinc gluconate at concentrations between 4.9 - 7.0 mM (2.2 – 3.2 mg/ml or 0.32 – 0.46 mg/ml of elemental Zn^{2+}) inhibited > 90% of *Giardia* growth when compared to non-treated cultures. Thus, in the case of giardiosis, *in situ* bioavailability of supplemented Zn should facilitate its high concentration at the

site of infection inhibiting the parasite growth. Nevertheless, as discussed by Surjawidjaja et al. (2004), this amount of Zn will be distributed in the entire gut, and some factors such as relative gut motility and water intake and retention, may lower the concentration of unabsorbed Zn in the gut. However, a small amount of Zn may remain effective to inhibit enteric pathogens growth.

Elucidation of the detailed mechanism of action of Zn on *G. duodenalis* pathogenesis should be addressed in the future. This study showed that Zn induces alterations in both form and movement of *Giardia* trophozoites and acts as an inhibitor of its growth *in vitro* at concentrations achievable in the gut. These findings suggested that supplemental levels of zinc in the host may alter pathogenic events of the parasite, which encourages further studies of the clinical effects of zinc administration in treatment of giardiosis to evaluate how zinc status influences the proliferation, pathogenicity and interaction between *Giardia*, and host.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

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Table 1. The addition of ZG solution to TYI-S-33 medium

TYI-S-33 (µl)	ZG (µl)	[ZG] (mg/ml)	$[Zn^{2+}]$ (mg/ml)	(mM)
1900	0	0	0	0
1880	20	0.3	0.05	0.7
1860	40	0.6	0.09	1.4
1840	60	1.0	0.14	2.1
1820	80	1.3	0.18	2.8
1800	100	1.6	0.23	3.5
1780	120	1.9	0.27	4.2
1760	140	2.2	0.32	4.9
1740	160	2.6	0.37	5.6
1720	180	2.9	0.41	6.3
1700	200	3.2	0.46	7.0

For a total volume of 2000 µl, each tube received an adjusted inoculum of $5x10^4$ *G. duodenalis* trophozoites in 100 µl TYI-S-33 medium.

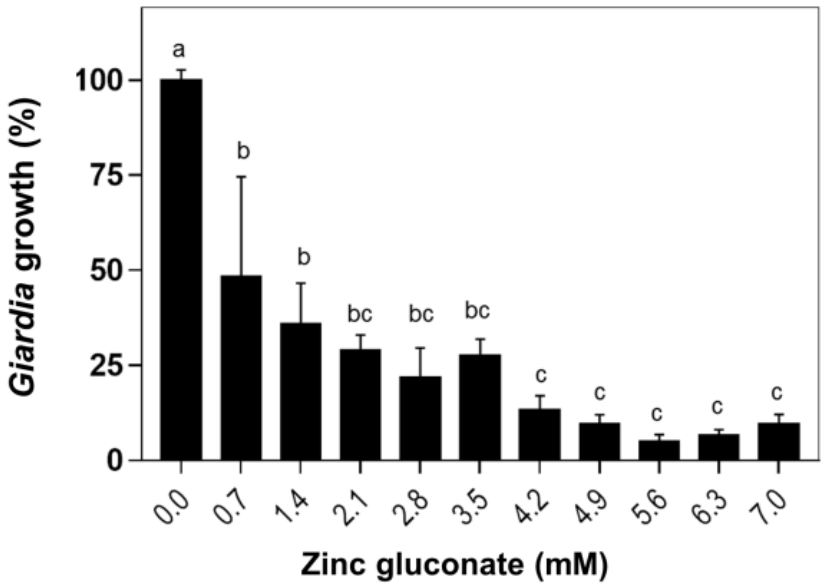

Fig. 1* ZG inhibits growth of *G. duodenalis. Anti-*Giardia* activity of ZG was determined on the *in vitro* growth of *Giardia* trophozoites after 48 h of exposure to different concentrations of ZG (0 – 7.0 mM). Results are presented as percentage of growth of the untreated trophozoites (control). Each point indicates the mean ± SD of parasite numbers determined in triplicate cultures.

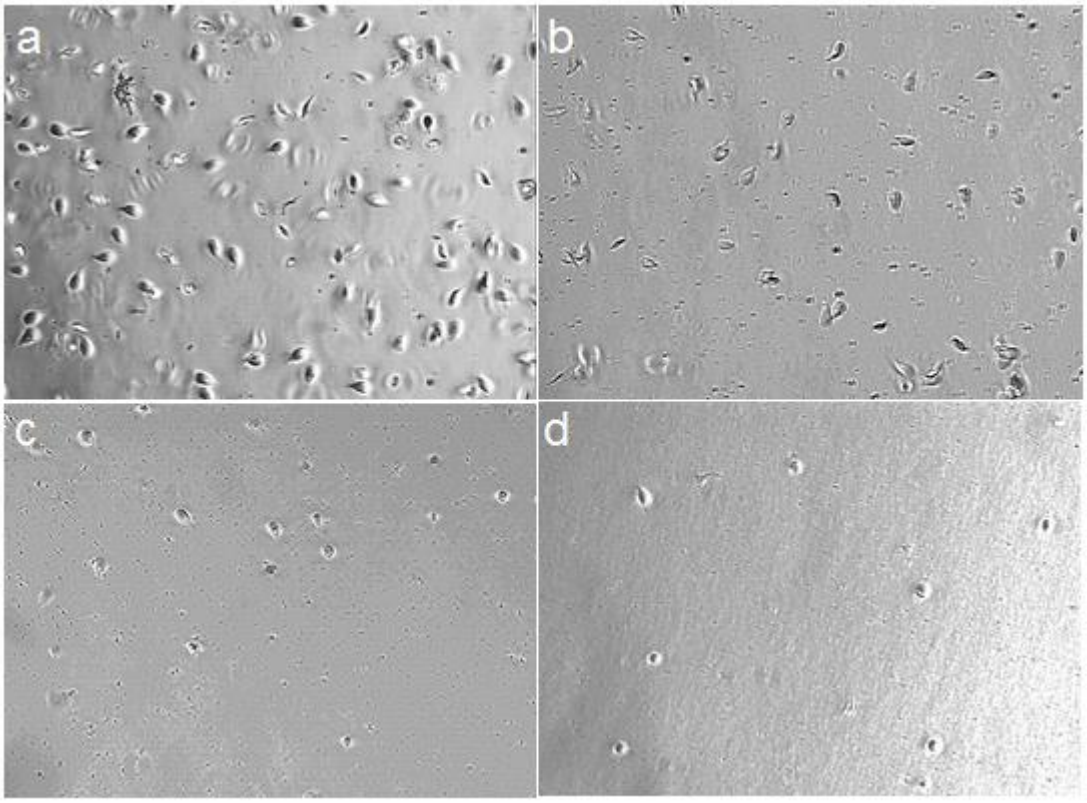

Fig. 2 **Effect of ZG on the growth of *G. duodenalis***. *Giardia* trophozoites after 48 h exposure at different concentrations of ZG, as seen under the light microscope (40x). (a) Non-treated control (b) ZG, 1.4 mM (c) ZG, 3.5 mM (d) ZG, 7.0 mM.

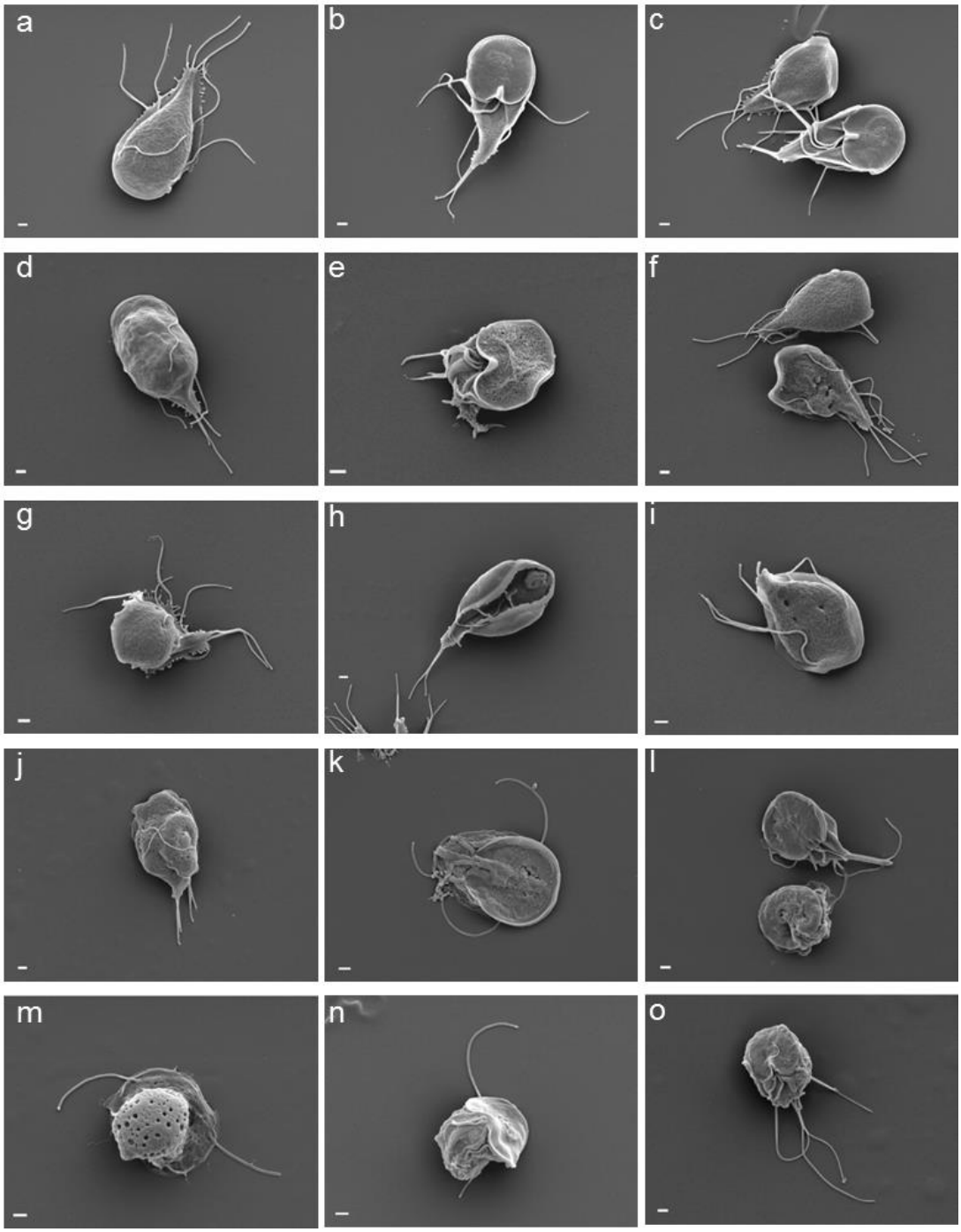

Fig. 3 **Scanning electron microscopy images showing morphological changes induced by ZG on *Giardia* trophozoites.** (a-c) Non-treated control, (d-i) ZG 1.4 mM, (j-o) ZG 4.9 mM. Controls (a-c) reveal the typical appearance of trophozoites with their pear-shaped cell body on the dorsal (a) and ventral (b) side, ventral disc and the characteristic arrangement of the eight flagella (c). ZG-treated trophozoites (d-o) show distorted shape and damaged membrane (l-o), membrane bending and a concave depression in the ventral region (h), as well as rounded cells with severe surface damage (m-o). Scale bar=1µm

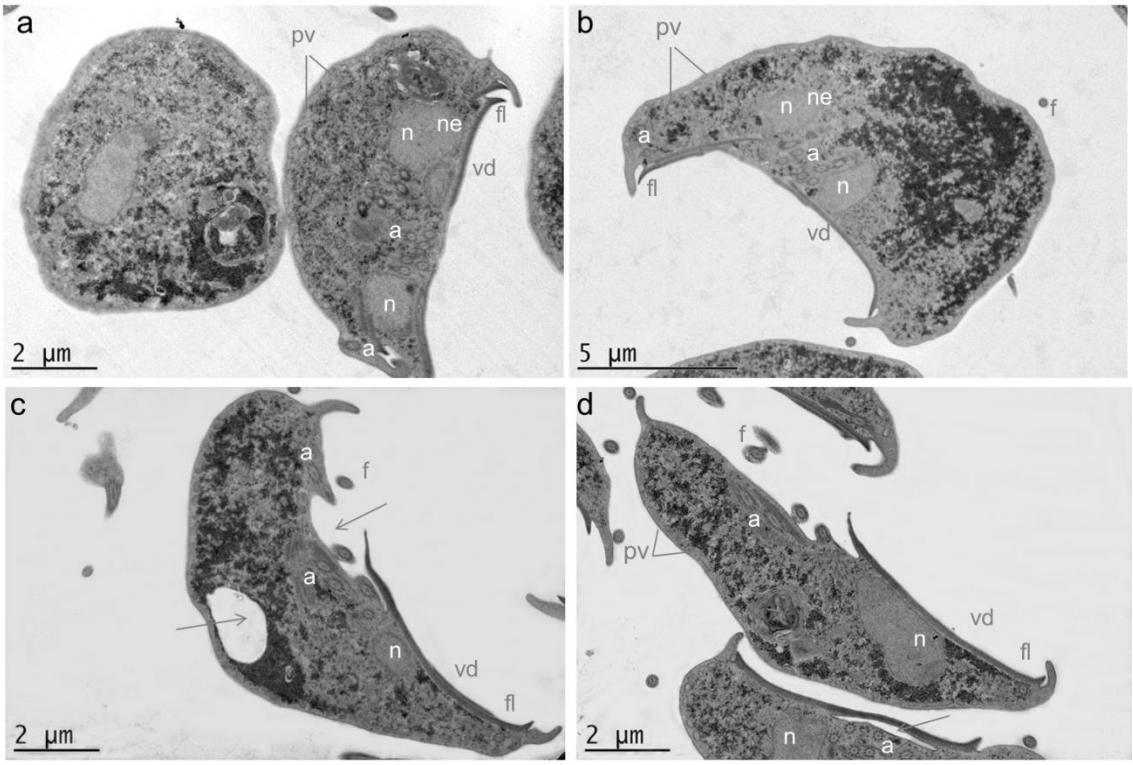

Fig. 4 **Transmission electron microscopy images showing ultrastructural changes induced by ZG on *Giardia* trophozoites.** (a-b) Control trophozoites which display normal morphology: the two nuclei (n), the ventral disk in the anterior face of the plasma membrane with protrusions of the cell body (fl); peripheral vesicles (pv) aligned in the cytoplasm down the plasma membrane, and the axonemes of flagella (a). (c-d) ZG-treated trophozoites showed formation of extensive empty areas in the cytoplasm (arrows) and accumulations of electrodense granular material. The adhesive disk was found displaced and in contact with the nuclei (d); flagella emergence was also altered with loss of some of them.

CAPITULO IV

Artículo:

Effect of zinc on *Giardia lamblia* pathogenicity: clinical, parasitological, and immunopathological study in the gerbil model

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En revisión interna

Effect of zinc on *Giardia lamblia* pathogenicity: clinical, parasitological, and immunopathological study in the gerbil model

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Abstract *Giardia lamblia* (*G. lamblia*) is increasingly recognized as a major cause of persistent diarrheal disease worldwide. We previously demonstrated that zinc gluconate (ZG) inhibits the growth and induces morphological and ultrastructural alterations on *Giardia* trophozoites in axenic culture. The benefits of zinc on host immunity are widely recognized. On this basis, it seemed worthwhile to explore how zinc deficiency and supplementation influence *Giardia* colonization and pathogenicity *in vivo*. Gerbils (*Meriones unguiculatus*) were randomly assigned into three groups: ZA (Zinc-adequate-diet); ZD (Zinc-deficient-diet) and ZS (zinc supplemented= ZA+50 mgZG/kg body weight/day, by gavage, starting 48 h before the establishment of infection), and then fed for 28d before oral inoculation with $1.5x10^6$ *Giardia* trophozoites; thereafter, the feeding was continued on the assigned diet until 30 days post-infection (dpi). Infection with *G. lamblia* was associated with impaired growth; in ZA gerbils significant adverse effects on body weight gain (BWG) were observed; ZD accentuated the severity of these growth decrements and significantly increased the amount of weight loss; ZS gerbils significantly improved BWG in both pre- and post-infection phases. In addition, ZD gerbils developed higher numbers of parasitosis earlier and exhibited prolonged infection; the administration of Zn was able to reduce significantly the intensity of infection at 15 dpi (5.6 $\log_{10}$ vs 6.4 $\log_{10}$ and 6.9 $\log_{10}$ for the ZS, ZA and ZD groups respectively; $P<0.05$); in ZS gerbils, there was an increase in the amount of mucus

covering the intestine; some of the recovered trophozoites were swollen or shrunken, while others were completely misshaped and entangled in thick mucus. By day 30 pi parasites were usually undetectable in ZA-ZS gerbils, but persisted in the ZD group (6.6 $\log_{10}$). Antibody titer increased as the infection progressed; while ZS enhanced anti-*G. lamblia* immune response earlier in infection (ZS 0.249 vs ZA 0.131, 5 dpi), ZD gerbil showed much smaller increases in anti-parasite IgG (ZD 0.249 vs ZA 0.525, 30 dpi), suggesting an impaired immune response. These data show a dual effect of zinc in benefitting the host while impairing *Giardia* pathogenicity. In the current study, we were able to demonstrate increased susceptibility to develop persistent infection and *G. lamblia*–potentiated growth decrements in ZD gerbils. In conclusion, ZS might exert a protective role in controlling *Giardia* infeciton.

Keywords: zinc deficiency, zinc supplementation, giardiasis, *Giardia lamblia*, gerbil

Introduction

Giardia lamblia (*G. lamblia*) is one of the most prevalent parasites in the world, common to humans and domestic and farm animals (Monis et al., 2009). In developed countries, it is a frequent cause of diarrhea in children in day care centers, institutionalized individuals, backpackers and travelers. In developing countries, about 200 million people have symptomatic giardiasis, with some 500,000 new cases reported annually (Adam, 2001; Savioli et al., 2006). *Giardia* has a simple life cycle consisting of infective cysts and vegetative trophozoites. Infection is transmitted by ingestion of cysts, which are passed in the feces (Luján et al., 1997). Trophozoites are responsible for the clinical manifestations associated with the disease, which vary from asymptomatic infections to acute or chronic diarrhea with malabsorption (Buret, 2008).

Giardiosis is usually self-limiting in immunocompetent individuals, indicating the presence of effective host defence mechanisms against the parasite (Eckmann, 2003), although chronic infections can occur in the absence of any apparent immunodeficiency (Nash, 1997). The host-parasite interactions that direct the outcome of *Giardia* infection remain poorly understood (Buret, 2008). Studies have demonstrated that the host immune response in control of primary infection by *G. lamblia* derives from a variety of immunological factors including CD4+ T cells and their cytokines, nitric oxide, intestinal mast cells and their products and the action of B cells and their antibodies (Abdul-Wahid and Faubert, 2008). Previous researchers have suggested that humoral immunity is important for elimination of the parasite, since hypogammaglobulinemic individuals (Ament and Rubin, 1972) and mice genetically deficient in functional B cells or treated with anti-IgM depleting antibody tend to present prolonged infections (Langford et al., 2002).

At present, controlling the disease is limited to chemotherapy (Gardner y Hill, 2001), but only a few drugs are available, and problems such as side effects, drug resistance (Upcroft et al., 1990) and a high rate of post-curative re-infection make this impractical in endemic areas. In view of the above, researchers have used immunostimulants or immunomodulatory agents such as vitamins and micronutriets in the management of giardiosis; the immune system is a highly proliferative, complex and integrated network of cells and organs, and therefore can be strongly influenced by these micronutrients and vitamins (Wellinghausen and Rink, 1998).

Zinc biology is a rapidly developing field, and recent research reveals zinc's strategic role in most organ systems. Zinc, an essential trace element, is required by all organisms and modulates the immune response, influencing cellular growth and affecting the development and integrity of immune system (Dardene, 2002). Generally, zinc deficiency has been shown to impair host defences to a variety of bacterial, parasitic, fungal and viral diseases (Van Eeckhout et al., 1976; Pekarek et al., 1977;

Shankar and Prasad, 1998; Wellinghausen, 2001), and zinc-deficient persons experience increased susceptibility to infection (Shankar and Prasad, 1998).

In 1993 giardiosis was reported for the first time as a risk factor for zinc malabsorption in children (Jendryzcko *et al., 1993).* Several studies conducted regarding trace elements in giardiosis have shown a significant decrease in zinc levels (Ertan et al., 2002; Demirci et al., 2003; Abou-Shady et al., 2011; Zarebavani et al., 2012; Yones et al., 2015; Lazarte et al., 2015). Quihui et al., (2010) investigated the association between giardiosis and zinc deficiency, concluding that infection with this parasite may be a risk factor for zinc deficiency in schoolchildren from northwestern Mexico. Zinc deficiency can increase the risk of a child to develop a more severe diarrheal illness following the ingestion of pathogens.

How zinc metabolism is compromised by *Giardia* is not well understood; as discussed in a recent review (Astiazarán-García et al., 2015), zinc status could be altered by intestinal malabsorption, organ redistribution or host-pathogen competition; the potential metal-binding properties of *Giardia* suggest unusual ways that the parasite may interact with its host.

Zinc deficiency can be reversed with zinc supplementation, and nutritional doses of zinc supplements could prevent alteration of the immune function and improve resistance to infections. The benefits of zinc on host immunity are widely recognized, and zinc supplementation has been shown to improve intestinal barrier function and reduce diarrhea in children (Bhutta et al., 2000; Black, 2003; Yakoob et al., 2011).

Zinc supplementation was recently found to reduce the rate of diarrhea caused by *Giardia* in children and to improve zinc status and upregulate humoral immune response in *Giardia*-infected mice (Long et al., 2007; Veenemans et al., 2012; Iñigo-Figueroa et al., 2013); *in vitro* and *in vivo*, zinc-salts enhanced the activity of bacitracin in a zinc-dose-dependent way (Andrews and Mylvaganam, 1994). Some of our previous work showed that zinc induces alterations in both form and movement of *G. lamblia* trophozoites and acts as an inhibitor of its growth *in vitro* at concentrations achievable in the gut. Zinc levels might be an influential factor determining susceptibility or resistance to giardiosis. These findings suggest that supplemental levels of zinc in the host may alter pathogenic events in the parasite, which encourages further studies of the clinical effects of zinc administration in treatment of giardiosis to evaluate how zinc status influences the proliferation and pathogenicity of *Giardia*, and the interaction with its host.

The major problem in understanding the influence of being zinc deficient, or zinc supplemented, on infection outcome is that the population is too heterogeneous in various aspects, incluiding dietary patterns, individual microbial and social environments. The characteristics of infections in adult humans are highly variable. Because the immune response elicited to infectious agents normally includes many redundancies, the ultimate consequence of zinc deficiency and supplementation in controlling infection needs to be established in an infected host.

Thus, we need to use laboratory animal models for explaining the impact of zinc deficiency or supplementation on infection, independant of the confounding heterogeneity in humans, and in which results are consistent and reproducible. Several experimental models have been used to evaluate the clinical profile and pathology of giardiosis (Hewlett et al., 1982; Craft, 1982; Belosevic et al., 1983; Yanke et al., 1998). Gerbils (*Meriones unguiculatus*) are one of the preferred animals, since they present high susceptibility to infections by oral inoculation of cysts and trophozoites, abundant cyst elimination in the feces and pathophysiological alterations similar to those observed in humans (Belosevic et al., 1983; Araújo et al., 2008).

It is important to stress that the ultimate indicators of the impact of zinc status on host immune function are the intensity, duration and severity of parasitic infection and not the number or function of immune cells. Otherwise, we may observe many specific immunological defects during zinc deficiency yet never understand whether they make a difference in the host's ultimate ability to resist or control infection.

This study was therefore undertaken to investigate the effects of zinc on host outcome, host immune response, and *Giardia* pathogenicity using an experimental model of giardiosis in gerbils.

Material and Methods

Experimental animals

This study involved 72 gerbils (*Meriones unguiculatus*), 4-6 weeks old and free of specific pathogens. The animals were supplied by the vivarium of Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV-IPN). Gerbils were kept in the vivarium of Centro de Investigación en Alimentación y Desarrollo, A.C., (CIAD, A.C.) under controlled atmosphere, at a temperature of 22°C and relative humidity of 40%, with 12-h photoperiods. Upon arrival at the facility gerbils were treated with metronidazole (Flagyl; 20 mg/gerbil/day, given by oral gavage for 3 consecutive days). This treatment ensured that the animals were free from all previous infections of the small intestine at the time of the experiment.

Animals were kept and handled separately under scrupulously aseptic working conditions. Care was taken in cleaning of cages and water bottles as well as the provision of water and chow to ensure that no other source of infection affected these animals throughout the experimental period. All animals were treated and cared for according to protocols approved by the Institutional Care and Use Committee at CIAD, A.C., as per Public Health Service policy and the Animal Welfare Act requirements.

Experimental design

Figure 1 show the experimental protocol used in the present study. The gerbils were divided into three groups, each comprising 12 animals:

- ZA. Zinc-adequate diet (TD.2016S Teklad Global Rodent Diet)
- ZD. Zinc-deficient diet (TD.85419 Teklad Custom Zinc Deficient Diet)
- ZS. Zinc-adequate diet + zinc supplement [50 mg zinc-gluconate/kg body weight/day, by gavage, 48 hours prior to infection and for the duration of the experiment].

To control for zinc levels in diet, gerbils were maintained on their respective dietary regimen, 4 weeks prior to infection and for the duration of the experiment (30 days post infection).

Following challenge with *Giardia lamblia* trophozoites, as described below, gerbils were monitored for food intake and weighed daily. Samples collected from these animals include blood (for serum) and duodenum/jejunum sections. Animals were humanely euthanized with chloroform followed by manual cervical dislocation. The parameters used to assess the effect of zinc treatment were as follows: change in body weight, change in food intake, parasite burden, and alterations in intestinal mucosa and antibody titre. The parameters were taken on day B, 0, 5, 15 and 30 post-infection.

Culture of Giardia lamblia trophozoites and gerbil inoculation

Giardia lamblia assemblage B strain (GS-M83-H7) trophozoites were obtained from the American Type Culture collection (ATCC 50581) and maintained in TYI-S-33 medium (Diamond et al., 1978) with modifications (Keister, 1983), supplemented with 10% bovine serum. Trophozoites were used for experimental inoculation and for preparation of the soluble antigen used in serological assays. For experimental inoculation, actively growing trophozoites (48–72 h culture) were sedimented after chilling the tubes in ice for 10 min and were finally suspended in PBS (pH 7.2); the inoculum was prepared by adjusting concentration of trophozoites ($1.5x10^6$) in 200 µl PBS. Inoculation was carried out via oral gavage using a cannula coupled to a 1 mL syringe.

Collection of data and blood samples

The average daily food intake was determined by subtracting the amount of food remaining in the feeding containers from the amount given the previous day. Blood samples from each animal were collected by cardiac puncture. The first collection was made 28 days before inoculation (B= Basal), the second on the day of inoculation (day

0) and subsequent ones at 5, 15 and 30 days post-inoculation (dpi). Blood samples were centrifuged at 3,000*x*g for 10 min to obtain the serum samples, which were stored at -20°C until use.

Infection kinetics, tissue parasite burden and pathological analysis

Gerbils were euthanised, the small intestine was removed, and a segment of the proximal small intestine (duodenum and jejunum) was cut into two segments. The first fragment was carefully opened longitudinally and then processed for scaning electron microscopy (SEM); intestinal tissue samples were immediately fixed in cold glutaraldehyde for 2 hours; after that, they were dehydrated through a graded ethanol series (50–100 %). Samples were mounted on stainless steel holders, CO_2 critical point dried (Tousimis Samdri 780), covered with gold in a JEOL JFC-1100 ionsputtering, and examined in a JSM-7100F field emission scanning electron microscope.

The second fragment was used to quantify the number of trophozoites adhering to the mucosa, following the method described by Belosevic et al. (1983). Briefly, the fragment was split longitudally and placed in 2 mL of sterile PBS. The sample was placed in a -4°C shaking incubator for 60 minutes under vigorous shaking (200 rpm) to dislodge the trophozoites from the gut. Trophozoites recovered from the mucosa were enumerated on a haemocytometer to obtain the concentration of trophozoites.

Determination of anti-G. lamblia IgG

Immunoenzymatic assays (ELISA) were carried out to determine the levels of IgG antibodies to *G. lamblia* in serum samples from gerbils. All the steps described below were previously standardized, using positive control sera from four age- and weight-matched gerbils inoculated with 10^6 trophozoites and the respective negative control sera obtained from the animals before inoculation. Secondary anti-mouse antibodies were used in this assay. Briefly, 96 well plates (Corning) were coated overnight with 50 μL (2.5 μg) of soluble *G. lamblia* antigen in 0.1 M sodium bicarbonate buffer pH 9.6. Soluble *G. lamblia* trophozoite antigens were obtained by using the method described by Gottstein *et al*. (1990) with slight modifications (Velázquez et al., 2005). Briefly, *G. lamblia* trophozoites from confluent cultures were harvested during log-phase by chilling on ice for 30 min. One hundred million trophozoites were washed three times with sterile phosphate buffer saline (PBS), resuspended in 1.5 mL of PBS, frozen (liquid nitrogen) and thawed (room temperature) three times, and then sonicated (30 cycles for 2 min (Brandon sonifier 250, Shelton, CT, USA) in the presence of protease inhibitor cocktail (23 mM/L 4(2-aminoethyl) benzenesulphonyl fluoride (AEBSF)), 0.3 mM/L pepstatin A, 0.3 mM/L E-64, 2 mM/L bestatin, and 100 mM/L sodium EDTA (Sigma, St. Louis, MO, USA). Cell debris was removed by centrifugation (10,000 *g* for 30 min).

The protein concentration of the soluble antigen preparation was determined by the Bradford method (Bio-Rad).

After overnight incubation with soluble *G. lamblia* antigen at 4°C, plates were washed with PBS-0.05% Tween 20 (PBST), and blocked with PBS-1% BSA for 1 h at room temperature and washed. Gerbil serum samples (diluted 1:10 in PBS 1% BSA) from both infected and non-infected gerbils were added to triplicate wells and incubated for 1 h at room temperature. After washing with PBST, antibody binding was detected with 50 μL of HRP-conjugated goat anti-mouse IgG (1:1000 diluted in PBS 1% BSA) (Sigma, St. Louis, MO, USA). After 1 h of incubation at room temperature, the plates were washed, and developed with 1 mL ABT-S in citrate buffer with 0.03% H_2O_2. Optical density was measured at 415 nm with an ELISA reader (Benchmark Microplate Reader, Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data on the weight gain, food intake, kinetics of parasite colonization and antibody levels between the groups were analysed using ANOVA and Tukey's test as a multiple comparison test, differences being considered significant at $P<0.05$. All calculations were run using NCSS 2000 (NCSS Statistical Software, Kaysville, UT, USA) and GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

Results and Discussion

It is well recognized that zinc is an essential trace element, influencing growth and affecting the development and integrity of the immune system. The use of oligoelements as zinc can be considered a tool in modulating the effectiveness of the immune response. The aims of the present work were to evaluate how zinc status influences the proliferation and pathogenicity of *Giardia*, and the interaction with its host.

In our study, zinc deficiency in gerbils occurred after 28 days on the particular zinc-deficient (ZD) experimental diet, as demonstrated by some of the typical signs of zinc deficiency: growth retardation, parakeratosis around the eyes, rough hair coat, scaly skin, alopecia and lesions on feet and tail (Figure 2B); these changes were not seen in zinc-adequate (ZA) fed gerbils (Figure 2A). Infection with *G. lamblia* was associated with impaired growth; significant adverse effects on body weight gain (BWG) (Table 1), but no significant effect on feed intake (Table 2) were observed in gerbils when compared with non-infected controls. ZD accentuated the severity of these growth decrements and significantly increased the amount of weight loss in gerbils after a single oral challenge of 10^6 *G. lamblia* trophozoites (Table 2, *$P<0.05$ vs. ZD non-infected control). On the other side, gerbils fed a diet with supplemental zinc (ZS) (zinc was daily and orally supplied in the form of zinc-gluconate) had significantly improved BWG in both phases, pre- and post-infection ($P<0.05$) when compared to un-supplemented

groups. These results are in agreement with our previous studies showing that there is a significant improvement in growth performance for *Giardia*-challenged mice fed a zinc-enriched diet (Iñigo-Figueroa et al., 2013). Thus, ZD children may be at increased risk of *G. lamblia*–potentiated growth decrements, and therefore could benefit from zinc supplementation.

In addition, ZD gerbils developed higher numbers of parasitosis earlier and exhibited prolonged infection (Figure 3). The administration of Zn was able to reduce significantly the intensity of *Giardia* infection (number of viable trophozoites in intestinal lumen) at 15 dpi (5.6 $\log_{10}$ vs 6.4 $\log_{10}$ and 6.9 $\log_{10}$ for the ZS, ZA and ZD groups respectively; $P<0.05$), the time when maximal intensity of infection is usually observed in this and similar models (Benyacoub et al., 2005; Humen et al., 2005; Goyal et al., 2011). Aspects of latent and patent periods, incluiding a self-limiting outcome during gerbil infection were similar to those observed by Belosevic et al., (1983) and also to some reported cases in human giardiosis (Vinayak et al., 1989). By 30 dpi parasites were usually no longer visible in the ZA and ZS gerbils, but persisted in the ZD group (6.6 $\log_{10}$). These data demonstrate that ZD enhances susceptibility to develop persistent infection in our gerbil model of giardiasis.

In the intestinal mucosa of ZA-infected gerbils, scanning electron microscopy revealed a large number of typical pear-shaped trophozoites, with smooth intact ventral and dorsal surfaces (Figures 5-7). Trophozoites were found grouped in clusters at the top and base of the villi (Figures 5A, 6A); some of these trophozoites fell into epitelial gaps (Figure 5A) or attached *in-situ* with their ventral disc projecting above the microvillus brush border (Figures 6B-C, 7A-B) while others appeared immersed in mucus sheets (Figures 5A-B). Circular markings were also observed on the epithelium of some villi, similar to the markings resulting from the detachment of trophozoite ventral disc (Figures 7A, 7C). On the 30th dpi no alterations were observed in the production of mucus nor were trophozoites present in the intestine of the gerbils (data not shown).

In ZS gerbils, there was a decrease in the epitelial gaps and an increase in the amount of mucus covering the intestine (Figures 11A, 13A-B). Mucous production plays an important role in the host's defense against giardiosis. The presence of mucins in the intestinal lumen has been related to the reduction of the ability for adhesion of the parasite to the intestinal epithelium (Cebra, 1999). The intensity of the infection might have been affected by the quantity of mucus enveloping the parasite and the consequent removal through intestinal peristaltism (Walker and Owen, 1990).

Some of the recovered trophozoites from ZS gerbils were swollen or shrunken, while others presented some irregularities, erosion and peeling (Figures 11A-B,). These results are in accordance with our previous work where zinc-gluconate in increasing concentrations, dose-dependantly, induced morphological and ultrstructural alterations on axenic cultures of *G. lamblia* trophozoites; Zn might interact with *Giardia* membranes resulting in cell membrane discontinuity, cytoplasm leakage and parasite swelling, as previously discussed.

ZS decreased parasite burden during the peak of infection when compared to ZA and ZD gerbils. The ultrastructural and morphological alterations induced by Zn on *G. lamblia* might have affected trophozoites attachment leading to their slipping and disintegration while in non-supplemented groups many trophozoites would still attach *in-situ*. By day 30 pi more progression of intestinal healing was observed, which was indicated by more closure of the epitelial gaps, and the marked decline in the number of trophozoites. Some of the remaining trophozoites were completely misshaped and entangled in thick mucus sheets (Figure 11B), showing irregular dorsal and ventral surfaces, while other trophozoites were swollen but still keeping their pear shape (Figure 11A).

Unlike the groups described above, in ZD gerbils, clusters of pear-shaped trophozoites were still seen at day 30 pi; some of the trophozoites were still attached *in situ* (Figure 10A), while others stayed immersed in mucus sheets (Figure 9A).

Zinc has been reported to influence host resistance mechanisms, thus altering the susceptibility to infectious diseases. Although *Giardia* maintains a strictly enteric life cycle, it does stimulate antigen-specific immune processes in peripheral sites. Oral administration of *G. lamblia* trophozoites to gerbils was able to induce systemic humoral immune responses (Figure 16); these responses were kinetically similar but quantitatively different at some time points in response to zinc level. However there was an apparent tendency to antibody persistance towards the end of the experiment.

As shown in Figure 16, circulating antibody levels against soluble antigens of trophozoites were detected by day 5 pi in ZS gerbils; from this point on, antibody titers increased as the infection progressed. In ZA and ZD gerbils, this increment in antibodies was detected until day 15 pi; however, by day 30 pi antibody levels were similar among ZS and ZA gerbils. ZD gerbils instead showed much smaller increases in IgG levels compared with the ZA-ZS groups.

Our data support the importance of the diet in regulating the intestinal milieu. Evidence suggesting that zinc deficiency impaired the immune response during the primary infection can be summarized as follows. First, trophozoites survival was highest in zinc-deficient gerbils; zinc deficiency was associated with lower reactions to *G. lamblia* antigens and decreased levels of IgG in circulating blood. Given that these specialized immune responses are mediated by Th2 cells, it could be suggested that the prolonged trophozoite survival in zinc-deficient gerbils during the primary infection was related to impaired Th2 response.

Although infected gerbils produce specific circulating antibodies against the parasite, the role of these molecules in protective immunity and the mechanisms that lead to their induction are not completely understood (Heyworth, 1992). Despite its maximum levels being detected after no trophozoites were found in the intestine, this immunoglobulin does not participate directly in the control of infection, since it occurs systemically, not acting on the parasite and/or its products in the intestinal lumen. This

probably means that serum IgG does not participate actively in the control of this parasite, but reflects the induction of the immune response to infection.

Given the main role of gut associated lymphoid tissues (GALT) in inducing and regulating immune responses to intestinal parasites, the responses observed in peripheral circulation were orchestrated by immunological events that occurred initially in the gut. There is a remarkable paucity of information about the specific effects of zinc on the GALT. We know that the parasite lives in the intestine, that the intestine is an important immunological organ and that cells primed in the GALT migrate through the mesenteric lymph nodes to the systemic circulation and then home back to the intestine. Our data clearly indicate that zinc deficiency and supplementation exerts effects on the systemic response, and that trophozoites are better able to survive in zinc-deficient gerbils, which suggests that the systemic immunosuppression caused by zinc deficiency translates back to the GALT. If anything, we expect the effects of zinc deficiency to be even more dramatic at the local intestinal level.

Cytokines are mediators that regulate the timing of initiation of an immune response, its duration, strength, and constitution. Work is in progress in this gerbil model to concencentrate on immunological processes occurring in inductive or effector sites of the GALT (cytokine production in MLN or Peyer's patches) and on immune mediators specifically unique to the gut mucosa (sIgA).

Conclusion

Zinc is a critical micronutrient in the prevention and treatment of *Giardia* infection. Our results showed delayed expulsion and higher burdens of *Giardia* trophozoites in gerbils fed a zinc-deficient diet; parasites seem better able to survive in the zinc-deficient hosts; ZD gerbils exhibited impaired IgG response and suggest that, similarly, ZD children may be at increased risk to develop a persistent infection and *G. lamblia*–potentiated growth decrements. ZS not only improved BWG and empowered host immunity, but also had effects on the trophozoite which may reduce parasite burden and decrease disease severity.

In the current study, we were able to demonstrate *in vivo*, increased susceptibility to develop persistent *Giardia* infection in ZD gerbils. Taking all evidence into account, the present study clearly highlighted and evidenced the importance of zinc during giardiosis. These observations should provide an impetus to further research on the understanding of how zinc deficiency promotes parasite survival, and the potential prophylactic-therapeutic uses of Zn for the treatment or modulation of human giardiosis. Zinc may not function as an antiparasite on its own, but it may assist other treatments or help in immunological manipulations that confer protection against giardiosis, preventing other pathological processes in the host.

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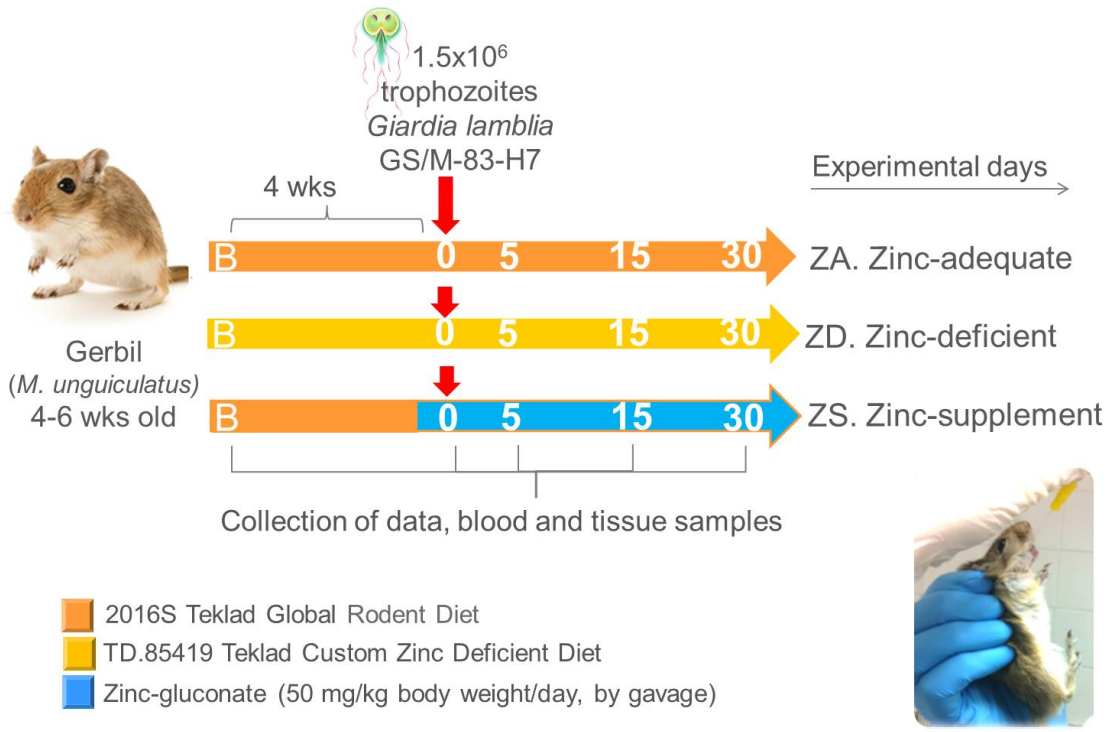

Figure 1. Experimental protocol used in the study. The gerbils were divided into three groups (ZA, ZD, ZS), each comprising 12 animals. In the control group (ZA), gerbils were fed with a zinc-adequate diet *ad libitum* for 28 days; on day 28, the gerbils were challenged orally with a single dose of *Giardia* trophozoites ($1.5x10^6$ trophozoites/animal); thereafter, zinc-adequate feeding was continued until day 30 post-infection (pi). In the zinc deficient (ZD) group, gerbils were fed with a zinc-deficient diet *ad libitum* for 28 days; on day 28, a single dose of *Giardia* trophozoites ($1.5x10^6$ trophozoites/animal) was given orally; thereafter, zinc-deficient feeding was continued until day 30 pi. In the zinc supplemented (ZS) group, gerbils were fed with zinc-adequate diet *ad libitum* for 26 days; 48 hours prior to infection, gerbils received a single dose of zinc gluconate (50 mg/kg body weight/day) by gavage; on day 28, a single dose of *Giardia* trophozoites ($1.5x10^6$ trophozoites/animal) was given orally; thereafter, zinc-adequate feeding and daily oral zinc supplementation was continued until day 30 pi. Following challenge with *Giardia lamblia* trophozoites gerbils were monitored for food intake and weighed daily; samples collected from these animals include blood (for serum) and intestinal tissue sections. The parameters were taken on day B (basal), 0 (establishment of infection), 5, 15 and 30 pi.

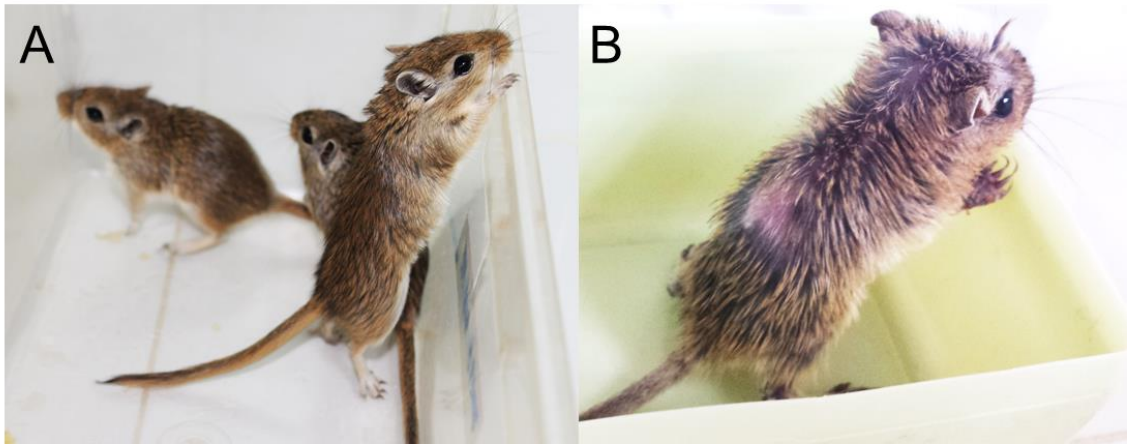

Figure 2. Clinical manifestations of zinc deficiency in gerbils. After 28 days on a zinc-deficient diet gerbils show the typical signs of zinc deficiency (B), as demonstrated by growth retardation, alopecia, and lesions on feet and tail, as compared to control ZA gerbils (A).

Table 1. Effect of zinc and infection on body weight gain (BWG) of gerbils (g)

Group		$\Delta_{B \rightarrow 0}$	$\Delta_{B \rightarrow 30}$
Zinc-adequate	Non-infected	+9.40 ± 2.46 [c]	+13.80 ± 1.74 [d]
	G. lamblia infected	+8.14 ± 2.08 [c]	+10.45 ± 1.23 [c]
Zinc-deficient	Non-infected	-2.05 ± 1.78 [a]	-2.75 ± 0.95 [a]
	G. lamblia infected	-2.44 ± 1.38 [a]	-6.50 ± 1.89 [b]
Zinc-supplement	Non-infected	+9.19 ± 1.20 [c]	+13.33 ± 1.02 [d]
	G. lamblia infected	+8.00 ± 2.39 [c]	+13.27 ± 1.52 [d]

Values represent means ± SD; values followed by different letters differ significantly ($p<0.05$)
BWG: $\Delta_{B \rightarrow 0}$ = [Weight at day 0] – [Basal weight], $\Delta_{B \rightarrow 30}$ = [Weight at day 30 pi] – [Basal weight]

Table 2. Effect of zinc and infection on average daily food intake (g)

Dpi	**Zinc-adequate**		**Zinc-deficient**		**Zinc-supplemented**	
	G (-)	G (+)	G (-)	G (+)	G (-)	G (+)
-28	7.5 ± 1.4	7.1 ± 2.1	7.3 ± 1.0	6.5 ± 2.2	7.3 ± 0.6	6.8 ± 0.3
-15	6.1 ± 1.2	5.9 ± 1.8	5.8 ± 1.2	5.6 ± 1.0	6.8 ± 0.9	6.3 ± 0.4
-5	5.5 ± 1.0	6.6 ± 1.7	6.0 ± 1.2	5.8 ± 1.6	6.8 ± 1.2	6.3 ± 1.0
0	6.0 ± 0.2	5.8 ± 0.8	5.5 ± 0.5	6.1 ± 1.4	6.6 ± 0.7	6.5 ± 0.2
5	6.8 ± 0.4	6.1 ± 1.6	5.8 ± 0.6	5.4 ± 0.7	6.2 ± 1.0	6.7 ± 0.5
15	5.4 ± 0.9	5.7 ± 0.8	5.7 ± 1.4	5.0 ± 0.8	6.9 ± 0.3	5.9 ± 0.5
30	7.0 ± 1.0	6.5 ± 1.1	5.9 ± 0.8	5.3 ± 0.6	7.3 ± 0.6	6.8 ± 0.3

Values represent means ± SD
dpi=days post-infection; G(-)=Non-infected; G(+)= *G. lamblia* infected

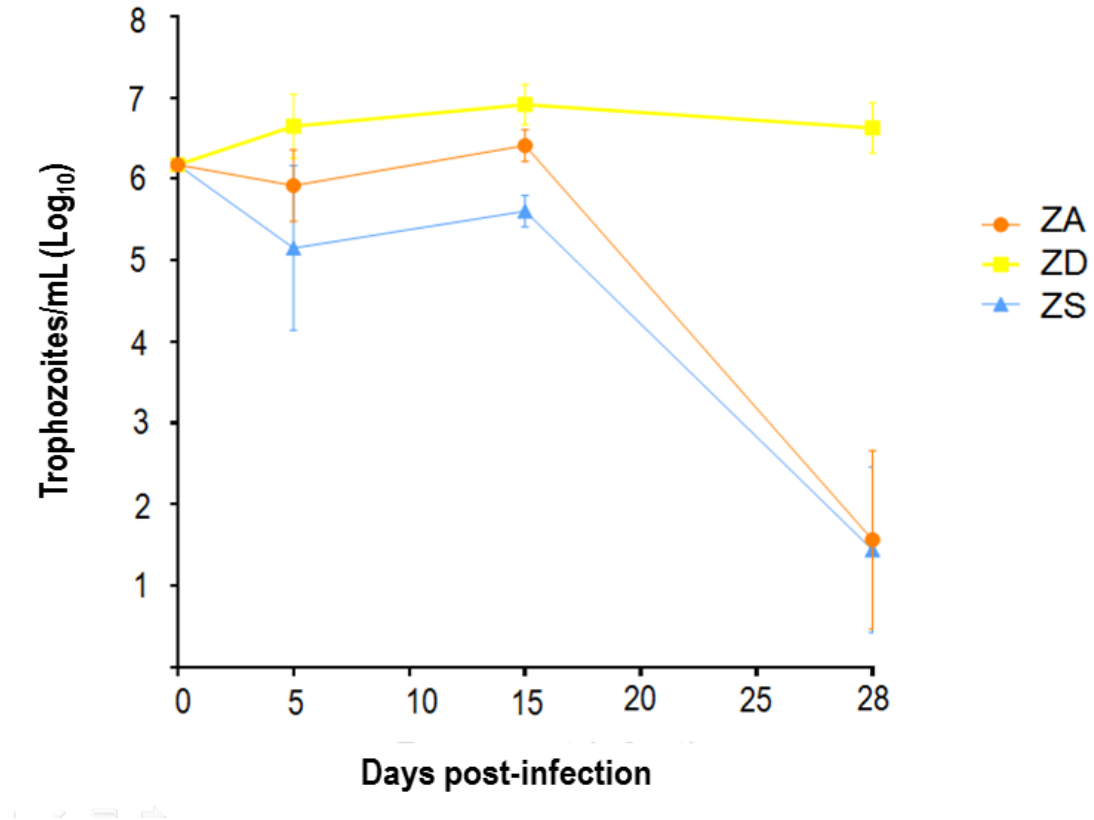

Figure 3. Mean values of trophozoites recovered from the intestinal mucosa of gerbils in each group per day. Each point represents the mean number of trophozoites recovered from three animals in each group-point. Values are expressed as mean ± SD.

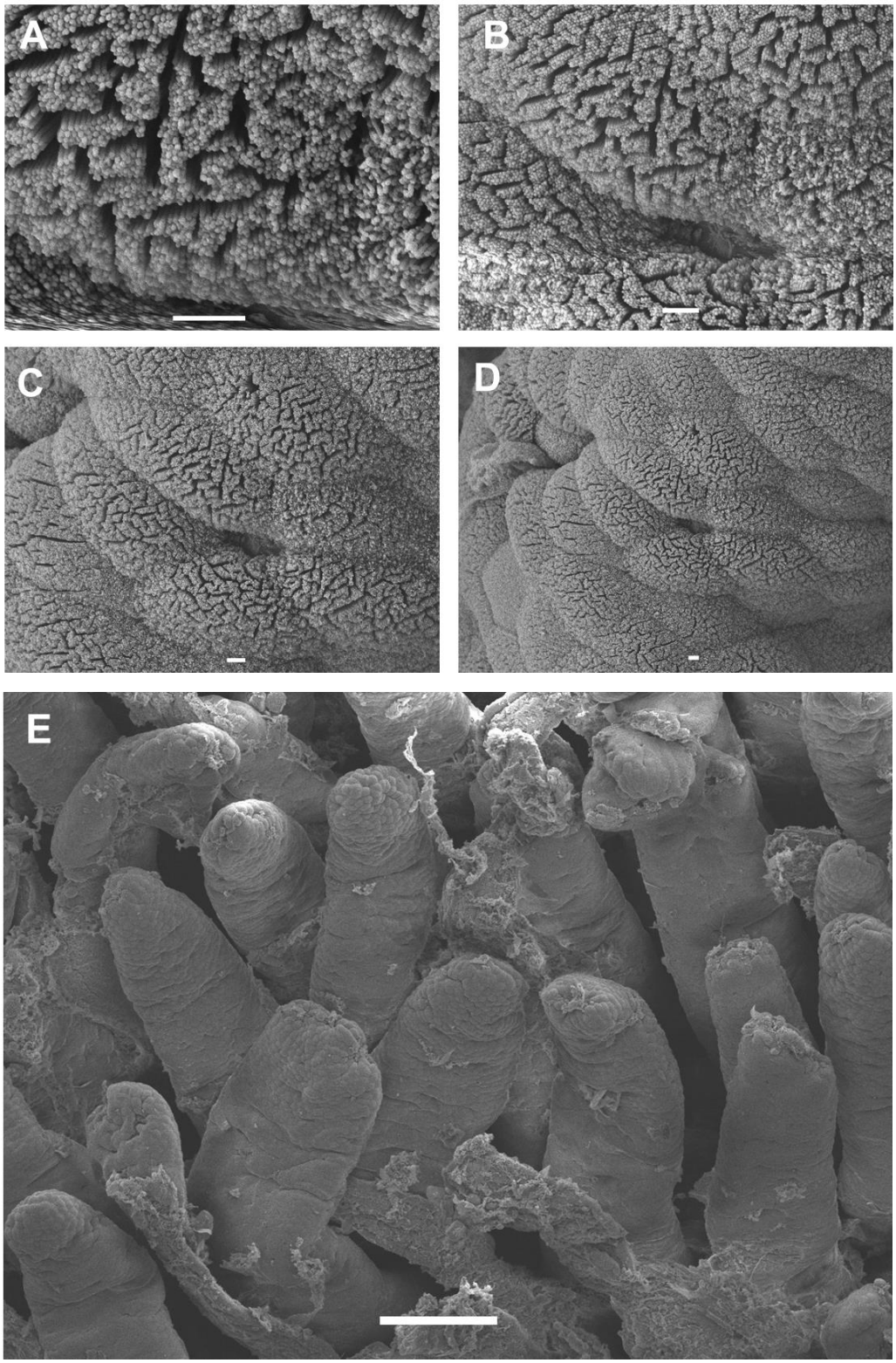

Figure 4. Scanning electron microscope images of intestinal mucosa from control gerbils, non-infected, zinc-adequate fed (Basal). Scanty mucus coating the intestinal mucosa. (A) x20000 (B) x10000 (C) x5000 (D) x3000 (E) x160; Bar (1μm for A-D, 100μm for E)

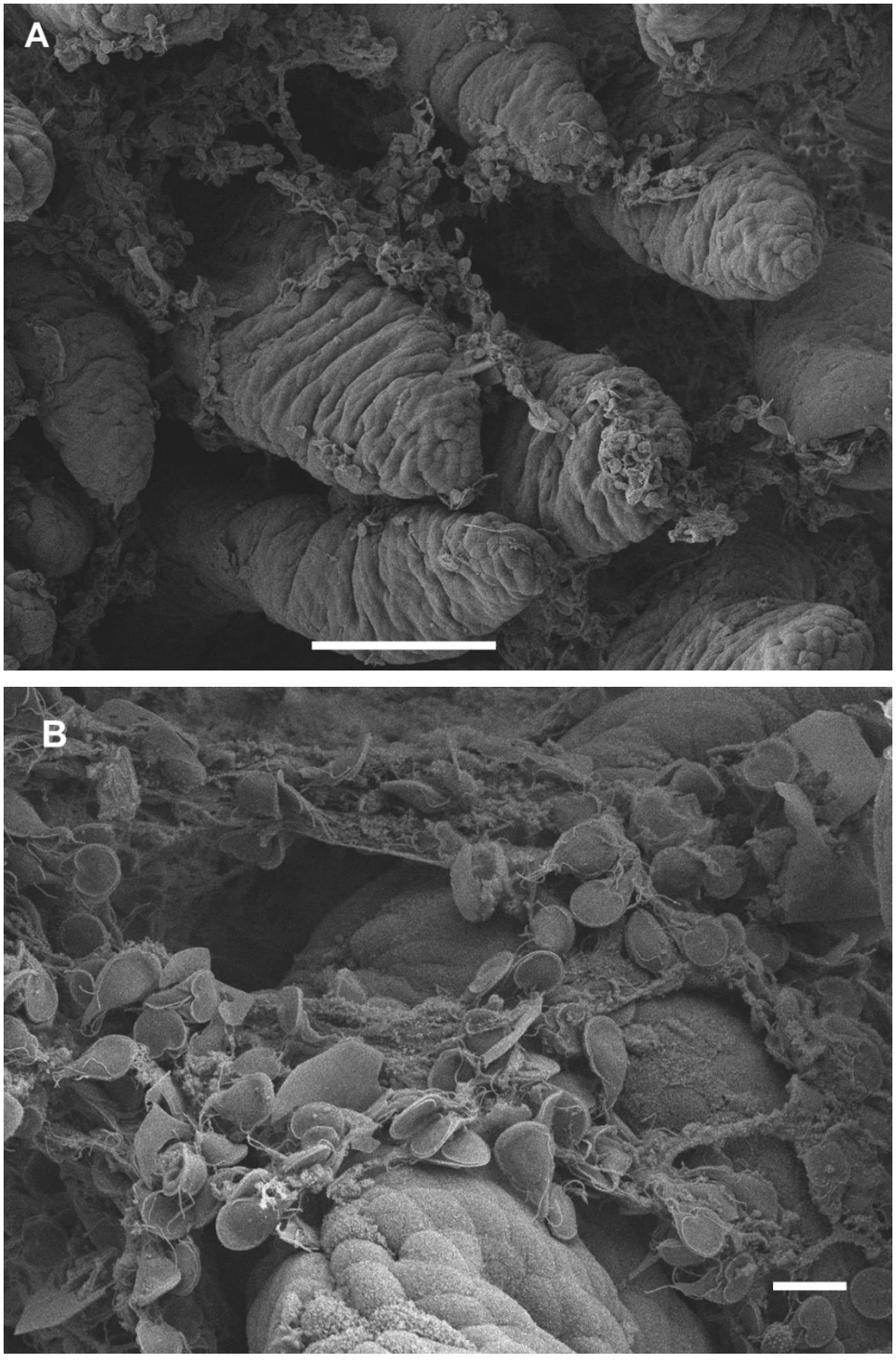

Figure 5. Scanning electron microscope images of zinc-adequate fed-*G. lamblia*-infected gerbils (ZA) 5 dpi, showing normal trophozoites with smooth intact ventral and dorsal surface; trophozoites immersed in mucus on the apical portion of the villus; clusters of trophozoites located at the base of the villosity (A) x250, bar 1 µm (B) x1000, bar 10 µm

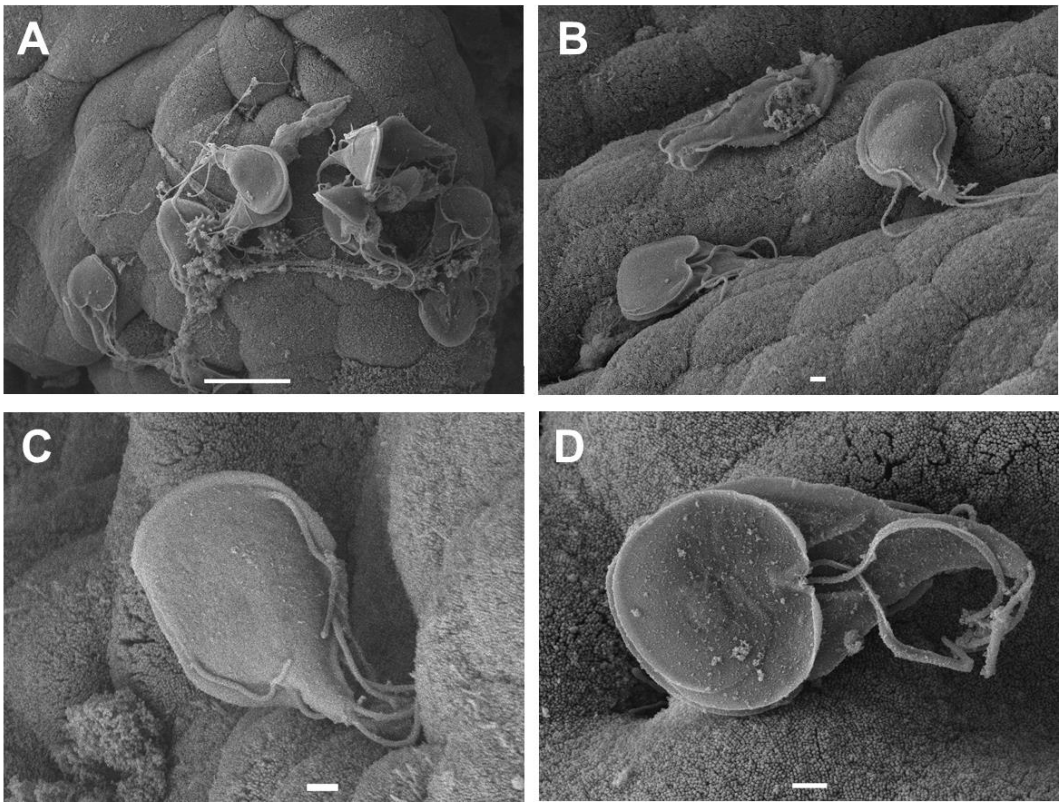

Figure 6. Scanning electron microscope images of zinc-adequate fed-G. *lamblia*-infected gerbils (ZA) 5 dpi. (A) Presence of trophozoites in the apical region of the villosity, with traces of mucus. (C, D) Normal trophozoites under higher magnification with smooth intact ventral and dorsal surface (A) x1900, bar 10 µm (B) x3500, bar 1 µm (C) x5500, bar 1 µm (D) x8000, bar 1 µm

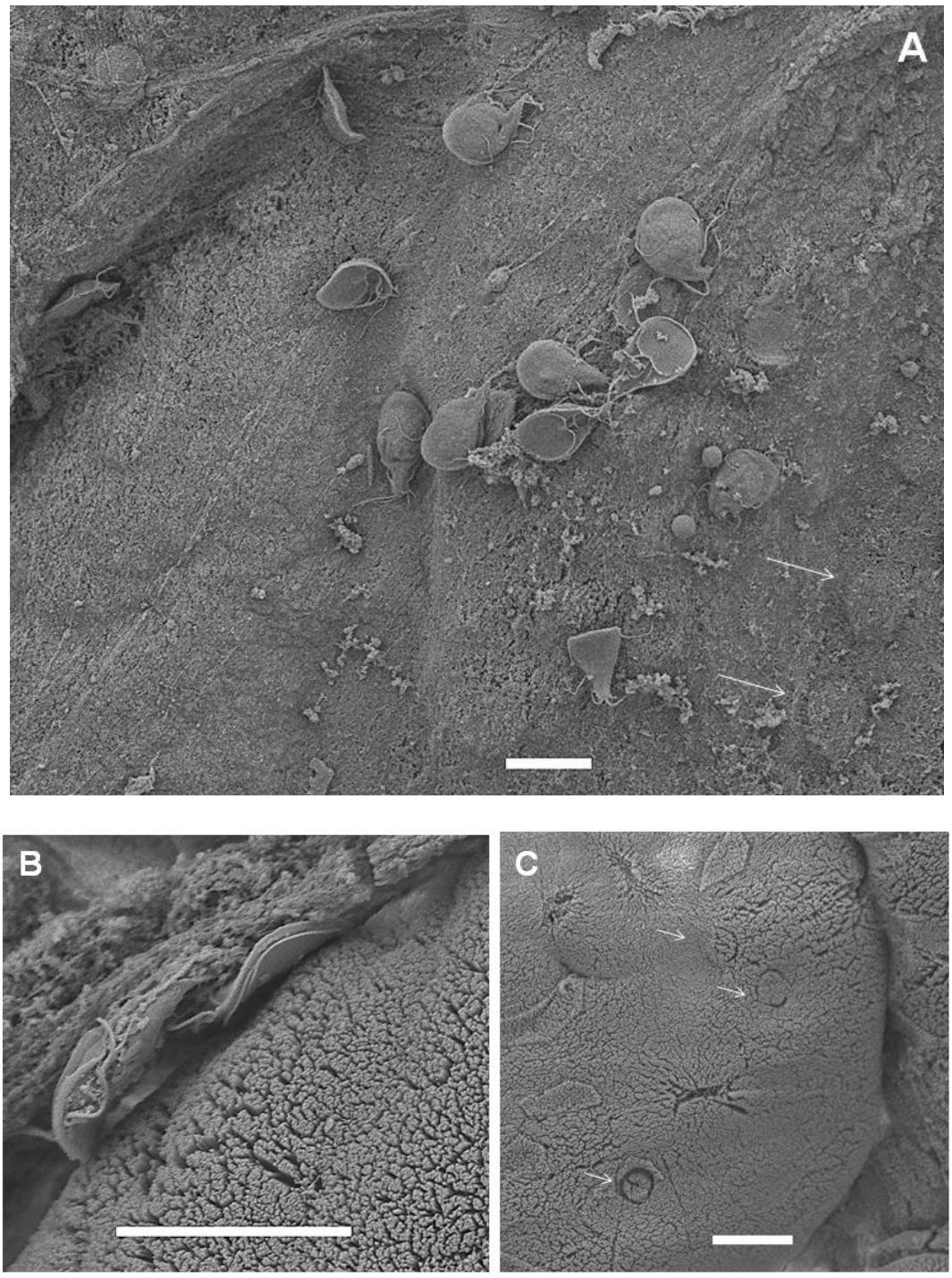

Figure 7. Scanning electron microscope images of zinc-adequate fed-*G. lamblia*-infected gerbils (ZA) 15 dpi, showing normal trophozoites with smooth intact ventral and dorsal surface**.** Intestinal mucosa show multiple epitelial gaps with trophozoites attached; note the presence of circular marking at the villosity (A and C, arrows) (A) x1000, bar 10 µm (B) x2500, bar 10 µm (C) x1300, bar 10 µm

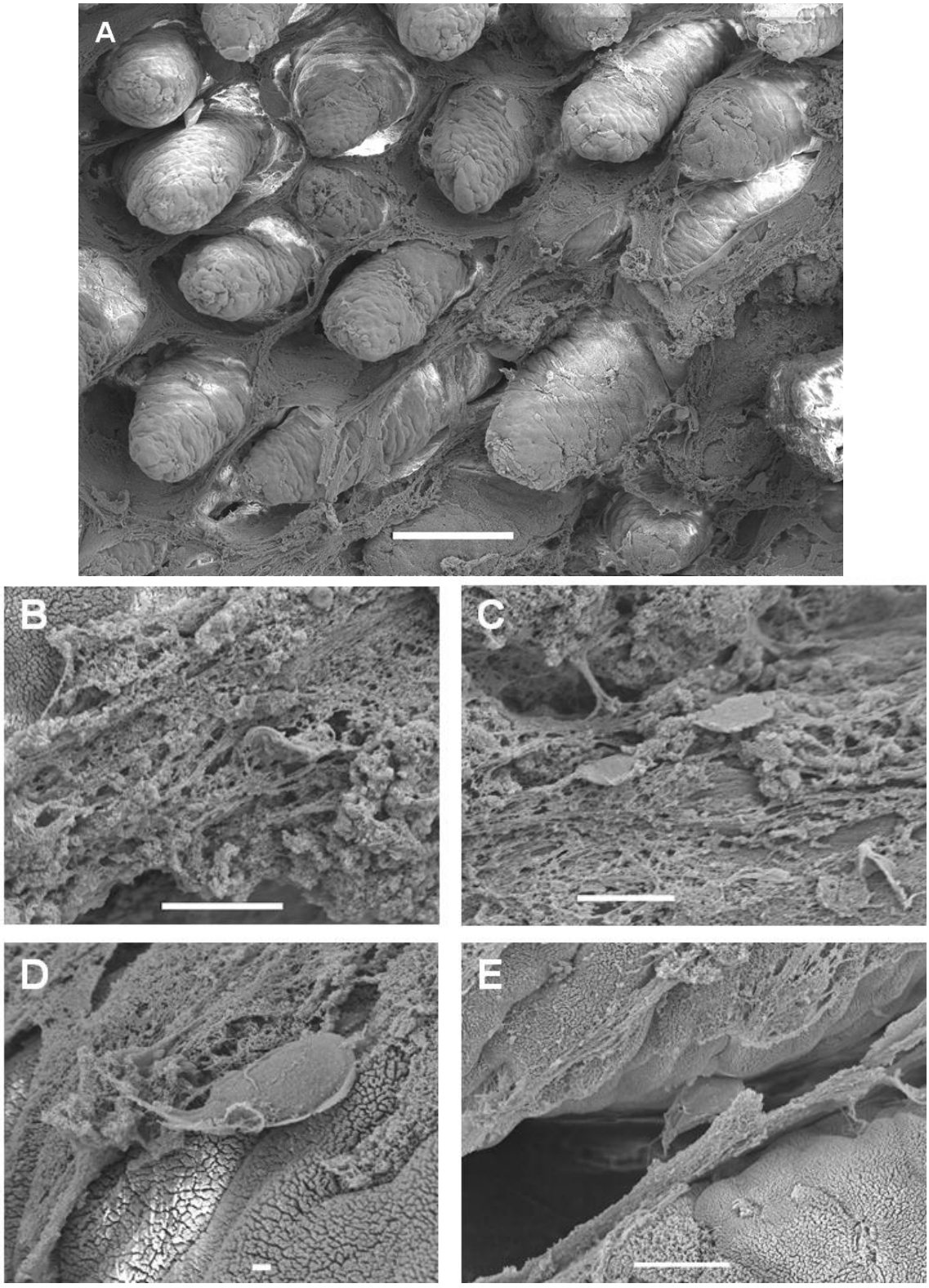

Figure 8. Scanning electron microscope images of zinc-deficient fed-*G. lamblia*-infected gerbils (ZD) 15 dpi. (A) x190, bar 100 µm (B) x2500, bar 10 µm (C) x2000, bar 10 µm (D) x3500, bar 1 µm (E) x2500, bar 10 µm

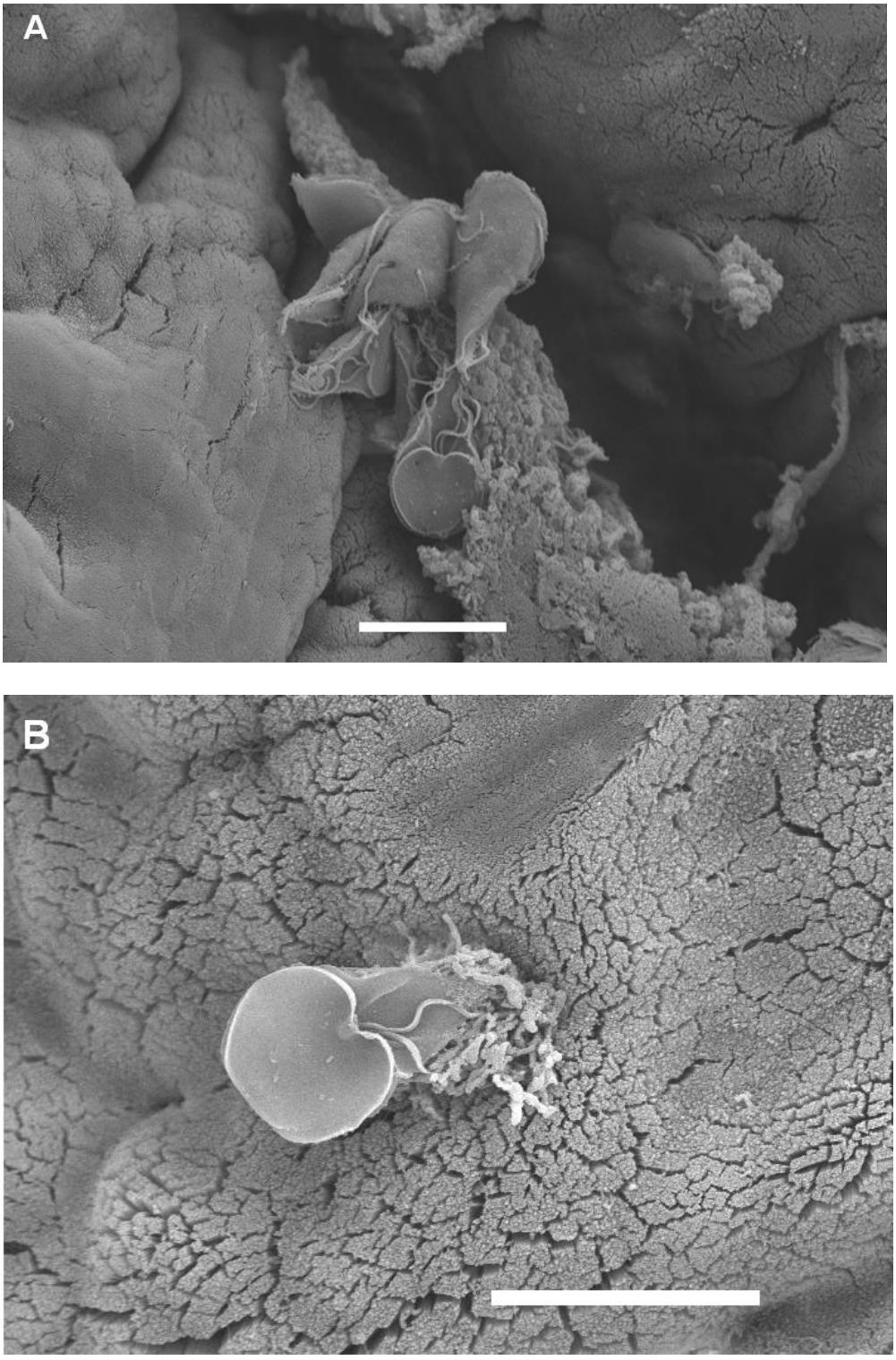

Figure 9. Scanning electron microscope images of zinc-deficient fed-*G. lamblia*-infected gerbils (ZD) 30 dpi. Note that trophozoites are still present in the gut (A) (B) x2000, bar 10 µm

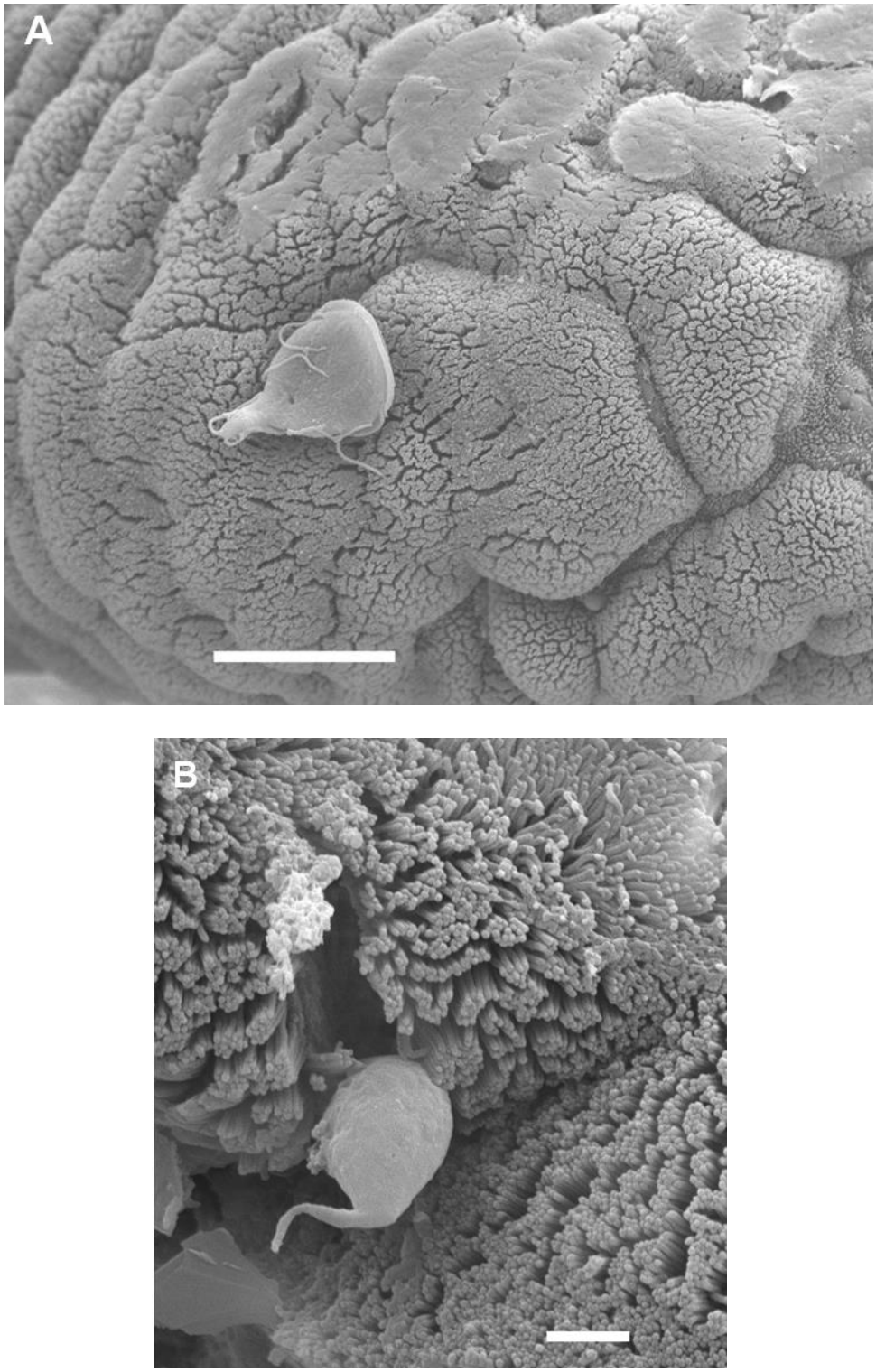

Figure 10. Scanning electron microscope images of zinc-deficient fed-*G. lamblia*-infected gerbils (ZD) 30 dpi, showing epitelial gaps and trophozoites still attached *in situ***.** (A) x2000, bar 10 µm (B) x10000, bar 1 µm

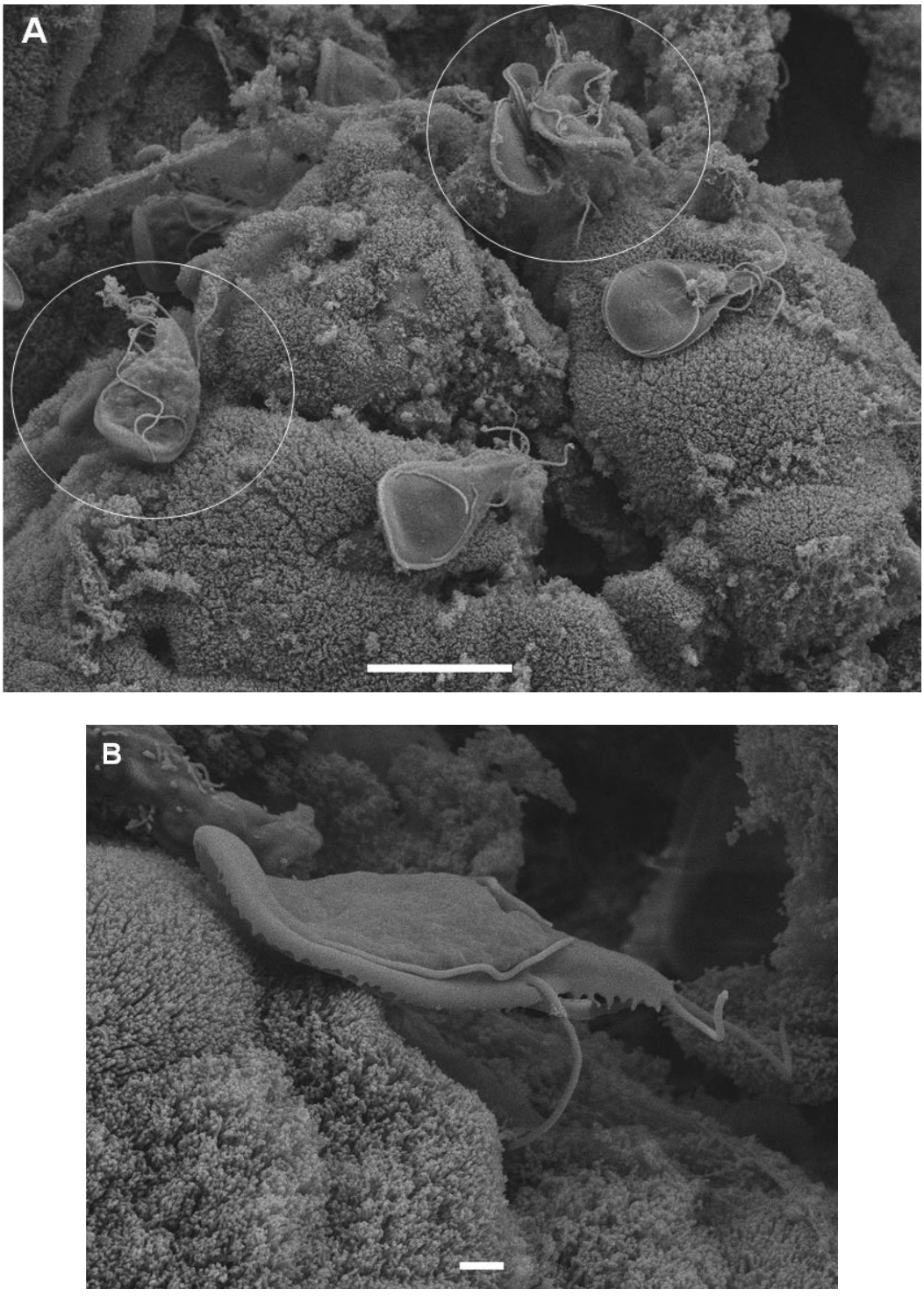

Figure 11. Scanning electron microscope images of zinc-supplemented *G. lamblia*-infected gerbils (ZS) 5 dpi, showing pear-shaped trophozoites with multiple erosions and irregularities on the outer surface (circles). (A) x1900, bar 10 µm (B) x7000, bar 1 µm

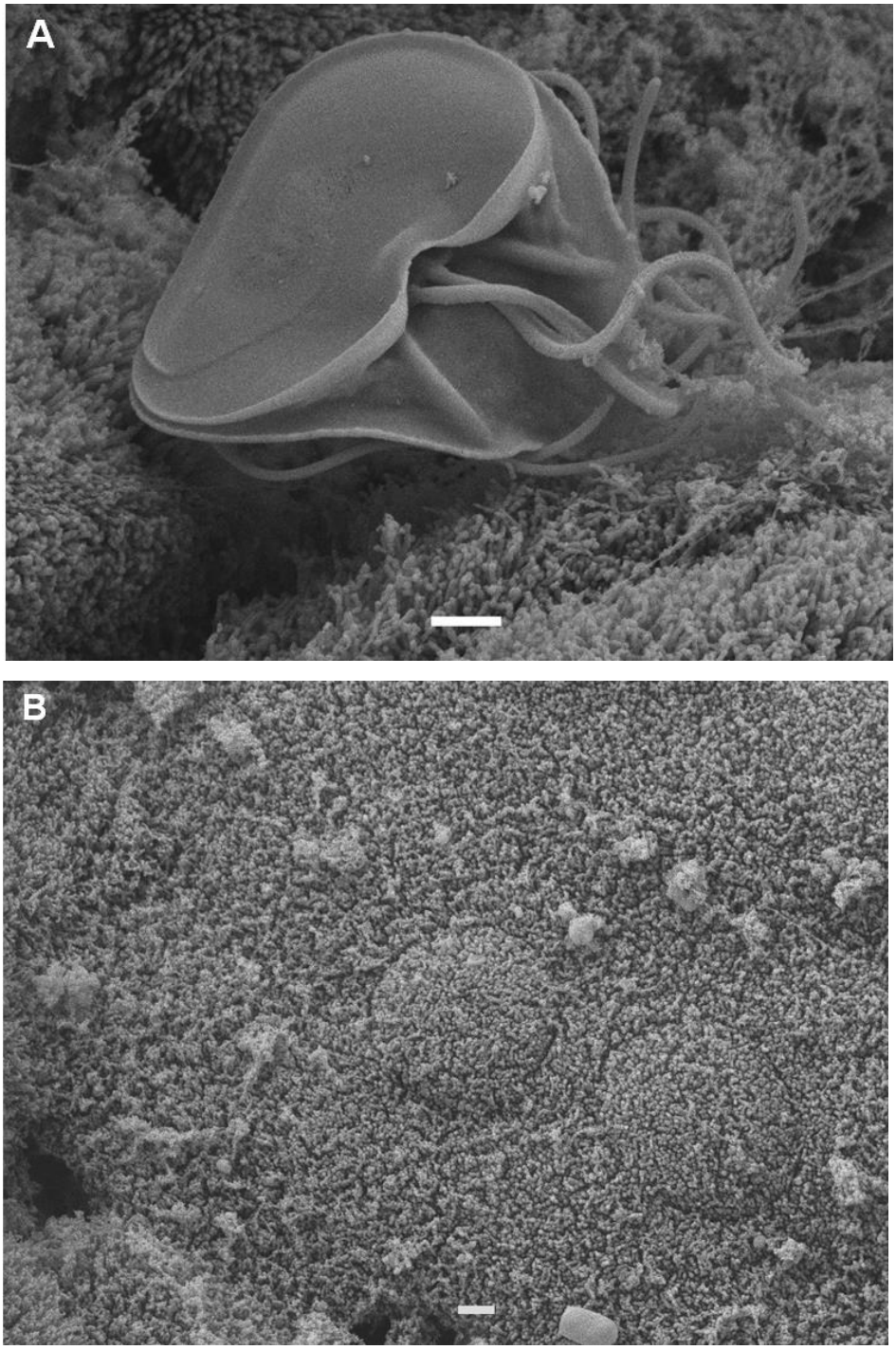

Figure 12. Scanning electron microscope images of zinc-supplemented-*G. lamblia*-infected gerbils (ZS) 5 dpi. Note the presence of circular marking at the villosity (A) x9500, bar 1 µm (B) x5000, bar 1 µm

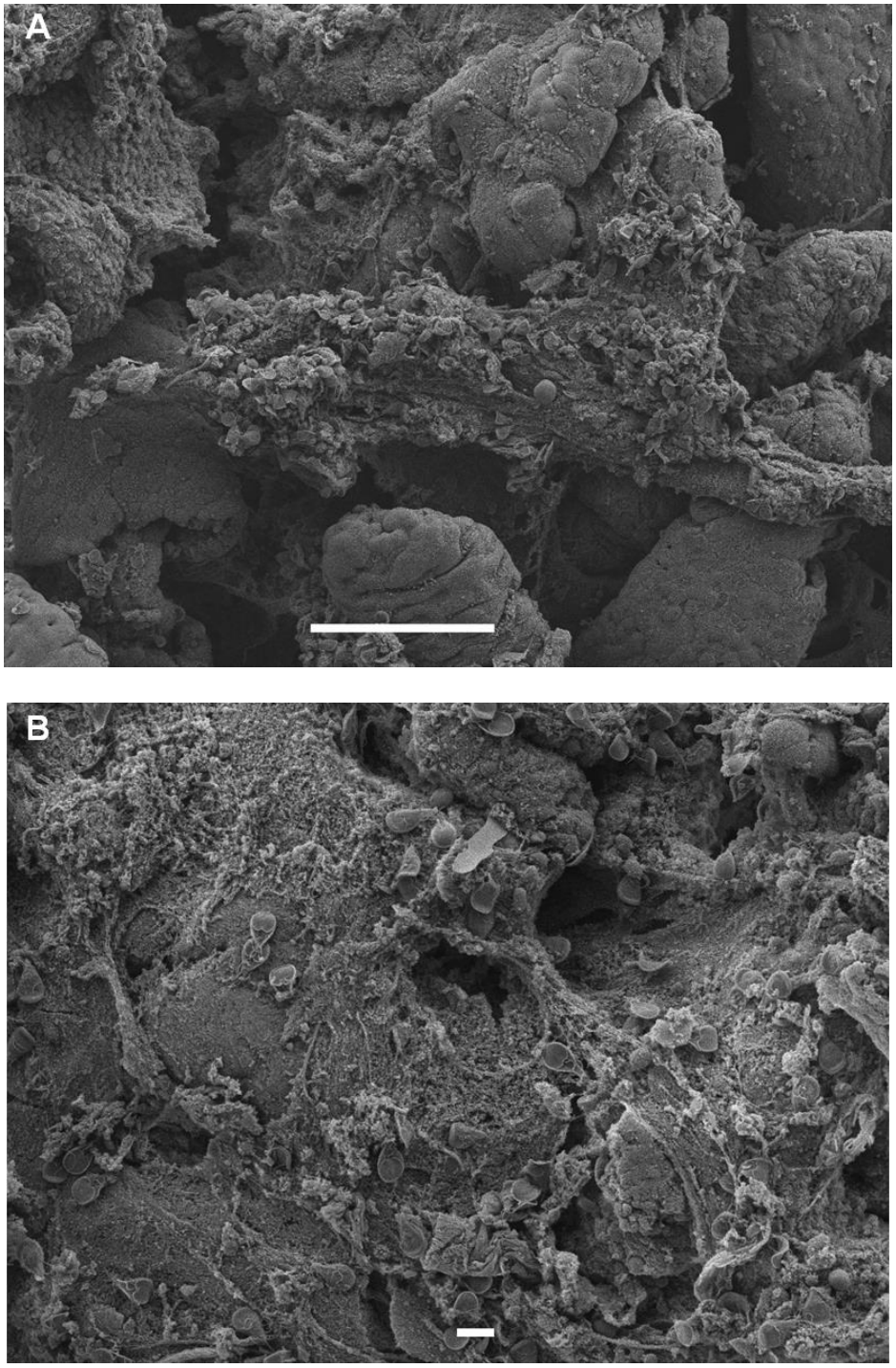

Figure 13. Scanning electron microscope images of zinc-supplemented *G. lamblia*-infected gerbils (ZS) 15 dpi. Note the dense layer of mucus covering the entire fragment; trophozoites immersed in mucus on the apical portion of the villus (A) x250, bar 100 µm (B) x850, bar 10 µm

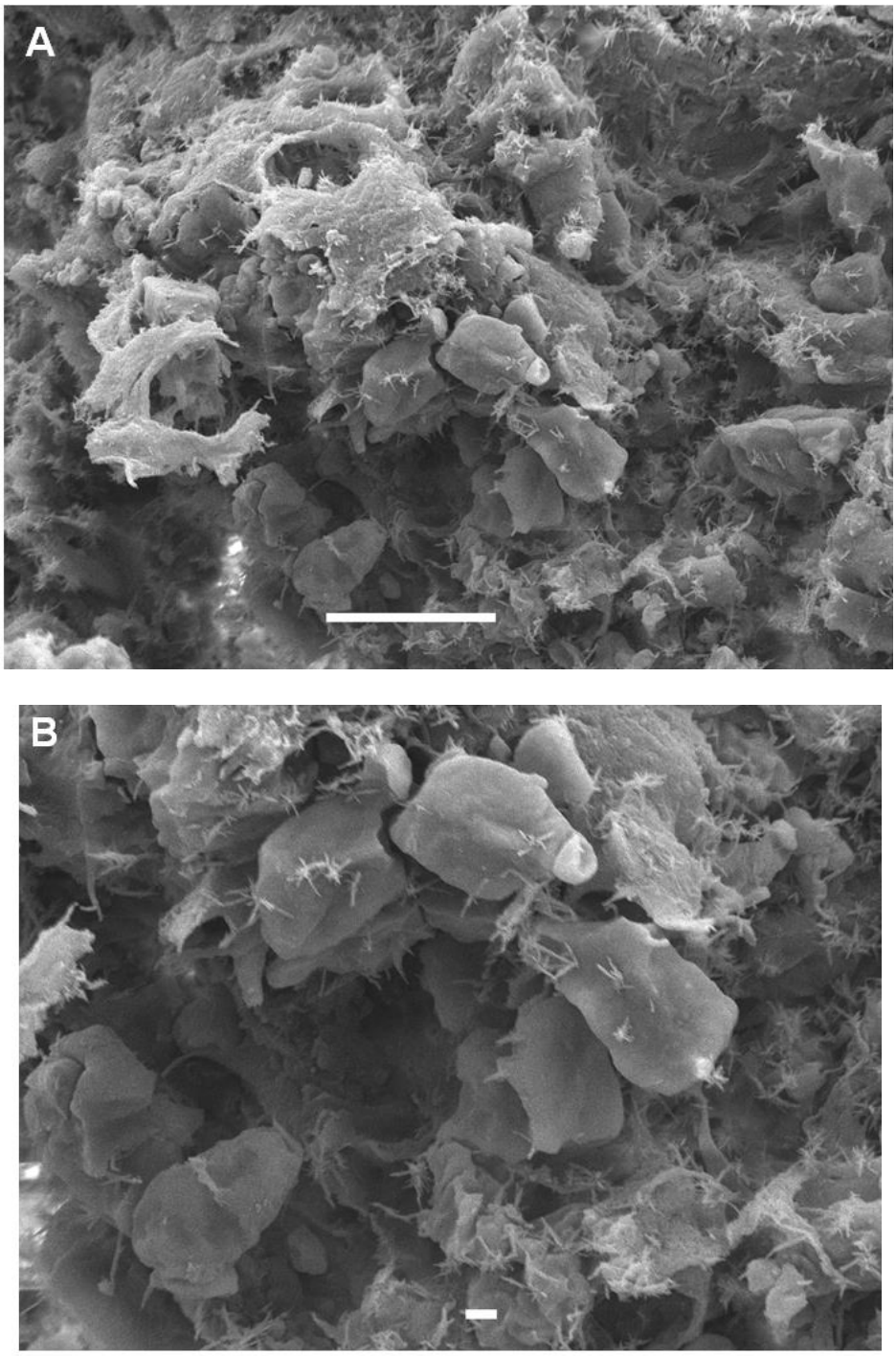

Figure 14. Scanning electron microscope images of zinc-supplemented *G. lamblia*-infected gerbils (ZS) 15 dpi, showing swollen pear-shaped trophozoites entangled in thick mucus sheets. (A) x2300, bar 10 µm (B) x4300, bar 1 µm

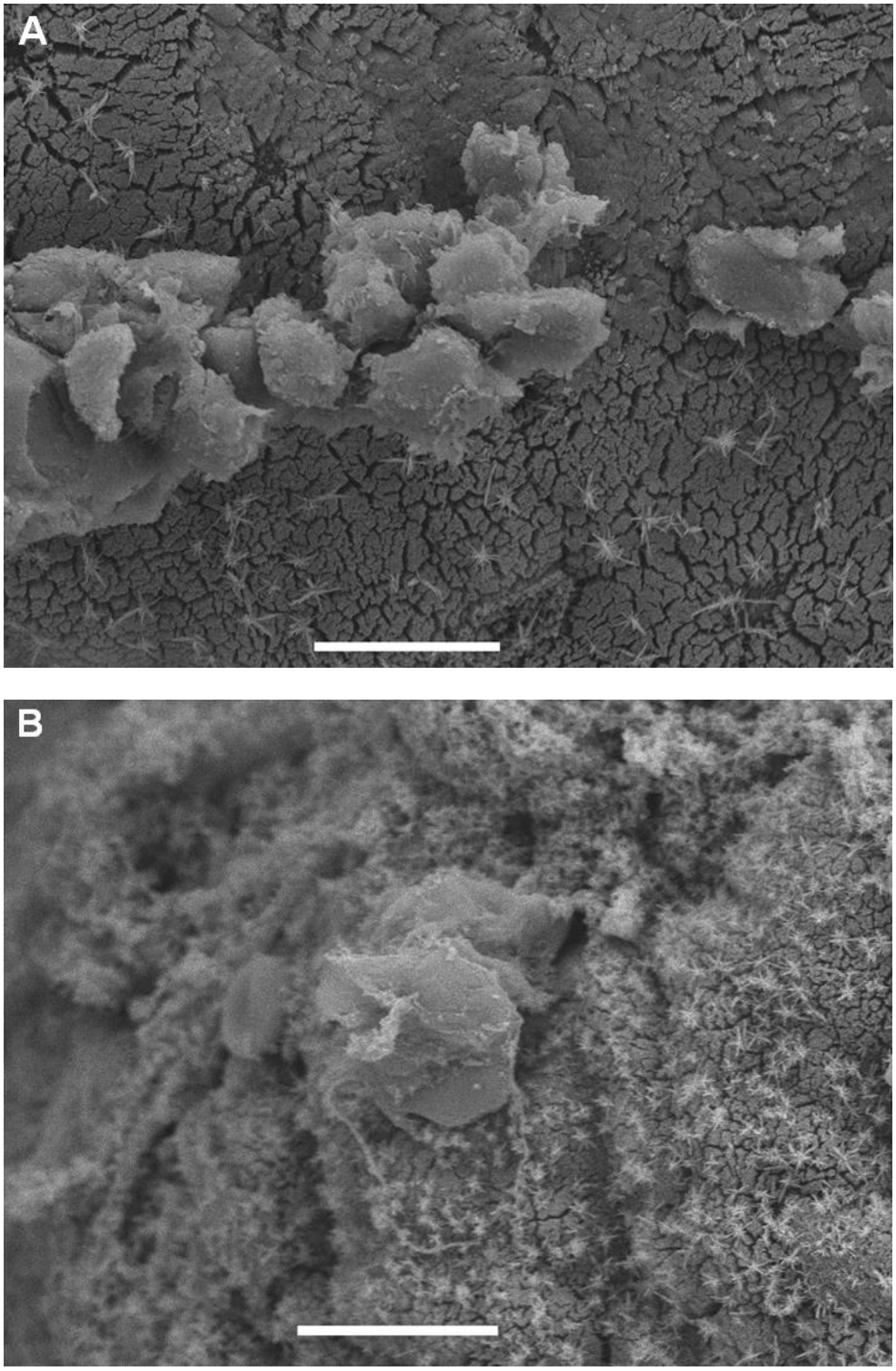

Figure 15. Scanning electron microscope images of zinc-supplemented *G. lamblia*-infected gerbils (ZS) 30 dpi, showing swollen trophozoites but still keeping their pear shape (A) x2500, bar 10 µm (B) x2700, bar 10 µm

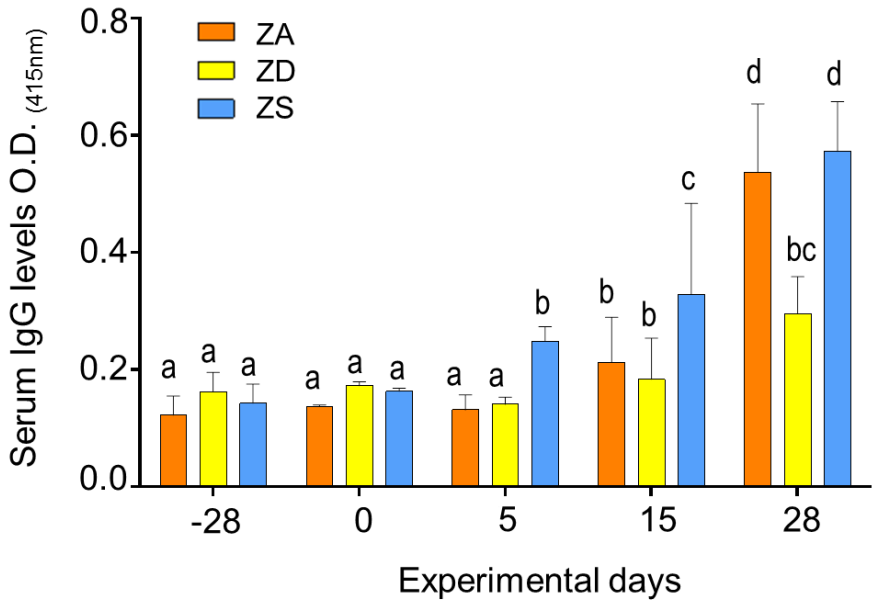

Figure 16. Pattern of induction of specific serum antibodies in *G. lamblia* infected gebils. Anti-*G. lamblia* serum antibodies were determined by ELISA in samples collected at different time intervals. Data shown are the mean values ± SD of 3-4 gerbils in each group. Zinc treatment is shown at the upper-left corner. Different letters represent significant differences throughout the infection or at specific time-points