Long-term survive of *Aliarcobacter butzleri* in two models symbiotic interaction with *Acanthamoeba castellanii*

Gustavo A. Medina1 · Sandra N. Flores-Martin2 · Wellison A. Pereira3 · Elías G. Figueroa4 · Neftalí H. Guzmán1 · Pablo J. Letelier1 · Marcela R. Andaur1 · Pilar I. Leyán1 · Rodrigo E. Boguen1 · Alfonso H. Hernández1 · Heriberto Fernández2

Received: 23 August 2022 / Revised: 23 August 2022 / Accepted: 25 August 2022
© The Author(s), under exclusive licence to Springer-Verlag GmbH Germany, part of Springer Nature 2022

**Abstract**

*Aliarcobacter butzleri* (formerly known as *Arcobacter butzleri*) is an emerging food-borne zoonotic pathogen that establishes in vitro endosymbiotic relationships with *Acanthamoeba castellanii*, a free-living amoeba. Previously, we described that this bacterium acts as an endocytobiont of *A. castellanii*, surviving for at least 10 days in absence of bacterial replication. Thus, the aim of this study was to evaluate the ability of *A. butzleri* to survive as a long-term endosymbiont of *A. castellanii* for 30 days in two models of symbiotic interaction with *A. castellanii*: (i) endosymbiotic culture followed by gentamicin protection assay and (ii) transwell co-culture assay. The results allow us to conclude that *A. butzleri* is capable of surviving as an endosymbiont of *A. castellanii* for at least 30 days, without multiplying, under controlled laboratory conditions. In addition, in the absence of nutrients and as both microorganisms remain in the same culture, separated by semi-permeable membranes, *A. castellanii* does not promote the survival of *A. butzleri*, nor does it multiply. Our findings suggest that the greater survival capacity of *A. butzleri* is associated with their endosymbiont status inside *A. castellanii*, pointing out the complexity of this type of symbiotic relationship.

**Keywords** *Acanthamoeba* · *Aliarcobacter* · Endosymbiosis

*Acanthamoeba castellanii* is a ubiquitous free-living amoeba (FLA) that plays an important role in the ecology of multiple ecosystems due to its participation in nutrient recycling, mainly in aqueous environments (Scheid 2014; Anderson et al. 2005). This protozoan feeds on bacteria, algae and yeasts, controlling the biomass of these organisms in the environment (Yousuf et al. 2013). However, some bacteria are resistant to amoebic phagocytosis and can survive and/or multiply inside FLA, being able to establish endosymbiotic relationships, mainly with *A. castellanii*. Some of these bacteria are considered to be clinically important pathogens for humans and other mammals, being collectively named ARB for amoebae-resistant bacteria (Schuster 2002; Greub and Raoult 2004; Anderson et al. 2005; Garcia-Sanchez et al. 2013; Mella et al. 2016; Balcunz and Scheid 2017).

*Aliarcobacter butzleri* [formerly known as *Arcobacter butzleri* (Oren and Garrity 2014)] is a small, curved, non-spore-forming Gram-negative rod, considered an emerging food-borne zoonotic pathogen worldwide, classified as a serious risk to humans (Vandamme et al. 1992; ICMSF 2002; Ramees et al. 2017). It is the species of the genus most frequently isolated from environmental water, food and human clinical samples, being associated with abortion and enteritis in animals, as well as diarrhea and occasional systemic infections in humans (Collado and Figueras 2011; Ferreira et al. 2015). *A. butzleri* and FLA can be frequently found in environmental water sources, where this bacterium
could enter in contact with \textit{A. castellanii}, acting as ARB and establishing endosymbiotic relationships, suggesting that amoebas may be potential environmental reservoirs and vehicles for \textit{A. butzleri} in different environmental water sources (Collado and Figueras 2011; Ferreira et al. 2015; Mella et al. 2016).

Previously, we described some of the mechanisms associated with the endosymbiotic process between \textit{A. butzleri} and \textit{A. castellanii} under controlled laboratory conditions (Fernández et al. 2012; Medina et al. 2014, 2019; Villanueva et al. 2016). This bacterium acts as an endocytobiont of \textit{A. castellanii} due to its ability to establish itself within amoebic vacuoles, surviving for at least 10 days in absence of bacterial replication. The adhesion of \textit{A. butzleri} to amoebas mainly involves membrane-associated galactose receptors present on the amoebic membrane, and the PI3K and RhoA pathways are involved in the bacterial internalization, where the tyrosine kinase-induced actin polymerization signal is essential in \textit{Acanthamoeba}-mediated bacterial uptake. Furthermore, \textit{A. butzleri} requires a biphasic transcriptional pattern of flagellar and putative virulence genes to establish an endosymbiotic relationship with \textit{A. castellanii}.

Despite advances in understanding the events involved between these microorganisms during the early stages of endosymbiosis, there are no studies that contribute to describe the behavior of \textit{A. butzleri} as a long-term endosymbiont of \textit{A. castellanii}. Moreover, the effect that amoebic factors would promote on \textit{A. butzleri} in the long-term under controlled laboratory conditions remains unknown. Therefore, the aim of this study was to analyze the ability of \textit{A. butzleri} to survive and multiply in two models of long-term symbiotic interaction with \textit{A. castellanii} for 30 days.

In this study, \textit{A. castellanii} T4 genotype originally isolated from a patient with keratitis (European Centre for Disease Prevention and Control—ECDC—Collection, BP 91/2760) was used in all the experiments. Trophozoites were maintained in log phase at 25 °C in T-25 tissue culture flasks with 10 mL of modified peptone–yeast–glucose (PYG) medium. Prior to the assays a suspension of $5 \times 10^6$ amoebae/mL in PBS 1X was adjusted as previously described by Medina et al. (2019). \textit{A. butzleri} ATCC 49616 and native strain HF2810, isolated from an environment water source, were used in all experiments. The routine cultures were performed on plates of Blood Agar Base No. 2 (Oxoid ™) supplemented with 5% sheep blood. The plates were incubated for 24 h at 30 °C under aerobic conditions. Prior to the assays, a suspension of each strain culture in exponential growth phase was adjusted to $5 \times 10^7$ bacteria/mL in PBS 1X as previously described by Medina et al. (2019).

Endosymbiotic culture assays were performed as previously described by Medina et al. (2019) and Jung et al. (2007), at two different multiplicities of infection (MOI), as follows: A suspension of $5 \times 10^5$ amoebas was inoculated with $5 \times 10^7$ bacteria (MOI 100) into a microcentrifuge tube (Eppendorf Tubes™) in a total volume of 1 mL PBS 1×. A second suspension of $6 \times 10^5$ amoebas was inoculated with $2.4 \times 10^6$ bacteria (MOI 40) in a total volume of 1 mL PBS 1×. Later, both samples were incubated for 1 h at 25 °C. A bacteria-free amoebas suspension was included as control. The intracellular survival of \textit{A. butzleri} was determined by the gentamicin protection assay with the addition of 50 μg/mL gentamicin during 1 h at 25 °C to kill extracellular bacteria. Subsequently, gentamicin was removed by twice step of centrifugation (2000 rpm x 10 min). The obtained pellet was suspended in 1 mL of PBS with 10 μg/mL of gentamicin on the flat side of the 3 mL cell culture tube (Thermo Scientific™) and kept at an inclination of approximately 45° at 25 °C for 1, 2, 5, 10, 15, 20, 25 and 30 days, followed by gentamicin treatment (50 μg/mL gentamycin at 30 °C for 1 h) to kill possible extracellular bacteria.

Again, gentamycin was removed by repeating the centrifugation step twice (2000 rpm x 2 min). Amoebas were counted to detect possible variations in the number of trophozoites in all the infection times. Pellets were suspended in 1 mL of PBS 1× containing sodium deoxycholate 0.5% and incubated at 30 °C for 10 min to lyse amoebae and release intracellular bacteria. The samples were then washed with PBS 1× by centrifugation at 2000 rpm for 2 min to remove the sodium deoxycholate. The viable bacteria were counted according to the protocol previously described by Medina et al. (2019). Briefly, 250 μL of sample was loaded into the first well of each row in a 96-well plate, and ten-fold serial dilutions were made using a multichannel pipette by transferring 20 μL from initial column into 180 μL of medium in the next column, mixing ten times, and repeating the process until completion of six dilutions. Pipette tips were changed between dilutions. Then, three replicates of 10 μL from each of the six dilutions were plated onto plates of Blood Agar Base No. 2 (Oxoid ™) supplemented with 5% sheep blood. The plates were incubated for 24 h at 30 °C under aerobicic conditions. Then, colonies were enumerated at the latest dilution where growth was observed. Colony count was calculated by multiplying the number of colonies observed by the corresponding dilution. Additionally, the amoebae infection percentage was calculated by counting in the Neubauer chamber at the times of infection.

Transwell co-culture assays were performed as previously described by Laskowski-Arce and Orth (2008) with modifications. Briefly, a suspension of $5 \times 10^7$ bacteria was deposited at the bottom of a 24-well tissue culture plate. Then, a 0.2 μm pore size transwell membrane (Anopore™NUNC™ Cell Culture) was inserted into each well of the 24-well tissue culture dish. Later, a suspension of $5 \times 10^5$ amoebas was added over the transwell membrane. The final volume of co-culture was adjusted to 2 mL PBS 1×. The transwell co-cultures were incubated in humid chamber at 25 °C for 1,
2, 5, 10, 15, 20, 25 and 30 days. The viable bacteria were counted as mentioned above, with the following modifications: prior to the bacterial count, the integrity of the transwell membrane was verified by seeding a volume of 10 μL from upper of the well onto plates of Blood Agar Base No. 2 (Oxoid™) supplemented with 5% sheep blood, incubated for 24 h at 30 °C under aerobiotic conditions. Then, the transwell membrane was removed and the well was exposed at -20° C for 3 min in order to detach the bacterial monolayer. The bacterial suspension was washed by centrifugation (2000 rpm × 2 min) and the obtained pellet was suspended in 1 mL of PBS. Then, the viable bacteria were counted according to the protocol mentioned above.

For both endosymbiotic culture and transwell co-cultures assays, experimental units were done in duplicate (biological duplicate), and each duplicate was performed in triplicate. Data were analyzed using Student’s *t* test and one-way ANOVA with GraphPad software. Values of *p* < 0.05 were considered statistically significant.

When evaluating the viability and multiplication capacity of *A. butzleri* as an endosymbiont of *A. castellanii* at different multiplicity of infection (MOI), it was observed that both strains of *A. butzleri* have the capacity to survive, for at least 30 days, under controlled conditions in endosymbiosis with *A. castellanii*, while the survival capacity of this bacterium was observed in all trials (Fig. 1). Previously, the ability of *A. butzleri* to survive inside *A. castellanii* for 240 h has been demonstrated, suggesting that it can resist amoeba digestion processes (Villanueva et al. 2016). From the results of this study, the ability of *A. butzleri* to survive, for at least 30 days, as an endosymbiont of *A. castellanii* is demonstrated for the first time. In this sense, Balczun and Scheid (2017) proposed that the relationship between microorganism/FLA would promote evolutionary processes associated with the development of pathogenicity and adaptation to human macrophages.

Significant differences were observed in the percentage of viable intra-amoebic bacteria dependent on the

---

**Fig. 1** Endosymbiotic culture assays. Graphics represent the results indicating the count of viable intra-amoebic bacteria expressed as log CFU/mL. a *A. butzleri* ATCC 49616 (MOI 1:40); b *A. butzleri* ATCC 49616 (MOI 1:100); c *A. butzleri* HF2810 (MOI 1:40); d *A. butzleri* HF2810 (MOI 1:100). Data were analyzed using Student’s *t* test through Graph Pad software. (*p* < 0.05; **p** < 0.01; ***p*** < 0.001)
MOI at which the tests were performed. For MOI 1:40, 1.75% (SD ± 0.34) of viable intra-amoebic bacteria was observed for ATCC 49619 and 1.62% (SD ± 0.21) for HF 2810. On the other hand, when performing the infection at a MOI of 1:100, a percentage of viable bacteria of 11.26% (SD ± 3.4) and 11.93% (SD ± 2.7) was obtained for the ATCC 49619 and HF 2810 strains, respectively. The results observed allow us to suggest that the culture of *A. butzleri* in endosymbiosis with *A. castellanii* has a more stable behavior at a MOI greater than or equal to 500,000 amoebas/mL. Therefore, this bacterial/amoeba ratio would be optimal for these tests.

When analyzing the behavior of *A. butzleri* ATCC 49619 as an endosymbiont of *A. castellanii* (for MOI 1:40) significant differences were observed in all counts after the first day of endosymbiosis (Fig. 1A). In contrast, no significant changes were observed in the count of viable intra-amoebic bacteria in the first 10 days after the initiation of endosymbiosis in the cultures made at MOI of 1:100 (Fig. 1B). Regarding the *A. butzleri* HF 2810 strain, significant differences were observed in the number of CFU/mL obtained after the first day of endosymbiosis initiation for MOI 1:40 (Fig. 1C), while for the second trial (MOI 1:100), no significant differences were observed in the count of viable intra-amoebic bacteria until day 5 after endosymbiosis began (Fig. 1D). Finally, the results indicate that both strains of *A. butzleri* do not undergo multiplication in endosymbiosis with *A. castellanii* since the count of viable intra-cellular bacteria (CFU/mL) decreased as the infection time increased, independent of the MOI to which the cultures were made.

This finding suggests that, under in vitro culture conditions for 30 days, *A. butzleri* is capable of surviving but not multiplying within *A. castellanii* and can be considered a stable relationship over time. Furthermore, we previously showed that during the early stages of endosymbiosis, there is not colocalization between amoebic vacuoles containing *A. butzleri* and mitochondria or ER vesicles of *A. castellanii*, which would contribute. Therefore, the lack of replication of *A. butzleri* may be associated with its inability to access nutrients that are found in endoplasmic reticulum vesicles and mitochondria.

In this context, two mechanisms have been proposed to explain the interaction of amoebas with bacteria. In the first of them, the amoeba acts as a reservoir where the bacteria are able to evade the defenses of the amoeba, which allows its survival and multiplication (Kebbi- Beghdadi and Greub 2014). The second mechanism proposes that the amoeba acts as a “Trojan horse”, allowing the bacterium to remain viable without multiplying in number, which would indicate that the microorganism has mechanisms that protect it from the lysosomal action of the amoeba (Mungroo et al. 2021). Based on this evidence, the observed results suggest that *A. castellanii* would participate as a transmission vehicle for *A. butzleri*.

To determine the viability and multiplication capacity of *A. butzleri* in the presence of *A. castellanii*, co-cultures using semi-permeable membranes were performed, allowing both microorganisms to be kept in the same culture but in separate compartments. This interaction model has been used to describe the symbiotic relationships between various bacteria and FLA. The results show that both strains of *A. butzleri* remain viable for at least 30 days (under controlled conditions) in the presence of *A. castellanii* (Fig. 2). For both strains, no significant differences were observed until day 25 of infection between the number of viable bacteria maintained in co-culture with respect to the control, which corresponded to a suspension of bacteria, free of amoebas, incubated under the same conditions as the co-cultures. On the other hand, on day 30 of infection significant differences were observed with respect to the control. As reported in the first interaction model analyzed,
no multiplication of A. butzleri strains was observed in the presence of A. castellanii (Fig. 2).

Available evidence regarding this second analyzed model suggests that the extracellular survival of bacteria would depend on a diffusible factor produced and secreted by the amoeba, which highlights the possibility that these microorganisms coexist in the environment, being considered as an interaction of a high level of complexity and dependent on each species. Thus, the observed results show that A. castellanii does not promote the survival of A. butzleri, nor its multiplication, as both microorganisms remain in the same culture separated by semi-permeable membranes. This indicates that, in the absence of nutrients, the amoeba would not produce any diffusible factor that positively or negatively alters the survival of the bacteria, at least during the first 25 days of incubation. Therefore, the greater survival capacity of A. butzleri is associated with the endosymbiont status of A. castellanii, which shows the complexity of this type of symbiotic relationship.

Finally, the results of this study allow us to conclude that A. butzleri is capable of surviving as an endosymbiont of A. castellanii for at least 30 days, without multiplying, under controlled laboratory conditions. In addition, in the absence of nutrients and as both microorganisms remain in the same culture, separated by semi-permeable membranes, A. castellanii does not promote the survival of A. butzleri, nor does it multiply.

Acknowledgements I would like to express my deep gratitude to Dra. Carola Otth, my co-sponsor professor who recently pass away, for their patient guidance, enthusiastic encouragement and useful critiques of this paper and the research behind.

Author contributions GM, WP and AH wrote the main manuscript text. GM, HF and SFM planned the experimental design. GM, PL and SFM performed the experiments. EF, NG and RB performed the statistical analyzes. PL and MA prepared Figs. 1 and 2.

Funding This work was supported by Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT, #1110202), Fondo Concurso Interno Línea Profondecyt Vicerrectoría de Investigación y Postgrado Universidad Católica De Temuco (VIPUCT, #2016PF-GM-03) and Fondo de Equipamiento (FEQUIP2019-CS-05) Vicerrectoría de Investigación y Posgrado Universidad Católica de Temuco.

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

References

Almsherqi Z, Hyde S, Ramachandran M, Deng Y (2008) Cubic membranes: a structure-based design for DNA uptake. J R Soc Interface 5:1023–1029. https://doi.org/10.1098/rsif.2007.1351
Anderson I, Watkins RF, Samuelson J et al (2005) Gene discovery in the genome. Protist 156:203–214. https://doi.org/10.1016/j.protis.2005.04.001
Balczun C, Scheid PL (2017) Free-living amoebae as hosts for and vectors of intracellular microorganisms with public health significance. Viruses. https://doi.org/10.3390/v9040065
Burdíková Z, Čapek M, Ostašov P et al (2010) Testate amoebae examined by confocal and two-photon microscopy: implications for taxonomy and ecophysiology. Microsc Microanal 16:735–746. https://doi.org/10.1017/S1431927610094031
Chen CY, Nace GW, Irwin PL (2003) A 6x6 drop plate method for simultaneous colony counting and MPN enumeration of Campylobacter jejuni, Listeria monocyogenes, and Escherichia coli. J Microbiol Methods 55:475–479. https://doi.org/10.1016/S0167-7012(03)00194-5
Collado L, Figueras MJ (2011) Taxonomy, epidemiology, and clinical relevance of the genus Arcobacter. Clin Microbiol Rev 24:174–192. https://doi.org/10.1128/CMR.00034-10
Fera MT, Maugeri TL, Gugliandolo C et al (2008) Induction and resuscitation of viable nonculturable Arcobacter butzleri cells. Appl Environ Microbiol 74:3266–3268. https://doi.org/10.1128/AEM.00059-08
Fernández H, Villanueva MP, Medina G (2012) Endosymbiosis of Arcobacter butzleri in Acanthamoeba castellanii. Rev Argent Microbiol 44:133
Ferreira S, Queiroz JA, Oleastro M, Domingues FC (2015) Insights in the pathogenesis and resistance of Arcobacter: a review. Crit Rev Microbiol. https://doi.org/10.3109/1040841X.2014.954523
García-Sanchez AM, Ariza C, Ubeda JM et al (2013) Free-living amoeba in sediments from the Lascaux Cave in France. Int J Speleol 42:9–13. https://doi.org/10.5038/1827-806X.42.1.2
Gilson PR, Yu X-C, Hareld D et al (2003) Two Dicystostelium orthologs of the prokaryotic cell division protein FtsZ localize to mitochondria and are required for the maintenance of normal mitochondrial morphology. Eukaryot Cell 2:1315–1326. https://doi.org/10.1128/EC.2.6.1315-1326.2003
Greub G, Raoult D (2004) Microorganisms resistant to free-living amoebae. Clin Microbiol Rev 17:413–433
International Commission for the Microbiological Specifications of Foods (2002) Microorganisms in foods 7—microbiological testing in food safety management, 1st edn. Springer, New York
Jung SY, Matin A, Kim KS, Khan NA (2007) The capsule plays an important role in Escherichia coli K1 interactions with Acanthamoeba. Int J Parasitol 37:417–423. https://doi.org/10.1016/j.ijpara.2006.10.012
Kakley MR, Velle KB, Fritz-Laylin LK (2018) Relative quantitation of polymerized actin in suspension cells by flow cytometry. Bio-Protoc. https://doi.org/10.21769/BioProtoc.3094
Kebbi-Beghdadi C, Greub G (2014) Importance of amoebae as a tool to isolate amoeba-resisting microorganisms and for their ecology and evolution: the Chlamydia paradigm. Environ Microbiol Rep 4:309–24
Khan NA, Siddiqui R (2014) Predator vs aliens: bacteria interactions with Acanthamoeba. Parasitology 141:869–874. https://doi.org/10.1017/S003118210100231X
Laskowski-Arce MA, Orth K (2008) Acanthamoeba castellanii promotes the survival of Vibrio parahaemolyticus. Appl Environ Microbiol 74:7183–7188. https://doi.org/10.1128/AEM.01332-08
Medina G, Flores-Martín S, Fonseca B et al (2014) Mechanisms associated with phagocytosis of Arcobacter butzleri by Acanthamoeba
Medina G, Neves P, Flores-Martin S et al (2019) Transcriptional analysis of flagellar and putative virulence genes of Arcobacter butzleri as an endocytobiont of Acanthamoeba castellanii. Arch Microbiol. https://doi.org/10.1007/s00203-019-01678-0

Mungroo M, Siddiqui R, Khan N (2021) War of the microbial world: Acanthamoeba spp. interactions with microorganisms. Folia Microbiol 66:689–699. https://doi.org/10.1007/s12223-021-00889-7

Mella C, Medina G, Toledo Z (2016) Interaction between zoonotic bacteria and free living amoebas. A new angle of an epidemiological polyhedron of public health importance. Austral J Vet Sci 10:1–10

Molofsky AB, Swanson MS (2004) Differentiate to thrive: Lessons from the Legionella pneumophila life cycle. Mol Microbiol 53:29–40

Oliver JD (2005) The viable but nonculturable state in bacteria. J Microbiol 43((spec 1)):93–100

Oren A, Garrity GM (2014) List of new names and new combinations previously effectively, but not validly, published. Int J Syst Evol Microbiol 64:1–5. https://doi.org/10.1099/ijs.0.060285-0

Pizarro-Cerdá J, Méresse S, Parton RG et al (1998) Brucella abortus transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. Infect Immun 66:5711–5724

Ramees TP, Dhama K, Karthik K et al (2017) Arcobacter: an emerging food-borne zoonotic pathogen, its public health concerns and advances in diagnosis and control—a comprehensive review. Vet Q 37:136–161. https://doi.org/10.1080/01652176.2017.1323355

Roy CR, Tilney LG (2002) The road less traveled: transport of Legionella to the endoplasmic reticulum. J Cell Biol 158:415–419. https://doi.org/10.1083/jcb.200205011

Scheid P (2014) Relevance of free-living amoebae as hosts for phylogenetically diverse microorganisms. Parasitol Res 113:2407–2417

Publisher’s Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.