**University of São Paulo
“Luiz de Queiroz” College of Agriculture**

**Isolation, identification and genotypic and phenotypic characterization
of *Arcobacter* spp. isolated from Minas frescal cheese and raw milk**

Melina Luz Mary Cruzado Bravo

Thesis presented to obtain the degree of Doctor in Science.
Area: Food Science and Technology

**Piracicaba
2020**

Melina Luz Mary Cruzado Bravo
Agroindustrial Engineering

**Isolation, identification and genotypic and phenotypic characterization
of *Arcobacter* spp. isolated from Minas frescal cheese and raw milk**
versão revisada de acordo com a resolução CoPGr 6018 de 2011

Advisor:
Prof.^a. Dra. **CARMEN JOSEFINA CONTRERAS CASTILLO**

Co-Advisor:
Prof. Dr. **LUIS ROBERTO COLLADO GONZÁLEZ**

Thesis presented to obtain the degree of Doctor in Science.
Area: Food Science and Technology

Piracicaba
2020

Dados Internacionais de Catalogação na Publicação
DIVISÃO DE BIBLIOTECA – DIBD/ESALQ/USP

Cruzado Bravo, Melina Luz Mary

Isolation, identification and genotypic and phenotypic characterization of *Arcobacter* spp. isolated from Minas frescal cheese and raw milk / Melina Luz Mary Cruzado Bravo. - - versão revisada de acordo com a resolução CoPGr 6018 de 2011. - - Piracicaba, 2020.

88 p.

Tese (Doutorado) - - USP / Escola Superior de Agricultura “Luiz de Queiroz”.

1. Patógeno emergente 2. Produtos lácteos 3. Genes de virulência 4. Resistência aos antibióticos 5. Biofilmes I. Título

DEDICATION

Dedico este trabajo a mis padres Isabel y Nery,
mis hermanitas Vanessa y Jheny,
mis sobrinos Alexa, Antonio y Nerick
y a mi querida Sunchubamba.

ACKNOWLEDGEMENTS

Es probable que olvide agradecer a algunas personas que me permitieron llegar a la culminación de este largo camino, por lo cual, pido disculpen mi memoria

A mis padres, Isabel e Nery, que sacándome del campo me dirón grandes oportunidades, por que su esfuerzo valió la pena papis

A mis hermanas Vanessa y Jheny, siempre me dieron fuerza y ánimos para seguir y por que también me han dado unos sobrinos geniales

Agradezco gratamente a mi gran amigo Erick Saldaña Villa, por toda la ayuda recibida, por las bromas, por ser la persona quien me inspiró a iniciar mi vida como investigadora, es a quien le debo el estímulo y las buenas vibras

A mi orientadora Carmen Contreras Castillo, quien ha sido importante en todo este camino, quien me dió la oportunidad de cambiar mi vida

A mi coorientador Luis Collado, un excelente profesional, amigo, por sus consejos, me ayudó demasiado en todo este camino, siempre le estaré eternamente agradecida, me dió la oportunidad de aprender junto a él y su equipo

Al profesor Rodrigo Bicalho, por brindarme una excelente oportunidad con el intercambio en la Universidad de Cornell (USA), un gran profesional y persona, mi agradecimiento y estima estarán siempre con él y su querida esposa Marcela Bicalho que me hicieron sentir en familia

A la sra. Denise Leme, por ser una segunda madre, por todos los consejos recibidos, por que me dió fuerza y compañía en los momentos más difíciles, muchas gracias por alimentarme y siempre ser tan gentil, por que siempre la tendré en mi corazón

A mis amigos los peruanitos: Carmencita, Claudio, Rafaél, Meliza, Dario, Fernando, Paola son mi familia, mi compañía, tenemos grandes recuerdos, alegrías, una amistad que perdurará por siempre

A mis colegas del laboratorio de *Higiene e Laticínios*: Giovani Henrique Mariano, Nicolle Padovani, Fernanda Muto, Thiago Sugizaki, Vitoria Bagarollo, Amanda geraldí, Mayara Cardoso por toda su ayuda y disposición en la colecta de material y toda la tormenta de análisis, gracias chicos!

A la Dra. Giovana Barancelli, una gran amiga y colega, siempre tan servicial ayudándome en todo momento, fuiste fundamental para llegar hasta aquí querida Gio

A las profesoras Daniele Maffei y Aline Cesar, por toda su ayuda y disposición

A mis colegas del Laboratorio de *Campylobacter* y *Helicobacter* de la Universidad Austral de Chile, a mi gran amiga, colega de pláticas y apoyo mutuo Sofía Ochoa, al gran Boris Vidal, el lindo y tierno Sebastián Ruiz, me sentí tan bien con ustedes chicos

A mi siempre querida y recordada Marjory Xavier Rodrigues, eres una linda persona, por toda la ayuda y enseñanza en los meses que nos tocó convivir durante mi intercambio en Cornell, y por que el trio Lab, volvió a encontrarse, con la presencia de nuestra querida profesora Nathália Cirone Silva. Ma querida me permitiste conocer grandes personas, Josi Silva, tan linda y servicial, una bellísima persona (la amo!!), el gran Leo Bringhenti, Tiago Tomazi, Ana Campos, Yasmim Pazini, Plínio Azevedo, Márcia Melo, amigos que voy a recordar y llevar conmigo siempre, me acogieron y me hicieron parte de Bicalho Lab, gracias infinitas

A mis queridos amigos de la Vila, Roni, Sibeles, Aline, Timoteo, Dani una grandiosa etapa de comida, baile e intercambio cultural, siempre los recordaré y extrañaré nuestros churros y reuniones de la nada, organizadas por el grandioso Roni, mi amigo, mi confidente mi cosita bonita

A mi miga sua louca, Yasmin Florentino, siempre tan ocurrente y buena persona, con un corazón de oro, me ayudas siempre querida Yasmi, me acogiste e hiciste que me sienta de tu familia, gracias por todo

A mis colegas de innumerables casas, por que mude muchas veces, Tatiana, Jenifer, Cezar, Fabio, siempre fui acogida y la convivencia fue genial

A mis últimos colegas de casa, ya dije que me mudé bastante, Julio y Denise, por toda su ayuda, me hacen sentir en casa, gracias chicos, les deseo lo mejor

Al departamento de *Agroindustria Alimentos e Nutrição* ESALQ- USP, a los excelentes profesionales que forman parte de esta gran institución

A la Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brasil) por la beca de 36 meses concedida

Al Concejo Nacional de Ciencia, Tecnología e Innovación Tecnológica – (CONCYTEC, Perú) por la subvención de 12 meses concedida con el contrato N° 125-2018-FONDECYT que me han permitido culminar mis actividades

EPIÍGRAFE

*“Mesmo quando tudo parece desabar, cabe a mim decidir entre rir ou chorar, ir ou ficar, desistir ou lutar;
porque descobri, no caminho incerto da vida, que o mais importante é o decidir”*

Cora Carolina

CONTENTS

RESUMO.....	9
ABSTRACT	10
INTRODUCTION	12
CHAPTER 1: LITERATURE REVIEW	14
1.1 <i>Arcobacter</i> GENUS	14
1.2 CLINICAL RELEVANCE OF <i>Arcobacter</i>	18
1.3 <i>Arcobacter</i> IN DAIRY PRODUCTS	18
LITERATURE CITED REVIEW	21
CHAPTER 2: Occurrence of <i>Arcobacter spp.</i> in Brazilian Minas frescal cheese and raw cow milk and its association with microbiological and physicochemical parameters	29
CHAPTER 3: Biofilm formation on contact surfaces and different media of <i>Arcobacter butzleri</i> isolates from dairy products	55
FINAL CONCLUSION	86
PUBLICATION LIST	87

RESUMO

Identificação e caracterização genotípica e fenotípica de *Arcobacter* spp. isolados em queijo minas frescal e leite bovino cru

Arcobacter é considerado um patógeno emergente de origem alimentar; associado a gastroenterites, diarreia persistente e bacteremia em humanos. Em animais pode provocar aborto, infertilidade e diarreia. O isolamento a partir de animais assintomáticos também é frequente, acreditando-se que possam ser seu reservatório natural. Alimentos de origem animal provavelmente são a principal rota de transmissão para humanos. Estudos realizados em pacientes com gastroenterite indicam que *Arcobacter* está na quarta posição dos patógeno com maior número de isolado, tendo sido reportado em diferentes países. Atualmente, no Brasil há uma grande ocorrência de doenças gastrointestinais sem identificação do agente etiológico. O isolamento de *Arcobacter* em queijo Minas frescal e leite bovino cru no estado de São Paulo, Brasil, identificou a ocorrência de um gênero anteriormente ignorado ou desconhecido. Neste trabalho, foram analisadas 98 amostras de queijo Minas frescal e 103 amostras de leite bovino cru, mostrando uma ocorrência de 10.2% e 16.5% respectivamente, todos os isolados foram identificados como *A. butzleri*, a presença de *Arcobacter* foi correlacionada com a umidade do queijo e com os coliformes totais e a contagem total de mesófilos no leite. Posteriormente, 36 cepas de *A. butzleri*, foram analisadas quanto à virulência, resistência aos antibióticos e a formação de biofilmes em microplaca. Finalmente, em oito destes isolados foi avaliada a formação de biofilmes sobre diferentes superfícies de contato comumente utilizadas em laticínios e a influência de outros fatores na produção de biofilmes de *A. butzleri*. Observou-se alta diversidade genotípica, sensibilidade aos antibióticos Ciprofloxacino, eritromicina e azitromicina. As cepas provenientes de leite cru foram as que apresentaram maior formação de biofilmes. A superfície de contato com maior adesão celular foi o aço inoxidável, utilizando Mueller Hinton como caldo de cultivo. Com a informação aqui obtida sobre os isolados de *Arcobacter* permite-se o desenvolvimento de estratégias mais eficientes para evitar a contaminação em alimentos prontos para o consumo como o queijo Minas frescal, verificar o tratamento térmico adequado, e verificar a limpeza e sanitização correta em tempos e aplicação de sanitizantes corretos. Pesquisas mais aprofundadas sobre a presença e atividade do gênero *Arcobacter* é necessária na cadeia completa de produtos lácteos, uma vez que o mesmo tem sido identificado com frequência nesses alimentos.

Palavras-chave: Patógeno emergente, Produtos lácteos, Biofilmes, Genes de virulência, Superfícies de contato

ABSTRACT

Isolation, identification and genotypic and phenotypic characterization of *Arcobacter* spp. isolated from Minas frescal cheese and raw milk

Arcobacter is considered an emerging foodborne pathogen which is associated with gastroenteritis, persistent diarrhea and bacteremia in humans. Similarly, in animals, it can cause abortion, infertility and diarrhea. Isolation of this pathogen from asymptomatic animals is also frequent as they are natural reservoirs. Animal foods are probably the main route of transmission to humans. Studies carried out in different countries have indicated that patients with gastroenteritis caused by *Arcobacter* is the fourth most common isolate. Currently, in Brazil, there is a great occurrence of gastrointestinal diseases without identification of the etiologic agent. *Arcobacter* was isolated from Minas frescal cheese and raw bovine milk in São Paulo, Brazil. In this work, 98 samples of Minas frescal cheese and 103 samples of raw bovine milk were analyzed and data showed 10.2% and 16.5% occurrence respectively. All isolates were identified as *A. butzleri* and the presence of *Arcobacter* was correlated with the moisture (cheese) and total coliforms and mesophilic bacteria counts (milk). Subsequently, 36 strains of *A. butzleri* were analyzed for virulence, resistance to antibiotics and the formation of biofilms. Then, the formation of biofilms in eight isolates was observed on different contact surfaces commonly used in dairy processing plants. Finally, the influence of other factors on the production of *A. butzleri* biofilm was evaluated. High genotypic diversity, sensitivity to ciprofloxacin antibiotics, erythromycin and azithromycin were observed. Strains from raw milk showed the highest biofilm formation ability. Stainless steel presented the greatest cellular adhesion. With the information obtained about *Arcobacter* isolates, it is possible to develop better strategies to avoid contamination in ready-to-eat foods, such as Minas fresal cheese, and to provide valuable information on proper heat treatment conditions, cleanliness and sanitation. Further research concerning the presence and activity of the *Arcobacter* genus is necessary in the whole dairy product chain.

Keywords: Emerging pathogen, Dairy products, Biofilms, Virulence-associated genes, Contact surfaces

INTRODUCTION

Arcobacter is an emerging pathogen initially isolated in the 70s using semi-solid leptospira medium (Beran, 1994; Ellis, Neill, O'Brien, Ferguson, & Hanna, 1977; Ellis, Neill, O'Brien, & Hanna, 1978; Neill, Ellis, & O'Brien, 1979). *Arcobacter* was named *Campylobacter cryaerophilus* because of its small curved rod shape, which is very similar to *Campylobacter* (Neill, Campbell, O'Brien, Weatherup, & Ellis, 1985; Solnick & Vandamme, 2001). After more than 10 years, in 1991, successive biochemical tests were required to differentiate *Campylobacter* strains and form a new genus, the so-called *Arcobacter* (Vandamme & De Ley, 1991; Vandamme et al., 1991, 1992).

Arcobacter species are associated with diarrhea in cattle, mastitis, abortions, infertility, and in humans they are associated with gastroenteritis and enteritis (Vandamme & De Ley, 1991; Vandamme et al., 1991, 1992). Four species are considered emerging foodborne pathogens: *A. butzleri*, *A. cryaerophilus*, *A. skirrowii* and *A. cibarius* (McGregor & Wright, 2015; Sasi Jyothsna, Rahul, Ramaprasad, Sasikala, & Ramana, 2013). These species are also frequently isolated from asymptomatic animals, who are natural reservoirs.

Through new generation sequencing, a high incidence of *Arcobacter* was observed in water samples (Collado & Figueras, 2011; Ghaju Shrestha et al., 2017). The International Commission on Microbiological Specifications for Foods (ICMSF) has considered *A. butzleri* an emerging pathogen because of its potential risk to human health. However, there are still few studies focusing on the pathogenicity of *Arcobacter* species (Cardoen et al., 2009).

Thus, increasing efforts to ensure food safety come in response to the increasing number of problems associated with food consumption and consumer concerns. Foodborne diseases are responsible for economic and social losses and have become increasingly important in public health (WHO, 2015). In Brazil, 66.8% of foodborne diseases are not identified (UVHA/MS, 2016), thus it is important to trace emerging pathogens, such as *A. butzleri*. Additionally, the lack of epidemiological data on *Arcobacter* spp. has implications for the control of the spread and treatment of possible health outcomes caused by this pathogen.

The objective of the present investigation was to evaluate the occurrence of *Arcobacter* in Minas frescal cheese and raw milk, relating its presence with some physicochemical and microbiological characteristics. Isolates were identified by PCR, PCR-RLFP, sequencing of the 16S rRNA and/or *rpoB* gene, genotypical characterization (ERIC-PCR, genes associated with virulence) and phenotypic characterization (resistance to antibiotics, biofilms formation on contact surfaces).

This investigation contributed with new information about an emerging pathogen still ignored in raw milk and dairy products. The data obtained will aid in the understanding of some factors that may be involved in the contamination of Minas frescal cheese. The generated data will provide insights on the presence of genes associated with resistance, resistance to antibiotics, and biofilm formation ability.

CHAPTER 1: LITERATURE REVIEW

1.1. *Arcobacter* genus

Taxonomy

Arcobacter and *Campylobacter* genera are closely related and are members of the *Campylobacteraceae* family. Waite et al. (2017) proposed to create the *Arcobacteraceae* family. *Helicobacter*, which belongs to the *Helicobacteraceae* family, is closely related to the two families mentioned above. The Scientific classification of bacteria is described below.

Phylum	Proteobacteria
Class	Epsilonproteobacteria
Order	Campylobacterales
Family	Campylobacteraceae
Genus	<i>Arcobacter</i>

Figure 1. Taxonomy of *Arcobacter* spp.

The genus *Arcobacter* has been created in 1991 from the subdivision of *Campylobacter* species into three large rRNA homology groups. One of these groups, composed of *Campylobacter aerotolerant* species, the creation of a new genus composed of *Arcobacter nitrofigilis* and *Arcobacter cryaerophilus* (Vandamme et al., 1991). Since then, the number of species has progressively increased from six species in 2008 to more than twenty-four in 2019 (<http://www.bacterio.net/arcobacter.html>). The species identified to date are presented in Table 1. Most species described so far have been isolated from seafood, livestock, marine environments and sewage. *Arcobacter* species are aerotolerant and able to grow at low temperatures (15 °C). Only five species have been identified in humans: *A. butzleri*, *A. cryaerophilus*, *A. thereius*, *A. skirrowii* and *Malacobacter (Arcobacter) mytili* (Banting &

Figueras Salvat, 2017; Vasiljevic et al., 2019). From these species, *A. butzleri* and *A. cryaerophilus* are probably the most clinically significant ones (Banting and Figueras 2017).

Table 1. List of *Arcobacter* species and their origin (continued)

Nº	<i>Arcobacter</i> species	First time isolated from	Reference
1.	<i>A. nitrofigilis</i>	Roots of <i>Spartina alterniflora</i>	(McClung, Patriquin, & Davis, 1983)
2.	<i>A. cryaerophilus</i>	Bovine abortus fetus	(Neill et al., 1985)
3.	<i>A. butzleri</i>	Human feces	(Kiehlbauch et al., 1991)
4.	<i>A. skirrowii</i>	Sheep feces	(Vandamme et al., 1992)
5.	<i>A. sulfidicus</i>	Coastal seawater	(Wirsén et al., 2002)
6.	<i>A. cibarius</i>	Chicken meat	(Kurt Houf, De Zutter, Verbeke, Van Hoof, & Vandamme, 2003)
7.	<i>A. halophilus</i>	Hypersaline lagoon	(Donachie, Bowman, On, & Alam, 2005)
8.	<i>A. mytili</i>	Mussels	(Collado, Cleenwerck, Van Trappen, De Vos, & Figueras, 2009)
9.	<i>A. thereius</i>	Porcine abortion	(K Houf et al., 2009)
10.	<i>A. marinus</i>	Seawater, seaweed and a starfish	(Kim, Hwang, & Cho, 2010)
11.	<i>A. tropharium</i>	Pig feces	(De Smet, De Zutter, & Houf, 2011)
12.	<i>A. defluvii</i>	Sewage samples	(Collado & Figueras, 2011)
13.	<i>A. molluscorum</i>	Shellfish	(Maria José Figueras, Collado, et al., 2011; Maria José Figueras, Levican, Collado, Inza, & Yustes, 2011)
14.	<i>A. ellisii</i> sp	Shellfish	(Maria José Figueras, Collado, et al., 2011; Maria José Figueras, Levican, et al., 2011)
15.	<i>A. venerupissp</i>	Shellfish	(Levican et al., 2012)
16.	<i>A. bivalviorum</i>	Shellfish	(Levican et al., 2012)
17.	<i>A. cloacae</i>	Sewage	(Levican, Alkeskas, Günter, Forsythe, & Figueras, 2013)
18.	<i>A. suis</i> sp	Pork meat	(Levican et al., 2013)

Note: Adapted from (Ramees et al., 2017)

Table 1. List of *Arcobacter* species and their origin (culmination)

Nº	<i>Arcobacter</i> species	First time isolated from	Reference
19	<i>A. anaerophilussp</i>	Estuarine sediment	(Sasi Jyothsna et al., 2013)
20	<i>A. ebronensis</i>	Mussels	(Levican, Rubio-Arcos, Martinez-Murcia, Collado, & Figueras, 2015)
21	<i>A. aquimarinus</i>	Sea water	(Levican et al., 2015)
22	<i>A. lanthieri</i>	Cattle and pig manure	(Whiteduck-Léveillé et al., 2015)
23	<i>A. pacificus</i>	Seawater	(Zhang, Yu, Wang, Yu, & Zhang, 2016)
24	<i>A. acticola</i>	Seawater	(Park, Jung, Kim, & Yoon, 2016)
25	<i>A. porcinus</i>	Pig and ducks	(M. J. Figueras, Pérez-Cataluña, Salas-Massó, Levican, & Collado, 2017)
26	<i>A. lekithochrous</i>	Great scallop (<i>Pecten maximus</i>) larvae and tank seawater	(Diéguez, Balboa, Magnesen, & Romalde, 2017)
27	<i>A. lacus</i>	Wastewater treatment plant	(Pérez-Cataluña, Salas-Massó, & Figueras, 2019)
28	<i>A. caeni</i>	wastewater treatment plant	(Pérez-Cataluña et al., 2019)
29	<i>A. peruensis</i>	Coastal waters	(Callbeck et al., 2019)

Note: Adapted from (Ramees et al., 2017)

Description of the genus *Arcobacter*

The morphology of *Arcobacter* spp. is very similar to that of *Campylobacter* spp. Both genera are gram negative and present non-sporogenic rods, usually curved, helical or with typical “S” or “seagull wing” arrangement, with approximately 0.2-0.9 µm of width and 0.5-3 µm in length. They grow in both microaerobic and aerobic atmosphere between 15 and 37 °C, and species can grow at 30 °C after primary isolation from a microaerobic atmosphere (Snelling, Matsuda, Moore, & Dooley, 2006).

Optimum growth of *Arcobacter* occurs under microaerophilic conditions (3-10% O₂) but the presence of hydrogen is not required. *Arcobacter anaerophilus* is anaerobic (Sasi Jyothsna et al., 2013) and grows in the presence of 1-2% NaCl and 1% (weight / volume) of vibrio static compound.

Figure 2. Typical colonies of *A. butzleri* in blood agar (A) and *Arcobacter* agar (B)

Arcobacter are oxidase and catalase positive, and some species are able to produce H_2S . Amino acids and organic acids are used as carbon sources instead of carbohydrates, (Sasi Jyothsna et al., 2013). They can survive at 4 °C, which is commonly found in retail and food processing establishments, but at 55 °C they are inactivated (Atanassova, Kessen, Reich, & Klein, 2008). Recently, *Arcobacter halophilus* and *Arcobacter marinus* were isolated from seawater and shellfish using *Arcobacter* broth supplemented with Cefoperazone, Amphotericin B and Teicoplanin (CAT) added with 2.5% NaCl (w / v) followed by streaking in marine agar. The introduction of this protocol made it possible to identify 40% more positive samples in seawater compared to the conventional method without the NaCl supplementation (Salas-Massó, Andree, Furones, & Figueras, 2016). This result emphasized the need for differentiated isolation procedures. Because *Arcobacter* is considered a difficult microorganism, the molecular identification of this species is currently the most recommended methodology (McVey, Kennedy, & Chengappa, 2013).

There is little information on *Arcobacter* virulence factors. Available research has evaluated adherence, pathogen invasion, toxin secretion, and Interleukin 8 (IL-8) production, factors that play an important role in eukaryotic cell infection (Collado & Figueras, 2011; Ferreira, Queiroz, Oleastro, & Domingues, 2015). *A. cryaerophilus* and *A. butzleri* are the two most exploited species for adhesive and invasive capacities, showing that they could be the causative agents of electrolyte and fluid accumulation (Fernández, Paillacar, Gajardo, & Riquelme, 1995). Tsang et al. 1996 reported the property of hemagglutinin in *A. butzleri*, which allows interaction with red blood cells and bacterial adhesion.

1.2. Clinical relevance of *Arcobacter*

Arcobacter infections are often confused with the outcomes of *Campylobacter* infection, which suggests an incidence underestimation of *Arcobacter* infections in consequence to the use of inappropriate methods for the detection and identification (Figueras et al., 2014).

Arcobacter is a potentially zoonotic genus and can be transmitted by contaminated food and water and the survival in the air has been recorded. Poultry and pigs are the most cited vehicles in the transmission chain. Epidemiological data have been poorly elucidated and require further specific studies. *Arcobacter* species are considered a potential pathogen for humans, especially children (Zerpa Larrauri et al., 2014). In studies conducted between 2008 and 2013, with 6,774 patients with symptoms of acute gastroenteritis and / or gastroenteritis, *Arcobacter* was the fourth most isolated pathogen (Van den Abeele, Vogelaers, Van Hende, & Houf, 2014).

Arcobacter genus has been reported in Belgium, Chile, Spain, the United States, France, Italy, New Zealand, Portugal and South Africa, among others. Feces and blood from individuals who have had gastroenteritis and bacteremia are the main focus of research. *Arcobacter* has also been isolated from tissues of bovine and porcine fetuses resulting from abortions, animal feces, poultry and pig carcasses, marine and terrestrial animal products, seawater, hypersaline lagoons and sewer pipes (Collado & Figueras, 2011). Almost no information is available regarding the correlation between *Arcobacter* genes and the underlying virulence mechanisms. Some studies have been developed in this context, but there is no formal definition of functional virulence when compared to *Campylobacter* species. However, several studies have shown the adhesion, invasion and cytotoxicity of *Arcobacter* pathogenic species in different cell lines (Collado & Figueras, 2011). In addition, it has recently been shown that *Arcobacter* species have several putative virulence genes that are similar to those of *Campylobacter jejuni* (Doudah, De Zutter, Vandamme, & Houf, 2010; Karadas et al., 2013; Levican, Collado, Yustes, Aguilar, & Figueras, 2014).

1.3. *Arcobacter* in dairy products

The dairy chain is complex and has a high probability of contamination by *Arcobacter*, mainly related to the unit operations used in the manufacture system and the high-water activity of end products, in addition to the high nutritional value that makes it suitable for

contamination. In multiple studies, *Arcobacter* has been identified in milk tanks, milking devices, barn floors and cattle feces (Serraino et al., 2013; Wesley et al., 2000; Yesilmen, Vural, Erkan, & Yildirim, 2014).

the high incidence of *Arcobacter* in dairy products and processing plants is a public health concern (Giacometti et al., 2015; Yesilmen et al., 2014). Nonetheless there are still few publications that report the presence of *Arcobacter* in dairy products (Table 2).

Table 2. *Arcobacter* spp. occurrence in raw milk and dairy products (continued)

Isolated Species	Country	Samples collected from	Occurrence (%) (n = total samples)	Reference
<i>A. butzleri</i>	Northern Ireland	tank raw milk	46 (n=101)	(Scullion, Harrington, & Madden, 2006)
<i>A. butzleri</i> <i>A. skirrowii</i>	Turkey	raw milk	6 (n = 50)	(Ertas, Dogruer, Gonulalan, Guner, & Ulger, 2010)
<i>A. butzleri</i> , <i>A. cryaerophilus</i>	Malaysia	raw milk	5.8 (n = 86)	(Shah, Saleha, Murugaiyah, Zunita, & Memon, 2012)
<i>A. butzleri</i> <i>A. cryaerophilus</i> <i>A. skirrowii</i>	Northern Spain	raw milk	73.3 (n = 12)	(Nieva-Echevarria et al., 2013)
<i>A. butzleri</i> <i>A. cryaerophilus</i>	Italy	Milk Filters	85.7 (n=49)	(Serraino et al., 2013)
<i>A. butzleri</i>	Italy	Surfaces with and without food contact, cheese, raw milk, cheese molds	49.5 (n=59)	(Giacometti et al., 2013)
<i>A. butzleri</i> <i>A. cryaerophilus</i>	Finland	raw milk	15 (n=177)	(Revez, Huuskonen, Ruusunen, Lindström, & Hänninen, 2013)
<i>A. butzleri</i> <i>A. cryaerophilus</i>	Italy	Surfaces with and without food contact, cheese, environment, water	29.3 (n=75)	(Serraino & Giacometti, 2014)
<i>Arcobacter</i> spp	Turkey	Raw milk	36 (n = 50)	(Yesilmen et al., 2014)
<i>Arcobacter</i> spp	Turkey	fresh cheese	56 (n = 56)	(Yesilmen et al., 2014)
<i>A. butzleri</i>	Italy	raw milk, milking system	80 (n=10)	(Giacometti et al., 2015)

Table 2. *Arcobacter* spp. occurrence in raw milk and dairy products (culmination)

Isolated Species	Country	Samples collected from	Occurrence (%) (n = total samples)	Reference
<i>A. butzleri</i> ; <i>A. skirrowii</i> ; <i>A. cryaerophilus</i> .	Turkey	Raw milk	23.9 (n=46)	(Elmali, Can, Elmali, & Can, 2016)
<i>A. butzleri</i> <i>A. cryaerophilus</i>	India	Raw milk, cheese	22 (n=50)	(Modi & Chatur, 2018)
<i>A. butzleri</i>	Italy	Raw milk	8 (n=37)	(Traversa et al., 2019)

On the other hand, the presence of *Arcobacter* in raw milk has been more investigated since 2006. Figure 3 shows the reports and countries in which *Arcobacter* has been identified and isolated from milk and dairy products. In Brazil, there are specific studies about the occurrence of *Arcobacter* in slaughterhouses and processing plants of chicken and pig. There are some studies concerning the occurrence of bacteria in retail products and commercialization points (Oliveira, 2016; Oliveira et al., 2001), but no publications are available in dairy products. Pianta et al. (2007) analyzed 188 raw bovine milk samples collected from 11 dairy farms located in different cities of Rio Grande do Sul, Brazil, and results showed a prevalence of 5%.

Figure 3. Distribution of *Arcobacter* in raw milk and dairy products around the world

Note: Green (Northern Ireland, Spain, Finland, Malaysia, Brazil). Pink (Italy, Turkey, India).

Reference: Table 2

LITERATURE CITED REVIEW

- Atanassova, V., Kessen, V., Reich, F., & Klein, G. (2008). Incidence of *Arcobacter* spp. in Poultry: Quantitative and Qualitative Analysis and PCR Differentiation. *Journal of Food Protection*, 71(12), 2533–2536. <https://doi.org/10.4315/0362-028X-71.12.2533>
- Banting, G., & Figueras Salvat, M. J. (2017). *Arcobacter* | Global Water Pathogen Project. <https://doi.org/http://www.waterpathogens.org/book/arcobacter>
- Beran, G. W. (1994). *Handbook of zoonoses*. Retrieved from https://books.google.com.br/books?id=wcE1AR_CLy8C&pg=PA187&lpg=PA187&dq=Arcobacter+mastitis&source=bl&ots=7Cv1vXCxdN&sig=VFFRtiSKMQdk1Ev5Ujgfuza_i9y&hl=pt-br&sa=x&ved=0ahukewjrwloftafvahuiujakhvgha5sq6aeitzag#v=onepage&q=arcobactermastitis&f=false
- Callbeck, C. M., Pelzer, C., Lavik, G., Ferdelman, T. G., Graf, J. S., Vekeman, B., ... Kuypers, M. M. M. (2019). *Arcobacter peruensis* sp. nov., a chemolithoheterotroph isolated from sulfide and organic rich coastal waters off Peru. *Applied and Environmental Microbiology*. <https://doi.org/10.1128/aem.01344-19>
- Cardoen, S., Van Huffel, X., Berkvens, D., Quoilin, S., Ducoffre, G., Saegerman, C., ... Dierick, K. (2009). Evidence-Based Semiquantitative Methodology for Prioritization of Foodborne Zoonoses. *Foodborne Pathogens and Disease*, 6(9), 1083–1096. <https://doi.org/10.1089/fpd.2009.0291>
- Collado, L., Cleenwerck, I., Van Trappen, S., De Vos, P., & Figueras, M. J. (2009). *Arcobacter mytili* sp. nov., an indoxyl acetate-hydrolysis-negative bacterium isolated from mussels. *International Journal of Systematic and Evolutionary Microbiology*, 59(6), 1391–1396. <https://doi.org/10.1099/ijs.0.003749-0>
- Collado, L., & Figueras, M. J. (2011). Taxonomy, Epidemiology, and Clinical Relevance of the Genus *Arcobacter*. *Clinical Microbiology Reviews*, 24(1), 174–192. <https://doi.org/10.1128/CMR.00034-10>
- De Smet, S., De Zutter, L., & Houf, K. (2011). Small ruminants as carriers of the emerging foodborne pathogen *Arcobacter* on small and medium farms. *Small Ruminant Research*, 97(1–3), 124–129. <https://doi.org/10.1016/j.smallrumres.2011.02.004>
- Diéguez, A. L., Balboa, S., Magnesen, T., & Romalde, J. L. (2017). *Arcobacter lekithochrous* sp. nov., isolated from a molluscan hatchery. *International Journal of Systematic and Evolutionary Microbiology*, 67(5), 1327–1332. <https://doi.org/10.1099/ijsem.0.001809>
- Donachie, S. P., Bowman, J. P., On, S. L. W., & Alam, M. (2005). *Arcobacter halophilus* sp. nov., the first obligate halophile in the genus *Arcobacter*. *International Journal Of Systematic And Evolutionary Microbiology*, 55(3), 1271–1277. <https://doi.org/10.1099/ijs.0.63581-0>
- Doudah, L., De Zutter, L., Vandamme, P., & Houf, K. (2010). Identification of five human and mammal associated *Arcobacter* species by a novel multiplex-PCR assay. *Journal of Microbiological Methods*, 80, 281–286. <https://doi.org/10.1016/j.mimet.2010.01.009>

- Ellis, W. A., Neill, S. D., O'Brien, J. J., Ferguson, H. W., & Hanna, J. (1977). Isolation of *Spirillum/Vibrio*-like organisms from bovine fetuses. *The Veterinary Record*, 100(21), 451–452. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/560078>
- Ellis, W. A., Neill, S. D., O'Brien, J. J., & Hanna, J. (1978). Isolation of spirillum-like organisms from pig fetuses. *The Veterinary Record*, 102(5), 106. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/416554>
- Elmali, M., Can, H. Y., Elmali, M., & Can, H. Y. (2016). Occurrence and antimicrobial resistance of *Arcobacter* species in food and slaughterhouse samples. *Food Science and Technology (Campinas)*, 37(ahead), 0–0. <https://doi.org/10.1590/1678-457X.19516>
- Ertas, N., Dogruer, Y., Gonulalan, Z., Guner, A., & Ulger, I. (2010). Prevalence of *Arcobacter* Species in Drinking Water, Spring Water, and Raw Milk as Determined by Multiplex PCR. *Journal of Food Protection*, 73(11), 2099–2102. <https://doi.org/10.4315/0362-028X-73.11.2099>
- Fernández, G. E., Paillacar, J., Gajardo, T., & Riquelme, A. (1995). *Toxigenic and Invasive Capacities: Possible Pathogenic Mechanisms in Arcobacter cryaerophilus*. Retrieved from <https://tspace.library.utoronto.ca/html/1807/22886/oc95128.html>
- Ferreira, S., Queiroz, J., Oleastro, M., & Domingues, F. (2015). Insights in the pathogenesis and resistance of *Arcobacter* : A review. *Critical Reviews in Microbiology*, 42(3), 364–383. <https://doi.org/10.3109/1040841X.2014.954523>
- Figueras, M. J., Pérez-Cataluña, A., Salas-Massó, N., Levican, A., & Collado, L. (2017). 'Arcobacter porcinus' sp. nov., a novel *Arcobacter* species uncovered by *Arcobacter* thereius. *New Microbes and New Infections*, 15, 104–106. <https://doi.org/10.1016/j.nmni.2016.11.014>
- Figueras, M.J, Levican, A., Pujol, I., Ballester, F., Rabada Quilez, M. ., & Gomez-Bertomeu, F. (2014). A severe case of persistent diarrhoea associated with *Arcobacter cryaerophilus* but attributed to *Campylobacter* sp. and a review of the clinical incidence of *Arcobacter* spp. *New Microbes and New Infections*, 2(2), 31–37. <https://doi.org/10.1002/2052-2975.35>
- Figueras, Maria José, Collado, L., Levican, A., Perez, J., Solsona, M. J., & Yustes, C. (2011). *Arcobacter molluscorum* sp. nov., a new species isolated from shellfish. *Systematic and Applied Microbiology*, 34(2), 105–109. <https://doi.org/10.1016/j.syapm.2010.10.001>
- Figueras, Maria José, Levican, A., Collado, L., Inza, M. I., & Yustes, C. (2011). *Arcobacter ellisii* sp. nov., isolated from mussels. *Systematic and Applied Microbiology*, 34(6), 414–418. <https://doi.org/10.1016/j.syapm.2011.04.004>
- Ghaju Shrestha, R., Tanaka, Y., Malla, B., Bhandari, D., Tandukar, S., Inoue, D., ... Haramoto, E. (2017). Next-generation sequencing identification of pathogenic bacterial genes and their relationship with fecal indicator bacteria in different water sources in the Kathmandu Valley, Nepal. *Science of The Total Environment*, 601–602, 278–284. <https://doi.org/10.1016/j.scitotenv.2017.05.105>
- Giacometti, F., Lucchi, A., Di Francesco, A., Delogu, M., Grilli, E., Guarniero, I., ... Serraino, A. (2015). *Arcobacter butzleri*, *Arcobacter cryaerophilus*, and *Arcobacter skirrowii* Circulation in a Dairy Farm and Sources of Milk Contamination. *Applied and Environmental Microbiology*, 81(15), 5055–5063. <https://doi.org/10.1128/AEM.01035-15>

- Giacometti, F., Serraino, A., Marchetti, G., Bonerba, E., Florio, D., Bonfante, E., ... Rosmini, R. (2013). Isolation of *Arcobacter butzleri* in environmental and food samples collected in industrial and artisanal dairy plants. *Italian Journal of Food Safety*, 2(3), 34. <https://doi.org/10.4081/ijfs.2013.e34>
- Houf, K., On, S. L. W., Coenye, T., Debruyne, L., De Smet, S., & Vandamme, P. (2009). *Arcobacter thereius* sp. nov., isolated from pigs and ducks. *International Journal Of Systematic And Evolutionary Microbiology*, 59(10), 2599–2604. <https://doi.org/10.1099/ijfs.0.006650-0>
- Houf, Kurt, De Zutter, L., Verbeke, B., Van Hoof, J., & Vandamme, P. (2003). Molecular Characterization of *Arcobacter* Isolates Collected in a Poultry Slaughterhouse. *Journal of Food Protection*, 66(3), 364–369. <https://doi.org/10.4315/0362-028X-66.3.364>
- Karadas, G., Sharbati, S., Hänel, I., Messelhäuser, U., Glocker, E., Alter, T., & Götz, G. (2013). Presence of virulence genes, adhesion and invasion of *Arcobacter butzleri*. *Journal of Applied Microbiology*, 115(2), 583–590. <https://doi.org/10.1111/jam.12245>
- Kiehlbauch, J. A., Brenner, D. J., Nicholson, M. A., Baker, C. N., Patton, C. M., Steigerwalt, A. G., & Wachsmuth, I. K. (1991). *Campylobacter butzleri* sp. nov. isolated from humans and animals with diarrheal illness. *Journal of Clinical Microbiology*, 29(2), 376–385. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/2007646>
- Kim, H. M., Hwang, C. Y., & Cho, B. C. (2010). *Arcobacter marinus* sp. nov. *International Journal Of Systematic And Evolutionary Microbiology*, 60(3), 531–536. <https://doi.org/10.1099/ijfs.0.007740-0>
- Levican, A., Alkeskas, A., Günter, C., Forsythe, S. J., & Figueras, M. J. (2013). Adherence to and invasion of human intestinal cells by *Arcobacter* species and their virulence genotypes. *Applied and Environmental Microbiology*, 79(16), 4951–4957. <https://doi.org/10.1128/AEM.01073-13>
- Levican, A., Collado, L., Aguilar, C., Yustes, C., Diéguez, A. L., Romalde, J. L., & Figueras, M. J. (2012). *Arcobacter bivalviorum* sp. nov. and *Arcobacter venerupis* sp. nov., new species isolated from shellfish. *Systematic and Applied Microbiology*, 35(3), 133–138. <https://doi.org/10.1016/j.syapm.2012.01.002>
- Levican, A., Collado, L., Yustes, C., Aguilar, C., & Figueras, M. J. (2014). Higher water temperature and incubation under aerobic and microaerobic conditions increase the recovery and diversity of *Arcobacter* spp. from shellfish. *Applied and Environmental Microbiology*, 80(1), 385–391. <https://doi.org/10.1128/AEM.03014-13>
- Levican, A., Rubio-Arcos, S., Martínez-Murcia, A., Collado, L., & Figueras, M. J. (2015). *Arcobacter ebronensis* sp. nov. and *Arcobacter aquimarinus* sp. nov., two new species isolated from marine environment. *Systematic and Applied Microbiology*, 38(1), 30–35. <https://doi.org/10.1016/j.syapm.2014.10.011>
- McClung, C. R., Patriquin, D. G., & Davis, R. E. (1983). *Campylobacter nitrofigilis* sp. nov., a Nitrogen-Fixing Bacterium Associated with Roots of *Spartina alterniflora* Loisel. *International Journal of Systematic Bacteriology*, 33(3), 605–612. <https://doi.org/10.1099/00207713-33-3-605>

- McGregor, A. C., & Wright, S. G. (2015). Gastrointestinal symptoms in travellers. *Clinical Medicine (London, England)*, 15(1), 93–95. <https://doi.org/10.7861/clinmedicine.15-1-93>
- McVey, D. S., Kennedy, M., & Chengappa, M. M. (2013). *Veterinary microbiology*. Retrieved from <https://books.google.com.br/books?id=ne57vqxi4koc&pg=pt435&lpg=pt435&dq=arcobacter+mastitis&source=bl&ots=rsljd6fgdw&sig=zyewjg0cdqr1fhru4olodf6pvvw&hl=pt-br&sa=x&ved=0ahukewjrwloftafvahuujakhvgha5sq6aeistae#v=onepage&q=arcobactermastitis&f=false>
- Modi, S., & Chatur, Y. (2018). Detection and Characterization of *Arcobacter* Species from Milk and Milk Products Procured from Anand City, Gujarat (India). *International Journal of Livestock Research*, 1. <https://doi.org/10.5455/ijlr.20171005053150>
- Neill, S. D., Campbell, J. N., O'Brien, J. J., Weatherup, S. T. C., & Ellis, W. A. (1985). Taxonomic Position of *Campylobacter cryaerophila* sp. nov. *International Journal of Systematic Bacteriology*, 35(3), 342–356. <https://doi.org/10.1099/00207713-35-3-342>
- Neill, S. D., Ellis, W. A., & O'Brien, J. J. (1979). Designation of aerotolerant *Campylobacter*-like organisms from porcine and bovine abortions to the genus *Campylobacter*. *Research in Veterinary Science*, 27(2), 180–186. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/523802>
- Nieva-Echevarria, B., Martinez-Malaxetxebarria, I., Girbau, C., Alonso, R., & Fernández-Astorga, A. (2013). Prevalence and Genetic Diversity of *Arcobacter* in Food Products in the North of Spain. *Journal of Food Protection*, 76(8), 1447–1450. <https://doi.org/10.4315/0362-028X.JFP-13-014>
- Oliveira, M. G. X. de. (2016). Caracterização genotípica de *Arcobacter* spp. isolados de carnes de aves comercializadas no Município de São Paulo - SP. Retrieved from <http://www.teses.usp.br/teses/disponiveis/10/10133/tde-23032016-105513/en.php>
- Oliveira, S. J. de, Moraes, H. L. de S., Kuchenbecker, B. S., Ikuta, N., Lunge, V., Fonseca, A., & Coiro, J. R. (2001). Isolation of *Arcobacter* spp from poultry carcasses, in Brazil. *Ciência Rural*, 31(4), 639–643. <https://doi.org/10.1590/S0103-84782001000400013>
- Park, S., Jung, Y.-T., Kim, S., & Yoon, J.-H. (2016). *Arcobacter acticola* sp. nov., isolated from seawater on the East Sea in South Korea. *Journal of Microbiology*, 54(10), 655–659. <https://doi.org/10.1007/s12275-016-6268-4>
- Pérez-Cataluña, A., Salas-Massó, N., & Figueras, M. J. (2019). *Arcobacter lacus* sp. nov. and *Arcobacter caeni* sp. nov., two novel species isolated from reclaimed water. *International Journal of Systematic and Evolutionary Microbiology*, 69(11), 3326–3331. <https://doi.org/10.1099/ijsem.0.003101>
- Pianta, C., Passos, D. T., Hepp, D., & Oliveira, S. J. de. (2007). Isolation of *Arcobacter* spp from the milk of dairy cows in Brazil. *Ciência Rural*, 37(1), 171–174. <https://doi.org/10.1590/S0103-84782007000100027>
- Ramees, T. P., Dhama, K., Karthik, K., Rathore, R. S., Kumar, A., Saminathan, M., ... Singh, R. K. (2017). *Arcobacter* : an emerging food-borne zoonotic pathogen, its public health concerns and advances in diagnosis and control – a comprehensive review. *Veterinary Quarterly*, 37(1), 136–161. <https://doi.org/10.1080/01652176.2017.1323355>

- Revez, J., Huuskonen, M., Ruusunen, M., Lindström, M., & Hänninen, M.-L. (2013). *Arcobacter* Species and Their Pulsed-Field Gel Electrophoresis Genotypes in Finnish Raw Milk during Summer 2011. *Journal of Food Protection*, 76(9), 1630–1632. <https://doi.org/10.4315/0362-028X.JFP-13-083>
- Salas-Massó, N., Andree, K. B., Furones, M. D., & Figueras, M. J. (2016). Enhanced recovery of *Arcobacter* spp. using NaCl in culture media and re-assessment of the traits of *Arcobacter marinus* and *Arcobacter halophilus* isolated from marine water and shellfish. *Science of The Total Environment*, 566–567, 1355–1361. <https://doi.org/10.1016/j.scitotenv.2016.05.197>
- Sasi Jyothsna, T. S., Rahul, K., Ramaprasad, E. V. V, Sasikala, C., & Ramana, C. V. (2013). *Arcobacter anaerophilus* sp. nov., isolated from an estuarine sediment and emended description of the genus *Arcobacter*. *International Journal Of Systematic And Evolutionary Microbiology*, 63(Pt 12), 4619–4625. <https://doi.org/10.1099/ij.s.0.054155-0>
- Scullion, R., Harrington, C. S., & Madden, R. H. (2006). Prevalence of *Arcobacter* spp. in Raw Milk and Retail Raw Meats in Northern Ireland. *Journal of Food Protection*, 69(8). Retrieved from <http://jfoodprotection.org/doi/pdf/10.4315/0362-028X-69.8.1986>
- Serraino, A., Florio, D., Giacometti, F., Piva, S., Mion, D., & Zanoni, R. G. (2013). Presence of *Campylobacter* and *Arcobacter* species in in-line milk filters of farms authorized to produce and sell raw milk and of a water buffalo dairy farm in Italy. *Journal of Dairy Science*, 96(5), 2801–2807. <https://doi.org/10.3168/jds.2012-6249>
- Serraino, A., & Giacometti, F. (2014). Short communication: Occurrence of *Arcobacter* species in industrial dairy plants. *Journal of Dairy Science*, 97(4), 2061–2065. <https://doi.org/10.3168/jds.2013-7682>
- Shah, A. H., Saleha, A. A., Murugaiyah, M., Zunita, Z., & Memon, A. A. (2012). Prevalence and Distribution of *Arcobacter* spp. in Raw Milk and Retail Raw Beef. *Journal of Food Protection*, 75(8), 1474–1478. <https://doi.org/10.4315/0362-028X.JFP-11-487>
- Snelling, W. ., Matsuda, M., Moore, J. ., & Dooley, J. S. . (2006). Under the Microscope: *Arcobacter*. *Letters in Applied Microbiology*, 42(1), 7–14. <https://doi.org/10.1111/j.1472-765X.2005.01841.x>
- Solnick, J. V, & Vandamme, P. (2001). Taxonomy of the *Helicobacter* Genus. In *Helicobacter pylori: Physiology and Genetics*. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/21290754>
- Traversa, A., Gallina, S., Martucci, F., Boteva, C., Baioni, E., Maurella, C., ... Decastelli, L. (2019). *Arcobacter* spp. in raw milk from vending machines in Piedmont and occurrence of virulence genes in isolates. *Italian Journal of Food Safety*, 8(4). <https://doi.org/10.4081/ijfs.2019.7859>
- Tsang, R. S. W., Luk, J. M. C., Woodward, D. L., & Johnson, W. M. (1996). Immunochemical characterization of a haemagglutinating antigen of *Arcobacter* spp. '. *FEMS Microbiology Letters*, 136, 209–2. Retrieved from http://ac.els-cdn.com/0378109796842029/1-s2.0-0378109796842029-main.pdf?_tid=10876438-7b21-11e7-9891-00000aabb0f27&acdnat=1502076940_b6bf76ce5cf2ca93f71a8c43a9d47993

- UVHA/MS. (2016). *Unidade de Vigilância das Doenças Transmissão Hídrica e Alimentos. Ministério da Saúde. Surtos de doenças transmitidas por alimentos no Brasil*. Retrieved from <http://u.saude.gov.br/images/pdf/2016/junho/08/Apresenta---o-Surtos-DTA-2016.pdf>
- Van den Abeele, A.-M., Vogelaers, D., Van Hende, J., & Houf, K. (2014). Prevalence of *Arcobacter* species among humans, Belgium, 2008-2013. *Emerging Infectious Diseases*, 20(10), 1731–1734. <https://doi.org/10.3201/eid2010.140433>
- Vandamme, P., & De Ley, J. (1991). Proposal for a New Family, Campylobacteraceae. *International Journal Of Systematic Bacteriology International Union of Microbiological Societies*, 41(3). Retrieved from <http://www.microbiologyresearch.org/docserver/fulltext/ijsem/41/3/ijis-41-3-451.pdf?expires=1501510378&id=id&accname=guest&checksum=FE5A92228C2134E74E0BDC6A4B3BED4C>
- Vandamme, P., Falsen, E., Rossau, R., Hoste, B., Segers, P., Tytgat, R., & De Ley, J. (1991). Revision of *Campylobacter*, *Helicobacter*, and *Wolinella* Taxonomy: Emendation of Generic Descriptions and Proposal of *Arcobacter* gen. nov. *International Journal of Systematic Bacteriology*, 41(1), 88–103. <https://doi.org/10.1099/00207713-41-1-88>
- Vandamme, P., Vancanneyt, M., Pot, B., Mels, L., Hoste, B., Dewettinck, D., ... Goossens, H. (1992). Polyphasic Taxonomic Study of the Emended Genus *Arcobacter* with *Arcobacter butzleri* comb. nov. and *Arcobacter skirrowii* sp. nov., an Aerotolerant Bacterium Isolated from Veterinary Specimens. *International Journal of Systematic Bacteriology*, 42(3), 344–356. <https://doi.org/10.1099/00207713-42-3-344>
- Vasiljevic, M., Fenwick, A. J., Nematollahi, S., Gundareddy, V. P., Romagnoli, M., Zenilman, J., & Carroll, K. C. (2019). First Case Report of Human Bacteremia With *Malacobacter (Arcobacter) mytili*. *Open Forum Infectious Diseases*, 6(7). <https://doi.org/10.1093/ofid/ofz319>
- Waite, D. W., Vanwonterghem, I., Rinke, C., Parks, D. H., Zhang, Y., Takai, K., ... Hugenholtz, P. (2017). Comparative Genomic Analysis of the Class Epsilonproteobacteria and Proposed Reclassification to Epsilonbacteraeota (phyl. nov.). *Frontiers in Microbiology*, 8, 682. <https://doi.org/10.3389/fmicb.2017.00682>
- Wesley, I. V., Wells, S. J., Harmon, K. M., Green, A., Schroeder-Tucker, L., Glover, M., & Siddique, I. (2000). Fecal Shedding of *Campylobacter* and *Arcobacter* spp. in Dairy Cattle. *Appl. Environ. Microbiol.*, 66(5), 1994–2000. <https://doi.org/10.1128/AEM.66.5.1994-2000.2000>
- Whiteduck-Léveillé, J., Lapen, D. R., Whiteduck-Léveillé, K., Tambong, J. T., André Lévesque, C., Cloutier, M., ... Khan, I. U. H. (2015). *Arcobacter lanthieri* sp. nov., isolated from pig and dairy cattle manure. *International Journal of Systematic and Evolutionary Microbiology*, 65(8), 2709–2716. <https://doi.org/10.1099/ijis.0.000318>
- WHO. (2015). WHO | Food safety. Retrieved August 1, 2017, from World Health Organization website: <http://who.int/mediacentre/factsheets/fs399/en/>
- Wirsén, C. O., Sievert, S. M., Cavanaugh, C. M., Molyneaux, S. J., Ahmad, A., Taylor, L. T., ... Taylor, C. D. (2002). Characterization of an autotrophic sulfide-oxidizing marine *Arcobacter* sp. that produces filamentous sulfur. *Applied and Environmental Microbiology*, 68(1), 316–325. <https://doi.org/10.1128/AEM.68.1.316-325.2002>

- Yesilmen, S., Vural, A., Erkan, M. E., & Yildirim, I. H. (2014). Prevalence and antimicrobial susceptibility of *Arcobacter* species in cow milk, water buffalo milk and fresh village cheese. *International Journal of Food Microbiology*, 188, 11–14. <https://doi.org/10.1016/j.ijfoodmicro.2014.07.006>
- Zerpa Larrauri, R., Alarcón Villaverde, J. O., Vigo, L., Percy, E., Patiño Gabriel, L., Reyes Dioses, A., & Alarcón León, M. J. (2014). Identificación de *Arcobacter* en heces de niños y adultos con/sin diarrea y en reservorios animales. *Anales de La Facultad de Medicina*, Vol. 75, pp. 185–187. Retrieved from http://www.scielo.org.pe/scielo.php?pid=S1025-55832014000200017&script=sci_arttext&tlng=pt
- Zhang, Z., Yu, C., Wang, X., Yu, S., & Zhang, X.-H. (2016). *Arcobacter pacificus* sp. nov., isolated from seawater of the South Pacific Gyre. *International Journal of Systematic and Evolutionary Microbiology*, 66(2), 542–547. <https://doi.org/10.1099/ijsem.0.000751>

CHAPTER 2: Occurrence of *Arcobacter* spp. in Brazilian Minas frescal cheese and raw cow milk and its association with microbiological and physicochemical parameters

This article has been submitted to: Food Control (2019)

Manuscript received at Food Control:

Received 26 July 2019;

Received in revised form 17 September 2019;

Accepted 19 September 2019

Available online 22 September 2019

Authors: Melina L.M. Cruzado-Bravo, Giovana V. Barancelli, Ana Paula Dini Andreote, Erick Saldaña, Boris Vidal-Veuthey, Luis Collado, Carmen J. Contreras-Castillo

<https://doi.org/10.1016/j.foodcont.2019.106904>

Occurrence of *Arcobacter* spp. in Brazilian Minas frescal cheese and raw cow milk and its association with microbiological and physicochemical parameters

Melina L.M Cruzado-Bravo¹, Giovana V. Barancelli¹, Ana Paula Dini Andreote², Erick Saldaña¹, Boris Vidal-Veuthey³, Luis Collado³, Carmen J. Contreras-Castillo¹

Affiliations:

1. Universidade de São Paulo, Escola Superior de Agricultura “Luiz de Queiroz”, Departamento de Agroindústria, Alimentos e Nutrição, Av. Pádua Dias 11, 13418-900 Piracicaba, São Paulo, Brazil
2. Universidade de São Paulo, Centro de Energia Nuclear na Agricultura, Avenida Centenário 303, 13400-970, Piracicaba, São Paulo, Brazil.
3. Universidad Austral de Chile, Institute of Biochemistry and Microbiology, Faculty of Sciences, , Campus Isla Teja, Valdivia, Chile.

ABSTRACT

Some *Arcobacter* species are considered emerging food-borne pathogens. However, their prevalence in dairy products is not well known. Therefore, the aim of the current research is to know the occurrence of *Arcobacter* species in Minas frescal cheese and raw cow milk and its potential association with physicochemical and microbiological parameters. Two hundred and one food samples (cheese, n = 98 and milk, n = 103) collected between 2016 and 2017 in São Paulo State, Brazil, were analyzed. *Arcobacter* was isolated using an enrichment broth with further filtration over *Arcobacter* agar. Colonies were characterized by ERIC-PCR, m-PCR and PCR-RFLP and, when was necessary, sequencing of 16S rRNA and *rpoB* genes were also assessed. Additionally, cheese samples were evaluated in relation to total coliform (TC), thermotolerant coliforms (TtC), coagulase positive staphylococci (CoPS) count and moisture. Raw milk samples were assessed for aerobic mesophilic bacteria count, TC, TtC, Dornic acidity, proteins, fats, lactose, defatted dry extract, total solids and somatic cell counts. The occurrence of *Arcobacter* was 10.2% (10/98) and 16.5% (17/103) in cheese and milk samples, respectively. All isolates were identified as *Arcobacter butzleri*. On the other hand, Multiple Factorial Analysis showed correlation between the presence of this species and the cheese moisture, while this pathogen was correlated with high counts of mesophilic microorganisms and TC in raw milk. No other evaluated parameter in cheese (TC, CoPS) and milk (TtC, Dornic acidity, proteins, fats, lactose, defatted dry extract, total solids and somatic cell counts) showed significant correlation. To date, this is the first report on *A. butzleri* in Minas frescal cheese, therefore its presence in dairy products could be considered a potential public health concern.

Keywords: Emerging pathogen, Microbiological and physicochemical analysis, Somatic cell counts, Milk components

Highlights

1. The occurrence of *Arcobacter butzleri* was 10.2% in Minas frescal cheese and 16.5% in raw cow milk
2. First report of *Arcobacter* in Minas frescal cheese in the state of São Paulo, Brazil.
3. High mesophilic bacteria and total coliforms count correlates with presence of *A. butzleri*, in raw cow milk samples

2.1 INTRODUCTION

The first published clinical report of *Arcobacter* isolation dates back to the 1970s in samples from pork abortions (Ellis, Neill, O'Brien, Ferguson, & Hanna, 1977). Since then, a significant advance in the taxonomy and epidemiology of this genus has been evidenced by the increase of related publications (Hsu & Lee, 2015).

The genus *Arcobacter* includes some species (*A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, and *A. thereius*) considered as emerging human pathogens (Banting & Figueras, 2017; Vandenberg et al., 2004). Those species can trigger several gastrointestinal disorders in humans, such as chronic diarrhea and bacteremia (Ferreira, Júlio, Queiroz, Domingues, & Oleastro, 2014; Webb et al., 2016). *Arcobacter butzleri* is the most clinically significant species for humans (Banting & Figueras, 2017) and it was classified as a serious hazard to human's health by the International Commission on Microbiological Specifications for Foods in 2002 (ICMSF, 2002). *Arcobacter* species have been isolated from different matrices, such as water and foods of animal origin -milk, meat products and shellfish (Collado & Figueras, 2011; Ghaju Shrestha et al., 2017).

In the dairy production chain, *Arcobacter* species have been isolated from different sources, such as cow fecal samples, raw milk and fresh cheese (Merga et al., 2011; Yesilmen, Vural, Erkan, & Yildirim, 2014). There are several possibilities for *Arcobacter* contamination in the dairy industry because the operations in this chain production are quite complex (Giacometti et al., 2015; Serraino & Giacometti, 2014). *Arcobacter* contamination has been investigated in bulk milk tanks (Elmali, Can, Elmali, & Can, 2016; Ertas, Dogruer, Gonulalan, Guner, & Ulger, 2010), milking equipment, barn floors, inline filters in milking machinery and, more recently, in cheese (Giacometti et al., 2015; Serraino et al., 2013; Serraino & Giacometti, 2014). However, in comparison with other foods, studies on dairy products are scarce and restricted to a small number of countries (Hsu & Lee, 2015).

The dairy sector is one of the main national social and economic activities in Brazil (Milanez et al., 2018), and taking into consideration that Brazil is the 5th largest producer of milk and the largest consumer of milk and dairy products in South America; regarding that information it is justifiable to investigate emerging pathogens in this production chain.

Minas frescal cheese, also known as white or fresh cheese, is one of the most consumed cheeses in Brazil. It is produced in all Brazilian states mainly by small and medium size dairy plants (ABIQ, 2019). Therefore, the main goal of the current research is to determine the occurrence of *Arcobacter* species in retailed Minas frescal cheese and in raw cow milk used for production of dairy products in the Northeast region of São Paulo State, Brazil.

2.2 MATERIALS AND METHODS

2.2.1 Sampling

Ninety-eight Minas frescal cheese samples were collected between December/2016 and July/2017: five samples from a cheese processing plant and 93 samples from nine local markets. Additionally, samples of bulk tank milk (n=103) were collected in 27 dairy farms from different Brazilian cities (Piracicaba, Brotas, São Pedro and Torrinha – São Paulo State), between August and December 2017, in which three samples per farm were collected. Samples were collected under aseptic conditions and transported under cold storage temperature (4 °C) to be analyzed for their microbiological and physicochemical properties within 12 h (milk) and 24 h (cheese).

2.2.2 Microbiological analysis

Aerobic mesophilic bacteria count, total coliforms (TC), thermotolerant coliforms (TtC) and coagulase-positive staphylococci (CoPS) counts were carried out following the methodology described by Downes & Itō (2001). All analyses were conducted in the Laboratory of “*Higiene e Laticínios*” in the “Escola Superior de Agricultura Luiz de Queiroz” (ESALQ), Universidade de São Paulo (USP), Piracicaba-SP, Brazil.

2.2.3 Physicochemical properties and somatic cell counts

Dornic acidity of raw milk and moisture of Minas frescal cheese were analyzed according to Brazilian normative (Brazil, 2006). Milk components (proteins, fats, lactose, defatted dry matter and total solids) and somatic cell counts (SCC) were analyzed in “*Clínica do Leite*” in the “*Departamento de Zootecnia*”, ESALQ, USP, Piracicaba-SP, Brazil. The milk composition was estimated by the infrared method – PO ANA 009 and SCC was estimated using flow cytometry (model Bentley Soma count 300, Bentley Instruments Inc., Chaska, MN, USA).

2.2.4 *Arcobacter* isolation

For *Arcobacter* isolation, 10 g of cheese or 10 mL of raw cow milk were placed in a sterile sampling bag and homogenized for one min in a stomacher with 90 mL of *Arcobacter* Enrichment Broth (AEB) (1.8% peptone, 0.5% NaCl and 0.01% g yeast extract) supplemented with three antibiotics: Cefoperazone (16 mg)-Amphotericin B (10 mg)-Teicoplanin (64 mg) (CAT supplement). Inoculated AEB were incubated under aerobic conditions at 30 °C for 48 h (Shah, Saleha, Murugaiyah, Zunita, & Memon, 2012). After incubation, 0.4 mL of the enriched medium was inoculated on *Arcobacter* broth supplemented with 1.4% agar and CAT using the membrane filtration method. Cellulose acetate membrane filters with a diameter of 47 mm and

a pore size of 0.45 µm were used to remove other enteric bacteria (Atabay, Aydin, Houf, Sahin, & Vandamme, 2003). These filters were aseptically removed 1 h after inoculation and the plates were incubated under aerobic conditions for up five days examining the plates every day (Collado, Cleenwerck, Trappen, De Vos, & Figueras, 2009a). From each plate in which the bacterial growth was detected, three to five suspected colonies were confirmed by Gram stain and streaked on new plates. Typical *Arcobacter* colonies (2–3 mm in diameter, circular and convex, beige to off-white color) were stored in 15% glycerol at -80 °C.

2.2.5 Molecular identification and typing

The bacterial DNA was extracted using InstaGene™ DNA Purification Matrix (Bio-Rad, Hercules, CA, USA). Identification at genus level was carried out using the PCR method described by Harmon & Wesley (1996). Positive (*A. butzleri* LMG 10828^T) and negative controls were included in every PCR run.

To eliminate clonal redundant strains in further analyses, the isolates were genotyped with Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR). Only isolates with different genotypes were further identified using the multiplex PCR (m-PCR) method as described by Doudah, De Zutter, Vandamme, & Houf (2010) and by the 16S rRNA gene Restriction Fragment Length Polymorphism (16S rRNA-RFLP) method described by Figueras, Levican, & Collado (2012). Reference strains *A. butzleri* LMG 10828^T, *A. cryaerophilus* LMG 9904^T and *A. skirrowii* LMG 6621^T; CECT 7203 *A. cibarius*, and LMG 24486^T *A. thereius* were included in each analysis.

The 16S rRNA or *rpoB* genes were amplified and sequenced using the universal primer and protocol as described by Harmon & Wesley (1996) and Collado, Cleenwerck, Trappen, De Vos, & Figueras (2009b), respectively, for strains that showed inconclusive identification using both identification methods, or when a different RFLP pattern defined for the type strains was obtained. The PCR products were purified using GenElute™ PCR Clean-Up Kit (Sigma-Aldrich), and the amplicons were sequenced bidirectionally using the same primers by an ABI Prism 3500 genetic analyzer (Applied Biosystems, Thermo Fisher Scientific, USA) with the BigDye terminator cycle sequencing kit (Applied Biosystems, Thermo Fisher Scientific, USA). DNA sequences were compared with the NCBI database using Blast Library to determine the identity of the species.

2.3 STATISTICAL ANALYSES

The association between *A. butzleri* and the Minas frescal cheese characteristics and raw milk was studied using Multiple Factorial Analysis (MFA). For cheeses, the microbiological characteristics (TC, TtC and CoPS), physicochemical characteristics (moisture) and the presence of *A. butzleri* were included in the data matrix. For milk samples, microbiological characteristics (TC, TtC and aerobic mesophilic bacteria count), physicochemical characteristics (composition of milk, Dornic acidity), somatic cell count and the presence *A. butzleri* were considered. Statistical analyses were performed using XLSTAT 2018 software for Microsoft Excel® (Microsoft®, WA, USA).

2.4 RESULTS

The presence of *Arcobacter* was observed in 10.2% (10/98) of Minas frescal cheese samples and in 16.5% (17/103) of raw milk samples (Table 1). The isolates (n=84) were genotyped by ERIC-PCR, resulting in 12 and 17 different profiles in 29 Minas frescal cheese and 55 raw milk isolates, respectively.

Sixteen isolates needed to be identified by sequencing the 16S rRNA gene, whereas three isolates (Q73C, LC34C, LC96G) were characterized by sequencing the *rpoB* gene. All isolates from both cheese and milk samples were identified as *A. butzleri*.

Relationship between microbiological and physicochemical characteristics and the presence of *A. butzleri* in Minas frescal cheese

According to the microbiological analysis performed on the Minas frescal cheese samples, 57.1% (4/7) exceeded 5×10^2 CFU/g of TtC (Table 2), which are the maximum limit tolerated by Brazilian legislation. In addition, it was observed that 42.9% (3/7) were greater than 5×10^2 CFU/g, which are the maximum limit allowed for CoPS (Brazil, 2001). The moisture of the cheeses was higher than 55% when *Arcobacter* was detected.

Figure 1 shows the representation of dimension 1 and 2 of the MFA run with the physicochemical and microbiological parameters of the Minas frescal cheese samples. The first two dimensions explained up to 55% of the total variance. It was possible to observe that the *A. butzleri* isolates had a slight positive correlation with the moisture content of the cheese (RV=0.012). However, no correlation was found between the presence of *A. butzleri* and TC, TtC and CoPS (Figure 1A). The distribution of the samples in which *A. butzleri* was detected are shown in Figure 1B. A clear pattern is observed: cheese samples negative for *A. butzleri* are located in the 1st dimension, whereas cheese samples positive for *A. butzleri* are distributed in

the 1st and 4th quadrants. The MFA analysis was carried out only in 62 out of the 98 Minas frescal cheese samples.

Relationship of the microbiological and physicochemical analyzes with the presence of *A. butzleri* in raw milk

In Table 3, it is possible to observe that 82.4% (14/17) of the samples of raw milk analyzed positive for *A. butzleri* were above the allowed limits for mesophilic aerobic microorganisms (3.0×10^5 CFU/mL) (Brazil, 2011). Similarly, 41.2% (7/17) of samples contaminated with *A. butzleri* exceeded the 5.0×10^5 cells/mL- which represents the maximum limit allowed for SCC. Regarding the milk components (proteins, fats, lactose, defatted dry extract and total solids) were within the parameters required by the same regulation (Brazil, 2011).

MFA was performed using the physicochemical and microbiological parameters of the raw milk samples, and results are shown in Figure 2. Overall, MFA explained 51.43% of the total variance using the first 2 dimensions. The presence of *A. butzleri* was correlated (RV = 0.12) with the aerobic mesophilic counts and TC, which means that the samples positive for *A. butzleri* showed high counts of aerobic mesophilic and TC (Figure 2A). However, no correlation was found between *A. butzleri* and TtC. Moreover, no correlation was observed between *A. butzleri* and SCC, chemical composition and Dornic acidity. It was possible to observe the correlation between the SCC and the milk composition (RV = 0.22), which is already known and widely reported (Lindmark-Månsson, Bränning, Aldén, & Paulsson, 2006; Malek dos Reis, Barreiro, Mestieri, Porcionato, & dos Santos, 2013). In Figure 2A, the position (dimensions 1 and 2) of the raw milk positive for *A. butzleri* is shown. In the 1st and 4th quadrants, the negative samples for *A. butzleri* are grouped, implying they present similar characteristics in terms of physicochemical parameters. On the other hand, positive samples for *A. butzleri* are spread over the 2nd and 3rd quadrants. To facilitate the presentation of the results in Figures 1B and 2B, positive samples for *A. butzleri* are highlighted and within an ellipse.

2.5 DISCUSSION

2.5.1 *A. butzleri* on Minas frescal cheese and raw milk

Research has shown that *A. butzleri* followed by *A. cryaerophilus* and *A. skirrowii* are the most frequently species isolated from milk and dairy products (Giacometti et al., 2013; Serraino & Giacometti, 2014; Yesilmen et al., 2014). These data agree with those found in the present

investigation (Table 1), considering that all isolates obtained from Minas frescal cheese and raw milk were identified as *A. butzleri*.

To date, this is the first report of *A. butzleri* isolated from Minas frescal cheese in São Paulo, Brazil. This product is a fresh cheese with a high moisture content ($> 55\%$) and is consumed as a ready to eat snack, and for the preparation of sandwich, salads, and other dishes. The current legislation regarding the manufacture of Minas frescal cheese in Brazil requires the pasteurization of milk to avoid the presence of pathogens (Brazil, 2001). However, as shown in Table 1, it was possible to isolate *Arcobacter* in 10.2% of the cheese samples. Yesilmen et al., (2014) isolated *Arcobacter* in 56% (28/50) from fresh village cheese produced with raw milk in Turkey. All analyzed cheeses had sanitary inspection stamp on the package. Thus, it is assumed that these samples were produced with pasteurized milk.

Giacometti et al. (2014) did not find *Arcobacter* in cheese samples produced with pasteurized milk in Italy. Based on these observations, we may infer that the milk used to manufacture the cheese samples studied herein probably did not undergo an adequate heat treatment or a post-pasteurization contamination occurred (Hilton, Mackey, Hargreaves, & Forsythe, 2001).

All Minas frescal cheese samples positive for *A. butzleri* had a moisture content higher than 55% (Table 2). The high moisture allied to the availability of nutrients in cheeses can promote a suitable environment for survival and growth of *A. butzleri*. In fact, Giacometti et al., (2015) observed the growth of *A. butzleri* and *A. cryaerophilus* in a similar type of fresh cheese (ricotta), showing that at 6 °C the bacterial population remained stable; on the other hand, the bacterial population increased when cheeses were stored at 12 °C.

Additionally, by means of the MFA, it was not possible to find a correlation between the presence of *A. butzleri* and TC, TtC and CopS in the cheese samples analyzed. However, these results should be interpreted considering the low number of samples analyzed, implying that further studies with a larger sample set should be conducted to better elucidate the correlation between the presence of *A. butzleri* in the Minas frescal cheese and other physicochemical and microbiological factors.

High counts of TC, TtC and CopS indicates process failures, such as cross contamination by inadequate flow in the processing cheese plants, insufficient time and temperatures of pasteurization, poor hygiene of the manipulators, abuse of storage temperature (Tortorello, 2003). Failures in hygiene procedures and microbial biofilms can also be a cause of post-heat treatment contamination (Marchand et al., 2012). Regarding the raw milk samples, 16.5% (16/103) were positive for *A. butzleri*, which is above the prevalence reported by Ertas et al.,

(2010) and Revez, Huuskonen, Ruusunen, Lindström, & Hänninen, (2013) in Turkey and Finland, respectively. The source of contamination of cheese is diverse. Milk is usually contaminated with microbial pathogens because of endogenous contamination – mastitis or from exogenous sources, such as the contamination during or after milking (e.g., feces contamination on the skin, udder/tits, environment) (Verraes et al., 2015).

The presence of *Arcobacter* in raw milk has been widely reported in multiple publications around the world (Shah et al., 2012; Van den Abeele, Vogelaers, Van Hende, & Houf, 2014). An investigation carried out by Logan, Neill, & Mackie, (1982) indicates that *A. cryaerophilus* could be a causative agent of mastitis; however, this hypothesis has not yet been proven. The only previous Brazilian report of *Arcobacter* in milk samples was made by Pianta, Passos, Hepp, & Oliveira, (2007), who obtained a lower prevalence (3.2%) compared to the data obtained in the current research. However, it is important to consider that those authors analyzed milk from subclinical mastitis cows.

Multivariate statistical analysis has been used to correlate data from different natures. By using MFA, it is possible to find a correlation between the presence of *A. butzleri* with the TC and the mesophilic count in the raw milk analyzed. Our results suggest that the presence of *A. butzleri* could have an environmental origin. The bacterium adapts to environmental conditions and its niche can be barnyard animals (Merga et al., 2013; Merga et al., 2011).

In fact, *A. butzleri* is quite common in cow feces (Kabeya et al., 2003; Shah et al., 2013; Vilar et al., 2010). Their prevalence ranged from 4 to around 40%, depending on the methodology used for detection (Golla et al., 2002; Grove-White, Leatherbarrow, Cripps, Diggle, & French, 2014; Vilar et al., 2010). Technical errors in the milking process, precarious hygiene conditions, and other factors may increase the risk of milk contamination by bacteria from environmental or fecal origin. However, in the present investigation, no correlation was found between the presence of *A. butzleri* and TtC, which could be a clear evidence of fecal contamination. Nevertheless, the correlation between *A. butzleri* with total coliforms (Figure 2) could lead to reinforce that the presence of *A. butzleri* is caused by environmental conditions.

However, in other environments, such as water, seawater and sewage, the correlation between *Arcobacter* and high counts of total coliforms was already reported (Collado, Inza, Guarro, & Figueras, 2008). Similarly, Lee, Agidi, Marion, & Lee, (2012) observed a significant correlation between *Arcobacter* and the human-specific fecal marker, *HuBac*, on the beaches of the lake Erie located in Ohio, USA. In foods, Salas-Massó, Figueras, Andree, & Furones, (2018) indicated that the presence of *E. coli* can predict the presence of pathogenic *Arcobacter* species in shellfish samples harvested from water with temperatures lower than 26.2 °C. It is

important to clarify, that this phenomenon has not been well elucidated because of the complexity of bacterial communities and due to the limited use of specific bacterial groups as indicators of *Arcobacter* species (Hsu, Mitsch, Martin, & Lee, 2017; Leight, Crump, & Hood, 2018).

The quality of milk is always associated with its physicochemical composition as well as its microbiological quality (Oliver, Jayarao, & Almeida, 2005). As shown in Table 3, milks that were positive for *A. butzleri* presented high somatic cell counts ($> 5.0 \times 10^5$ somatic cells/mL), exceeding the limits of 10^5 somatic cells/mL, thus indicating contaminated udders (Lindmark-Månsson et al., 2006). By analyzing MFA (Figure 2A), the factor SCC did not show a correlation with the presence of *A. butzleri*, which reinforces the hypothesis that the presence of *A. butzleri* in raw milk could have an environmental origin.

As already stated by other authors (Hamann & Kromker, 1997; Schukken, Wilson, Welcome, Garrison-Tikofsky, & Gonzalez, 2003), the somatic cells count always have a correlation with the chemical composition of the milk, which is observed in Figure 2A. In relation to Dornic acidity, which is also an important indicator of microbiological quality (Gargouri, Hamed, & Elfeki, 2013), no correlation was found with *A. butzleri*.

To guarantee microbiological quality, milk should be maintained at refrigerated temperatures after proper pasteurization (USDA, 2019). The current Brazilian regulation (Brazil, 2011) stipulates that the raw milk tanks should be kept at 4 – 7 °C in dairy farms, to avoid the multiplication of pathogenic microorganisms. Giacometti et al., (2014) observed that when UHT milk was stored at 6 °C, the *A. butzleri* remained stable, whereas when the storage temperature was increased to 20 °C, *A. butzleri* counts increased considerably.

2.6 CONCLUSION

This work shows the occurrence of *A. butzleri* in Minas frescal cheese and raw milk in the state of São Paulo, Brazil. This is the first published report on *A. butzleri* isolation in Brazilian frescal cheese. The occurrence of *A. butzleri* can be a public health problem considering that it is a ready to eat product. It is important to highlight that infectious dose and the behavior of this bacterium in food are not yet well comprehended. Additionally, the results of the present investigation point out the need to control *A. butzleri* from the post-milking period. A continuous microbiological control of milk should be carried out in the whole chain of production and distribution of the Minas frescal cheese.

Acknowledgments

Melina L. M. Cruzado-Bravo and Erick Saldaña received the support of the Consejo Nacional de Ciencia, Tecnología e Innovación Tecnológica CONCYTEC, Peru (CIENCIACTIVA programme, PhD scholarship contracts: No. 241-2018-FONDECYT, No. 104-2016-FONDECYT, respectively). The authors are grateful also to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) for the financial support.

LITERATURE CITED

- ABIQ. (2019). Associação Brasileira das Indústrias de Queijo. Retrieved June 25, 2019, from Associação Brasileira das Indústrias de Queijo website:
https://www.abiq.com.br/queijos_ler.asp?codigo=1925&codigo_categoria=16&codigo_subcategoria=37
- Atabay, H. I., Aydin, F., Houf, K., Sahin, M., & Vandamme, P. (2003). The prevalence of *Arcobacter* spp. on chicken carcasses sold in retail markets in Turkey, and identification of the isolates using SDS-PAGE. *International Journal of Food Microbiology*, 81(1), 21–28. [https://doi.org/10.1016/S0168-1605\(02\)00163-0](https://doi.org/10.1016/S0168-1605(02)00163-0)
- Banting, G., & Figueras, S. (2017). *Arcobacter*. In J.B. Rose and B. Jiménez-Cisneros (Ed.), *Water Pathogens Project* (Global). Retrieved from <http://www.unesco.org/openaccess/terms-use-ccbysa-en>
- Brazil. (2001). *Agência Nacional de Vigilância Sanitária. Resolução RDC no. 12, de 02.01.01: regulamento técnico sobre padrões microbiológicos para alimentos* (p. 37). p. 37. Retrieved from http://portal.anvisa.gov.br/documents/33880/2568070/RDC_12_2001.pdf/15ffddf6-3767-4527-bfac-740a0400829b

- Brazil. (2006). *Ministério da Agricultura, Pecuária e Abastecimento Secretaria de Defesa Agropecuária Instrução Normativa Nº 68, de 12 Dezembro de 2006 O Secretário de Defesa Agropecuária, do Ministério da Agricultura, Pecuária e Abastecimento* (p. 141). p. 141. Retrieved from <https://wp.ufpel.edu.br/inspleite/files/2016/03/Instrução-normativa-nº-68-de-12-dezembro-de-2006.pdf>
- Brazil. (2011). *Ministério da Agricultura, Pecuária e Abastecimento. Gabinete do Ministro. Instrução Normativa Nº 62, de 29 de Dezembro de 2011* (p. 24). p. 24. Retrieved from <https://www.apcbrh.com.br/files/IN62.pdf>
- Collado, L., Cleenwerck, I., Van Trappen, S., De Vos, P., & Figueras, M. J. (2009a). *Arcobacter mytili* sp. nov., an indoxyl acetate-hydrolysis-negative bacterium isolated from mussels. *International Journal Of Systematic And Evolutionary Microbiology*, 59(6), 1391–1396. <https://doi.org/10.1099/ijms.0.003749-0>
- Collado, L., Cleenwerck, I., Van Trappen, S., De Vos, P., & Figueras, M. J. (2009b). *Arcobacter mytili* sp. nov., an indoxyl acetate-hydrolysis-negative bacterium isolated from mussels. *International Journal Of Systematic And Evolutionary Microbiology*, 59(6), 1391–1396. <https://doi.org/10.1099/ijms.0.003749-0>
- Collado, L., & Figueras, M. J. (2011). Taxonomy, Epidemiology, and Clinical Relevance of the Genus *Arcobacter*. *Clinical Microbiology Reviews*, 24(1), 174–192. <https://doi.org/10.1128/CMR.00034-10>
- Collado, Luis, Inza, I., Guarro, J., & Figueras, M. J. (2008). Presence of *Arcobacter* spp. in environmental waters correlates with high levels of fecal pollution. *Environmental Microbiology*, 10(6), 1635–1640. <https://doi.org/10.1111/j.1462-2920.2007.01555.x>
- Doudah, L., De Zutter, L., Vandamme, P., & Houf, K. (2010). Identification of five human and mammal associated *Arcobacter* species by a novel multiplex-PCR assay. *Journal of Microbiological Methods*, 80, 281–286. <https://doi.org/10.1016/j.mimet.2010.01.009>

- Downes, F. P., & Itō, K. (2001). *Compendium of methods for the microbiological examination of foods* (4th ed.). Washington D.C: American Public Health Association (APHA).
- Ellis, W. A., Neill, S. D., O'Brien, J. J., Ferguson, H. W., & Hanna, J. (1977). Isolation of *Spirillum/Vibrio*-like organisms from bovine fetuses. *The Veterinary Record*, 100(21), 451–452. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/560078>
- Elmali, M., Can, H. Y., Elmali, M., & Can, H. Y. (2016). Occurrence and antimicrobial resistance of *Arcobacter* species in food and slaughterhouse samples. *Food Science and Technology (Campinas)*, 37(ahead), 0–0. <https://doi.org/10.1590/1678-457X.19516>
- Ertas, N., Dogruer, Y., Gonulalan, Z., Guner, A., & Ulger, I. (2010). Prevalence of *Arcobacter* Species in Drinking Water, Spring Water, and Raw Milk as Determined by Multiplex PCR. *Journal of Food Protection*, 73(11), 2099–2102. <https://doi.org/10.4315/0362-028X-73.11.2099>
- Ferreira, S., Júlio, C., Queiroz, J. A., Domingues, F. C., & Oleastro, M. (2014). Molecular diagnosis of *Arcobacter* and *Campylobacter* in diarrhoeal samples among Portuguese patients. *Diagnostic Microbiology and Infectious Disease*, 78(3), 220–225. <https://doi.org/10.1016/j.diagmicrobio.2013.11.021>
- Figueras, M. J., Levican, A., & Collado, L. (2012). Updated 16S rRNA-RFLP method for the identification of all currently characterised *Arcobacter* spp. *BMC Microbiology*, 12(1), 292. <https://doi.org/10.1186/1471-2180-12-292>
- Gargouri, A., Hamed, H., & Elfeki, A. (2013). Analysis of raw milk quality at reception and during cold storage: Combined effects of somatic cell counts and psychrotrophic bacteria on lipolysis. *Journal of Food Science*, 78(9), 1405–1411. <https://doi.org/10.1111/1750-3841.12188>

Ghaju Shrestha, R., Tanaka, Y., Malla, B., Bhandari, D., Tandukar, S., Inoue, D., ...

Haramoto, E. (2017). Next-generation sequencing identification of pathogenic bacterial genes and their relationship with fecal indicator bacteria in different water sources in the Kathmandu Valley, Nepal. *Science of The Total Environment*, 601–602, 278–284.

<https://doi.org/10.1016/j.scitotenv.2017.05.105>

Giacometti, F., Lucchi, A., Di Francesco, A., Delogu, M., Grilli, E., Guarniero, I., ...

Serraino, A. (2015). *Arcobacter butzleri*, *Arcobacter cryaerophilus*, and *Arcobacter skirrowii* Circulation in a Dairy Farm and Sources of Milk Contamination. *Applied and Environmental Microbiology*, 81(15), 5055–5063. [https://doi.org/10.1128/AEM.01035-](https://doi.org/10.1128/AEM.01035-15)

15

Giacometti, F., Serraino, A., Marchetti, G., Bonerba, E., Florio, D., Bonfante, E., ... Rosmini,

R. (2013). Isolation of *Arcobacter butzleri* in environmental and food samples collected in industrial and artisanal dairy plants. *Italian Journal of Food Safety*, 2(3), 34.

<https://doi.org/10.4081/ijfs.2013.e34>

Giacometti, F., Serraino, A., Pasquali, F., De Cesare, A., Bonerba, E., & Rosmini, R. (2014).

Behavior of *Arcobacter butzleri* and *Arcobacter cryaerophilus* in Ultrahigh-Temperature, Pasteurized, and Raw Cow's Milk Under Different Temperature Conditions. *Foodborne Pathogens and Disease*, 11(1), 15–20.

<https://doi.org/10.1089/fpd.2013.1597>

Golla, S. C., Murano, E. A., Johnson, L. G., Tipton, N. C., Cureington, E. A., & Savell, J. W.

(2002). Determination of the Occurrence of *Arcobacter butzleri* in Beef and Dairy Cattle from Texas by Various Isolation Methods. *Journal of Food Protection*, 65(12), 1849–1853. <https://doi.org/https://doi.org/10.4315/0362-028X-65.12.1849>

- Grove-White, D. H., Leatherbarrow, A. J. H., Cripps, P. J., Diggle, P. J., & French, N. P. (2014). Temporal and farm-management-associated variation in faecal pat prevalence of *Arcobacter* spp. in ruminants. *Epidemiology and Infection*, 142(04), 861–870. <https://doi.org/10.1017/S095026881300160X>
- Hamann, J., & Kromker, V. (1997). Livestock Production Science Potential of specific milk composition variables for cow health management 1. *Livestock Production Science*, 48, 201–208.
- Harmon, K. ., & Wesley, I. . (1996). Identification of *Arcobacter* isolates by PCR. *Letters in Applied Microbiology*, 23(4), 241–244. <https://doi.org/10.1111/j.1472-765X.1996.tb00074.x>
- Hilton, C. L., Mackey, B. M., Hargreaves, A. J., & Forsythe, S. J. (2001). The recovery of *Arcobacter butzleri* NCTC 12481 from various temperature treatments. *Journal of Applied Microbiology*, 91(5), 929–932. <https://doi.org/10.1046/j.1365-2672.2001.01457.x>
- Hsu, T., & Lee, J. (2015). Global Distribution and Prevalence of *Arcobacter* in Food and Water. *Zoonoses and Public Health*, 62(8), 579–589. <https://doi.org/10.1111/zph.12215>
- Hsu, T., Mitsch, W., Martin, J., & Lee, J. (2017). Towards sustainable protection of public health: The role of an urban wetland as a frontline safeguard of pathogen and antibiotic resistance spread. *Ecological Engineering*, 108, 547–555. <https://doi.org/10.1016/J.ECOLENG.2017.02.051>
- ICMSF. (2002). Microorganisms in food. Microbiological testing in food safety management. International Commission on Microbiological Specifications for Foods (ICMSF). New York NY USA: Kluwer Academic Publ.

- Kabeya, H., Maruyama, S., Morita, Y., Kubo, M., Yamamoto, K., Arai, S., ... Mikami, T. (2003). Distribution of *Arcobacter* species among livestock in Japan. *Veterinary Microbiology*, 93(2), 153–158. [https://doi.org/10.1016/S0378-1135\(02\)00312-7](https://doi.org/10.1016/S0378-1135(02)00312-7)
- Lee, C., Agidi, S., Marion, J. W., & Lee, J. (2012). *Arcobacter* in Lake Erie beach waters: an emerging gastrointestinal pathogen linked with human-associated fecal contamination. *Applied and Environmental Microbiology*, 78(16), 5511–5519. <https://doi.org/10.1128/AEM.08009-11>
- Leight, A., Crump, B., & Hood, R. (2018). Assessment of Fecal Indicator Bacteria and Potential Pathogen Co-Occurrence at a Shellfish Growing Area. *Frontiers in Microbiology*, 9, 384. <https://doi.org/10.3389/fmicb.2018.00384>
- Lindmark-Månsson, H., Bränning, C., Aldén, G., & Paulsson, M. (2006). Relationship between somatic cell count, individual leukocyte populations and milk components in bovine udder quarter milk. *International Dairy Journal*, 16(7), 717–727. <https://doi.org/10.1016/j.idairyj.2005.07.003>
- Logan, E. F., Neill, S. D., & Mackie, D. P. (1982). Mastitis in dairy cows associated with an aerotolerant campylobacter. *The Veterinary Record*, 110(10), 229–230. <https://doi.org/10.1136/VR.110.10.229>
- Malek dos Reis, C. B., Barreiro, J. R., Mestieri, L., Porcionato, M. A. de F., & dos Santos, M. V. (2013). Effect of somatic cell count and mastitis pathogens on milk composition in Gyr cows. *BMC Veterinary Research*, 9(1), 67. <https://doi.org/10.1186/1746-6148-9-67>
- Marchand, S., De Block, J., De Jonghe, V., Coorevits, A., Heyndrickx, M., & Herman, L. (2012). Biofilm Formation in Milk Production and Processing Environments; Influence on Milk Quality and Safety. *Comprehensive Reviews in Food Science and Food Safety*, 11(2), 133–147. <https://doi.org/10.1111/j.1541-4337.2011.00183.x>

- Merga, J. Yvette, Williams, N. J., Miller, W. G., Leatherbarrow, A. J. H., Bennett, M., Hall, N., ... Winstanley, C. (2013). Exploring the Diversity of *Arcobacter butzleri* from Cattle in the UK Using MLST and Whole Genome Sequencing. *PLoS ONE*, 8(2), e55240. <https://doi.org/10.1371/journal.pone.0055240>
- Merga, J Y, Leatherbarrow, A. J. H., Winstanley, C., Bennett, M., Hart, C. A., Miller, W. G., & Williams, N. J. (2011). Comparison of *Arcobacter* isolation methods, and diversity of *Arcobacter* spp. in Cheshire, United Kingdom. *Applied and Environmental Microbiology*, 77(5), 1646–1650. <https://doi.org/10.1128/AEM.01964-10>
- Milanez, A. Y., Guimarães, D. D., Maia, G. B. da S., Martins, P. do C., Lima, G. S., Oliveira, S. J. de M., ... Freitas, V. M. F. de. (2018). *Desafios para a exportação brasileira de leite* (pp. 45–114). pp. 45–114. Retrieved from <https://web.bndes.gov.br/bib/jspui/handle/1408/16060>
- Oliver, S. P., Jayarao, B. M., & Almeida, R. A. (2005). Foodborne Pathogens in Milk and the Dairy Farm Environment: Food Safety and Public Health Implications. *Foodborne Pathogens and Disease*, 2(2), 115–129. <https://doi.org/10.1089/fpd.2005.2.115>
- Pianta, C., Passos, D. T., Hepp, D., & Oliveira, S. J. de. (2007). Isolation of *Arcobacter* spp from the milk of dairy cows in Brazil. *Ciência Rural*, 37(1), 171–174. <https://doi.org/10.1590/S0103-84782007000100027>
- Revez, J., Huuskonen, M., Ruusunen, M., Lindström, M., & Hänninen, M.-L. (2013). *Arcobacter* Species and Their Pulsed-Field Gel Electrophoresis Genotypes in Finnish Raw Milk during Summer 2011. *Journal of Food Protection*, 76(9), 1630–1632. <https://doi.org/10.4315/0362-028X.JFP-13-083>

- Salas-Massó, N., Figueras, M. J., Andree, K. B., & Furones, M. D. (2018). Do the *Escherichia coli* European Union shellfish safety standards predict the presence of *Arcobacter* spp., a potential zoonotic pathogen? *Science of The Total Environment*, 624, 1171–1179. <https://doi.org/10.1016/J.SCITOTENV.2017.12.178>
- Schukken, Y. H., Wilson, D. J., Welcome, F., Garrison-Tikofsky, L., & Gonzalez, R. N. (2003). Monitoring udder health and milk quality using somatic cell counts. *Vet. Res*, 34, 579–596. <https://doi.org/10.1051/vetres:2003028>
- Serraino, A., Florio, D., Giacometti, F., Piva, S., Mion, D., & Zanoni, R. G. (2013). Presence of *Campylobacter* and *Arcobacter* species in in-line milk filters of farms authorized to produce and sell raw milk and of a water buffalo dairy farm in Italy. *Journal of Dairy Science*, 96(5), 2801–2807. <https://doi.org/10.3168/jds.2012-6249>
- Serraino, A., & Giacometti, F. (2014). Short communication: Occurrence of *Arcobacter* species in industrial dairy plants. *Journal of Dairy Science*, 97(4), 2061–2065. <https://doi.org/10.3168/jds.2013-7682>
- Shah, A. H., Saleha, A. A., Murugaiyah, M., Zunita, Z., & Memon, A. A. (2012). Prevalence and Distribution of *Arcobacter* spp. in Raw Milk and Retail Raw Beef. *Journal of Food Protection*, 75(8), 1474–1478. <https://doi.org/10.4315/0362-028X.JFP-11-487>
- Shah, A. H., Saleha, A. A., Zunita, Z., Murugaiyah, M., Aliyu, A. B., & Jafri, N. (2013). Prevalence, Distribution and Antibiotic Resistance of Emergent *Arcobacter* spp. from Clinically Healthy Cattle and Goats. *Transboundary and Emerging Diseases*, 60(1), 9–16. <https://doi.org/10.1111/j.1865-1682.2012.01311.x>

- Tortorello, M. L. (2003). Indicator Organisms for Safety and Quality—Uses and Methods for Detection: Minireview. *Journal of AOAC International*, 86(6), 1208–1217. Retrieved from <https://www.ingentaconnect-com.ez67.periodicos.capes.gov.br/content/aoac/jaoac/2003/00000086/00000006/art00017>
- USDA. (2019). Refrigeration and Food Safety. Retrieved June 24, 2019, from United States Department of Agriculture. Food Safety and Inspection Service website: https://www.fsis.usda.gov/shared/PDF/Refrigeration_and_Food_Safety.pdf
- Van den Abeele, A.-M., Vogelaers, D., Van Hende, J., & Houf, K. (2014). Prevalence of *Arcobacter* species among humans, Belgium, 2008-2013. *Emerging Infectious Diseases*, 20(10), 1731–1734. <https://doi.org/10.3201/eid2010.140433>
- Vandenberg, O., Dediste, A., Houf, K., Ibekwem, S., Souayah, H., Cadranel, S., ... Vandamme, P. (2004). *Arcobacter* species in humans. *Emerging Infectious Diseases*, 10(10), 1863–1867. <https://doi.org/10.3201/eid1010.040241>
- Verraes, C., Vlaemynck, G., Van Weyenberg, S., De Zutter, L., Daube, G., Sindic, M., ... Herman, L. (2015). A review of the microbiological hazards of dairy products made from raw milk. *International Dairy Journal*, 50, 32–44. <https://doi.org/10.1016/j.idairyj.2015.05.011>
- Vilar, M. J., Peña, F. J. G., Pérez, I., Diéguez, F. J., Sanjuán, M. L., Rodríguez-Otero, J. L., & Yus, E. (2010). Presence of *Listeria*, *Arcobacter*, and *Campylobacter* spp. in dairy farms in Spain. *Berliner Und Munchener Tierarztliche Wochenschrift*, 123(1–2), 58–62. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/20135911>

- Webb, A. L., Boras, V. F., Kruczkiewicz, P., Selinger, L. B., Taboada, E. N., & Inglis, G. D. (2016). Comparative Detection and Quantification of *Arcobacter butzleri* in Stools from Diarrheic and Nondiarrheic People in Southwestern Alberta, Canada. *Journal of Clinical Microbiology*, 54(4), 1082–1088. <https://doi.org/10.1128/JCM.03202-15>
- Yesilmen, S., Vural, A., Erkan, M. E., & Yildirim, I. H. (2014). Prevalence and antimicrobial susceptibility of *Arcobacter* species in cow milk, water buffalo milk and fresh village cheese. *International Journal of Food Microbiology*, 188, 11–14. <https://doi.org/10.1016/j.ijfoodmicro.2014.07.006>

List of Tables and Figures

Table 1. Occurrence of *A. butzleri* isolated from Minas frescal cheese and raw milk

Samples	N° of identified colonies			Identified species and genotypes	
	Positives (%)	<i>Arcobacter</i> spp	Non- <i>Arcobacter</i>	N° Colonies	<i>A. butzleri</i>
Type samples					N° of ERIC patterns
cheese (n=98)	10 (10.2)	29	93	15	12
raw milk (n=103)	17 (16.5)	55	104	21	17
Total (n=201)	27 (13.4)	84	197	36	29

Table 2. Samples of Minas frescal cheese positive to *A. butzleri*

Note: UD= Undetermined. NMP= most probable number. CoPS: Coagulase-positive staphylococci. **In bold**, samples that are above the limit tolerated by Brazilian legislation (BRAZIL, 2001).

Sample code	Total Coliforms MPN/g	Thermotolerant coliforms MPN/g	CoPS CFU/g	Moisture %
Q5	UD	UD	UD	UD
Q27	UD	UD	UD	UD
Q33	UD	UD	UD	UD
Q61	1.6 x 10 ³	7.8 x 10²	1.3 x 10⁶	88.1±1.53
Q72	8.1 x 10 ²	8.1 x 10²	4.1 x 10⁷	76.9±1.05
Q73	2.4 x 10 ³	24 x 10 ²	< 10	85.3±1.36
Q74	1.7 x 10	1.7x10	< 10	71.9±2.25
Q75	4.5	<1.8	< 10	80.0±5.21
Q78	2.4 x 10 ³	1.4 x 10³	8.9 x 10⁶	59.7±1.63
Q79	2.4 x 10 ³	1.0 x 10³	< 10	69.8±6.61

Table 3. Samples of raw milk positive to *A. butzleri*

Sample code	Dornic acidity	Total Coliforms MPN/mL	Thermotolerant coliforms MPN /mL	Aerobic mesophilic bacteria count CFU/mL	fat (% m/m)	Protein (% m/m)	Lactose (% m/m)	ST (% m/m)	Solids-not-fat (% m/m)	SCC/ m
L22	16.0±0.00	>1.1x10 ⁴	1.4x10	1.8x10 ⁵ ±0.51	3.75	3.40	4.36	12.54	8.79	5.2 x10⁵
L23	16.0±0.47	>1.1x10 ⁴	<3	7.2x10⁶±1.20	2.06	3.16	4.52	10.71	8.65	8.5 x10 ⁴
L24	13.0±0.81	9.2x10	2.3x10	7.3x10 ³ ±2.33	3.35	3.07	4.65	12.07	8.72	2.4 x10 ⁵
L27	15.0±0.00	>1.1x10 ⁴	2.3x10	5.3x10⁶±0.61	3.81	3.28	4.55	12.64	8.83	1.1 x10⁶
L28	15.0±0.47	>1.1x10 ⁴	3.6	2.5x10⁶±0.82	3.02	3.09	4.71	11.80	8.78	5.3 x10⁵
L29	14.5±0.23	>1.1x10 ⁴	<3	2.8x10⁶±0.54	2.91	2.69	4.10	10.69	7.78	2.2 x10 ⁵
L34	15.0±0.00	4.30x10 ²	<3	6.6 x10⁵±0.39	3.95	3.17	4.35	12.51	8.56	5.4 x10⁵
L40	16.0±0.00	>1.1x10 ⁴	3.6	6.3x10⁷±1.34	4.83	3.70	4.01	13.62	8.79	1.5 x10⁶
L46	16.0±0.00	1.1x10 ⁴	<3	7.3x10⁵±0.91	3.10	3.34	4.55	12.00	8.90	5.3 x10 ⁴
L47	16.0±0.00	4.3x10	4.3x10	1.2x10 ⁵ ±1.33	3.02	3.20	4.70	11.90	8.88	4.6 x10 ⁴
L48	17.0±0.47	>1.1x10 ³	<3	4.5x 10⁵±0.22	3.33	3.26	4.72	12.28	8.95	7.7 x10 ⁴
L63	14.0±0.23	1.1x10 ³	3.6	3.6x 10⁵±0.40	3.43	2.96	4.35	11.72	8.29	5.4 x10⁵
L70	15.0±0.00	>1.1x10 ³	3.6	1.4x 10⁶±0.27	2.70	3.31	4.44	11.44	8.74	1.7 x10 ⁵
L79	14.0±0.23	3.8x10 ²	3.6	1.3x10⁷±1.69	3.10	2.73	4.00	10.89	7.79	2.8 x10 ⁵
L82	17.0±0.47	2.4x10 ³	<3	1.5x10⁷±0.71	2.82	3.15	4.59	11.50	8.68	2.6 x10 ⁵
L96	11.5±0.47	2.4x10 ³	2.4x10 ²	3.1x10⁷±0.54	1.98	2.49	3.23	8.86	6.88	5.0 x10 ⁵
L101	15.5±0.23	1.1x10 ³	9.2	2.8x10⁶±0.92	3.51	3.11	4.36	12.01	8.50	5.5 x10⁵

Note: NMP= Most Probable Number, CFU= Colony Forming Unit, ST=, SCC=Somatic Cell Count. **In bold**, samples that are above the limit tolerated by Brazilian legislation (BRAZIL, 2011).

Figure 1. Multiple factor analysis (MFA) of Minas frescal cheese samples for dimensions 1 and 2. **(A)** Red lines represent the microbiological analyses, blue line represents the physical analyses and green line represent the positive *A. butzleri* samples. **(B)** Position of positive samples for *A. butzleri*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article). Note: CoPS= Coagulase-positive staphylococci.

Figure 2. Multiple factor analysis (MFA) of raw milk samples for dimensions 1 and 2. **(A)** Red lines represent the physicochemical analyses, blue lines represent the microbiological analyses, green line represent samples positive for *A. butzleri* and purple line represent somatic cell count. **(B)** Position of positive samples for *A. butzleri*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article). Note: SCC= somatic cell count.

CHAPTER 3: Biofilm formation on contact surfaces and different media of *Arcobacter butzleri* isolates from dairy products

Authors: Melina L.M Cruzado-Bravo¹, Giovana V. Barancelli¹, Erick Saldaña², Giovanni Henrique Mariano¹, Nicolle F.A Padovani¹, Luis Collado³, Carmen J. Contreras-Castillo¹

Affiliations:

1. Universidade de São Paulo, Escola Superior de Agricultura “Luiz de Queiroz”, Departamento de Agroindústria, Alimentos e Nutrição, Av. Pádua Dias 11, 13418-900 Piracicaba, São Paulo, Brazil
2. Facultad de Ingeniería Agroindustrial, Universidad Nacional de Moquegua (UNAM), Moquegua, Peru.
3. Universidad Austral de Chile, Institute of Biochemistry and Microbiology, Faculty of Sciences, Isla Teja, Valdivia, Chile.

Corresponding author:

Carmen Josefina Contreras Castillo

Universidade de São Paulo, Escola Superior de Agricultura “Luiz de Queiroz”, Departamento de Agroindústria, Alimentos e Nutrição, Av. Pádua Dias 11, 13418-900 Piracicaba, São Paulo, Brazil

Address: Avenida Padua Dias 11 CEP 13418-900, Piracicaba, São Paulo, Brazil, Phone +55 19 99909844, fax +55 19 346006

E-mail: ccastil@usp.br, mcruzado@usp.br

ABSTRACT

Biofilms are complex microbial ecosystems formed by one or more species of microorganisms and are a latent problem of contamination in the food industry. *Arcobacter butzleri* is considered an emerging human pathogen, since can trigger several gastrointestinal disorders in humans, such as chronic diarrhea and bacteremia. The objective of this research was to evaluate the formation of *A. butzleri* biofilms and their association with some intrinsic and extreme factors. Biofilm formation was quantified in 96-well microplate evaluating cell concentration (10^6 , 10^7 and 10^8 UFC/mL) and time of incubation (48 h and 96 h) for 36 *A. butzleri* strains isolated from Minas frescal cheese and raw milk in São Paulo State, Brazil. The presence of virulence genes *cadF*, *ciaB*, *cjl349*, *irgA*, *hecA*, *hecB*, *mviN*, *pldA*, *tlyA* and *iroE*, was detected by PCR. The antibiotics penicillin G (PEN), tetracycline (TET), neomycin (NEO), ciprofloxacin (CIP), erythromycin (E), and azithromycin (AZI) were tested by disk diffusion technique according to CLSI. Subsequently, eight selected field strains and LMG 10828^T, were evaluated to assess biofilms formation on two contact surfaces (stainless steel and polypropylene), with two culture broths: whey and Mueller Hinton (MH) and time of incubation (48 h and 96 h). The milk strains showed the highest biofilm formation, while at 48 h it was possible to quantify more biofilms than at 96 h, additionally the greatest biofilms formation was at the concentration 10^8 CFU/mL. All strains presented the genes *pldA*, *ciaB*, *cadF*, *tlyA* and *mviN*, while the lowest prevalences were for *irgA* and *iroE*. The greatest antibiotics resistance was for PEN (94.4%), while 100% of the strains were sensitive for CIP and AZI, and more than 90% of the strains were sensitive to TET, NEO and E. Greatest formation of biofilms was observed on stainless steel and using MH as a culture broth. The two methodologies used to quantify biofilms had a similarity of 80%. This study provides information about biofilm formation of *A. butzleri*, evidence their virulence potential based on genes presence and antibiotic resistance. The results justify further research with respect to *Arcobacter* biofilm role in food safety.

Key words: surface contact, dairy products, food safety, emerging human pathogen

Highlights

1. Strains from raw milk showed more biofilm formation than those from cheese
2. Virulence genes *pldA*, *ciaB*, *cadF*, *tlyA* and *mviN* were presented in all strains.
3. Relative frequency of the genes evaluated was slightly related to the resistance to the antibiotics evaluated but not to the formation of biofilms.
4. Strains were able to use the whey to form biofilms on contact surfaces, being larger on stainless steel coupons.

3.1 INTRODUCTION

A natural process in which microorganisms inhabit are biofilms, in fact, 99% of the microorganisms on the Earth are estimated to exist in sessile aggregates, and more than 80% of the bacterial habitats have been estimated to comprise biofilms (Mizan et al. 2015). If we can make an analogy, biofilm would be the cities that humans building to live protected and in community. Just as we have roads, to communicate bacterial biofilms have channels and three-dimensional structures that allow the entry of nutrients, for their survival as a community.

Due to the ability that microorganisms have to adhere to inert surfaces used in different environments, such as the area of human and animal medicine, in food processing and water treatment (Harro et al. 2010), this represents a public health concern especially if pathogens are present. It is noted that many foodborne outbreaks have been associated with biofilms (Srey et al. 2013).

The emergent pathogen *Arcobacter butzleri* can trigger several gastrointestinal disorders in humans, such as chronic diarrhea and bacteremia and is the best known of the 29 species conforming this genus. The *Arcobacter* morphology is taxonomically similar to *Campylobacter*, i.e: is a small curved or spiral cells, motile and non-sporulated. However, arcobacters differ from campylobacters by their ability to grow in aerobic environment and at lower temperatures (15 °C compared with 30 °C) (Mansfield & Forsythe 2000).

The presence of *A. butzleri* has already been widely reported in raw cow's milk, dairy products, processing plants and from fresh cheeses (Serraino et al. 2013; Yesilmen et al. 2014; Shirzad Aski et al. 2016). An important point would be to understand how this pathogen would enter to the dairy processing plants. Recent studies, have demonstrated the presence of *A. butzleri* in raw milk and therefore this would be the way of enter to the dairy production (Traversa et al. 2019; Cruzado-Bravo et al. 2020). Subsequently, due to probable failures in the process, such as cross-contamination, inadequate heat treatment makes it possible to isolate *A. butzleri* in dairy products ready for consumption.

Despite the increase in the number of *A. butzleri* studies, the pathogenicity of this species has been little explored (Miller et al. 2007; Doudah, de Zutter, et al. 2012; Karadas et al. 2013). The complete genome sequence of the strain RM4018 showed several putative virulence genes, some of them presenting homology with determinants of *Campylobacter jejuni* (Miller et al. 2007). The presence of *cadF*, *ciaB*, *cj1349*, *irgA*, *hecA*, *hecB*, *mviN*, *pldA*, *tlyA*, *iroE* genes have been explored in strains isolated from milk and dairy products (Ferreira et al. 2013; Girbau et al. 2015; Piva et al. 2017; Traversa et al. 2019) and has been observed high presence of genes *cadF*, *ciaB*, *cj1349*, *mviN*, *pldA* and *tlyA*.

Another important point in the characterization of new isolates is to know their sensitivity or resistance to the antibiotics used in milk production and in human infections treatments. In relation with this, recent publications showed low resistance in isolates of *A. butzleri* from cows, milk, retail food, as sensitive to erythromycin and ciprofloxacin which are the first and second -line antibiotics used in Campylobacteriosis treatments respectively (Yesilmen et al. 2014; Shirzad Aski et al. 2016; Vicente-Martins et al. 2018).

The present work aimed to characterize the pathogenic potential as well as antibiotic resistance of *A. butzleri* strains from raw cow milk and Minas frescal cheese in the São Paulo State, Brazil. For this purpose, the presence of ten genes associated with virulence was correlated with the resistance of six antibiotics with the production of biofilms in 96-well microplates. In addition, two methodologies were compared to quantify the production of biofilms formed on contact surfaces widely used in the dairy products industries.

3.2 MATERIALS AND METHODS

3.2.1 Isolates and growth conditions

A total of 36 *A. butzleri* strains belonging to the bacterial collection from the *Higiene e laticínios* laboratory (*Escola superior de Agricultura “Luiz de Queiroz”*, São Paulo University) which were isolated from Minas frescal cheese (n=15) and raw milk (n=21) (Cruzado-Bravo et al. 2020) were here characterized. The *A. butzleri* strain LMG 10828^T was used as control.

Bacteria were maintained at -80 °C, therefore to recover them two successive culture were made on blood agar base (Oxoid CM55, Hampshire, UK) supplemented with 5% (v/v) defibrinated sheep blood (Newprov, Curitiba, Brazil) and incubated at 30 °C under aerobic conditions for 48 h.

3.2.2 Screening strains of *A. butzleri* for the ability to form a biofilm

The ability of 36 *A. butzleri* strains to form biofilms was assayed using a method previously described by Ferreira et al. (2013), with minor modifications. Inoculum preparation was performed using bacterial cells grown on blood agar base supplemented with 5% (v/v) defibrinated sheep blood (30 °C/48 h) under aerobic condition. Bacterial population was standardized using the McFarland scale (0.5; $\sim 10^8$ CFU/mL) and from that concentration 2 successive dilutions were made in Mueller Hinton (MH) broth. Of these bacterial suspension ($\sim 10^8$, $\sim 10^7$ and $\sim 10^6$ CFU/mL), 200 μ L aliquot were placed in 96-well microtiter polystyrene plates and incubated for 48 h and 96 h at 25 °C in aerobic conditions. As a negative control it was used MH broth without bacterial inoculation.

After incubation, staining with crystal violet (CV) was performed according to Teh et al. (2010), with some variations, after drying, the wells were stained with 200 μ L of 0.1% of CV and left on the bench for 15 min. Then, the CV was removed, the wells were washed three times with sterile distilled water and dried for another 15 min at 42 °C. Then were resolubilized (200 μ L) with a 33% (v/v) glacial acetic acid solution. After that, the solubilized CV were transferred to new 96-well plates and the optical density (OD) was measured at 600 nm using a plate reader (Vitor™ X3, Perkin Elmer, USA). The average of triplicates was used applied in further analysis.

3.2.3 Detection of virulence genes

The bacterial DNA was extracted using InstaGene™ DNA Purification Matrix (Bio-Rad, Hercules, CA, USA). The DNA samples were stored at -20 °C until further analysis. Polymerase chain reaction (PCR) was performed to detect the genes *cadF*, *ciaB*, *cjl349*, *irgA*, *hecA*, *hecB*, *mviN*, *pldA*, *tlyA* and *iroE*.

DNA amplification was carried out using 1X PCR buffer, 1U GoTaq® Hot Start Polymerase (Promega Corporation, Madison, USA), 1.3 - 2.0 mM MgCl₂ (Promega Corporation, Madison, USA), 10 pmol of each primer described for Doudah et al. (2012) and Karadas et al. (2013), 200 μ M of deoxynucleotides (Promega Corporation, Madison, USA), template DNA (~ 40 ng), and ultrapure water to complete the volume to 25 μ L. Positive (*A. butzleri* LMG 10828^T) and negative (ultrapure water) controls were used in every PCR run. The PCR conditions were the following; initial denaturation (94°C for 4 min), followed by 30 amplification cycles consisting of 45 sec at 94°C (denaturation), 45 sec at 56°C (primer annealing), and 2 min at 72°C (primer extension). A primer extension step (72°C for 7 min) followed the final amplification cycle.

Analysis of the PCR products were carried out by agarose gel 1.0% (1.2% w/v) with ethidium bromide (0.3 µg/mL) in TBE buffer 0.5X (45 mmol/L Tris-Borate; pH 8.0, 1mmol/L EDTA). Images of gels were captured using Image LabTM Software and Molecular Imager Gel DocTM XR (BioRad Laboratories, Hercules, CA, USA).

3.2.4 Antimicrobial susceptibility testing

The antimicrobial susceptibility of 36 *A. butzleri* isolates was tested by disk diffusion technique according to Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI 2015) for *Campylobacter jejuni/coli*. The selected antibiotics were chosen based on their use in the dairy production and included penicillin G (PEN, 10 µg), tetracycline (TE, 30 µg), neomycin (NEO, 30 µg), ciprofloxacin (CIP, 5 µg), erythromycin (E, 15 µg), and azithromycin (AZI, 15 µg) (Laborclin, Brazil).

The isolates were grown in blood agar base with 5% defibrinated sheep blood and incubated at 30° C under aerobic conditions for 48 h. Bacterial colonies from fresh pure culture were mixed with sterile saline solution (0.85%) to prepare the turbidity of each inoculum, which was adjusted to McFarland (0.5; ~10⁸ CFU/mL) standards. Bacteria from each suspension were inoculated on Muller-Hinton agar with 5% defibrinated sheep blood using a sterile cotton-tipped swab. After that antibiotic discs were dispensed, incubation of the plates took place in aerobic atmosphere at 30 °C for 48 h and the diameter of the inhibition zones was measured with calipers. Since there are no breakpoints for *Arcobacter* species, the zones of inhibition were recorded and interpreted according to CLSI guide lines (CLSI 2015) for *Campylobacter jejuni/coli* and *Enterobacteriaceae*.

3.2.5 Assay of biofilm formation on surfaces contact

Coupons, whey and bacterial suspension preparations

For sanitary stainless-steel polish 304 and polypropylene coupons with 12 mm in diameter were used as surfaces for *A. butzleri* biofilm formation. The coupons were washed and sanitized as described by Cruzado-Bravo et al. (2019).

The whey used, was obtained from the production of fresh cheese, sterilized at 121 °C for 15 min and filtered to remove residues, therefore, the whey for biofilm assay was transparent and similar to a common laboratory culture broth.

Bacterial suspension preparation was performed in the same way as screening strains a 1 mL aliquot of bacterial suspension (~10⁸ CFU/mL) preparing in MH broth and whey separately was placed in a 24-well plate containing a cleaned and sterilized coupon. Microplates were

incubated at 25 °C for 48 h, and 96 h under aerobic conditions, and each treatment was developed in triplicate. For the treatments with 96 h of incubation, the culture broths were renewed after 48 h of incubation.

Quantification of biofilm with crystal violet

After incubation, the coupons stained with CV was performed according to Teh et al. (2010). Coupons were removed aseptically from a new 24-well plate and the same methodology for CV, was carried out. The solubilized CV (200 µL) were transferred to new 96-well plates and OD was measured.

Bacterial counting of adhered cells and biofilm quantification

The coupons were transferred to tubes with 5 mL of peptone water 0.1% (w/v). The coupons immersed in peptone water were subjected to sonication at 40 kHz (sonication bath, Sonicador Ultra Cleaner 800A, Unique®, São Paulo, Brazil) for 30 °C/3 min to detach cells from the inert support, by additional vortexing for one minute. The bacterial suspensions obtained were used for serial dilution. A volume of 0.01 mL was then plated on blood agar by the drop plate method. The procedure was carried out in triplicate and plates were incubated at 30 °C/48 h. The bacterial count was performed according to the method using for Martin et al. (2016).

3.3 STATISTICAL ANALYSES

To the production of *A. butzleri* biofilms in a 96-well microplate, an analysis of variance (ANOVA) for the optical density (OD) was applied considering a factorial design (37x3x2) was used, with strains (n = 37), cell concentration (~ 10⁸, ~ 10⁷ and ~ 10⁶ CFU/mL) and incubation time (48 h and 96h) as source of variation. Significant factors (p≤0.05) were subjected to multiple comparisons using the Duncan test. The detection of genes was analyzed by the Cochran's Q test and Multiple McNemar (Bonferroni) test. Genetic relationships were assessed using Multiple Factor (MFA), the antibiotic's resistance was depicted by Principal Component Analysis (PCA) and the frequency of genes was analyzed by Principal Coordinates Analysis (PCoA). For the contact surfaces biofilm formation, a factorial ANOVA (7x2x2x2) was also used, with strains (n = 7), growth media (MH, whey), contact surfaces (stainless steel and polystyrene) and incubation time (48 h and 96h) as factors. Significant factors (p≤0.05) were then subjected to multiple comparisons using the Duncan test. Biofilm production relationships between strains and method for evaluating biofilm formation were assessed using

MFA. Statistical analyses were performed using XLSTAT 2018 software for Microsoft Excel® (Microsoft®, WA, USA) and R environment (R Core Team, 2017).

3.4 RESULTS

The quantification of biofilm in 96-well microplate was performed with the 36 field strains from dairy products and the type strain (LMG 10828^T) of the species *A. butzleri*, in the same way the presence of 10 virulence genes was investigated, as well as resistance test to six antibiotics.

According to the ANOVA carried out on the OD obtained in the biofilm formation assays, we observed that there was a significant difference ($p < 0.05$) between cell concentrations and incubation time for the strains used. Comparing the three cell concentrations, greater biofilm formation was observed at the concentration 10^8 CFU/mL demonstrated in Supplementary Table S1. In the case of incubation time, higher ODs were quantified at 48 h of incubation (Figure 1B). The strains with the highest production of biofilms were LC82A, Q78A, Q5, LC63A2, LC70C, LC63B1 and LMG 10828^T while the strains with less formation were LC40A, LC46A and Q74D as shown in Figure 2.

Supplementary Table S1 resumes the interaction between cell concentration and incubation time with respect to OD. Interactions with 10^8 CFU/mL were those that obtained the highest production of biofilms.

Analyzing the relative frequency of virulence-associated genes present in *A. butzleri*, all field strains presented the *pldA*, *ciaB*, *tlyA* and *mviN* genes and less than 10% of the strains presented the *irgA* and *iroE* genes (Table 1). In Supplementary Figure 1SA, it can see how the genes which have greatest presence are grouped in the center of the quadrants, clearly distinguishing the less present genes

On the other hand, antibiotic resistance was assessed using the diffusion disk method and breakpoints were analyzed according to the CLSI for *Campylobacter jejuni/coli* and *Enterobacteriaceae*. Table 2 shows the results of the antibiograms. All field strains were sensitive for AZI and CIP. Resistance to PEN was observed in 34 field strains (94.4%). The most resistant strains were from raw cow milk. Supplementary Figure 1SB represents the inhibition zone. It can be observed that the size of the closest inhibition zone was the NEO and AZI, and all strains were sensitive to those antibiotics, the proximity of the vectors indicates similar sensitivity. The most sensitive antibiotics are relatively close (ERI, AZI, NEO and CIP), while the PEN and TET inhibition zone that were lower and indicated resistance, are relatively far from the sensitive strain group.

In Supplementary Figure 2S, elaborated with the relative frequency of the genes, the inhibition zone and the OD obtained of the biofilm quantification in 96-well microplate, can be observed that the presence of the genes investigated and antibiotic resistance are close, indicating a slight correlation. However, it cannot be observe correlation with OD.

Selection of strains for biofilm test on contact surfaces

After the 96-well microplate biofilm test, antibiograms and virulence patterns, 8 field strains (cheese $n = 4$, raw milk $n = 4$) were selected, according to the statistical analysis shown in Figure 2. Of them, two field strains highly-forming biofilms and two strains of low biofilm formation were selected. This criterion was both field strains of Minas frescal cheese and raw milk, in addition the strain LMG 10828^T was added to the biofilm test on contact surfaces. The detailed information of each strain is shown in Table 3.

The versatility of the *A. butzleri* strains was evaluated by quantifying biofilms formed on contact surfaces using whey as a culture broth for their production.

The data obtained, both from OD and cell count ($\log \text{CFU}/\text{cm}^2$) were analyzed by 5% ANOVA, as shown in Supplementary Table 2S. All the statistically analyzed factors were different except the culture medium in the case of the OD response variable. The strains LC63A2 and LMG10828^T were the ones that showed the highest biofilm formation (OD) and for the cell count response variable was the LC63A2 and Q78A strains. The Q33A strain of Minas frescal cheese was the strain with the lowest biofilm formation. The arrangement of the strains obtained by means of the MFA analysis is shown in Supplementary Figure 3S, showing that the strains with the highest biofilm formation were located in I and IV quadrants.

The growth medium had a clear influence on the formation of *A. butzleri* biofilms (Figure 3A) when the cell count was analyzed as a response variable. MH was the culture medium that presented the highest adhesion cell counts, while when analyzing OD as response variable, no significant difference was observed between the culture media. This indicates that the strains of *A. butzleri* can use efficiently or in the same way the nutrients present in the whey and the MH medium to develop the biofilms.

Analyzing the incubation time factor and its effect on the cell count response variable, it was observed that with all strains the effect of time was significant ($p < 0.5$), being the highest counts were obtained with 96 h incubation (Figure 3B). On the other hand, the effect of the time on OD as a response variable, had a different behavior: on average the greatest formation of biofilms at 48 h was in whey, while in 96 h it was in MH. In general, the formation of biofilms in 48 h was greater than in 96 h, analyzing the OD as a response variable.

The surfaces used also had a significant effect (Figure 3C), greater biofilm formation on stainless steel was observed. On average the strains reached 6.4 log CFU/cm², while in polypropylene it was 5.5 log CFU/cm².

The comparison of the two response variables analyzed (OD and cell count) are shown in Figure 4, these variables were analyzed using MFA, in order to observe the correlation between both methodologies. As seen in Figure 4B, the two response variables had a correlation of 80% (RV = 0.8), which means that the two techniques are similar in 80% and allow to quantify and to obtaining highly similar results.

3.5 DISCUSSION

In the present study, 36 strains isolated from dairy products by Cruzado-Bravo et al. (2020) were characterized. For this, all strains were evaluated for the formation of biofilms in 96-well microplate, resistance to antibiotics and the presence of genes associated with virulence. Subsequently, eight strains were selected to evaluate the biofilms formation on contact surfaces commonly used in the food industry with different culture media, incubation time.

Considering that biofilms formation in surfaces is a normal behavior of microorganisms for survival, this become a critical problem in the food industry, since they can be a source of contamination. Eradication of this problem using sustainable and environmentally friendly techniques remains critically important, and therefore, it is receiving great attention from researchers around the world (Gule et al. 2016). In the same way, the number of investigations studying different bacterium and their ability to form biofilms has increased (Bridier et al. 2015). Thus, the information obtained to address the problems of biofilms has been improving, as for example in the design of new materials that prevent or reduce the formation of biofilms, besides the hygiene techniques. Knowing the natural processes such as the structure of bacterial biofilms and factors that directly affect those processes such as time, temperature, type of microorganisms helps to improve this control.

The results showed that in the case of *A. butzleri* the bacterial population had an effect on the production of biofilms, observing that the highest biomass production was with an initial population of 10⁸ CFU/mL (Figure 1), similar results were found by Rosenberg et al. (2008), who analyzed the formation of *Salmonella enterica* biofilms in different concentrations of the inoculum.

Which respect for the incubation time evaluation, on average, the greatest production of biofilms occurred at 48 h of incubation. However, in strains with high biofilm production, the

same amount of biomass was observed in both times evaluated. These results suggest that the limitation of nutrients could reduce the accumulation of biofilms in 96 h of incubation (Chen et al. 2013). These results can be attributed to the natural processes of biofilms, and their natural detachment (Mizan et al. 2015).

The presence of the *ciaB*, *pldA*, *tlyA*, *mviN*, *tlyA*, *cj1349* and *cadF* genes is frequently reported in *A. butzleri* isolated from food, animals, environments as well as in clinical strains (Doudah, de Zutter, et al. 2012; Ferreira et al. 2013; Girbau et al. 2015). Similar results were obtained for the milk and cheese isolates analyzed in the present investigation as shown in Table 1 and schematized by PCoA in Supplementary Figure 1SA. The genes detected are associated with adhesion (*cadF*, *cj1349*), invasion (*ciaB*), phospholipase (*pldA*) and hemolysin (*tlyA*) (Miller et al. 2007).

On the other hand, the genes reported less frequently, to be depending on the origin of the isolates are *iroE*, *irgA*, *hecA* and *hecB* (Doudah, De Zutter, et al. 2012; Ferreira et al. 2013; Ferreira et al. 2017; Rathlavath et al. 2017). *IroE* is a siderophore esterase found also in uropathogenic *Escherichia coli*, *irgA* gene coding for an iron-regulated outer membrane protein; the gene *hecA*, a member of the filamentous hemagglutinin (FHA) family; and the gene *hecB*, coding for a hemolysin activation protein (Miller et al. 2007). As observed in Table 1, only one strain from Minas frescal cheese presented the *irgA* gene and 3/36 strains presented the *iroE* gene, unlike the isolates analyzed by Lehmann et al. (2015) from fish and poultry meat where about 35% and 40% of the strains studied had the *irgA* and *iroE* genes respectively, percentages higher than those reported in the present investigation.

To evaluate antibiotic resistance, we used: beta lactam (penicillin), aminoglycosides (neomycin), quinolone (ciprofloxacin) and macrolides (erythromycin and azithromycin). The antibiotics PEN, TET and NEO, were selected because they are frequently used in dairy cows, in both prevention and curative treatments, while CIP and ERY, these antimicrobials were selected because they are the first-line drugs for the treatment of *Campylobacter* infection.

As can be seen in Table 2, 94.4% (34/36) of field strains were resistant to beta-lactam, similarly it was observed by Yesilmen et al. (2014), who reported 80% (8/10) of PEN resistance in raw milk (bovine origin) and fresh village cheese samples. High resistance to this antibiotic may be associated with the presence of suspected β -lactamases described in *A. butzleri* using complete genome sequencing (Miller et al. 2007).

The sensitivity to antibiotics ERY, CIP, TET and AZI in strains of *A. butzleri* from clinically healthy cattle, ready-to-eat packaged vegetables, beef, seafood, water, in-line milk has already been reported in more than 90% of isolates analyzed by Serraino et al. (2013);

Shirzad Aski et al. (2016) and Rathlavath et al. (2017). Similar results were obtained in the present investigation as shown in Table 2, with all field strains showing great sensitivity to mentioned antibiotics.

Supplementary Figure 2S shows the MFA elaborated with the relative frequency of the genes, the size of the zone of inhibition and the OD obtained in the quantification of biofilms in 96-well microplate. We observe that the presence of the investigated genes and the resistance to antibiotics (zone of inhibition) are close, indicating a slight correlation that is, the size of the zones of inhibition presented by field strains, which were mostly greater than 25 mm, measures that suggest sensitivity to the antibiotics evaluated were slightly correlated with the presence of the ten genes investigated. Which, was already shown in other investigations (Doudah, de Zutter, et al. 2012; Ferreira et al. 2013; Karadas et al. 2013; Girbau et al. 2015; Ferreira et al. 2019). In fact, a statistical analysis was not performed to observe this correlation, however, they show similar results to those presented in this research, such as the percentage of genes detected and high sensitivity to the same antibiotics that were analyzed.

However, we do not observe a correlation with OD (Supplementary Figure 2S), it is likely that the factors that affect the formation of biofilms are not necessarily the genes analyzed in the present investigation, which suggests the presence of other mechanisms such as those suggested by Pereira-Medrano et al. (2013) who associate the presence of the *flaA* gene with greater adhesion and subsequent formation of biofilms. Additionally, the expression of the *flaA* gene is directly related to temperature (Meinersmann et al. 1997).

Medina et al. (2019), suggest that the biphasic transcription of genes (*ciaB*, *cadF*, *mviN*, *pldA*) and flagellar genes (*flaA*, *flab*, *flgH*, *motA*) are necessary for *A. butzleri* to establish an endosymbiotic relationship with *Acanthamoeba castellanii*, and that after the established relationship, the transcription of these genes decrease, thereby inducing the importance of the expression of these genes for the survival and fixation of *A. butzleri* within the host cell.

Biofilms formation on contact surfaces

The *A. butzleri* strains used were isolated in milk and cheese, therefore, is important to take it to conditions that would be found in a dairy process plant.

Two contact surfaces commonly used were selected, stainless steel and polypropylene, materials present both in the machinery and in the utensils used to produce cheese. We also evaluate how *A. butzleri* could take advantage of the residues present in the cheese processing environment, such as whey that is a by-product from cheese production. The whey was used as a culture broth and compared to a commercial media (MH broth), to observe the versatility and metabolic plasticity of the strains to use different culture media.

Supplementary Figure 3S show the disposition of the nine strains evaluated (8 field strains + 1 type strain) is schematized by MFA analysis. Can be observed that the strains found in I and IV quadrants are those that formed the largest number of biofilms (within the ellipse). The average and the statistical difference among them are shown in Table 3. The strains evaluated exhibited a significant difference, as observed by Ferreira et al. (2013); Girbau et al. (2017) and Ferreira et al. (2018), that found significant difference among the *Arcobacter* strains studied. The LMG 10828^T strain, was also evaluated by Ferreira et al. (2013) and, as in the present investigation, showed high ability to form biofilm. Meanwhile, the field strain LC63A2 from raw milk, also showed a great ability to biofilm. Interesting to point out that this is a strain resistant to three antibiotics tested (TET, PEN, ERI) and presents six genes investigated (*pdlA*, *ciaB*, *cadF*, *tlyA*, *cjl349* and *mviN*).

Among the extrinsic factors that affect the formation of bacterial biofilms is the culture medium (Phillips 2016). An investigation carried out by Moltz & Martin (2005), using *L. monocytogenes*, showed that the medium had a great effect when biofilms were quantified, showing that the nutritionally poor means were those with greater biomass obtained.

On average biofilm formation was higher having MH as a culture medium (Figure 6A). However, we have shown that the *A. butzleri* strains evaluated were able to use the whey as a culture medium to biofilm formation. As it was already shown in the isolation of the strains used (Cruzado-Bravo et al. 2020), the presence of *Arcobacter* correlated slightly with the high moisture in the cheeses, as we know that moisture is from the whey, *Arcobacter* can use the nutrients present in the whey to maintain or increase its population.

With the tests carried out on the eight field strains used, we show that *A. butzleri* can use the whey as a culture medium to increase its population. Whey is known to be a rich medium and residue that can remain in cheese processing plants if sanitation processes fail may favor de biofilm formation inside the plants.

Incubation time (48 h and 96 h) to quantify the biofilms was significantly different (Table 5). When the biofilms on the contact surfaces were quantified, the culture broths were renewed at 48 h, in order to avoid nutrient depletion at 96 h of evaluation. In Figure 6B can be observed that with all strains the viable cell count was greater than 96 h of incubation, probably because the culture broth was renewed. Similar results were obtained (Assanta et al. 2002), which evaluated the biofilm formation of *Arcobacter* at different times (1, 4, 24, 48 and 72 h) at 4°C and 20°C, obtaining greater bacterial populations at 72 h of incubation.

Kjeldgaard et al. (2009), showed that the strain type *A. butzleri* ATCC 49616T (=LMG 10828^T), was able to form biofilms on stainless steel using chicken meat juice medium (CMJ)

and Brain Heart Infusion (BHI) as a culture medium. The authors were obtained cell count between 1.6 log CFU/cm² and 4.6 log CFU/cm², lower than those shown by field strains analyzed in the present investigation. We obtained cell counts > 2 log CFU/cm², the strain LC63A2 (raw milk) presented up to 8 log CFU/cm² of adhered cells on stainless steel (Figure 3C). Our results, probably were because a higher incubation temperature was used and the initial bacterial population was also higher than that used by Kjeldgaard et al. (2009).

Surface energy of the contact materials is another extremely important extrinsic factor that interferes in biofilm formation. Assanta et al. (2002), evaluated the biofilm of *Arcobacter* on stainless steel, plastic and copper, observing greater biofilms formation on stainless steel. The authors made the measurement of the surface energy of the materials used, and verified the highest surface energies in the following sequences: stainless steel > copper > plastic, which has a direct relationship with the biofilm formation. Similar results were obtained in the present investigation, as shown in Figure 3C, with greatest formation of biofilms observed in *A. butzleri* was on stainless steel.

Due to this type of problems with high surface energy materials, the design of new materials is high, obviously taking into account the practicality, safety and durability of stainless steel, but especially in the type of coating (Gule et al. 2016).

Therefore, the information obtained in this study with field strains contribute to better know the bacteria and its biofilm formation abilities that will allow us to further optimize the strategies to control this microbiological hazard in the dairy industries.

3.6 CONCLUSION

Bacterial biofilms are complex structures that confer protection and resistance to microorganisms, so this is challenging problem for the food industry in nowadays. In the case of emerging pathogens such as *A. butzleri*, the presence of biofilms represents a public health concern, taking into account that they can contaminate fresh cheeses and other ready-to-eat products. This study provides a better understanding on the bacteria and its biofilm formation characteristics and contribute with information to mitigate biofilm formation in food processing plants. The information obtained with the present investigation, such as the presence of virulence genes, resistance to antibiotics among other factors that would affect the formation of biofilms stress the need to develop better strategies, such as the design of new materials, cleaning and adequate sanitation in short times and with the right cleaning products. In addition, the results of this research indicate the need to characterize field strains of *A. butzleri* throughout the entire dairy chain.

LITERATURE CITED

- Assanta MA, Roy D, Lemay MJ, Montpetit D. 2002. Attachment of *Arcobacter butzleri*, a new waterborne pathogen, to water distribution pipe surfaces. *J Food Prot.* 65:1240–1247.
- Bridier A, Sanchez-Vizueté P, Guilbaud M, Piard JC, Naïtali M, Briandet R. 2015. Biofilm-associated persistence of food-borne pathogens. *Food Microbiol.* 45:167–178.
- Chen X, Suwarno SR, Chong TH, McDougald D, Kjelleberg S, Cohen Y, Fane AG, Rice SA. 2013. Dynamics of biofilm formation under different nutrient levels and the effect on biofouling of a reverse osmosis membrane system. *Biofouling* [Internet]. [cited 2019 Dec 5]; 29:319–330. Available from:
<http://www.tandfonline.com/doi/abs/10.1080/08927014.2013.772141>
- CLSI. 2015. Clinical and Laboratory Standards Institute – CLSI. Perform Stand Antimicrob susceptibility Test (25th informational Suppl – M100- S25) Wayne CLSI.
- Cruzado-Bravo MLM, Barancelli G V., Dini Andreote AP, Saldaña E, Vidal-Veuthey B, Collado L, Contreras-Castillo CJ. 2020. Occurrence of *Arcobacter* spp. in Brazilian Minas frescal cheese and raw cow milk and its association with microbiological and physicochemical parameters. *Food Control.* 109:106904.
- Cruzado-Bravo MLM, Silva NCC, Rodrigues MX, Silva GOE, Porto E, Sturion GL. 2019. Phenotypic and genotypic characterization of *Staphylococcus* spp. isolated from mastitis milk and cheese processing: Study of adherence and biofilm formation. *Food Res Int* [Internet]. [cited 2019 Sep 26]; 122:450–460. Available from:
<https://www.sciencedirect.com/science/article/pii/S0963996919302406>

- Doudah L, de Zutter L, Baré J, De Vos P, Vandamme P, Vandenberg O, Van den Abeele A-M, Houf K. 2012. Occurrence of putative virulence genes in *Arcobacter* species isolated from humans and animals. J Clin Microbiol [Internet]. [cited 2019 Sep 19]; 50:735–41. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22170914>
- Doudah L, De Zutter L, Baré J, De Vos P, Vandamme P, Vandenberg O, Van Den Abeele AM, Houf K. 2012. Occurrence of putative virulence genes in *Arcobacter* species isolated from humans and animals. J Clin Microbiol. 50:735–741.
- Ferreira S, Correia DR, Oleastro M, Domingues FC. 2018. *Arcobacter butzleri* ciprofloxacin resistance: Point mutations in DNA gyrase a and role on fitness cost. Microb Drug Resist. 24:915–922.
- Ferreira S, Fraqueza MJ, Queiroz JA, Domingues FC, Oleastro M. 2013. Genetic diversity, antibiotic resistance and biofilm-forming ability of *Arcobacter butzleri* isolated from poultry and environment from a Portuguese slaughterhouse. Int J Food Microbiol. 162:82–88.
- Ferreira S, Luís Â, Oleastro M, Pereira L, Domingues FC. 2019. A meta-analytic perspective on *Arcobacter* spp. antibiotic resistance. J Glob Antimicrob Resist. 16:130–139.
- Ferreira S, Oleastro M, Domingues FC. 2017. Occurrence, genetic diversity and antibiotic resistance of *Arcobacter* sp. in a dairy plant. J Appl Microbiol. 123:1019–1026.
- Girbau C, Guerra C, Martínez-Malaxetxebarria I, Alonso R, Fernández-Astorga A. 2015. Prevalence of ten putative virulence genes in the emerging foodborne pathogen *Arcobacter* isolated from food products. Food Microbiol. 52:146–149.
- Girbau C, Martinez-Malaxetxebarria I, Muruaga G, Carmona S, Alonso R, Fernandez-Astorga A. 2017. Study of biofilm formation ability of foodborne *Arcobacter butzleri* under different conditions. J Food Prot. 80:758–762.

- Gule NP, Begum NM, Klumperman B. 2016. Advances in biofouling mitigation: A review. Crit Rev Environ Sci Technol [Internet]. [cited 2019 Dec 4]; 46:535–555. Available from: <http://www.tandfonline.com/doi/full/10.1080/10643389.2015.1114444>
- Harro JM, Peters BM, O'May GA, Archer N, Kerns P, Prabhakara R, Shirtliff ME. 2010. Vaccine development in *Staphylococcus aureus* : taking the biofilm phenotype into consideration. FEMS Immunol Med Microbiol [Internet]. [cited 2018 May 23]; 59:306–323. Available from: <https://academic.oup.com/femspd/article-lookup/doi/10.1111/j.1574-695X.2010.00708>.
- Karadas G, Sharbati S, Hänel I, Messelhäuser U, Glocker E, Alter T, Götz G. 2013. Presence of virulence genes, adhesion and invasion of *Arcobacter butzleri*. J Appl Microbiol [Internet]. [cited 2019 Sep 19]; 115:583–590. Available from: <http://doi.wiley.com/10.1111/jam.12245>
- Kjeldgaard J, Jørgensen K, Ingmer H. 2009. Growth and survival at chiller temperatures of *Arcobacter butzleri*. Int J Food Microbiol [Internet]. [cited 2019 Dec 31]; 131:256–9. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/19297052>
- Lehmann D, Alter T, Lehmann L, Uherkova S, Seidler T, Götz G. 2015. Prevalence, virulence gene distribution and genetic diversity of *Arcobacter* in food samples in Germany. Berl Munch Tierarztl Wochenschr [Internet]. [cited 2019 Dec 7]; 128:163–8. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25876277>
- Mansfield LP, Forsythe SJ. 2000. *Arcobacter butzleri*, *A. skirrowii* and *A. cryaerophilus* — potential emerging human pathogens. Rev Med Microbiol [Internet]. [cited 2019 Dec 11]; 11:161–170. Available from: <https://insights.ovid.com/article/00013542-200011030-00006>
- Medina G, Neves P, Flores-Martin S, Manosalva C, Andaur M, Otth C, Lincopan N, Fernández H. 2019. Transcriptional analysis of flagellar and putative virulence genes of *Arcobacter butzleri* as an endocytobiont of *Acanthamoeba castellanii*. Arch Microbiol. 201:1075–1083.

- Meinersmann RJ, Helsel LO, Fields PI, Hiett KL. 1997. Discrimination of *Campylobacter jejuni* isolates by *fla* gene sequencing. J Clin Microbiol [Internet]. [cited 2019 Dec 9]; 35:2810–4. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/9350739>
- Miller WG, Parker CT, Rubenfield M, Mendz GL, Wösten MMSM, Ussery DW, Stolz JF, Binnewies TT, Hallin PF, Wang G, et al. 2007. The Complete Genome Sequence and Analysis of the Epsilonproteobacterium *Arcobacter butzleri*. Fairhead C, editor. PLoS One [Internet]. [cited 2019 Dec 5]; 2:e1358. Available from: <http://dx.plos.org/10.1371/journal.pone.0001358>
- Mizan MFR, Jahid IK, Ha S Do. 2015. Microbial biofilms in seafood: A food-hygiene challenge. Food Microbiol. 49:41–55.
- Moltz AG, Martin SE. 2005. Formation of biofilms by *Listeria monocytogenes* under various growth conditions. J Food Prot. 68:92–97.
- Pereira-Medrano AG, Knighton M, Fowler GJS, Ler ZY, Pham TK, Ow SY, Free A, Ward B, Wright PC. 2013. Quantitative proteomic analysis of the exoelectrogenic bacterium *Arcobacter butzleri* ED-1 reveals increased abundance of a flagellin protein under anaerobic growth on an insoluble electrode. J Proteomics. 78:197–210.
- Phillips CA. 2016. Bacterial biofilms in food processing environments: a review of recent developments in chemical and biological control. Int J Food Sci Technol [Internet]. [cited 2019 Dec 26]; 51:1731–1743. Available from: <http://doi.wiley.com/10.1111/ijfs.13159>
- Piva S, Gariano GR, Bonilauri P, Giacometti F, Decastelli L, Florio D, Massella E, Serraino A. 2017. Occurrence of putative virulence genes on *Arcobacter butzleri* isolated from three different environmental sites throughout the dairy chain. J Appl Microbiol [Internet]. [cited 2019 Dec 5]; 122:1071–1077. Available from: <http://doi.wiley.com/10.1111/jam.13403>
- Rathlavath S, Kohli V, Singh AS, Lekshmi M, Tripathi G, Kumar S, Nayak BB. 2017.

- Virulence genotypes and antimicrobial susceptibility patterns of *Arcobacter butzleri* isolated from seafood and its environment. *Int J Food Microbiol.* 263:32–37.
- Rosenberg LE, Carbone AL, Römling U, Uhrich KE, Chikindas ML. 2008. Salicylic acid-based poly(anhydride esters) for control of biofilm formation in *Salmonella enterica* serovar Typhimurium. *Lett Appl Microbiol* [Internet]. [cited 2019 Dec 5]; 46:593–599. Available from: <http://doi.wiley.com/10.1111/j.1472-765X.2008.02356.x>
- Serraino A, Florio D, Giacometti F, Piva S, Mion D, Zanoni RG. 2013. Presence of *Campylobacter* and *Arcobacter* species in in-line milk filters of farms authorized to produce and sell raw milk and of a water buffalo dairy farm in Italy. *J Dairy Sci.* 96:2801–2807.
- Shirzad Aski H, Tabatabaei M, Khoshbakht R, Raeisi M. 2016. Occurrence and antimicrobial resistance of emergent *Arcobacter* spp. isolated from cattle and sheep in Iran. *Comp Immunol Microbiol Infect Dis.* 44:37–40.
- Srey S, Jahid IK, Ha S Do. 2013. Biofilm formation in food industries: A food safety concern. *Food Control.* 31:572–585.
- Teh KH, Flint S, French N. 2010. Biofilm formation by *Campylobacter jejuni* in controlled mixed-microbial populations. *Int J Food Microbiol* [Internet]. [cited 2019 Sep 19]; 143:118–124. Available from: <https://www.sciencedirect.com/science/article/pii/S0168160510004472>
- Traversa A, Gallina S, Martucci F, Boteva C, Baioni E, Maurella C, Chiavacci L, Benvenuto E, Ferrero I, Ferrero E, et al. 2019. *Arcobacter* spp. in raw milk from vending machines in Piedmont and occurrence of virulence genes in isolates. *Ital J Food Saf* [Internet]. [cited 2019 Dec 11]; 8. Available from: <https://www.pagepressjournals.org/index.php/ijfs/article/view/7859>
- Vicente-Martins S, Oleastro M, Domingues FC, Ferreira S. 2018. *Arcobacter* spp. at retail food from Portugal: Prevalence, genotyping and antibiotics resistance. *Food Control.* 85:107–112.

Yesilmen S, Vural A, Erkan ME, Yildirim IH. 2014. Prevalence and antimicrobial susceptibility of *Arcobacter* species in cow milk, water buffalo milk and fresh village cheese. Int J Food Microbiol. 188:11–14.

List of Tables and Figures

Table 1. Presence of virulence genes in *A. butzleri* from Minas frescal cheese and raw milk

Strains origin	Genes n (%)									
	<i>ciaB</i>	<i>pldA</i>	<i>tlyA</i>	<i>mviN</i>	<i>cadF</i>	<i>cjl349</i>	<i>hecB</i>	<i>hecA</i>	<i>iroE</i>	<i>irgA</i>
cheese (n= 16)	15 (100)	15 (100)	15 (100)	15 (100)	15 (100)	14 (93.3)	3 (20)	2 (13.3)	1 (6.7)	1 (6.7)
milk (n=21)	21 (100)	21 (100)	21 (100)	21 (100)	20 (95.2)	21 (100)	7 (33.3)	7 (3.3)	2 (9.5)	0 (0)
Total (n= 36)	36 (100) a	36 (100) a	36 (100) a	36 (100) a	35 (97.2) a	35 (97.2) a	10 (27.8) b	9 (25) b	3 (8.3) b	1 (2.8) b

Note: Difference statistically significant ($P < .05$) is indicated by different letter according to Cochran's Q test.

Table 2. Antibiotic resistance in *A. butzleri* from Minas frescal cheese and raw milk

Antibiotics (concentration)	Minas frescal Cheese (n= 15)			Raw milk (n = 21)			Total (n= 36)		
	R n (%)	I n (%)	S n (%)	R n (%)	I n (%)	S n (%)	R n (%)	I n (%)	S n (%)
Penicillin G (10 µg)	13 (86.7)	-	2 (13.3)	21 (100)	-	-	34 (94.4)	-	2 (5.5)
Tetracycline (30 µg)	-	-	15 (100)	2 (9.5)	-	-	2 (5.5)	-	34 (94.4)
Neomycin (30 µg)	-	-	15 (100)	1 (4.8)	1 (4.8)	1 (4.8)	1 (2.8)	1 (2.8)	34 (94.4)
Ciprofloxacin (5 µg)	-	-	15 (100)	-	-	-	-	-	36 (100)
Erythromycin (15 µg)	-	-	15 (100)	3 (14.3)	-	-	3 (8.3)	-	33 (91.7)
Azithromycin (15 µg)	-	-	15 (100)	-	-	-	-	-	36 (100)

Note: R= Resistant, I= Intermediate resistant, S= Sensitive

Table 3. Strains information used in the biofilm formation tests on contact surfaces and the average obtained with the two methodologies used to quantify biofilms

Code	origin	Virulence patterns	Antibiotics			Biofilm on contact surfaces		
			Resistance	Intermediate	Sensible	average OD	average UFC/cm ²	Log
LMG 10828 ^T	clinical	<i>hecA pdlA irgA ciaB hecB cadF iroE tlyA cjl349 mviN</i>	PEN	TET, ERI	NEO, CIP, AZI	1.23±0.06 *	6.93±0.35	
LC 63A2	raw milk	<i>pdlA ciaB cadF tlyA cjl349 mviN</i>	TET, PEN, ERI		NEO, CIP, AZI	1.27±0.04 *	8.00±0.37 *	
Q5	cheese	<i>hecA pdlA irgA ciaB hecB cadF tlyA mviN</i>			TET, PEN, NEO, CIP, ERI AZI	1.17±0.10	7.60±0.55 *	
Q78A	cheese	<i>pdlA ciaB cadF tlyA cjl349 mviN</i>			TET, PEN, NEO, CIP, ERI, AZI	1.14±0.08	7.37±0.44	
LC 40A	raw milk	<i>pdlA ciaB cadF tlyA cjl349 mviN</i>	PEN		TET, NEO, CIP, ERI, AZI	1.08±0.12	4.67±1.47	
Q74d	cheese	<i>pdlA ciaB cadF tlyA cjl349 mviN</i>	PEN		TET, NEO, CIP, ERI, AZI	1.02±0.14	5.90±1.38	
LC 82A	raw milk	<i>pdlA ciaB cadF tlyA cjl349 mviN</i>	PEN		TET, NEO, CIP, ERI, AZI	0.98±0.05	4.31±1.61	
LC 46A	raw milk	<i>hecA pdlA ciaB hecB cadF tlyA cjl349 mviN</i>	PEN		TET, NEO, CIP, ERI, AZ	0.95±0.04	4.76±1.61 **	
Q33a	cheese	<i>pdlA ciaB cadF tlyA cjl349 mviN</i>	PEN		TET, NEO, CIP, ERI, AZI	0.82±0.09 **	4.16±1.76 **	

Note: OD= optical density. PEN= penicillin G, TET= tetracycline, NEO= neomycin, CIP=ciprofloxacin, ERI= erythromycin, AZI=azithromycin. (*)=Difference statistically significant (P<.05) is indicated by different letter according to Duncan's test

Supplementary Material- Tables

Table S1. Average optical density of the interaction of the factors cell concentration and time in the *A. butzleri* biofilm formation in 96-well microplate

Factor interaction (cell concentration *time)	OD Average
On 10 ⁸ CFU/ml *48h	1.40±0.12 a
10 ⁸ CFU/ml*96h	1.37±0.03 a
10 ⁷ CFU/ml*48h	1.12±0.02 b
10 ⁶ CFU/ml*48h	1.10±0.04 b
10 ⁷ CFU/ml*96h	0.98±0.05 c
10 ⁶ CFU/ml*96h	0.89±0.02 d

Note: Difference statistically significant (P< .05) is indicated by different letter according to Duncan's test

Table S2. ANOVA test for the factors evaluated in the biofilms formation on contact surfaces comparing the two methodologies used to quantify the biofilms formation

Factor	P*	
	OD	Log CFU/cm ²
Strain (n=9)	<0.0001	<0.0001
Broth media (whey, MH)	0.64	0.0004
Time (48h, 96h)	<0.001	<0.0001
Contact surface (SS, PP)	0.006	<0.0001

Note: OD= optical density, MH=Mueller Hinton broth, SS=stainless steel, PP=Polypropylene. P*= p value obtained by the ANOVA test.

Figure 1. Biofilms formation of *A. butzleri* strains in a 96-well plate with different cell concentrations and incubation times.

Note: Difference statistically significant ($P < .05$) is indicated by different letter according to Duncan's test. LC= raw milk strain. Q = Cheese strain

Figure 2. Average biofilms formation of *A. butzleri* strains in a 96-well plate

Note: SS=stai

Figure 3. Average biofilm formation of *A. butzleri* on surfaces contact with culture media and incubation time.

Note: CV=Crystal Violet

Figure 4. Average biofilm formation on contact surfaces considering cell count and crystal violet methodologies (A). MFA of the methodologies used in A. *butzleri* strains.

Supplementary Material- Figures

Figure 1S. Figure 1S. (A) PCoA on the frequency of genes, (B) PCA on the antibiotic's resistance.

Figure 2S. MFA representing the correlation between the factors evaluated in the 36 strains of *A. butzleri* from dairy products.

Figure 3S. MFA of *A. butzleri* strains of biofilm formation on contact surfaces

FINAL CONCLUSION

The presence of *Arcobacter* in Minas Frescal cheese and raw cow milk in São Paulo State, is reported for the first time in the present investigation. The presence of the emerging pathogen *A. butzleri* in fresh cheeses ready for consumption can be a public health problem, given that it is a highly consumed cheese in Brazil. It is important to address the presence of these bacteria with the physicochemical and microbiological characteristics; with this we can suggest improvements in the processes and the way to address pollution problems

The characterization of the isolates from milk and dairy products allowed to obtain information still unknown and unpublished, such as the presence of genes associated with virulence, resistance to antibiotics. Something very important we addressed the problem of *A. butzleri* biofilms. Bacterial biofilms remain a latent problem of contamination in the food industry, these biofilms are complex structures that confer protection, therefore, more resistance.

It is important to highlight that the infectious dose and the behavior of this bacteria in food is not yet well understood and it is necessary to continue exploring. In addition, the results of the present investigation indicate the need to control *A. butzleri* from the period after milking. Continuous microbiological control of milk should be carried out throughout the production and distribution chain of Minas frescal cheese and other high-consumption dairy products.

Additionally, the strains isolated in the analyzed matrices needed to be much more explored, it would be ideal to perform the complete sequencing of the genomes and compare with other strains which would allow us to understand a little more about this emerging pathogen.

PUBLICATION LIST

Publications

2020 Juan D. Rios-Mera, Erick Saldaña, **Melina L. M. Cruzado-Bravo**, Mariana M. Martins, Iliani Patinho, Miriam M. Selani, Dominique Valentin, Carmen J. Contreras-Castillo. Sensory and instrumental aspects of texture of NaCl-reduced beef burger. *Meat Science*.

2020 Melina L.M Cruzado-Bravo, Giovana V. Barancelli, Ana Paula Dini Andreote, Boris Vidal-Veuthey, Luis Collado, Carmen J. Contreras-Castillo. Occurrence of *Arcobacter* spp. in Brazilian Minas frescal cheese and raw cow milk and its association with microbiological and physicochemical parameters. *Food Control*.

2019 Cruzado-Bravo, M.L.M., Silva, Nathália Cristina Cirone, Rodrigues, Marjory Xavier, Silva, Gabriela Oliveira E, Porto, Ernani, Sturion, Gilma Lucazechi. Phenotypic and genotypic characterization of *Staphylococcus* spp. isolated from mastitis milk and cheese processing: Study of adherence and biofilm formation. *Food Research International*.

2019 Juan D. Rios-Mera, Erick Saldaña, **Melina L.M. Cruzado-Bravo**, Iliani Patinho, Miriam M. Selani, Dominique Valentin, Carmen J. Contreras-Castillo. Reducing the sodium content without modifying the quality of beef burgers by adding micronized salt. *Food Research International*.

2018 Cruzado-Bravo, M.L.M.; Silva, N.C.C.; Rodrigues, M.X.; Saldaña E.; Contreras-Castillo, C.J.; Sturion, G.L. Biopelículas de *Staphylococcus* spp. sobre acero inoxidable utilizando leche y brain heart infusion broth como medios de cultivo. *Scientia Agropecuaria*.

2018 Andrea I Estevez-Garcia, Giovana V Barancelli, **Melina LM Cruzado-Bravo**, Tanja Opriessnig Juan M. Villalobos-Salcedo, Carmen J Contreras-Castillo. The role of pork in human infections. *Veterinary Ireland Journal*.

2017 Rodrigues Marjory Xavier, Silva, Nathália Cristina Cirone, Trevilin Júlia Hellmeister, **Cruzado Melina Mary Bravo**, Mui Tsai Siu, Duarte Fábio Rodrigo Sanches, Castillo, Carmen J. Contreras, Canniatti-Brazaca, Solange Guidolin, Porto, Ernani. Molecular characterization and antibiotic resistance of *Staphylococcus* spp. isolated from cheese processing plants. *Journal of Dairy Science*.

2017 Marjory Xavier Rodrigues, Nathália Cristina Cirone Silva, Júlia Hellmeister Trevilin, **Melina Mary Bravo Cruzado**, Tsai Siu Mui, Fábio Rodrigo Sanches Duarte, Carmen J. Contreras Castillo, Solange Guidolin Canniatti-Brazaca and Ernani Porto. Antibiotic resistance and molecular characterization of *Staphylococcus* species from mastitic milk. *African Journal of Microbiology Research*.

Technical article

2019 Giovana Verginia Barancelli, **Melina Luz Mary Cruzado Bravo**, Adriana Muniz. *Listeria monocytogenes* em produtos cárneos. Anuario 2020 da Avicultura Industrial. Nº 11/2019, ANO 111. Ed. 1294

Posters

Melina L.M Cruzado-Bravo, Saldaña Villa Erick, Giovana V. Barancelli, Luis Collado, Carmen J. Contreras-Castillo. Biofilm formation, virulence and antimicrobial resistance of *Arcobacter butzleri* isolated from Minas frescal cheese and raw milk. 13° Simpósio Latino Americano de Ciência de Alimentos (SLACA) 2019. Campinas, Brazil.

Cruzado-Bravo M.L.M, Giovana Verginia Barancelli, Giovanni Henrique Mariano, Luis Collado, Carmen J. Contreras Castillo. Occurrence of *Arcobacter butzleri* in minas frescal cheese and raw milk of northeast region of São Paulo State, Brazil. 20th *Campylobacter, Helicobacter* and Related Microorganisms conference, CHRO 2019. Belfast, North Ireland, UK.

Melina Luz Mary Cruzado Bravo, Vitoria Bagarollo Veiga, Giovana Verginia Barancelli, Fabio Pratavieira, Nicolle Ferraz de Arruda Padovani and Carmen Contreras Castillo. Behavior of *Salmonella* in chicken breast at different times and temperatures. Joint Event on 3rd International Conference on Digital Pathology & 7th Global Summit on Microbiology Research, 2018. Madrid, Spain.

Nicolle Ferraz de Arruda Padovani, Mayara Cardoso da Rosa, Thiago Sugizaki dos Santos, **Melina Luz Mary Cruzado Bravo**, Denise de Almeida Leme Baptista, Rosalina de Fátima Ocangne, Giovana Verginia Barancelli, Carmen Josefina Contreras Castillo y Daniele Fernanda Maffei. Aprendizagem extracurricular em microbiologia dos alimentos: experiência de um grupo de extensão universitária”. 4° USP Graduate Conference 2018, São Paulo – SP, Brazil.

Padovani, N.F. A., Santos, T. S., **Cruzado-Bravo, M. L. M.**, Barancelli, G. V., Da Silva, N., Contreras-Castillo, C.J. occurrence of *Arcobacter spp.* in frozen or chilled chicken retail cuts and carcasses. 29° CONGRESSO BRAZILIAN MICROBIOLOGY - CBM 2017. Foz do Iguaçu, PR, Brazil.

Santos, T.S., **Cruzado-Bravo, M.L.M**, Padovani, N.F.A, Varancelli, G.V., Contreas-Castillo.J.C. microbiological quality of water samples from piracicaba – sp. 29° CONGRESSO BRAZILIAN MICROBIOLOGY - CBM 2017. Foz do Iguaçu, PR, Brazil.

Barancelli, G.V, **Cruzado-Bravo M.L.M**, Padovani, N.F.A, Oliveira, C.A.F, Hofer, E3, Contreras-Castillo C.J. Behavior of *Listeria monocytogenes* strains in brine simulating commercial conditions. 29° CONGRESSO BRAZILIAN MICROBIOLOGY - CBM 2017. Foz do Iguaçu, PR, Brazil.

Cruzado-Bravo M.L.M, Muto, F. A, Padovani, N.F.A, Barancelli, G.V, Contreras-Castillo C.J. *Arcobacter* isolated from cheese processing plant and commercial samples of minas frescal cheese. 29° CONGRESSO BRAZILIAN MICROBIOLOGY - CBM 2017. Foz do Iguaçu, PR, Brazil.

Geraldi, A.C., **Cruzado-Bravo, M.L.M.**, Padovani, N. F. A, Barancelli, G. V., Contreras-Castillo, J.C. Coagulase positive staphylococcus, total and thermotolerant coliforms in minas frescal cheese from market in piracicaba, sp, Brazil. 29° CONGRESSO BRAZILIAN MICROBIOLOGY - CBM 2017. Foz do Iguaçu, PR, Brazil.