

The ability of *Aliarcobacter butzleri* strains isolated from foods of animal origin in Costa Rica to form biofilm

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Abstract

Aliarcobacter butzleri is a zoonotic emerging food and waterborne pathogen widely distributed in nature. It is present in food processing environments and can easily be spread through the food industry because of its ability to form biofilm. The aim of this work was to determine the ability of strains isolated in Costa Rica from different food matrixes of animal origin to form biofilm. Thirty-eight *A. butzleri* strains previously isolated and identified from animal origin products were analyzed using the method described by Stepmovic *et al.* (2000), in three culture broths, brain heart infusion broth, Boer broth and Houf broth.

Results showed that 67% of poultry origin strains, 62.5% of meat origin strains and just 8% of milk origin strains showed ability to form biofilm.

The findings of this study confirm the adherence ability of *A. butzleri* to form biofilm, a characteristic that can promote dispersion and cross contamination along food industry processing lines.

Introduction

Biofilm is defined as a thick layer of organisms, including bacteria, fungi and protists which aggregate in order to form a colony. This colony attaches to a surface with a slime layer and has a very important activity on the protection of microorganisms, promoting their growth and survival. Once formed, biofilms provide a protective surrounding environment, developing a complex structure that allows transfer of nutrients to bacteria and removal of waste products. Also, biofilm provides protection to bacteria in stressful environments, such

as those in food industries and slaughterhouses. (Chmielewski and Frank, 2003)

The ability to form biofilm has been associated with bacterial virulence, colonization, environmental survival and even antibiotic resistance (Gaynor *et al.* 2007).

Furthermore, biofilms have been described as an important source of pathogenic bacteria (Parsek and Sigh, 2003). Indeed, biofilms have been implicated in various infections, including many foodborne outbreaks have been linked to it (Dewangan and Bhadania, 2015), and *Arcobacter* is not an exception.

The ability of bacteria to adhere to and grow on abiotic surfaces is a critical issue for industries with suitable environments for biofilm formation (Girbau *et al.* 2017) including food industries, where organic and inorganic residues easily accumulate. Product contact with these biofilms can lead to health problems if contaminated by pathogenic bacteria (Mafu *et al.* 2011).

Aliarcobacter are Gram negative rods taxonomically included in the family Campylobacteriaceae (Silha *et al.* 2019). This genus comprises several species, some of them considered as pathogenic for human beings.

Aliarcobacter butzleri is a zoonotic emerging food and waterborne pathogen widely distributed in nature, frequently isolated from water, animals, and foods of animal (Girbau *et al.*, 2017) and vegetable origin (Mottola *et al.*, 2016; Gonzalez *et al.*, 2019; Girbau *et al.*, 2017). It has been commonly isolated from food processing and handling facilities (Ferreria *et al.*, 2013). Also, it is present in food processing environments and can be persistent in equipment associated with the food industry (Giacometti *et al.*, 2015). This persistence has been associated with biofilm formation by several researchers (Ferreira *et al.*, 2013). Adherence to different surfaces can favour cross contamination and bacterial spread onto food.

Transmission of this bacterium is supported by various inherent bacterial properties, including its ability to form biofilm under different environmental conditions. It is known that this bacterium can form biofilm on stainless steel, copper and plastic surfaces, materials commonly used on food industry (Assanta *et al.*, 2002).

Arcobacter spp. can be the cause of gastroenteritis in human beings, especially *A. butzleri*, which has been included in the list of dangerous microorganisms for human health by the International Commission of Microbiological Specifications for Food (ICMSF) (Banting and Figueras, 2017).

Considering the increasing risk this bacterium represents for public health and the

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fact that it can easily be spread through the food industry, it is important to analyze the ability of biofilm formation of strains isolated from different foods of animal origin, in this case in Costa Rica. The aim of this study was to determine the ability of 28 *A. butzleri* isolates recovered from animal origin products from Costa Rica to form biofilm.

Materials and methods

Bacterial strains

Thirty-eight *A. butzleri* strains were studied. These were previously isolated and identified from animal origin products, including poultry meat (n=18), bovine meat (n=8) and milk (n=12). Poultry strains were isolated from chicken viscera and carcasses (Garcia *et al.*, 2013; Fallas *et al.*, 2014). Bovine meat samples were isolated from minced meat (Córdoba *et al.*, 2017) and unpasteurized milk samples from the Northern region of the country. These

strains were kept frozen at -70°C at the Food Microbiology Laboratory, University of Costa Rica.

Before analysis, all strains were transferred from stock cultures to trypticase soy broth and incubated aerobically at 30°C for 24 h. After incubation, each strain was adjusted to a final optical density (OD) 600 nm of 0.1 using sterile peptonized water 0.1%.

Biofilm formation assay and biofilm quantification

The ability of *A. butzleri* strains to form biofilms was determined using the method described by Stepanovic *et al.* (2000) in three different opportunities. Briefly, 96-well flatbottomed plastic tissue culture plates with lid (Thermo Scientific) were inoculated with three different culture broths, brain heart infusion broth (row 1), Boer broth (row 2) and Houf broth (row 3). Each row of media was inoculated, in triplicate, with 20ul of the inoculum previously prepared. The last two wells of each row were used as negative controls containing only broth. Each plate was incubated for 72h at 25°C under aerobic conditions. After this period, the content of each well was aspirated and washed three times with 250ul sterile physiological saline solution. Plates were vigorously shaken in order to remove all non-adherent bacteria. The remaining attached bacteria were fixed with 300 ul methanol and after 15 min, plates were emptied and left to dry and stained for 5 min with crystal violet 0.2%v/v in 20% ethanol (300 ul/well) for 5 min. After this period, plates were rinsed off with tap water for three times in order to eliminate any excess of stain and then air dried. The dye that had adhered to biofilm was suspended in 250 uL/well glacial acetic acid (33%v/v). The optical density (OD) of each well was measured at 570 nm using an automated Epoch Biotek microplate reader. Each measurement was carried out in triplicate

Adherence capability was graded into

four different categories: non-adherent (-), weakly adherent (+), moderately adherent (++) and strongly adherent (+++) based on the OD obtained as described by Stepanovic *et al.* (2000).

The cut off OD (COD) was defined as three standard deviations above the mean OD of the negative control. If the OD obtained was under this value, the strain was classified as a non-producer. If the OD was greater than cut off and less than 2 COD, the strain was classified as weakly adherent. Moderately adherent strains were those strains that presented an OD smaller than 4 COD, and strong biofilm producing strains were those that presented an OD greater than 4COD.

The results were statistically evaluated with SPSS 19.0 statistical package software. Statistical significance was determined by chi-square-one-way analysis of variance (ANOVA). Results were considered as significant at $p < 0.05$.

Results

Results obtained showed that 67% of poultry meat strains, 62.5% of bovine meat strains and just 8% of milk strains showed an ability to form biofilm ($p < 0.05$). The grading of this adherence is shown on Table 1.

Brain heart infusion broth was the medium that promoted most frequently the formation of biofilm compared with Boer and Houf broths, in which most of the strains evaluated promoted weak or non-adherent properties.

In brain heart infusion broth 67% of poultry strains showed adherence, and 7/18 of these were classified as strong adherents. Similar results were shown by meat origin strains, of which 62% showed some kind of adherence, and 3/8 were strong adherents.

Only one of the strains isolated from milk showed adherence and this was in Boer broth.

Discussion and conclusions

The persistence of microorganisms in food processing environments, including potentially pathogenic bacteria is a current growing public health problem worldwide.

A. butzleri transmission could possibly be enhanced by its ability to adhere to abiotic surfaces. Bacterial ability to adhere to and grow on abiotic surfaces is a critical issue for industries that provide a suitable environment for biofilm formation (Abdahha *et al.*, 2014).

In this study, a total of 17/38 strains (45%) showed ability to form biofilms. These results are lower than the ones reported by Ferreira *et al.* (2014) and Kjeldgaard *et al.* (2009) who report a 100% adhesion on polystyrene surfaces, but greater than the 19.7% reported by Ferreira (2013) from strains isolated from poultry and the environment in a Portuguese slaughterhouse.

Our results showed that most of the *A. butzleri* isolates coming from poultry and bovine meat samples showed adherent characteristics, varying from weak to strong. Only 33% of poultry meat samples and 38% of bovine meat samples did not show any *in vitro* adherence.

For poultry meat samples, 39% were strongly adherent, a result that contrasts with the ones reported by Ferreira *et al.* (2013), where only one isolate was strongly adherent (Ferreira *et al.*, 2013). The 28% of the strains isolated from bovine meat also were also characterized as strongly adherent.

The ability to form biofilm can vary greatly among strains of the same species depending on extrinsic factors such as nutrient availability, environmental conditions and surface properties (Girbau *et al.*, 2017). In this respect, it is important to point out that even weakly adherent isolates can produce biofilm and therefore be difficult to eliminate from the food chain (Srey *et al.*, 2013). Also, a weakly adherent bacterium may become strongly adherent depending

Table 1. The ability of *Aliarcobacter butzleri* strains isolated from three animal sources to form biofilm in different culture broths.

Culture broth		Non-adherent, n (%)	Weakly adherent, n (%)	Moderately adherent, n (%)	Strongly adherent, n (%)
Poultry meat n=18	Brain heart infusion broth	6 (33)	3 (17)	2 (11)	7 (39)
	Boer broth	18 (100)	0	0	0
	Houf broth	17 (94)	1 (6)	0	0
Bovine meat n=8	Brain heart infusion broth	3 (38)	1 (12)	1 (12)	3 (38)
	Boer broth	8 (100)	0	0	0
	Houf broth	8 (100)	0	0	0
Milk n=12	Brain heart infusion broth	12 (100)	0	0	0
	Boer broth	11 (92)	1 (8)	0	0
	Houf broth	12 (100)	0	0	0

on environmental conditions, as demonstrated in this study. *A. butzleri* strains growing in a rich medium such as brain heart infusion broth showed stronger adherent capacities than when they were cultured in selective media, such as Boer and Houf media.

Nutrient rich media do not support optimal biofilm formation, but nutritionally poor environments do promote biofilm formation as described by Reeser *et al.* 2007. Nevertheless, the presence of inhibitory substances in media, as those present in Houf and Boer media, decrease its formation.

Several researchers have described a high prevalence of *Arcobacter* spp on chicken carcasses and on slaughterhouse surfaces, but the isolation from intestinal content is very low or null. (Hanning *et al.*, 2008, Ferreira *et al.*, 2013). Biofilm formation could be the explanation for these discrepancies. This bacterium may reside and proliferate in the slaughterhouse environment and contaminate meat production even under chilled conditions (Ferreira *et al.*, 2013).

A. butzleri has been isolated from cow's milk with lower prevalence than that found in poultry and meat. Pianta *et al.*, isolated just one strain of this bacterium in 188 milk samples from dairy cows (Piant *et al.*, 2007).

The *A. butzleri* strains isolated from milk analyzed in this work showed a very small ability to form biofilm, but few samples were analyzed and further research needs to be done in order to understand this behavior.

In this study biofilm formation from strains isolated from milk was low, just one of 12 strains showed a weak ability. Similar results were reported by Girbau *et al.* (2017) where 3/15 milk isolates showed the ability to form biofilm. Chi square test showed no relationship between the adhesion ability of the isolates and the source ($p > 0,05$), except for milk, but only a small number of isolates were analyzed.

Stainless steel is a material widely used in the food industry, including poultry, meat and milk processing, because it has some of the most suitable properties for materials used for food equipment (Dewangan and Bhadania, 2015). Few studies have been conducted on the biofilm formation of *A. butzleri* on stainless steel surfaces. Asanta *et al.* (2002) and Kjeldgaard *et al.* (2009) demonstrated the adhesion capability of this bacterium to this material. Further research is planned for assessing the ability of *A. butzleri* to form biofilm on the materials present in food lines, particularly stainless steel.

The findings of this study confirm the

feared ability of *A. butzleri* to produce biofilm, a characteristic that can cause dispersion and cross contamination along food industry processing lines. Studies on biofilm formation are still scarce, further research is planned to assess the importance of biofilm formation as a virulence determinant and the development of natural or synthetic products that could inhibit it.

Data obtained in this work contributes to the understanding of the survival, persistence and dissemination mechanisms of this bacterium and the potential risk it represents for human public health.

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