Effects of short-chain fatty acids on *Aliarcobacter butzleri*'s virulence

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Dedicatory

To my family

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Resumo Alargado

Aliarcobacter butzleri tem sido comumente associada a doenças prevalentes tanto em humanos como animais e como tal é classificado como um risco moderado para a saúde humana. *A. butzleri* pode ser encontrada em diversos ambientes, tais como solos, águas e até mesmo a cadeia alimentar, sendo esta uma grande fonte de transmissão e contaminação. Esta bactéria tem sido associada a doenças gastrointestinais diarreicas e não diarreicas, cujos principais sintomas podem incluir dores abdominais, náuseas e febre. Atualmente, diversos trabalhos abordam o potencial papel patogénico deste microrganismo, porém os seus mecanismos de virulência ainda não se encontram suficientemente explorados de modo a clarificar a sua patogenicidade, nomeadamente no que diz respeito à sua resposta aos mecanismos de resistência à colonização do hospedeiro.

Os ácidos gordos de cadeia curta (AGCC) são ácidos gordos orgânicos produzidos pela fermentação microbiana de carboidratos não digeríveis no intestino, e são responsáveis por manter a saúde intestinal e metabólica do Homem, funcionando como mecanismo de resistência à colonização por agentes patogénicos. Considerando que para chegar ao seu local preferencial de colonização, o intestino, *A. butzleri* tem de ultrapassar diversas barreiras, sendo uma dessas os AGCC, este trabalho teve como objetivo avaliar os efeitos dos AGCC na virulência e sobrevivência desta bactéria.

Deste modo, para avaliar o comportamento das estirpes de *A. butzleri*, selecionadas para este estudo (n=8), na presença de AGCC, inicialmente foi realizada a análise de curvas de crescimento com uma duração de 48 horas, na presença e ausência destes compostos. Com isto foi possível verificar que os isolados são maioritariamente afetados pelas concentrações mais elevadas de AGCC, apresentando em geral um aumento da fase lag ou mesmo uma inibição de crescimento, em concentrações como 130 mM ou 172 mM de uma mistura de AGCC. No entanto, este comportamento parece ser dependente de estirpe.

Para avaliar de que forma estes compostos podem influenciar os mecanismos de virulência de *A. butzleri*, procedeu-se seguidamente ao estudo da influência de AGCC na expressão dos genes putativos de virulência geralmente associados a fatores de virulência deste microrganismo (*cadF, ciaB, flaA* e *luxS,* associados às capacidades de adesão e invasão, motilidade e formação de biofilme). Para tal, usou-se a metodologia de transcrição reversa seguida de PCR em tempo real (RT-qPCR), através do qual se observou que quando na presença de concentrações de AGCC semelhantes às encontradas no intestino, a expressão de alguns genes sofre alteração, como *flaA* e *luxS* que mostraram ser sobre-expressos na presença da maioria das concentrações usadas. O mesmo comportamento foi verificado com *ciaB* na presença de 130 mM de AGCC, consequentemente sugerindo um possível efeito dos AGCC nos fatores de virulência da bactéria.

A. butzleri é uma bactéria móvel com a presença de um flagelo na sua extremidade, e como tal a motilidade é um fator de virulência bastante importante. Assim, a motilidade bacteriana foi analisada pelo método de difusão em agar a 4 % na presença de AGCC, verificando-se uma diminuição da motilidade na presença das concentrações mais elevadas de AGCC, na maioria dos isolados testados.

A capacidade de formação de biofilmes de *A. butzleri* é outro dos fatores bastante investigados, uma vez que este é responsável por aumentar a resistência da bactéria a fatores extrínsecos potencialmente prejudiciais à sua sobrevivência. O método de violeta de cristal em placas de 24 poços na presença de AGCC foi a metodologia selecionada para efetuar a avaliação desta capacidade. Os resultados mostraram um comportamento dependente da estirpe, onde os isolados de *A. butzleri* W12-1, DQ40A1 e INSA2756 foram afetados positivamente pelos AGCC, verificando-se um aumento da capacidade de formação de biofilme. Contrariamente ao verificado com os isolados anteriores, as estirpes INSA3800 e INSA4015 apresentaram uma redução na formação de biofilme na presença das concentrações de AGCC utilizadas.

Por último, procedeu-se também à avaliação do efeito de *A. butzleri* sob a influência de AGCC em células de adenocarcinoma colorretal humano, Caco-2. Este estudo consistiu na realização de ensaios de adesão e invasão, onde a linha celular foi infetada pela bactéria em solução com os AGCC, com uma multiplicidade de infeção de 100. A estirpe representativa utilizada mostrou uma redução da capacidade de adesão e invasão na presença das concentrações de 90 e 130 mM das misturas de AGCC.

Concluindo, é possível afirmar, através dos resultados obtidos, que *A. butzleri* é afetado pelos AGCC de forma dependente de estirpe, e que os AGCC apresentam um efeito modulatório na sua sobrevivência e virulência. Estes compostos, presentes ao longo do intestino, têm um papel importante durante o processo de infeção, podendo ser explorado na modulação da virulência *A. butzleri*.

Palavras-chave

Aliarcobacter butzleri; Ácidos gordos de cadeia curta; Virulência; Genes putativos de virulência; Motilidade; Biofilmes; Viabilidade celular; Adesão; Invasão.

Abstract

Aliarcobacter butzleri has commonly been associated with diseases in both humans and animals and is therefore classified as a moderate hazard to human health. This bacterium may be found in various environments on a large scale, such as soils, waters and the food chain, being a major source of transmission and contamination. Moreover, it has been associated with diarrheal and non-diarrheal gastrointestinal diseases, whose main symptoms include abdominal pain, nausea and fever. Despite the research available on this microorganism, its response to host's resistance colonization mechanisms remain underexplored. Short-chain fatty acids (SCFA) have been taken into study as mechanisms of resistance to colonization and are produced through the microbial fermentation of non-digestible carbohydrates in the intestine, being responsible for the maintenance of intestinal and metabolic health in humans. As SFCA are one of the multiple adverse conditions *A. butzleri* faces until it reaches its preferred colonization site, the intestine, this work aims to evaluate the effects of SCFA on the virulence and survival of this bacterium. Therefore, the bacterium's growth, gene expression, motility, biofilm formation and adhesion and invasion abilities were evaluated with different types and concentrations of SCFA. The majority of the assays were conducted with eight *A. butzleri* isolates and, in general, lower concentrations showed no significant effect on bacterial growth, whereas the higher concentrations revealed an increase of the lag phase and/or growth inhibition, in a strain-dependent manner. Regarding the evaluation of putative virulence genes expression, it was observed an overexpression by most concentrations tested for the *flaA* and *luxS* genes. At a concentration of 130mM_SCFA, the *ciaB* gene also displayed an overexpression. The motility of most strains used in the study presented a decrease in this virulence factor. Also, the results of the biofilm formation of this bacterium showed a strain-dependence considering the concentration used. Furthermore, the SCFA tested appeared to affect the bacterium's ability to adhere and invade the Caco-2 cell line, showing a trend to reduce these virulence features. Altogether, through the analysis of the results obtained from this study, it is noticeable that SCFA present a modulatory effect on *A. butzleri'* survival and virulence, in a strain-dependent form. Regardless, it is possible to conclude that SCFA have an important role during the infection process, with the modulation of *A. butzleri'* virulence being a point for further investigation.

Keywords

Aliarcobacter butzleri; Short-chain fatty acids; Virulence; Putative virulence genes; Motility; Biofilms; Cell viability; Adhesion; Invasion.

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List of Abbreviations

CFU – Colony forming unit

DMEM - Dulbecco's modified Eagle medium

EHEC – Enterohemorrhagic *Escherichia coli*

ICMSF - International Commission on Microbiological Specification for Food

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NaCl – Sodium chloride

OD – Optical density

PBS – Phosphate buffered saline

SA – Sodium acetate

SB – Sodium butyrate

SCFA – Short-chain fatty acids mixtures

SP – Sodium propionate

TSA - Tryptic Soy Agar

TSB - Tryptic Soy Broth

Chapter I - Introduction

1.1. *Aliarcobacter Genus*

1.1.1. Taxonomical Classification and History

Arcobacter first ever isolation was accomplished in 1977, by Ellis *et al.* and until 2018, the previously denominated *Arcobacter* genus belonged to the *Campylobactereaceae* family integrated in the heterogeneous class Epsilonproteobacteria (Vandamme *et al.*) and comprised 33 recognized species (Mateus *et al.*, 2021). Nevertheless, in the last years, this genus has been passing through a controversial taxonomical reorganization (Waite *et al.*, 2017, 2018; Ferreira, Oleastro and Domingues, 2019; On *et al.*, 2020). Pérez-Cataluña et al. (2018) has proposed the division of *Arcobacter* genus into six different genera and one candidate, *Arcobacter*, *Aliarcobacter* gen. nov., *Pseudoarcobacter* gen. nov., *Haloarcobacter* gen. nov., *Malacobacter* gen. nov., *Poseidonibacter* gen. nov., and *Arcomarinus* gen. nov, being part of a new family, *Arcobacteraceae*, included in the class *Campylobacteria*. Due to this reclassification, *Arcobacter butzleri* was renominated as *Aliarcobacter butzleri* and included in the genus *Aliarcobacter* (Pérez-Cataluña *et al.*, 2018; Ferreira, Oleastro and Domingues, 2019), which comprises nine validated species, such as *A. butzleri, Aliarcobacter cibarius, Aliarcobacter cryaerophilus, Aliarcobacter lanthieri, Aliarcobacter skirrowii, Aliarcobacter thereius, Aliarcobacter trophiarum*, *Aliarcobacter vitoriensis* and *Aliarcobacter faecis* (Pérez-Cataluña *et al.*, 2018; Alonso *et al.*, 2020; Chieffi, Fanelli and Fusco, 2020). Despite, the division of the original genus *Arcobacter* into different genera is being heavily discussed, with some authors suggesting reverting back to the original single genus due to a lack of significant biological or clinical differences (On *et al.*, 2020), *A. butzleri* will be referred as belonging to the *Aliarcobacter* genus, since this is the still validated nomenclature.

1.1.2. General Characteristics of *Aliarcobacter spp.*

Species from the *Aliarcobacter* genus are Gram-negative often S shaped, spirally curved rods with 0.2–0.5 µm in diameter and 1–3 µm long. It is also a motile species due to its single polar flagellum (Collado and Figueras, 2011; Pérez-Cataluña *et al.*, 2018).

Aliarcobacter spp. can be differentiated from *Campylobacter* spp. by its ability to grow in aerobic conditions at lower temperatures (Collado and Figueras, 2011). Growth occurs at 15 to 42 ºC, it is chemoorganotrophic, oxidase and catalase positive and no growth can occur at 4% NaCl. Carbohydrates are not fermented, nitrate is usually reduced to nitrite, it is positive for the hydrolysis of indoxyl acetate and negative for urease. Growth does not occur in the presence of 2,3,5- triphenyltetrazolium chloride (0.04%, wt/vol) or glycine (1% wt/vol). Some species may grow in the presence of safranin (0.05% wt/vol) or oxgall (1% wt/vol). Fluorescent pigments are not produced, and some species are susceptible to cefoperazone (64 mg/L). Range of DNA G+C content is 26.4–29.4 mol% (Pérez-Cataluña *et al.*, 2018). Among the species of this genus, *A. butzleri* has been most commonly associated with animal and human disease (Mateus *et al.*, 2021), being classified by the International Commission on Microbiological Specification for Food as a moderate hazard to human health (ICMSF, 2018).

1.2. *Aliarcobacter butzleri's* distribution along different environments and hosts and its routes of transmission

Species belonging to the *Aliarcobacter* genus can be found in various environments, including soils, water sources like rivers, lakes, groundwater, seawater, plankton, as well as wastewater treatment plants or even effluent water after treatment (Collado and Figueras, 2011; Ferreira, Oleastro and Domingues, 2019). Globally, the *Arcobacteraceae* family has a prevalence of 69.2% in aquatic settings and is highly prevalent in low-income countries (90%). Its highest prevalence was found to be in the wastewater entering wastewater treatment plants (Venâncio *et al.*, 2022). *Aliarcobacter* spp. are present in environmental waters, which may be a source of contamination that can be caused by faecal pollution (Ferreira, Oleastro and Domingues, 2019). *A. butzleri* has also been isolated from different sources of drinking water, such as reservoir influents, raw water in treatment plants (Jacob, Lior and Feuerpfeil, 1993) and water collected from a well (Rice *et al.*, 1999). A study has demonstrated *A. butzleri's* ability to survive longer throughout different temperature changes in water, supporting its potential role as a waterborne pathogen and suggesting that water can act as a reservoir and be considered as a potential source of contamination to humans as well as animals (Van Driessche and Houf, 2008). Contaminated water has also been confirmed as a source of human infection in countries with ineffective water supplies (Taylor *et al.*, 2016).

Along the food chain, *Aliarcobacter* genus is found to have a wide distribution, making it a major source of contamination and transmission. It has a global prevalence of 30%, with Portugal having an even higher prevalence of 51.7%. However, when observing the level of economic development, it is surprising to observe that the countries with higher income levels exhibit a greater prevalence, reaching an estimated value of 34.2% (Mateus *et al.*, 2021). *A. butzleri* is an emergent foodborne pathogen that can arise at any stage of the food chain and is the most frequent species isolated from most types of food products, especially chicken, pork and beef meat (Houf *et al.*, 2003; Aydin *et al.*, 2007), being poultry the one with highest incidence (Mateus *et al.*, 2021). Therefore, the ingestion of contaminated food or water is considered the main route of transmission of *A. butzleri* to humans and associated to arcobacteriosis illness (Fong *et al.*, 2007; Collado and Figueras, 2011). Since some animals can be carriers of *Aliarcobacter* spp., the transfer from manure when used as fertilizer in agriculture (Ferreira, Oleastro and Domingues, 2017) or even the faecal contamination on slaughterhouses or cross-contamination in food processing facilities, may create various routes of contamination throughout the food chain (Collado and Figueras, 2011; Ferreira *et al.*, 2013). Also, the presence of *Aliarcobacter* species, namely *A. butzleri*, in raw food products, such as vegetables, and animal origin products at the various stages of processing and retail, but also in ready-to-eat food point for the role of the food products as a potential source of contamination (González and Ferrús, 2011; Hausdorf *et al.*, 2013; Balamurugan, Ahmed and Chambers, 2013; Giacometti *et al.*, 2014, 2015; Vicente-Martins *et al.*, 2018). These may be aggravated by *A. butzleri*'s ability to grow and survive in food products, for example in dairy products, in cases of thermal abuse and optimal storage temperatures (Giacometti *et al.*, 2015).

Apart from the previously mentioned possible routes and sources of contamination by *A. butzleri*, transmission may also occur due to contact with infected animals, especially dogs and cats, which may lead to a spread of this pathogen to humans. *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* have been shown as the mostly found species in humans as well as in domestic animals, being *A. butzleri* the most common in cats (Fera *et al.*, 2009; Goni *et al.*, 2017). *A. butzleri* was also related to diarrhoea and enteritis in pigs, horses, and cattle (Collado and Figueras, 2011).

Another suggested transmission route of *A. butzleri* has been the person-to-person contact, with the first proposal of this occurring in 1983 in a nursery in Italy, where an outbreak among 10 children from different ages and grades and a teacher occurred, with them suffering from abdominal cramps and some even experienced vomiting and fever. Faecal samples were collected and *A. butzleri* was identified in all of them (Vandamme *et al.*, 1992). Another situation that suggests this type of transmission is the first reported case of *A. butzleri* transmission *in utero*, where a 34-year-old mother, that did not appear to show any signs of illness gave birth to a baby with bacteraemia (On, Stacey and Smyth, 1995).

Among the species belonging to *Arcobacteracea* genus, some have been associated with gastrointestinal infections in humans, such as *A. butzleri, A. cryaerophilus*, *A. skirrowii*, *A. thereius*, *A. lanthieri* and *A. mytili* (Kerkhof *et al.*, 2021).

Several studies have been taken to investigate the diversity and prevalence of *Aliarcobacter* spp., for example, a study with 1862 samples collected in Lithuania was undertaken. Among these, 1200 samples from human stool were collected from patients exhibiting symptoms of gastroenteritis, while the remaining belonged to chicken meat, raw cow milk and environmental waters. The results indicated that 11.2% of the samples tested positive for *Aliarcobacter* spp., with *A. butzleri* being the most frequently isolated species (92.3%) followed by *A. cryaerophilus* (7.7%). Notably, the highest prevalence of *Aliarcobacter* spp., was observed in chicken meat (36%), environmental waters (28.1%), raw cow milk (25%), ready-to-eat salad mixes (7.1%) and human stool (1.7%) (Uljanovas *et al.*, 2021). A different study conducted in Germany involved the analysis of 4636 human stool samples, where in 0.85% of the outpatients' samples and in 0.40% of hospitalized patients were positive for *Aliarcobacter* spp.. The predominant species was *A. butzleri,* constituting 67% of the cases, being the only species isolated from hospitalized patients, followed by *A. cryaerophilus* (28%) and *A. lanthieri* (6%) (Brückner *et al.*, 2020). Despite this prevalence in humans , *A. butzleri* has been described as the fourth most common *Campylobacter*-like organism in diarrheal faeces, namely in Portugal ((Vandenberg *et al.*, 2004; Collado *et al.*, 2013; Ferreira, Júlio, *et al.*, 2014))

1.3. Pathogenicity and virulence factors of *Aliarcobacter butzleri*

A. butzleri was isolated originally from humans and animals with diarrhoea (Kiehlbauch *et al.*, 1991; Collado and Figueras, 2011) and it has been associated with diarrheal gastrointestinal disease with symptomatology, including acute or chronic watery diarrhoea, abdominal cramps and nausea (Mateus *et al.*, 2021), but has also been related with non-diarrheal gastrointestinal illness marked by vomiting, fever, nausea and abdominal pain (Ferreira *et al.*, 2016). Among the possible diseases caused in humans, it has been associated also with endocarditis, bacteraemia and peritonitis. Furthermore, *Aliarcobacter* spp. has often been isolated from intestinal tracts and faecal samples of many farm animals and can cause abortions, diarrhoea, and mastitis (Collado and Figueras, 2011).

Several epidemiological and case studies have reported the association of *A. butzleri* to disease, yet our understanding of their pathogenesis remains limited (Collado and Figueras, 2011; Chieffi, Fanelli and Fusco, 2020). The presence of putative virulence genes in the genome of *A. butzleri* has been highlighted due to their potential involvement in the epidemiology of this bacterium's infections (Uljanovas *et al.*, 2021).

1.3.1. Presence of putative virulence genes

Research on the prevalence of putative virulence genes in *Aliarcobacter* spp. is limited and exhibits geographic fragmentation. The absence of comprehensive surveillance studies prevents a definitive evaluation of the prevalence of putative virulence genes in this bacterium. Given that these genes may play a role in the colonization and infection mechanisms, the elevated detection rates imply a pathogenic potential in *A. butzleri* isolates (Piva *et al.*, 2017).

The examination of *A. butzleri* RM4018's genome has unveiled the existence of ten putative virulence-associated genes, namely *cadF*, *cj1349*, *ciaB*, *mviN*, *pldA*, *tlyA*, *irgA*, *hecA*, *hecB* and *iroE*, which were considered due to their homology to virulence factors found in other pathogenic microorganisms (Miller *et al.*, 2007). These putative virulence genes may be implicated in various mechanisms, for instance, *cadF* and *cj1349*, encode for fibronectin binding proteins from the outer membrane and promote adherence to host cells, *ciaB* contributes to the host cell invasion, while *mviN* is recognized for encoding a protein essential for peptidoglycan synthesis. In turn, *pldA* encodes an outer membrane phospholipase A, associated with haemolytic activity and *tlyA* is linked to erythrocyte lysis and is important in the adhesion to Caco-2 cells in *C. jejuni*. *HecA* encodes an adhesin protein, and *hecB* encodes a haemolysin activation protein (Douidah *et al.*, 2012; Ferreira *et al.*, 2016). Additionally, *irgA* and *iroE* encode iron-regulated proteins from the outer membrane (Uljanovas *et al.*, 2021).

Several studies, performed to gain a better understanding of the putative virulence genes present in *A. butzleri*, reveal consistent findings. For instance, in an analysis of 208 isolates collected from food, water and human stool, the predominant virulence genes were *cadF* (100%), *ciaB* (100%), *cj1349* (99%), *tlyA* (99%), *mviN* (97.9%) and *pldA* (95.8%) (Uljanovas *et al.*, 2021). A different study, involving a total of 212 isolates obtained from three distinct dairy production sites (farms, industrial dairy plants and artisanal dairy plants), identified *ciaB* (100%), *mviN* (98%), *tlyA* (98%), *cj1349* (97%), *pldA* (97%) and *cadF* (96%) as the most commonly detected genes. The consistent presence of these genes in this and prior studies suggests that they may serve as competitive factors contributing to *A. butzleri* 's dissemination within diverse host populations and environmental sites (Piva *et al.*, 2017).

The capacity for biofilm formation has also been associated with the expression of certain genes, including *flaA*, known to be implicated in bacterial motility, and *luxS*, likely linked to Quorum sensing. Despite the lack of characterization of these virulence genes in *A. butzleri*, a recent study has shed light on their potential significance. In this study, these genes appear to play a positive role in the biofilm formation ability of this bacterium, emphasizing their importance in understanding this bacterium's pathogenicity (Salazar-Sánchez *et al.*, 2022). The previously mentioned gene, *flaA*, along with the *flaB* gene, are responsible for the synthesis of flagellins. These flagellins are primarily associated with the formation of the flagellum which is linked to bacterial motility, underscoring their significance as crucial virulence factors across various microorganisms (Ho *et al.*, 2008).

1.3.2. Motility

Most bacteria possess in one or both extremities a flagellum, assembled by flagellins, which are protein subunits responsible for cell motility, chemotaxis, protein secretion, biofilm formation, colonization and invasion of host cells, making the bacterial flagella an important virulence factor (Haiko and Westerlund-Wikström, 2013).

Flagellar motility enables bacteria to adapt to different environments, allowing them to escape adverse conditions and migrate to more favourable surroundings. This motility also plays a crucial role in successfully competing with other microorganisms (Duan *et al.*, 2013).

A. butzleri is a motile bacterium possessing a single polar flagellum, which is an essential virulence factor for colonization (Isidro *et al.*, 2020). The flagellum comprehends two flagellin proteins, FlaA and FlaB, nonetheless Ho et al. (2008) demonstrated that *flaA* is the only gene necessary for motility in *A. butzleri*, while *flaB* is not required (Ho *et al.*, 2008). Furthermore, the *flaA* gene expression has been suggested to be necessary for a maximum adherence and invasion of eukaryotic cells (Medina *et al.*, 2019).

1.3.3. Biofilm Formation ability

The biofilm formation process comprises distinct stages. Upon encountering stressful conditions, such as unfavourable temperature, antibiotic exposure or nutrient deficits, planktonic bacterial cells initiate a reversible adherence to the surfaces and to each other.

Subsequently, the adhered bacteria produce exopolysaccharide (EPS), and adhesins, contributing to their irreversible attachment. Microcolonies are later formed, and as they continue to grow and produce EPS, the biofilm matures. Ultimately, when the biofilm reaches a critical mass, cells detach from the outermost layer, leading to the dispersion of planktonic cells from the biofilm (Salazar-Sánchez *et al.*, 2022). Biofilms are characterised as ubiquitous and exist on biotic and abiotic surfaces (Berne *et al.*, 2018). Additionally, they have been correlated to bacterial virulence, environmental survival, antibiotic resistance and colonization, which makes them a notable factor in the bacterial survival and persistence in numerous environments (Ferreira *et al.*, 2013).

It has been demonstrated that *A. butzleri* exhibits the capability to adhere to various materials and form biofilm under diverse conditions, potentially leading to cross-contamination and dissemination along food chain. Biofilm formation is influenced by factors, such as environmental conditions, nutrient availability, surface properties and varies greatly among strains (Girbau *et al.*, 2017). In-depth studies on the biofilm formation of this species revealed that the majority of the isolates were classified as weakly adherent. Nonetheless, this observation still supports the premise that *A. butzleri* is a biofilm-forming bacterium, present in slaughterhouses, and there is a possibility that it is capable of incorporating already established biofilms as a secondary colonizer. Furthermore, it has been suggested that biofilm formation could potentially explain the discrepancies of this bacterium's prevalence between the chicken's gut and their carcasses (Ferreira *et al.*, 2013). Moreover, *A. butzleri* possesses the capability of producing biofilm across a broad temperature range, from as low as 5°C to as high as 37°C. This adaptability allows this bacterium to survive, proliferate and form biofilms in chilled environments, which may lead to its establishment in food processing settings (Kjeldgaard, Jørgensen and Ingmer, 2009).

1.3.4. Adhesion and Invasion ability to eukaryotic cells

Adhesion constitutes a crucial step in bacterial pathogenicity, playing a pivotal role in establishing infections. It is essential for processes such as colonization, invasion, intracellular multiplication, dissemination to other tissues and persistence within host cells. In order to sustain an effective infection, pathogens employ various strategies to invade the host, evade and resist immune responses and induce damage to host cells. It has been demonstrated that *Aliarcobacter* spp. possesses the capability to adhere and invade to eukaryotic cells, in addition to producing toxins capable of causing damage to these cells (Ferreira *et al.*, 2016).

The adhesion and invasion characteristics of *Arcobacteraceae* species have been investigated across various species using multiple cell lines, such as Hep-2, HeLa, INT407, CHO and Caco-2. The research has indicated that there is variability in adhesion, invasion and toxicity based on the species' origin and the specific cell line utilized (Karadas *et al.*, 2013). In a particular study, it was observed that 14 out of the 16 *Arcobacteraceae* species examined could adhere to Caco-2 cells, and 10 out of the 16 also exhibited an invasive phenotype. It is important to underline that all *A. butzleri* strains demonstrated adhesion to the human intestinal cells, and the majority was also able to perform a successful invasion (Levican *et al.*, 2013), altogether highlighting the pathogenic potential of *A. butzleri.*

1.4. Colonization Resistance Mechanisms

After entering the host's organism, *A. butzleri* will face several barriers that need to overcome to reach its colonization site, where the colonization resistance, the ability of gut symbionts to establish a stable community within the host that defends off the growth of pathobionts and prevents the invasion of non-native bacteria, becomes crucial. This defence mechanism is vital in maintaining the integrity of the host's gastrointestinal ecosystem. (Bohnhoff, Drake and Miller, 1954).

Nonetheless, nowadays this term refers to various interconnected aspects, including increased tolerance to existing infections, resistance to initial infection and infection clearance (Pickard *et al.*, 2017). As illustrated in Figure 1, several mechanisms contribute to colonization resistance, such as secondary bile acids, bacteriocins, nutrient competition, mucus layers, bacteriophages and short-chain fatty acids (SCFA) (Ducarmon *et al.*, 2019).

Secondary bile salts possess antibacterial properties that indirectly influence colonization resistance (Termén *et al.*, 2008). Moreover, certain bacteria, including *Lactococcus, Lactobacillus* and some *Enterobacteriaceae*, produce short, noxious peptides that prevent the colonization and growth of other species, known as bacteriocins (Rea *et al.*, 2010; Rebuffat, 2011). Also, when exogenous bacteria enter the host, they must compete with local bacteria for available nutrients and resources in the gut to ensure their survival (Maltby *et al.*, 2013). The inner and outer mucus layers constitute another important resistance mechanism. The inner layer serves as a physical barrier that keeps the bacteria from penetrating and reaching the epithelial cells, therefore avoiding an inflammatory response (Johansson, Holmén Larsson and Hansson, 2011). As bacterial and host enzymes are able to hydrolyse the luminal edge of this layer, a looser outer layer is formed, supporting a denser and metabolically distinct community, including bacteria without specialized mucolytic abilities (Li *et al.*, 2015). Bacteriophages have a high selective role, therefore, targeting only specific bacterial strains. This selectivity serves as a means to protect the existing microbiota, selectively affecting pathogens while leaving beneficial bacteria unharmed (Wittebole, De Roock and Opal, 2014). Lastly, SCFA are capable of altering intracellular pH and metabolic processes, thereby influencing the growth of invasive bacteria (Cummings *et al.*, 1987).

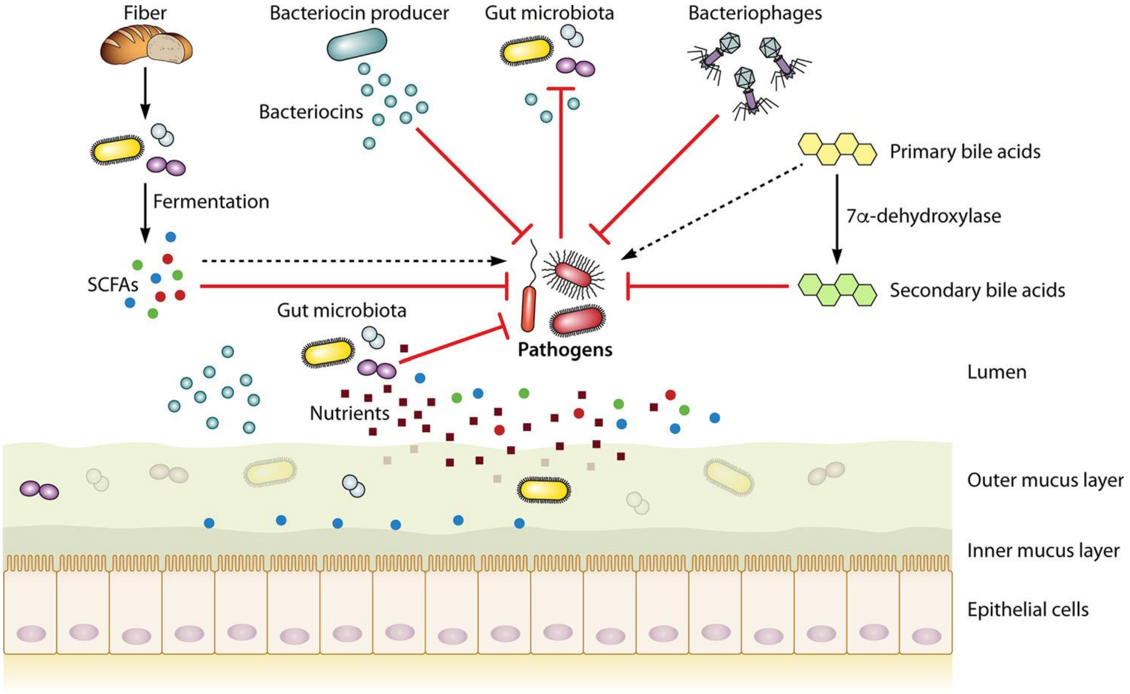


Figure 1. Outline of gut microbiota-mediated colonization resistance mechanisms, from Ducarmon et al. (2019).

Some colonization resistance mechanisms have been investigated with *Aliarcobacter* spp.. Notably, *A. butzleri* has demonstrated the ability to survive bile salts at physiological concentrations, found in the human gastrointestinal tract. Bile salts have also been observed to modulate virulence factors such as bacterial motility, biofilm formation and associated phenotype (Mateus *et al.*, 2023). In a different study, bacteriocin-like compounds, among antibacterial metabolites, were found to be produced by the selected human faecal *Bifidobacterium* strains. These compounds exhibit inhibitory effects against Gram-negative bacteria, including *Aliarcobacter* spp..

This represents the first report of such antimicrobial activity of *Bifidobacterium* against this species (Collado, Hernández and Sanz, 2005). Furthermore, more observations have been made regarding *A. butzleri*'s ability to penetrate and move through the mucus, suggesting it capacity to colonize the mucosa by adhering and proliferating within the human mucus (Buzzanca *et al.*, 2021).

1.5. Short-Chain Fatty Acids

Short Chain Fatty Acids are described as fatty acids with fewer than 6 carbons and are produced through the microbial fermentation of indigestible carbohydrates in the colon. Indigestible food components, such as soluble dietary fibre and prebiotics, undergo fermentation by the gut microbiota, leading to the production of SCFA. The most commonly found SCFA in the intestine include acetate (C2), propionate (C3) and butyrate (C4) (Blaak *et al.*, 2020; Pace *et al.*, 2021).

SCFA are extremely significant in preserving the gut and metabolic health, through the stimulation of mucus synthesis, the production of antimicrobial peptides and also in maintaining the integrity of the tight junction complex, therefore reducing epithelial permeability. The production of SCFA is indispensable for ensuring gut integrity, as it contributes to the maintenance of the intestinal barrier function and aid in intestinal inflammation under pathological conditions. The regulatory role of SCFA in gut integrity is due to their capability of regulating the luminal pH, act as an energy source for epithelial cells, influencing mucosal immune function and mucus production. Furthermore, SCFA have an impact on motility and gastrointestinal transit (Blaak *et al.*, 2020). The concentrations and variability at which SCFA are present in the intestine depend on various factors, such as the composition of the intestinal microbial communities, influenced by host genetic factors, intestinal inflammation, and notably, host's dietary patterns (Pace *et al.*, 2021).

SCFA are known to be rapidly absorbed following ingestion, impacting their concentration in the gut. Estimates suggest concentrations of 70 – 140 mM in the proximal colon, where they are predominantly released through fermentation, 20 – 70 mM in the distal colon and 20 – 40 mM in the distal ileum (Tan *et al.*, 2014). More specific site-based considerations, taking this study into account, revealed concentrations of approximately 13±6 mmol/Kg in the terminal ileum, 80±11 - 198 mmol/Kg in the caecum, and 97 - 131±9 mmol/Kg in the descending colon (Herold *et al.*, 2009).

1.5.1. Short Chain Fatty Acids' Biosynthesis, Absorption and Distribution

SCFA serve as the end product of multiple fermentation reactions, as represented in Figure 2, involving the breakdown of dietary fibre into monosaccharides, a process facilitated by enzymes from the gut microbiota (Koh *et al.*, 2016).

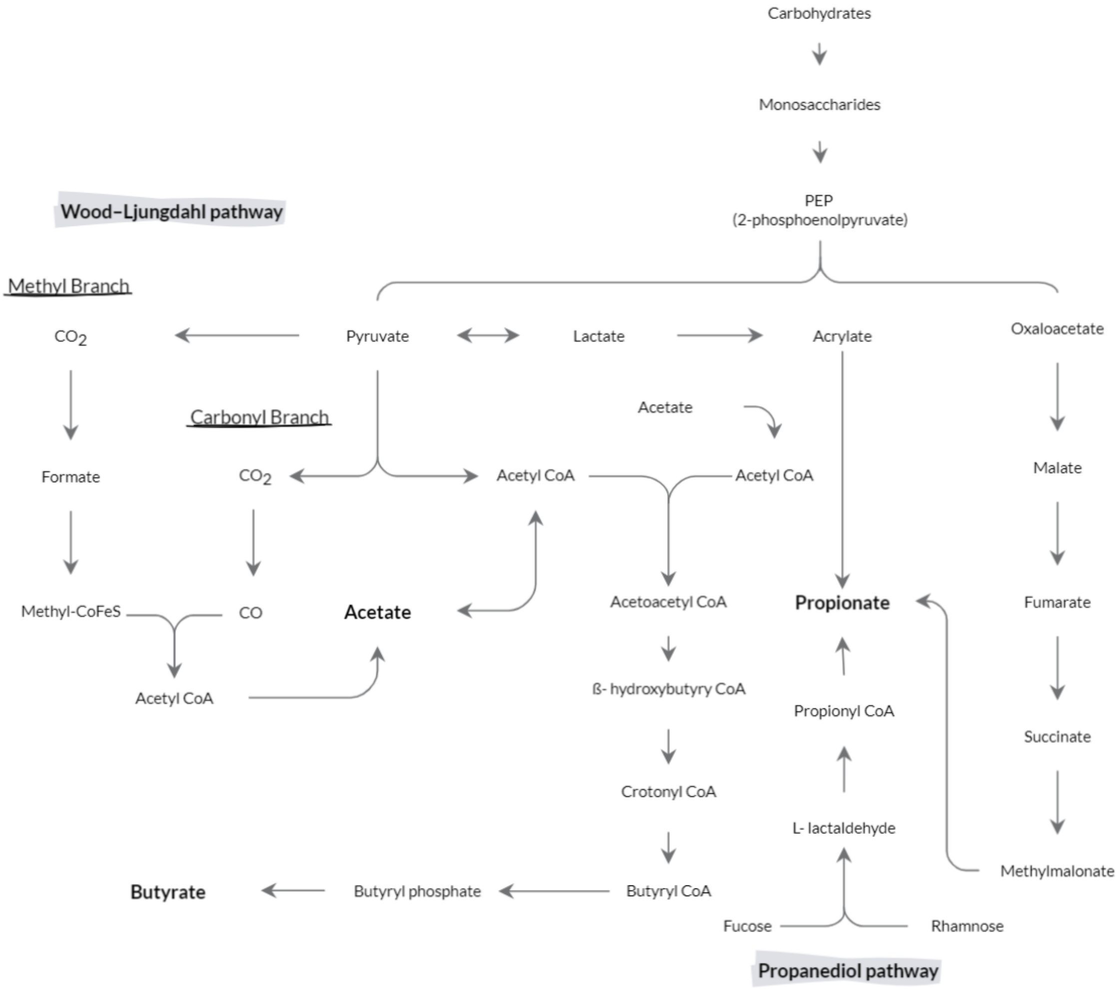


Figure 2. Biosynthesis of acetate, propionate and butyrate. Adapted from Den Besten et al. (2013).

Acetate can be produced through two main pathways. In the first pathway, acetate is formed from pyruvate via acetyl-CoA. The second pathway involves the Wood-Ljungdahl pathway where CO_2 is reduced to formate or is transformed into CO. Subsequently, when CO is combined with a methyl group there is a production of acetyl-CoA. Ultimately, both intermediates will turn into acetate (Ragsdale and Pierce, 2008).

Propionate, on the other hand, is generated through the conversion of succinate to methylmalonyl-CoA. Additionally, propionate can be produced from acrylate with lactate serving as a precursor. Another pathway that leads to the formation of propionate is the propanediol pathway, where fucose and rhamnose are used as substrates to the L-lactaldehyde (Hetzel *et al.*, 2003; Scott *et al.*, 2006).

Finally, butyrate can be produced through the condensation of two molecules of acetyl-CoA, resulting in a reduction to butyryl-CoA. Additionally, it can be produced when microorganisms existing in the gut utilize lactate or acetate, thereby preventing the accumulation of lactate and contributing to a healthy intestinal environment (Louis *et al.*, 2004; Vital, Howe and Tiedje, 2014).

Research has indicated that the production of butyrate in the gut is attributed to *Firmicutes*, which are Gram-positive bacteria, while acetate and propionate are produced by *Bacteroidetes*, classified as Gram-negative bacteria (Louis and Flint, 2009, 2017). Specific examples of bacteria capable of producing these SCFA are provided in Table 1.

Table 1. Short-chain fatty acids and their bacterial producers, from Mirzaei et al. (2022).

Short-chain Fatty Acid	Bacterial Producer	References
Acetate	*Akkermansia muciniphila*	(Duncan *et al.*, 2002); (Derrien *et al.*, 2004); (Kendrick *et al.*, 2010); (Rivière *et al.*, 2016); (Louis and Flint, 2017); (Feng, Ao and Peng, 2018); (Bui *et al.*, 2019); (Gu *et al.*, 2020); (Iljazovic *et al.*, 2021)
	Bacteroidetes	
	Bifidobacterium spp.	
	Clostridium spp.	
	Blautia hydrogenotrophica	
	Ruminococcus spp.	
	Prevotella spp.	
	Coprococcus spp.	
Propionate	*Veillonella parvula*	(Reichardt *et al.*, 2014); (Shimizu *et al.*, 2018)
	Bacteroides eggerthii	
	Bacteroides fragilis	
	Ruminococcus bromii	
	Eubacterium dolichum	
Butyrate	*Faecalibacterium prausnitzii*	(Louis and Flint, 2009); (Louis and Flint, 2017); (Walker *et al.*, 2014)
	Clostridium leptum	
	Eubacterium rectale	
	Roseburia spp.	
	Eubacterium hallii	
	Anaerostipes spp.	

The concentrations of the different SCFA vary along the gastrointestinal tract, reaching the highest levels in the caecum and proximal colon and decreasing in the distal colon (Cummings *et al.*, 1987). Butyrate has been identified as a preferred energy source for colonocytes and is primarily absorbed in the gut epithelium (Morrison and Preston, 2016). On the other hand, the other absorbed SCFA flow into the portal vein, where propionate is later metabolized in the liver and acetate can be utilized by muscle cells for energy production. Consequently, acetate is the most predominant SCFA in the peripheral circulation (Cummings *et al.*, 1987; Qian *et al.*, 2022).

1.5.2. SCFA's impact on the host

Gut microbiota is able to maintain constant communication with host cells, influencing multiple cellular mechanisms through the synthesis of SCFA, such as cell proliferation and differentiation (Park *et al.*, 2016).

SCFA play an important role in regulating the functions of Enteroendocrine cells (EEC), by stimulating and modulating gut hormone secretion and production. Additionally, they modulate gene expression of peptide YY and increase the number of EEC. These multiple functions of SCFA in the host's physiology, including gut homeostasis, are influenced by changes in gut hormones (Larraufie *et al.*, 2017).

SCFA' primarily function is to maintain the environment suitable to commensal bacteria and prevent the growth of pathogens. Nonetheless, their impact on the host's immunological responses and barrier functioning has received significant attention throughout the last years (Kelly *et al.*, 2015). Acetate, propionate and butyrate demonstrate the ability to penetrate through the cell membrane by passive diffusion, exerting favourable effects on intestinal epithelial cells (IECs) and immune cells. Butyrate, for instance, has been shown to restore intestinal barrier function, induce proliferation in healthy colonocytes and promote apoptosis in transformed cells (Rechkemmer and von Engelhardt, 1988; Lupton, 2004; Yan and Ajuwon, 2017). While these SCFA play a crucial role in the mutual communication between the immune system and the microbiome, it remains unclear whether they act as a signal promoting tolerance to the host-associated microbiome or contribute to the reduction of inflammatory reactions (Chang *et al.*, 2014).

The maintenance of gut integrity is crucial for the host, as it ensures the preservation of mucosal homeostasis. To maintain this integrity, the epithelial barrier functions as a separation between the gut luminal contents and the host (Voltolini *et al.*, 2012). Additionally, mucus forms a protective barrier between the luminal environment and the epithelium, in order to prevent bacteria from penetrating the epithelium, reducing the likelihood of occurring inflammation (Tolhurst *et al.*, 2012). Disruption of gut integrity can lead to a number of disorders, including colorectal cancer, irritable bowel syndrome, celiac disease and inflammatory bowel disease (Voltolini *et al.*, 2012).

In addition to their effects on the epithelium, SCFA might have a role in maintaining symbiosis, thereby supporting gut integrity. They possess the ability to stimulate the growth of symbionts by decreasing luminal pH and prevent pathobionts' growth (Roy *et al.*, 2006). Butyrate has been demonstrated to expedite the formation of tight junction proteins in Caco-2 cell monolayers, *in vitro*, without changing the levels of expression, hence improving transepithelial resistance (TER), which is a marker of gut integrity (Tolhurst *et al.*, 2012).

1.5.3. Short Chain Fatty Acids' Role in Bacterial Pathogenicity

Researchers began looking into SCFA' microbicidal potential as early as the 1930s. Around that time, it was discovered that SCFA might possess antimicrobial (Peck *et al*, 1939) and antifungal activities (Hoffman, Schweitzer and Dalby, 1939). Ever since, a fair amount of research has been done on SCFA' microbicidal activity and it is nowadays known that they are able to either inhibit or delay the growth of a variety of microorganisms, including *Enterococcus faecalis* (Jeong *et al.*, 2019)*, Pseudomonas aeruginosa* (Ghorbani *et al.*, 2015)*, Mycobacterium tuberculosis* (Coussens, Wilkinson and Martineau, 2015)*, Cryptococcus neoformans* and many others (Nguyen *et al.*, 2011). It has also been shown that they are powerful modulators of virulence factors (Lamas *et al.*, 2019).

Considering that Gram-positive bacteria exhibit greater resistance to lower internal pH, due to their ability to withstand acidic conditions, they are generally more resilient to SCFA compared to Gram-negative bacteria. To exert an antibacterial effect, substantial quantities of SCFA and acid pH are required (Roe *et al.*, 1998; Russell and Diez-Gonzalez, 1998). Additionally, studies have demonstrated that propionate is able to permeate Gram-negative bacteria's cell membrane, triggering an acid stress response through the shift in cytoplasmatic pH, which inhibits bacterial growth (Jacobson *et al.*, 2018). The inhibitory effect requires low extracellular pH, and regardless of metabolic differences dependent on the microorganisms and the SCFA, all SCFA must enter the cell (Russell and Diez-Gonzalez, 1998). SCFA are able to permeate the cell membrane, when undissociated at low pH levels, and subsequently dissociate once in contact with the alkaline cytoplasm. This dissociation increases the amount of SCFA anions and protons inside the cell, disrupting the balance of anions, affecting cell metabolism, lowering the intracellular pH and altering the osmotic balance (Roe *et al.*, 1998).

In the context of dysbiosis, inflammation can be triggered by numerous pathogens capable of detecting and utilizing SCFA for their own pathogenicity (Spiga *et al.*, 2017; Gillis *et al.*, 2018).

1.5.3.1. Gram-Positive Bacteria

As discussed above, SCFA may have an impact on microorganisms' behaviour including Gram-positive bacteria, like *Listeria monocytogenes*. Elevated concentrations of butyrate have been shown to regulate and reduce the production of certain virulence factors, such as Listeriolysin O and provide protection to the bacterium against host defence mechanisms (Sun et al., 2012). Additionally, propionate has been demonstrated to potentially enhance the macrophages' antimicrobial activities, thereby limiting the growth of intracellular pathogens, such as this one (Weis, 2019). Furthermore, in the *Enterococcus* genus, the effects of SCFA appear to vary among bacterial species, possibly because each SCFA targets a distinct pathway, specific to each species. Notably, propionate has been observed to effectively prevent *Enterococcus faecalis'* growth. Similar growth inhibitory effects were also observed in *Enterococcus faecium* and *Enterococcus hirae*, suggesting that propionate likely influences these bacteria's metabolism (Jeong et al., 2019).

1.5.3.2. Gram-Negative Bacteria

As previously mentioned, SCFA are capable of influencing bacterial gene expression, and are sensed by pathogens, such as Enterohemorrhagic *Escherichia coli* (EHEC), a Gram-negative bacterium, which has been shown to utilize these metabolites for proliferation and virulence (Ohira *et al.*, 2013; Li *et al.*, 2018; Miyamoto *et al.*, 2017). In an acidic medium, SCFA, particularly acetate, can affect and inhibit *E. coli's* growth potentially leading to a decrease in cytoplasmatic pH (Prohaszka, 1980; Roe *et al.*, 2002). Butyrate, in particular, has been shown to be responsible for improving bacterial adhesion to Caco-2 cells and increasing the expression of virulence genes responsible for the colonization of the gut, such as *LEE* genes (Nakanishi *et al.*, 2009) and *flhDC* genes (Tobe, Nakanishi and Sugimoto, 2011). It has also been demonstrated that all SCFA may induce the flagella synthesis in this species (Tobe, Nakanishi and Sugimoto, 2011). Additionally, another study showed that acetate produced by probiotic *Bifidobacteria*, could act *in vivo* to protect the host's epithelial cells from infection (Fukuda *et al.*, 2012). Moreover, propionate and butyrate have been found to promote invasiveness in a Caco-2 cell line, by upregulating the respective virulence genes (Pace *et al.*, 2021).

SCFA are able to decrease intracellular pH, which reduces *Salmonella*'s motility, and its flagellar motor can also be slowed down. Additionally, they are also capable of decreasing the formation of biofilm by this bacterium through the undermining of intercellular communication and polysaccharides synthesis (Amrutha, Sundar and Shetty, 2017; Lamas et al., 2019).

Recent findings by Goodman *et al.*, 2020, indicate that *Campylobacter jejuni* has the ability to detect and perceive the butyrate produced by the gut microbiota. Previous studies demonstrated that when this bacterium is pre-treated with SCFA, the invasion of Caco-2 cells is prevented. On the other hand, when Caco-2 cells are treated beforehand with SCFA, the invasion is significantly reduced (van Deun *et al.*, 2008). However, another study involving pre-treatment of Caco-2 cells with butyrate, resulted in a decrease in adhesion but not invasion by this bacterium, suggesting that butyrate may be capable of protecting differentiated Caco-2 cells from early stages of infection by *C. jejuni* (Cresci, Mayor and Thompson, 2017). In a distinct study, evidence has shown that *C. jejuni* not only is able to sense the SCFA but can also modulate different levels of expression of genes that might be influenced by these metabolites along the intestinal tract. The genes affected by SCFA, known to encode catabolic enzymes and proteins responsible for the transport and utilization of amino acids, contributing to the bacterium's metabolism and growth, were highly up-regulated in *C. jejuni* in the lower part of the intestinal tract, where these metabolites are abundant, making the colonization more efficient (Luethy *et al.*, 2017).

Despite the crucial role of SCFA as a resistance mechanism against pathogens, some bacteria may adapt and develop strategies for modulating the expression of virulence genes. This adaptive response enhances the effectiveness of host colonization (Mirzaei *et al.*, 2022). Additionally, SCFA have also been shown to modulate the migration and activity of immune cells, which leads to a decline in host injury and infection. Hereby promoting a higher chance of survival for both the host and SCFA-producing bacteria (Maslowski *et al.*, 2009; Tan *et al.*, 2014)

Undoubtedly, it is clear that SCFA offer vital connections between the gut microbiota, the host and pathogens. Their anti-inflammatory properties position them as potential therapeutic options for idiopathic inflammatory bowel diseases, including Crohn's disease and Ulcerative colitis. SCFA also show promise in addressing inflammatory pathological illnesses, such as cancer and infections (Pace *et al.*, 2021).

Chapter II - Objectives

Aliarcobacter butzleri is a Gram-negative enteropathogen distributed among various environments, while also being associated with diarrhoeal and non-diarrhoeal diseases in humans and has been classified as a moderate hazard to human health by the International Commission on Microbiological Specification for Food. Considering its pathogenic potential, to reach its colonization site, the gastrointestinal tract must be crossed, here it will find multiple adversities and hostile environments, such as short chain fatty acids. As a subject that remains understudied, the main objective of this work was to clarify the effect of SCFA on the *A. butzleri*'s virulence potential.

In order to respond to this objective, the following specific objectives were established:

- To evaluate the effect of SCFA in *A. butzleri* growth and survival;
- To assess the regulation of the *cadF*, *ciaB*, *flaA* and *luxS* virulence genes by exposure of *A. butzleri* to SCFA;
- To analyse the SCFA' effect in motility and biofilm formation by *A. butzleri*;
- To study the effect that SCFA have on the adhesion and invasion of *A. butzleri* to Caco-2 cell line.

Chapter III - Materials and Methods

3.1. Strains used in this study

In order to perform this study, eight *A. butzleri* isolates from distinct origins were used; two strains were isolated from the food industry (DQ40A1 and AB28/11), two originated from water samples (A11-1 and W12-1) and four from human faeces (INSA2756, INSA2999, INSA3800 and INSA4015), as can be found in Table 2. To proceed with more detailed assays, *A. butzleri* DQ40A1 strain was selected for further studies, considering it as a well characterized isolate.

Table 2. *Aliarcobacter butzleri's* strains used in this study, their origin and year of isolation.

Strain	Origin	Date of isolation	Reference
A11-1	River Water	2016	(Isidro *et al.*, 2020)
W12-1	Water Source	2019	(Venâncio, 2021)
AB28/11	Poultry Carcass Neck Skin	2011	(Ferreira *et al.*, 2013)
DQ40A1	Dairy Plant Equipment Surface	2015	(Ferreira *et al.*, 2017)
INSA2756	Human Faeces	2017	Not published
INSA2999	Human Faeces (Gastroenteritis)	2017	Not published
INSA3800	Human Faeces (Leukopenia)	2019	Not published
INSA4015	Human faeces (Diarrhoea, vomiting and abdominal cramps)	2020	Not published

3.2. Short chain fatty acids and mixtures used in the study

In order to perform the assays in this study, three SCFA were tested, sodium acetate (≥ 99%, Merck, Germany), sodium butyrate (≥ 98%, ThermoFisher Scientific, Germany) and sodium propionate (99%, ThermoFisher Scientific, Germany). Furthermore, during the work, mixtures of the SCFA with composition representative of various parts of the intestine were used, and the composition are presented in Table 3.

Table 3. Composition of the SCFA Mixtures

	30mM of SCFA	**90mM of SCFA**	**130mM of SCFA**	**172mM of SCFA**
Sodium Acetate	20mM	49.5mM	71.5mM	95mM
Sodium Propionate	2.5mM	31.5mM	45.5mM	60mM
Sodium Butyrate	2.5mM	9mM	13mM	17mM

From this point forward, when the abbreviation SCFA is mentioned in this study, it is referring to the mixtures composed by Sodium Acetate (SA), Sodium Propionate (SP) and Sodium Butyrate (SB) (Table 3).

3.3. Storage and growth conditions

Strains were preserved in cryogenic vials in Brain Heart Infusion with 20% (v/v) glycerol at -80ºC. Before use, the strains were inoculated in Tryptic Soy Agar (TSA) plates and incubated in microaerophilic conditions at 37 ºC for 24 h. After incubation, the inoculated plates were stored at 4 ºC for a maximum of two weeks. The microaerophilic environment (6% O_2, ±7.1% CO_2 and 3.6% H_2) was achieved using an Anoxomat system (Anoxomat AN2CTS, Mart Microbiology B.V., Drachten, Netherlands).

Previously to each assay, *A. butzleri* has been inoculated in TSA plates, from the plate stored at 4 ºC, in microaerophilic conditions for 24 h at 37 ºC. After the 24 h incubation, overnight cultures (~16 h) in 10 mL of Tryptic Soy Broth (TSB; VWR, Belgium) with an initial optical density at 620 nm (OD_{620nm}) of 0.02 were carried in an orbital shaker at 100 rpm and 37 ºC under microaerophilic conditions.

3.4. Analysis of the effect of SCFA on the growth of *A. butzleri*

To evaluate the *A. butzleri* strains behaviour in the presence of SCFA, an overnight culture was prepared as described in the previous subsection (3.3) and used for the growth curves construction. Thus, 100 µL of each SCFA or mixture of SCFA solutions in TSB or NaCl in TSB, used as an osmolarity control, was added to a 96-well polystyrene microplate, followed by 100 µL of the bacterial overnight culture with a dilution of 1/50 Additionally, both negative and positive controls were added to the plate, TSB and TSB with inoculum, respectively. For *A. butzleri* DQ40A1, the OD_{620nm} was measured every 2 hours starting at 0 h up to 24 h and then at 30 and 48 h. For the other seven *A. butzleri* strains, the OD_{620nm} was measured at 0, 12, 24 and 48 h. The assay was also carried out with minimal medium, composed of ammonium sulfate (1.0 g/L), di-potassium phosphate (7.0 g/L), glucose (1.0 g/L), magnesium sulphate (0.1 g//L), mono-potassium phosphate (2.0 g/L) and sodium citrate (0.5 g/L) at a pH of 7.0 ±0.2, as a substitute for TSB. The results were analysed through the construction of growth curves considering the OD_{620nm} versus time, with triplicates of each condition in each of the independent assay, with at least three independent assays tested for each condition.

3.5. RNA Extraction and Reverse transcription quantitative real-time PCR (RT-qPCR)

To evaluate the regulation of some of the putative virulence genes, described for *A. butzleri,* by SCFA, RT-qPCR was used according to Mateus *et al.*, (2023) with a few modifications.

Initially, the *A. butzleri* strain DQ40A1 was grown according to the subsection 3.3 and posteriorly diluted at 1:50 in TSB with 0, 30, 90 and 130 mM of SCFA mixtures and the corresponding NaCl concentrations, until reaching the mid-exponential phase (for 12 hours or 16 hours for the 130 mM of SCFA mixture). Total RNA from the culture cells was then extracted, starting by centrifugating the culture (11000 ×g for 5 min), washing the cells with NaCl 0.85%, adding 500 µL of TripleXtractor reagent (GRiSP, Portugal) and freezing at -80°C until use. RNA extraction was performed according to the manufacturer's instructions, TripleXtractor (GriSP, Portugal). The extracted RNA was suspended in 43 µL of Diethylpyrocarbonate water.

The RNA collected was later quantified with the assistance of a nanospectophotometer and a 1% agarose gel electrophoresis was performed, for 30 minutes at 120V, in order to confirm the integrity of the samples. A treatment with DNase I was executed with the DNase I, RNase-free kit (Thermo Scientific, United States of America), following the instructions from the manufacturer. This was followed by cDNA synthesis, using Xpert cDNA Synthesis Mastermix kit (GriSP, Portugal), using 1 µg of RNA.

RT-qPCR was performed with NZYSpeedy qPCR Green Master Mix (2×) kit (NZY Tech, Portugal) for evaluating the expression of the *cadF*, *ciaB*, *flaA* and *luxS* genes. For the amplification reaction, a reactional mixture was prepared for each gene, with 5 µL of master mix (2×), 0.4 µM of each set of primers, that correspond to the *cadF*, *ciaB*, *flaA* and *luxS* genes and the 16S rRNA gene (Table 4), and 1 µL of cDNA. Ultrapure water was added until a final volume of 10 µL was reached.

Table 3. Oligonucleotide sequences in RT-qPCR.

Primers	Target Gene	Sequence	Reference
cadF_F	*cadF*	5'-CTCCAGTTGCTGCACCAAAA-3'	(Mateus *et al.*, 2023)
cadF_R		5'-CCAATATTGTCAACTTTTGCACC-3'	
ciaB_F	*ciaB*	5'-TTGGCAAACTTCATGGACTGC-3'	(Mateus *et al.*, 2023)
ciaB_R		5'-AGCAGTAATTCCTCCATGTCCT-3'	
flaA_F	*flaA*	5'-AGTTGCACCAGCTGACATTT-3'	(Medina *et al.*, 2019)
flaA_R		5'-AGTTGGTGAAGGAAGTTCCGA-3'	
luxS_F	*luxS*	5'-GAGCACCTTTTTGCTGGATT-3'	(Mateus *et al.*, 2023)
luxS_R		5'-TTCCAAGCAACTGCAACTTC-3'	
P338_F	*16S rRNA*	5'-ACTCCTACGGGAGGCAGCAG-3'	(Muyzer, de Waal and Uitterlinden, 1993)
P518_R		5'-ATTACCGCGGCTGCTGG-3'	

Lastly, the samples were incubated in a CFX Real-Time PCR System (Bio-Rad, United States of America) following the succeeding program: 2 min at 95°C, 40 cycles for 5 s at 95°C, 30 s at 60°C and 5 s in crescent gradient to 95°C. The ΔΔCt method was used to analyse the relative expression of the genes, using the *16S rRNA* gene, which is constitutively expressed. The assay was carried out with two duplicates of each condition in at least three experiments done independently.

3.6. Motility evaluation

As previously mentioned, motility is one of *A. butzleri* main virulence factors, therefore, it would be important to evaluate the effect of SCFA in this virulence factor. This assay was performed as described by Ferreira *et al.*, (2018) with slight modifications.

Firstly, TSB plates with 0.4% agar and the desired concentration of the SCFA and NaCl have been prepared, the day before and stored at 25°C. A control plate was also prepared, without any added compounds. After the preparation of each strain, as mentioned in the subsection 3.3., the cells were collected through centrifugation (8000 ×g for 5 minutes) and resuspended in fresh TSB medium to achieve ~10^8 CFU/mL. Then, 5 µL of the suspension was inoculated by stabbing the centre of the semisolid plates and incubated in microaerophilia at 37 °C. The motility halos were measured after 24 and 48 h. The assay was performed at least three times independently for all the eight *A. butzleri* strains.

3.7. Biofilm formation ability

The biofilm formation assay was performed as described by Ferreira *et al.*, (2013) with minor modifications. Firstly, the *A. butzleri* strains were prepared as described before (subsection 3.3). Subsequently, a suspension of the overnight culture was prepared adjusting to a cellular concentration of ~10^8 CFU/mL with TSB containing SCFA, and the respective NaCl concentrations to be tested. The 24- well polystyrene plates (VWR, Belgium) were inoculated with 1 mL of each suspension and incubated in microaerophilic conditions at 37 °C for 48 h. After the incubation, the OD_{620nm} was measured with a microplate reader (Biorad, xMark) and the medium was removed carefully. The plates were then incubated at 55 °C to dry the wells and fixate the bacteria through heat for 1 h. Afterwards, 1 mL of 0.1% (w/v) crystal violet was added to each well for 15 min at room temperature. The unbound crystal violet was removed, and every well was washed with distilled water three times. The plates were again dried at 55 °C this time for 15 minutes. Crystal violet that was bound to the biofilms was then solubilized with 1 mL of a 30% methanol/10% acetic acid in water solution. Lastly, the absorbance at 570 nm was measured to determine the biofilm formation. Both negative and positive controls were also added to the plate. The assay was carried out with three replicates of each condition at least three times, independently.

3.8. Effects of SCFA on the adhesion and invasion of *A. butzleri* to a Caco-2 cell line

The effect of SCFA on the adhesion and invasion of *A. butzleri* was tested on a Caco-2 cell line, a human colorectal adenocarcinoma immortalized cell line, following assay was performed according to Ferreira, Queiroz, *et al.*, (2014) with few alterations.

3.8.1. Caco-2 cell line culture

Firstly, the Caco-2 human intestinal epithelial cells were maintained in tissue culture flasks with Dulbecco's modified Eagle medium (DMEM) high glucose supplemented with

10% (v/v) of foetal bovine serum, 1% (v/v) of non-essential amino acids, 100 µg/mL of streptomycin and 100 U/mL of penicillin at a pH of 7.2, at 37 ºC in 5% CO_2 and 95% air and the medium was exchanged every two days until the 80% semiconfluent state was achieved. At this point, Caco-2 cells were trypsinized and the viable cells were counted. Afterwards, to prepare the cells for the experiment, the cells were seeded in 24- well polystyrene plates (VWR) at 4.5×10^4 cells per well for 7 days at 37 ºC in 5% CO_2 and 95% air.

3.8.2. Cytotoxicity of SCFA

In the interest of understanding if the SCFA used in the adhesion and invasion assays were cytotoxic at the tested concentrations, the cell viability evaluation was executed according to van Deun *et al.*, (2008) with slight modifications.

After the plating and incubation of the cells in the 24-wells plates under the conditions used in the adhesion and invasion assays, the wells were washed two times with PBS and the 500 µL of the SCFA's mixtures dissolved in complete DMEM without antibiotic were added and incubated for 24 h.

The following day, the medium was removed and 500 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) at 0.5 mg/mL, dissolved in DMEM without antibiotic and FBS, was added to the wells and incubated for 3 h at 37 ºC in 5% CO_2 and 95% air. Later, the solution was removed, and the formazan crystals formed were dissolved with 500 µL of dimethylsulfoxide, and the absorbance was measured at 570 nm with a reference filter at 690 nm.

3.8.3. Adhesion and invasion assays

The bacterial strain was grown according to the subsection 3.3 and used for adjusting the bacterial suspensions in DMEM without antibiotic and with or without the mixtures of SCFA (30 mM, 90 mM and 130 mM) at 37 ºC, so that a multiplicity of infection (MOI) of 100 was possible. The *A. butzleri* cellular count was done by serial dilution and drop plate method in TSA plates (Chen, Nace and Irwin, 2003) and Caco-2 cells by trypsinization of the cells followed by counting. Then, the Caco-2 cells in the 24-wells plate were washed 2 times with 500 µL of PBS and 500 µL of the bacterial suspensions was added to each well. The plate was incubated for 3 hours at 37 ºC in 5% CO_2 and 95% air so that the adhesion and invasion could occur. After the 3 h, the plate was washed 3 times with 500 µL of PBS to eliminate the bacteria that did not adhere or invade the cells, and 500 µL of

Triton X-100 at 0.1% (V/V) was added and left for 10 minutes to lyse the eukaryotic cells. This suspension was then diluted and plated for posterior counting of bacteria.

Regarding invasion, after infection of Caco-2 cells for 3 h, the wells were washed 3 times with PBS, and 500 µL of gentamycin at 125 µg/mL (m/V) was added and incubated for 1 h, to kill any bacteria that did not entered the cells. The wells were washed thrice, and eukaryotic cells lysed with 500 µL of Trinton X-100 at 0.1% (V/V) for 10 minutes and the serial dilutions were plated, as previously. All the plates were incubated for 48 h at 37 ºC in a microaerophilic environment. Three replicates for each assay were performed in at least three independent measurements for the DQ40A1 strain and each tested condition.

Chapter IV - Results and Discussion

4.1. Effect of the SCFA on *Aliarcobacter butzleri'*s growth

Based on the aforementioned information highlighting the important role of SCFA as a colonization resistance mechanism against pathogens, it becomes apparent that SCFA can exert diverse effects on multiple microorganisms. These compounds are well known for their ability to provide protection and a decline in infection of the host, as a colonization resistance mechanism (Maslowski *et al.*, 2009; Cresci, Mayor and Thompson, 2017). However, despite the protection SCFA provide to host cells, some pathogens are able to adapt and improve its colonization ability of the host, mainly associated with reduced concentrations of butyrate, or even utilizing the metabolites as nutrients for growth (Gillis *et al.*, 2018; Mirzaei *et al.*, 2022). Among gastrointestinal pathogens, *A. butzleri* is identified as an emergent moderate hazard enteropathogen by the International Commission on Microbiological Specification for Food (ICMSF, 2018; Mateus *et al.*, 2021), yet there is a notable scarcity of information regarding its pathogenesis, but also its specific response to SCFA, thereby demanding for further research. Considering the objective of this research of elucidating on the effects of SCFA on this bacterium's virulence, the first step was the assessment of the bacterium's growth when exposed to SCFA.

The growth patterns of the eight strains under study was evaluated in the presence of different concentrations of SA, SP, SB and SCFA by recording each strain's growth curve considering at least four time points (Figures 3A to 3E). NaCl was used as an osmolarity control and the minimum medium to assess the potential use of SA, SP, SB and SCFA as a substrate by the bacterium (Figures 3F to 3I). The study in the presence of SCFA was also performed with different pH values in order to simulate different *in vivo* conditions found along the intestine, pH 7.18-7.30 and pH 6.5 (Figures 3D and 3E, respectively). The results for the *A. butzleri* DQ40A1 strain are presented in Figure 3, and the remaining results can be found in Annex I subcategorized by strain.

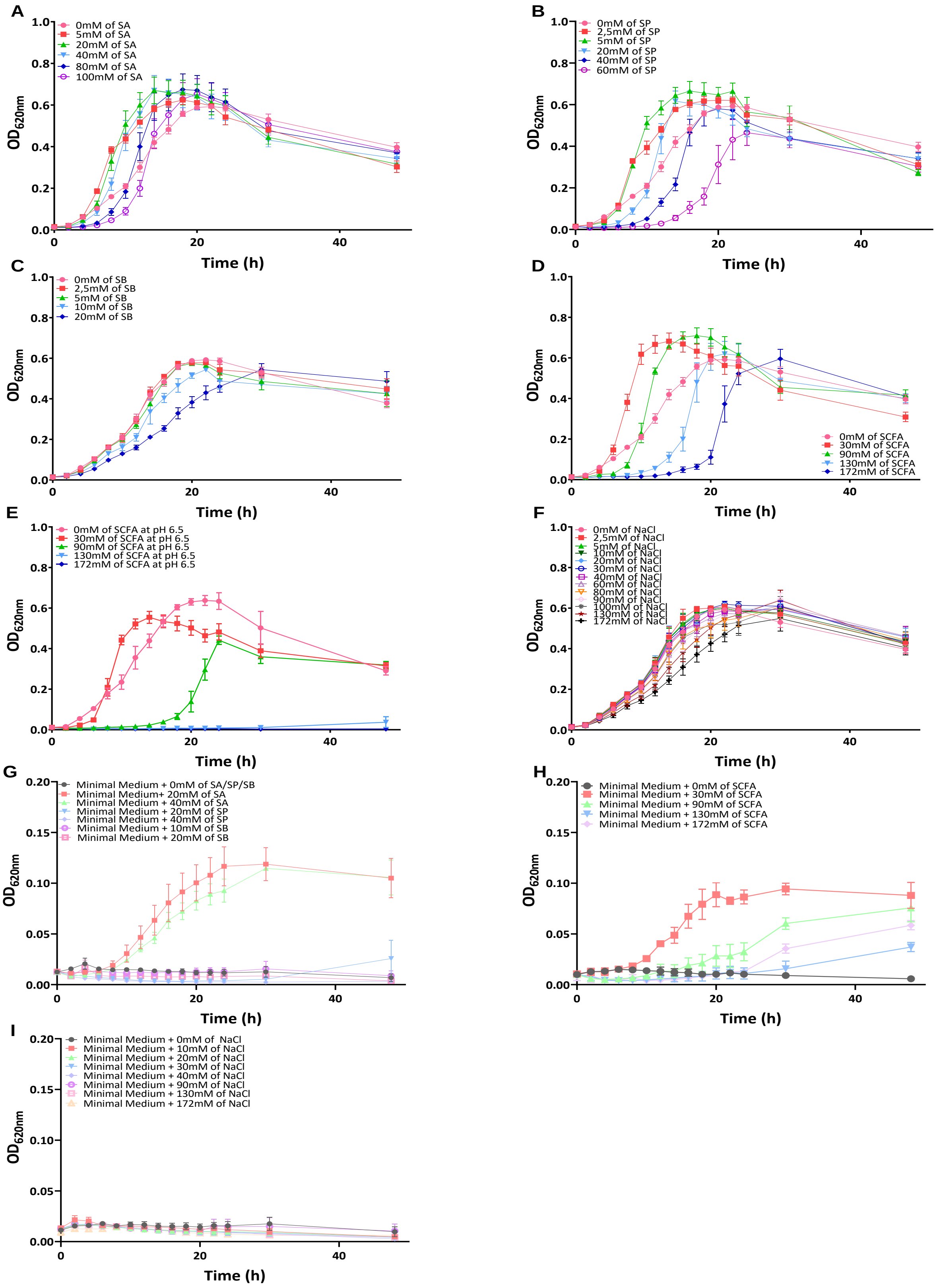


Figure 3. Growth curves for the *Aliarcobacter butzleri* DQ40A1 strain in the presence of different concentrations of SCFA, such as sodium acetate (SA) (A), sodium propionate (SP) (B), sodium butyrate (SB) (C), Mixtures of the three SCFA (D), Mixtures of the three SCFA at a pH adjusted to 6.5 (E), different concentrations of NaCl (F), to exclude the effects of the osmolarity, SA, SP and SB in Minimal Medium (G), Mixtures of the SCFA in Minimal Medium (H) and NaCl in Minimal Medium (I). The data represents the mean ± the standard error of the mean of at least three independent results. Results were analysed using a two-way ANOVA test (statistical analysis is presented in table S1 of the Annex I).

As displayed in Figure 3, *A. butzleri* DQ40A1's growth was assessed with different concentrations of SCFA, measured every two hours. This strain served as a reference for subsequent assays and as a comparison with the remaining seven strains.

Regarding the effect of SA, it was generally observed a potentiated growth with 5, 20, 40 and 80 mM during the exponential phase. However, the highest concentration analysed had no significant effect on this strain's growth (Figure 3A).

Identically, SP concentrations of 2.5, 5 and 20 mM increased the proliferation of the strain over the exponential phase (Figure 3B). An increase of the lag phase was verified with 40 and 60 mM_SP, followed by reduced growth after 8 h. Overall, SB demonstrated no discernible effect at lower concentrations, however, a slight reduction in growth was observed at a 20 mM concentration (Figure 3C).

When the results from the remaining seven *A. butzleri* isolates are considered, higher concentrations of SA generally led to an increase in the lag phase and/or a reduction in growth. Whereas other tested concentrations had no significant impact on the behaviour of the strains (Figure S1A to S7A of Annex I).

Concerning the SP effect, strains A11-1, W12-1 and INSA2999 showed increased bacterial growth at lower concentrations (Figures S1B, S2B and S5B, respectively). This was also verified with AB28/11 at 5 mM_SP (Figure S3B). Strains W12-1, AB28/11, INSA2756 and INSA2999 showed an increased lag phase and reduced growth at 60 mM_SP, similarly to the DQ40A1 strain's results (Figures S2B to S5B and 3D, respectively). In turn, strains from water-origin appeared potentially less affected by SP, while INSA3800 and INSA4015 were more affected by higher SP concentrations, thus indicating a strain-dependence effect (Respectively, Figures S1B, S2B, S6B and S7B).

The growth profile obtained for lower concentrations of SB was similar to the results presented for *A. butzleri* DQ40A1 (Figure 3C). However, the strain W12-1 presents higher growth at 48 h with 2.5 mM (Figure S2C). In turn, 5 mM_SB reduced growth in the A11-1, W12-1 and INSA2756 strains (Figures S1C, S2C and S4C). Overall, all the strains' growth was affected by 20 mM_SB, while AB28/11, INSA2999 and INSA3800 strains were less affected at 10 mM_SB, as presented, respectively, in Figures S3C, S5C and S6C.

Some studies carried out with *E. coli* have shown that SA is capable of inducing growth inhibition when the strain is incubated with high concentrations, such as 200 mM for the study conducted by Landwall and Holme (1977) and 128 mM in the study performed by Pinhal *et al.* (2019). These studies are in line with the observation taken from the results of the seven strains present in Figures S1 to S7 of Annex I, at a concentration of 100 mM_SA. In turn, the observed inhibitory effect of SP has been reported for other pathogens, being observed a slower growth rate and prolonged lag phase for *Salmonella* Typhimurium, associated with the disruption of intracellular pH homeostasis caused by higher concentrations of propionate (propionic acid)(12.5 mM and 25 mM) (Jacobson *et al.*, 2018). Butyrate, in the form of butyric acid was also studied in order to understand its influence on bacterial growth, and Namkung *et al.* (2011) concluded that butyrate was able to inhibit the bacterial growth of both *Clostridium perfringens* and *S.* Typhimurium in a concentration-dependent manner, such as observed by us in the present study.

Considering that varying concentrations of SCFA may be encountered along the intestine (Tan et al., 2014), we proceeded with the evaluation of its effect on bacterial growth, using the range and composition parameters, previously described by (Herold *et al.*, 2009). Moreover, for *A. butzleri* DQ40A1, we also tested pH of 6.5 to simulate *in vivo* colonic conditions (Zhang *et al.*, 2020). In fact, a notable distinction arises when comparing Figure 3D (pH of 7.18-7.30) and Figure 3E (pH 6.5). At a pH of 7.18-7.30, we observed that 30 and 90 mM_SCFA stimulated bacterial growth, during the exponential and stationary phases, whereas the 130 and 172 mM_SCFA extended the lag phase. Conversely, at a pH of 6.5, Figure 3E shows earlier growth at 10 h with 30 mM_SCFA, an increased lag phase with 90 mM_SCFA and a total inhibition of growth with the two higher concentrations. This discrepancy suggests that pH influences how this bacterium responds to SCFA, with varying pH levels along the gastrointestinal tract potentially eliciting different effects on the microorganisms, such as previously observed by Ghorbani *et al.*, (2015), with *P. aeruginosa*. In this study, it was verified that this bacterium's growth was affected by SCFA in a concentration and pH-dependent manner, as the growth was reduced with decreasing pH in the presence of SCFA. Furthermore, Zhang *et al.* (2020), also observed that the colonic conditions (pH 6.5 and 65-123 mM_SCFA) presented bacterial growth inhibition in *E. coli*. This inhibition aligns with the findings of *A. butzleri*'s DQ40A1 strain with the 130 and 172 mM concentrations. These results suggest that *A. butzleri* responds differently to SCFA based on environmental conditions, emphasizing the importance of considering pH levels and SCFA concentrations when studying bacterial growth dynamics.

The remaining seven strains' behaviour was analysed with mixtures of SCFA at pH of 7.18-7.30 (Figures S1 to S7 of Annex I). Only A11-1 and INSA2999 strains showed a similar behaviour to the DQ40A1 strain at 30 mM_SCFA (Figures S1D, S5D and 3D). At a concentration of 90 mM, there appeared to be no similarities between the DQ40A1 strain and the remaining seven strains. Regarding higher concentrations, overall, all the isolates had their growth inhibited with 130 and 172 mM_SCFA.

Studies considering the effect of SCFA on other bacterial species have been conducted to understand their outcome on bacterial growth. In a study with *P. aeruginosa,* low concentrations of SCFA (0.5-2.5 mM) provided a boost during mid-log phase whereas, higher concentrations (25-50 mM) were able to decrease bacterial growth (Ghorbani *et al.*, 2015). Furthermore, according to Zhang *et al.* (2020), *E. coli* showed growth potentiation at ileal conditions (pH 7.4 and 12 mM_SCFA), which corresponds to some strains' behaviour with the 30 mM concentration used in this study, such as A11-1, AB28/11, DQ40A1 and INSA 2999 , respectively demonstrated in Figures S1D, S3D, 3D and S5D. In case of infection by these pathosymbionts, it would be expected that the composition of the microbiota responsible for the production of the different concentrations of SCFA could be altered, suggesting a reduction in its concentration, which would allow a more favourable environment for the growth of enteropathogens in the intestine (Zhang *et al.*, 2020).

Regarding Figure 3F, as previously referred, NaCl showed no significant effects on bacterial growth at lower concentrations, but there appears to be a decrease in growth at the exponential phase with 130 and 172 mM_NaCl. Some strains presented a comparable behaviour as the DQ40A1 strain, due to their low susceptibility to NaCl, except at higher concentrations, such as A11-1, AB28/11, INSA2999, INSA3800 and INSA4015 (Figures S1E, S3E, S5R, S6E and S7E, respectively). The growth of *A. butzleri* in various concentrations of NaCl has been previously investigated by D'sa and Harrison (2005), who demonstrated that this species is able to thrive in a wide range of NaCl concentrations, ranging from 0.09% (15.4 mM) to 3.5% (590 mM) of NaCl, showing its resilience and ability to withstand higher osmotic differences.

In order to investigate the potential positive effect of SCFA on *A. butzleri*'s growth, bacterial growth was also tested in conditions with a minimal medium. In fact, minimal medium usually does not allow the growth of certain bacteria that need specific elements to grow (Bonnet *et al.*, 2020), as it was observed for all the *A. butzleri* tested strains. Therefore, the impact that SCFA may have on bacterial growth in minimal medium was evaluated by supplementing minimal medium with SA, SP, SB, SCFA and NaCl, as osmotic control, at various concentrations. Under incubation in minimal medium, the growth of *A. butzleri* DQ40A1 strain was clearly influenced by both concentrations of SA (20 mM and 40 mM), as evident in Figure 3G. A similar effect was observed with 30 and 90 mM_SCFA, showing an increase in growth. However, the highest concentrations only appeared to have an improvement in growth at around 48 h (Figure 3H). Overall, NaCl showed no significant effect on the growth in minimal medium, except for a slight decrease in growth at concentrations of 130 and 172 mM, as shown in Figure 3I.

Regarding these results in Figure 3, the strain whose behaviour is most similar to DQ40A1 was AB28/11. The remaining strains, although having differences among each other, share some results with this strain, such as a potentiation effect with SA, presented in Figures S1F to S7F, and 30 mM_SCFA for A11-1, 30 and 90 mM for the strains INSA2756, INSA2999 and INSA4015 (Figures S1G, S4G, S5G and S7G, respectively). W12-1 and INSA3800 also presented a potentiation effect by the 30, 90 and 130 mM_SCFA (Figures S2G and S6G). DQ40A1 was the only strain that did not show any influence by SP at any concentration tested (Figure 3G).

Upon the analysis of the results, it is to notice that SA may possess an enhancing effect on this strain's growth acting as a carbon source, as has been previously reported for *A. butzleri* (Buzzanca *et al.*, 2023). Similar concentrations of SA are used in the 30 and 90 mM_SCFA, indicating that SA could also be responsible for the growth potentiation effect of the mixtures. Other bacteria have been shown to be able to grow in minimal medium supplemented with acetate, such as *P. aeruginosa* (Kretzschmar, Schobert and Görisch, 2001) or *E. coli* (Oh *et al.*, 2002), also due to their ability to use this substrate as a carbon source.

When considering the osmolarity effect, Figure 4, by establishing a correlation in function of NaCl, the 30 and 90 mM_SCFA maintained their potentiation effect at early times (12 h), as NaCl displayed no significant effect, and its behaviour resembled the TSB control (absence of SCFA/NaCl) (Figures 3D and 3F). Whereas a growth inhibition was noted at 130 and 172 mM_SCFA, evident from the low percentage at 12 h of incubation, which subsequently increased in the following incubation periods associating with the end of the extended lag phase previously verified with the higher concentrations (Figure 4). This aligns with the results from Figure 3D, suggesting that NaCl may have no discernible effect on this strain's growth, as it maintains its behaviour towards SCFA, despite showing a reduction in growth with higher concentrations compared to the TSB control in Figure 3F. Regarding the remaining seven strains under study, the profiles differed from the DQ40A1 strain, having several significant differences in growth when compared in function of NaCl. Despite the differences, 5 strains (W12-1, AB28/11, INSA 2756, INSA3800 and INSA4015) displayed behavioural similarities in the presence of high concentrations of SCFA when compared to TSB control, although exhibiting a susceptibility to NaCl at a concentration of 130 mM (Figure S8).

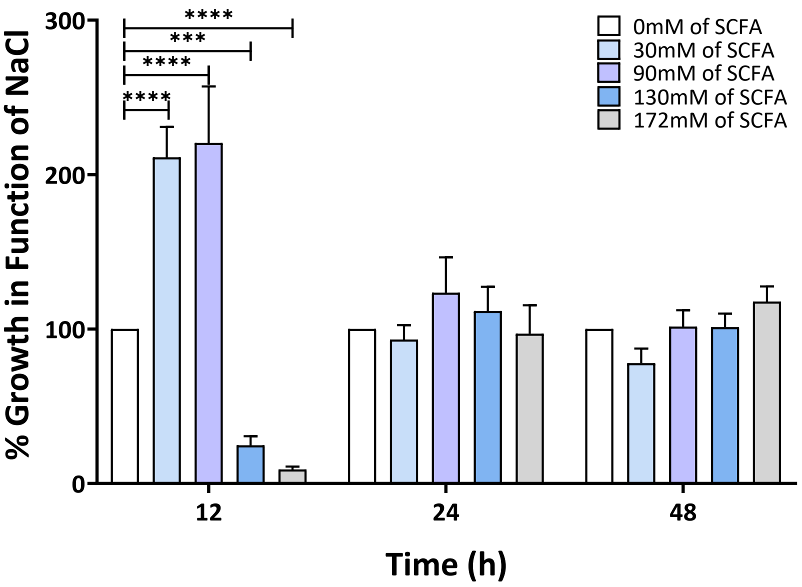


Figure 4. Percentage of growth of the *Aliarcobacter butzleri* DQ40A1 strain in the presence of different concentrations of SCFA mixtures in function with growth in the correspondent NaCl concentration. The data represents the mean ± the standard error of the mean of at least three independent results. Results were analysed using a two-way ANOVA test. ***p<0.001, ****p<00001.

Due to the noticeable differences in the seven strains' behaviour towards the osmotic control results, it would be fair to assume that NaCl, as an osmotic control, might influence bacterial growth, therefore resulting in alterations when compared to the SCFA. Overall, despite the variability of these results, it is clear that SCFA individually or in mixture have an effect on *A. butzleri's* growth behaviour, especially given their described role as a colonization resistance mechanism. This effect could be linked to higher concentrations, that serve to repress the bacterial growth when the pathogen reaches the intestine.

If taking the incubation period of 24 h in Figure 5, the growth behaviour seems to be strain-dependent and no correlation with the origin of the strains was found. For example, no profile similarity between water strains (A11-1 and W12-1) was observed, such as in the case of food-origin strains (AB28/11 and DQ40A1) (Figures 5A, 5B, 5C and 5D, respectively). Moreover, when comparing the human strains, notable differences among them were observed. The *A. butzleri* strain INSA2999 presented in Figure 5F, showed higher tolerance to SCFA and NaCl than the remaining strains, while all the strains were more susceptible to 130 and 172 mM_SCFA comparing with the same NaCl concentration. *A. butzleri* INSA2756 not only was affected by most SCFA concentrations but by the NaCl concentrations as well, indicating potential susceptibility to osmolarity effects (Figure 5E). In turn, *A. butzleri* INSA3800 and INSA4015 strains showed similar susceptibility for both SCFA and NaCl (Figures 5G and 5H).

Overall, the results of this assay underscore the diverse and strain-specific behaviours of *A. butzleri*, suggesting that the high genetic variability observed within this species may be more relevant for the tolerance profile than an origin correlation among the strains.

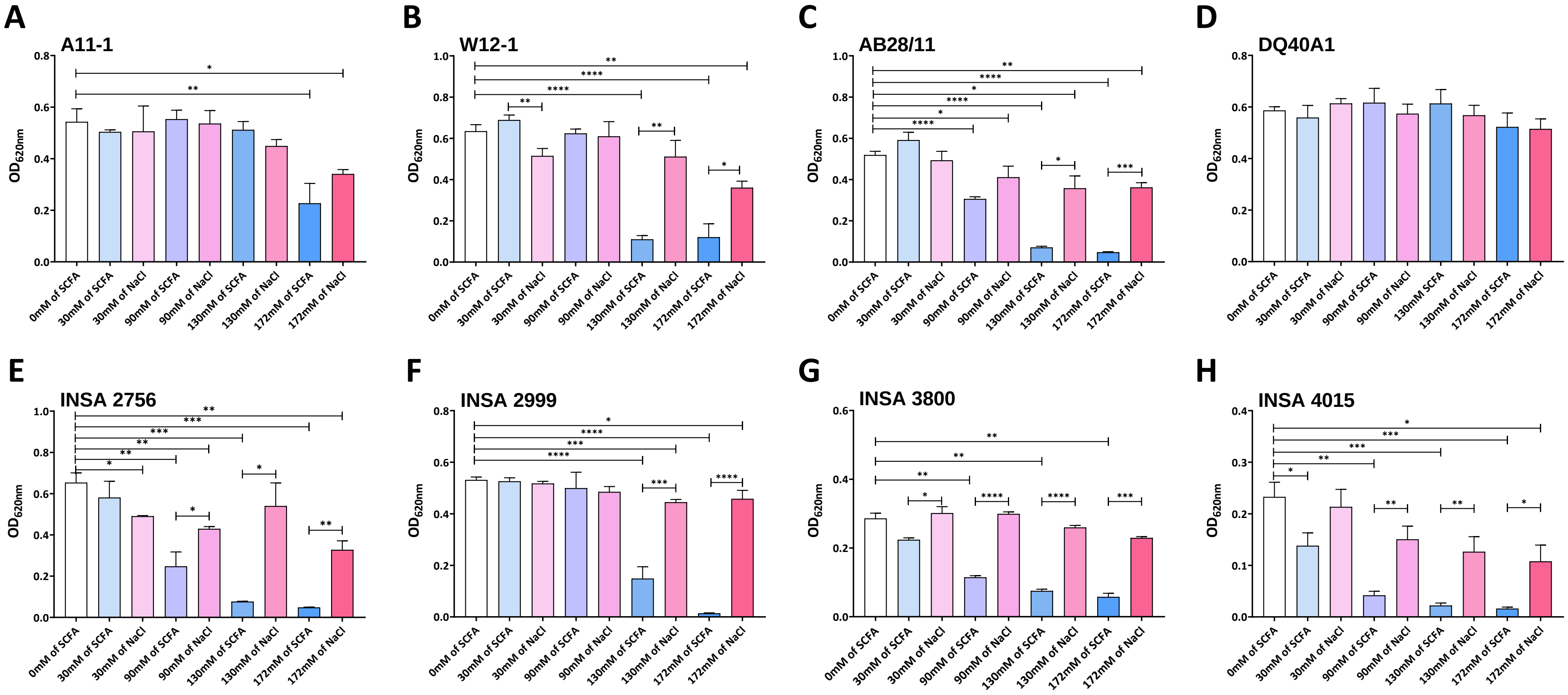


Figure 5. Bacterial growth of the eight *Aliarcobacter butzleri* strains used in this study, in presence of SCFA mixtures and respective NaCl concentrations, at 24 hours, A11-1- isolate (A); W12-1 isolate (B), AB28/11 isolate (C), DQ40A isolate (D), INSA2756 isolate (E), INSA2999 isolate (F), INSA3800 isolate (G) and INSA4015 isolate (H). The data represents the mean ± the standard error of the mean of at least three independent results. Results were analysed using a Student's t- test against absence of SCFA and the respective NaCl osmotic controls. *p<0.05, **p<0.01, ***p<0.001, ****p<00001.

4.2. Effect of SCFA on the gene expression of *Aliarcobacter butzleri*

Aliarcobacter virulence genes have been the subject of numerous investigations, owing to their potential role in the infection and colonization abilities of this bacterium (Levican *et al.*, 2013; Mateus *et al.*, 2023). Despite pointed as putative virulence genes, the significance of these genes in the virulence of bacteria underscores the imperative to explore and understand their functions thoroughly.

In this research, particular attention was directed towards putative virulence genes, with a focus on assessing their expression variability when *A. butzleri* is exposed to SCFA and NaCl, which served as an osmotic control. The analysis was performed by quantitative real-time PCR.

This assay was carried out with three of the SCFA mixtures, 30, 90 and 130 mM concentrations, along with the corresponding NaCl concentrations. The 172 mM concentration was excluded from this assay due to its negative impact on the overall growth behaviour of the strains under study. Furthermore, the remaining three concentrations seemed to represent properly the different concentrations along the intestine.

Four genes were selected, namely *cadF*, *ciaB*, *flaA* and *luxS*, given their studied correlation with motility, biofilm formation, adhesion and invasion, which are important for this study. The *cadF* and *ciaB* genes have been taken under study to possibly understand the adhesion and invasion abilities in *A. butzleri,* however no correlation has been found yet (Levican *et al.*, 2013); *flaA* is involved in motility (Ho *et al.*, 2008), being the flagella not only responsible for motility, but is also involved in biofilm formation, adhesion and invasion (Haiko and Westerlund-Wikström, 2013; Salazar-Sánchez *et al.*, 2022). The gene *luxS* has been associated with quorum sensing, a process of bacterial cell-to-cell communication important for biofilm formation, and appears to have a positive role in *A. butzleri’s* biofilm formation capability (Papenfort and Bassler, 2016; Salazar-Sánchez *et al.*, 2022).

The findings illustrated in Figure 6A show the fold change of the selected virulence genes in the presence of SCFA, where *ciaB* gene demonstrated overexpression in the presence of 90 and 130 mM_SCFA, along with 90 and 130 mM_NaCl. These results indicate a gene regulation by both SCFA and NaCl, however, when normalizing fold change in presence of SCFA in terms of NaCl concentration, an upregulation was noted only at a concentration of 130 mM_SCFA (Figure 6B). However, the overexpression verified, could suggest an augmented ability to invade host cells when incubated with SCFA at 90 and 130 mM concentrations or 130 mM if it is analysed in function of NaCl.

Furthermore, genes *flaA* and *luxS*, exhibited significant enhancement in response to the SCFA at a 90 mM concentration, which maintained its behaviour when in function of the osmotic controls in Figure 6B. The *flaA* showed a slight reduction in expression in Figure 6A when in the presence of 30 mM_NaCl suggesting a gene regulation under the effect of this osmotic concentration, which can correlate with the upregulation of this gene's expression at 30 mM_SCFA (Figure 6B). Furthermore, *luxS* also presented an enhanced expression at 130 mM_SCFA (Figure 6A and 6B). These upregulations could imply a potential for increased motility and biofilm formation at these concentrations due to the presence of SCFA.

Previous studies examining the impact of SCFA on bacterial behaviour have yielded divergent outcomes. For SP and SB, at a concentration of 20 mM, an effect of upregulation was observed for the transcription of flagellar genes in Adherent-Invasive *Escherichia coli,* while concurrently downregulating the capsule assembly and transport genes (Pace *et al.*, 2021). When taken into consideration the individual concentrations of SP and SB present in the mixtures used in the current study, it is possible to estimate a resemblance with the 90 mM concentration of SCFA, containing 31.5 mM_SP and 9 mM_SB, which upregulated the expression of *flaA,* similarly to the results of the mentioned study. A separate study involving *E. coli*, contradicts the results from the previous and current study, reporting that colonic conditions (pH 6.5 and 65 mM to 123 mM_SCFA mixture) led to a downregulation of the virulence gene expression, responsible for motility, adhesion and invasion (Zhang *et al.*, 2020). However, given that the pH used in the present study is 7.18-7.30, which differs from the above-mentioned study, it would be possible to assume a connection between the pH variation and the divergent results.

Considering the importance of virulence gene expression, analogous investigations were undertaken with *Salmonella* Typhimurium. One study established a reduction in expression of the genes responsible for invasion (*hilA, invF* and *sipC*) by 30 mM_SP and SB, with 30 mM_SA demonstrating the ability not only to increase this expression but also to restore it in a mutant strain at a pH of 6.7. They also evaluate mixtures of SCFA at a concentration of 30 mM (25 mM_SA; 2.5 mM_SP; 2.5 mM_SB) and 200 mM_SCFA (110 mM_SA; 70 mM_SP; 20 mM_SB), which showed similar effects as the single SCFA. In fact, 30 mM_SCFA resulted in the same conclusions taken from the SA alone, and 200 mM were found to decrease the invasion gene expression, as did the SP and SB alone (Lawhon *et al.*, 2002). In turn, considering *ciaB* gene as an invasion coding, these results do not correlate with what can be observed in Figure 6A and 6B, as there was a noticeable increase in the expression of the *ciaB* gene, both at 90 mM (49.5 mM_SA; 31.5 mM_SP; 9 mM_SB) and 130 mM_SCFA (71.5 mM_SA; 45.5 mM_SP; 13 mM_SB), possibly due to the SA concentrations. An alternative study with this bacterium, reported the upregulation of crucial invasion and biofilm formation related virulence genes in the presence of acidic acid and butyric acid, resulting in higher virulence (Lamas *et al.*, 2019). Despite the fact that the acid forms were used instead of SA and SB, these results are in fact more similar to the results of present study, than the previous ones, as there is also an overexpression of invasion related genes. Regarding the biofilm formation, these results also correspond to the conclusion taken from the present study, where an upregulation in the associated virulence genes, such as *luxS* and *flaA*, is observed.

The mentioned observations suggest a potential influence by SCFA on the expression of *A. butzleri*'s putative virulence genes.

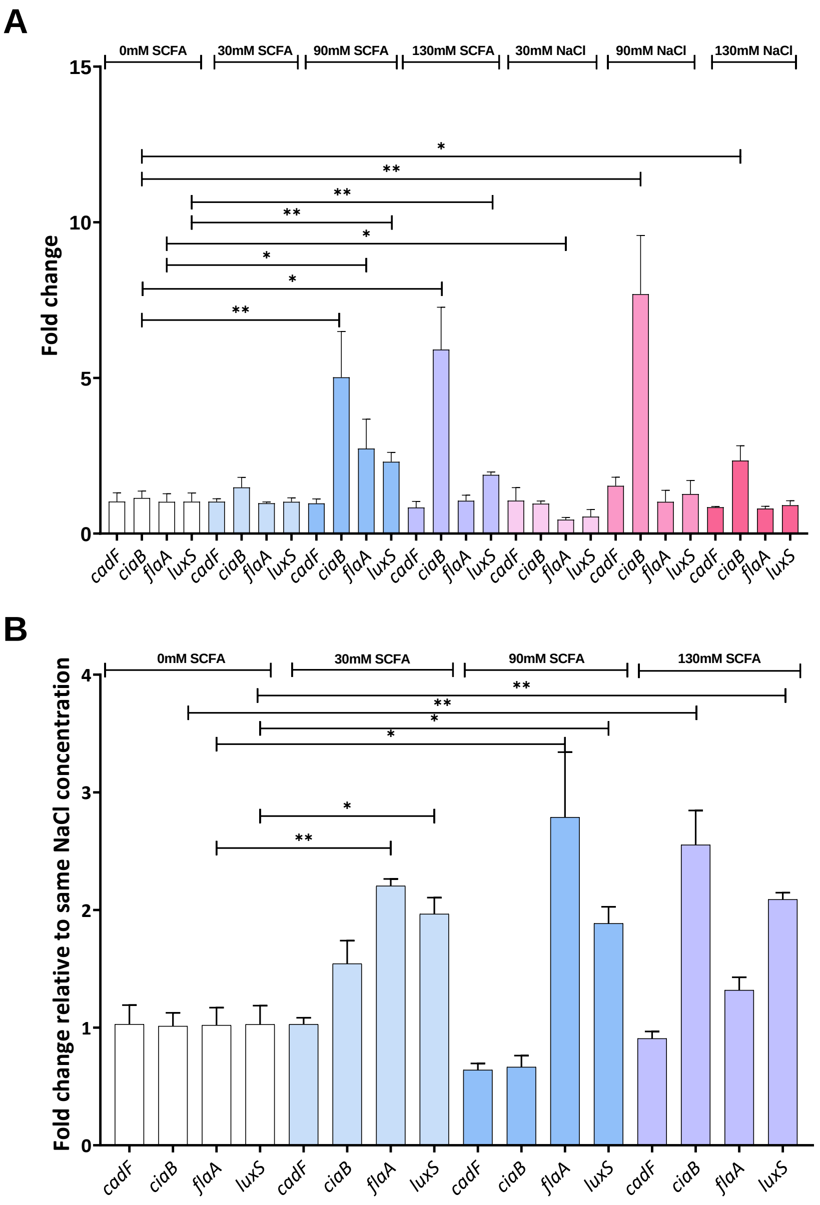


Figure 6. Relative expression of *cadF*, *ciaB*, *flaA* and *luxS* genes of *Aliarcobacter butzleri* DQ40A1 in the presence and absence of SCFA mixture and correspondent NaCl concentration. Fold change comparing with absence of compounds (A) and fold change of gene expression corresponding to SCFA exposure in function of the corresponding NaCl concentration (B). The data represents the mean ± the standard error of the means of at least three independent results. Results were analysed using a Student's t- test against absence of SCFA and the respective NaCl osmotic controls. *p<0.05, **p<0.01.

4.3. Effect of SCFA on the motility evaluation of *Aliarcobacter butzleri*

The assessment of motility stands as an extremely important virulence factor to incorporate in this study, offering insights into its impact on this bacterium's virulence, given that bacterial motility allows microorganisms to navigate, change environments and evade adverse conditions. This adaptive capability allows them to colonize diverse environments, facilitating the adhesion and invasion of epithelial cells, thereby increasing their virulence and survival (Duan *et al.*, 2013).

Consequently, the bacterial motility evaluation was conducted with SA, SP, SB, as well as SCFA and NaCl for the *A. butzleri* DQ40A1 strain, and with the mixtures of SCFA and NaCl for the remaining strains comprised in this study. This analysis aimed to discern whether these compounds would induce any consequences on this virulence factor.

Figure 7 represents the results of the motility assay of the *A. butzleri* DQ40A1 strain when exposed to SCFA. Some discrepancies between the presence of NaCl and SCFA were verified, with 40 mM_SA and SP at 24 h but also 20 and 40 mM_SP at 48 h showing a significant decrease in motility when compared to corresponding concentrations of NaCl (Figures 7A and 7B, respectively). The concentration of 40 mM_SP led to a decrease of motility at both 24 and 48 h when compared with the absence of SCFA and NaCl in Figure 7B, unlike the other tested conditions. Nonetheless, SB showed no discernible effect on the motility of this strain. When the percentage of motility is compared in function of NaCl, 40 mM_SP maintained its behaviour at 24 h, as it already displayed a significant decrease compared to NaCl. In turn, when in function of the effect observed with NaCl, all tested compounds and concentrations, with the exception of 20 mM_SA and 10 mM_SB, appeared to reduce motility of *A. butzleri* DQ40A1 at 24 h, while at 48 h of incubation only SB showed no significant reduction on the motility halo (Figure 7E). Despite not being observed an effect of NaCl on motility halo, there seems to be an influence of osmolarity on the strain's behaviour and motility.

When analysing the results of the effect of SCFA, it can be seen that at 90 and 130 mM a clear reduction of the motility of this strain occurred, which is also visible when in function with the NaCl concentration (Figures 7D and 7F, respectively). Interestingly, the inhibition profile is similar to the one observed for SP, pointing for a potential role of this compound on the noticed effect (Figure 7B). These results also show that the behaviour of the strain appears to vary with the osmolarity.

It is important to note that an overexpression of the *flaA* gene was observed in presence of 30 and 90 mM_SCFA, when in function of NaCl. FlaA is acknowledged for its role in the motility capability of the bacterium (Ho *et al.*, 2008). Thus, initially, an enhanced expression of this gene would suggest that the motility ability would increase in the presence of these compounds, as demonstrated by Lackraj *et al.* (2016), where both motility and flagellar expression were increased by 30 mM_SCFA and decreased by 90 mM_SCFA, therefore suggesting a proportional relation between these virulence factors. However, the motility assay results presented in the current study did not corroborate this expectation, as it appears to exist an inverse relationship with the expression of virulence genes and motility. Furthermore, the conclusions taken from these results are similar to what was observed by Santos (2023), who showed an increase in the expression of the *flaA* gene by the SCFA mixture at 30 and 90 mM, when compared to the NaCl controls, and a consequent reduction in the *A. butzleri* DQ40A1's motility in the same conditions. However, more studies should be carried out in order to understand this bacterium's behaviour towards motility and flagellar gene expression.

Regarding the results in Figure 7, when in comparison to the other seven strains used in this study, it is possible to understand that there are many similarities between strains, with all strains being affected by 90 and 130 mM_SCFA, with a reduced motility being observed, except for INSA3800 that showed no significant differences in its behaviour by the SCFA (Figure S14). Furthermore, INSA2999 is noticeably the most affected by the highest SCFA concentration with no motility ability observed (Figure S13). Also, the negative impact on the motility of INSA4015 strain could be related to a decrease in bacterial growth caused by the 130 mM concentration (Figure S15). The results from INSA3800 strain showed that this is the only strain where the percentage of motility appeared to be increased by 130 mM_SCFA in function of NaCl, indicating a possible effect from osmotic conditions (Figure S14B).

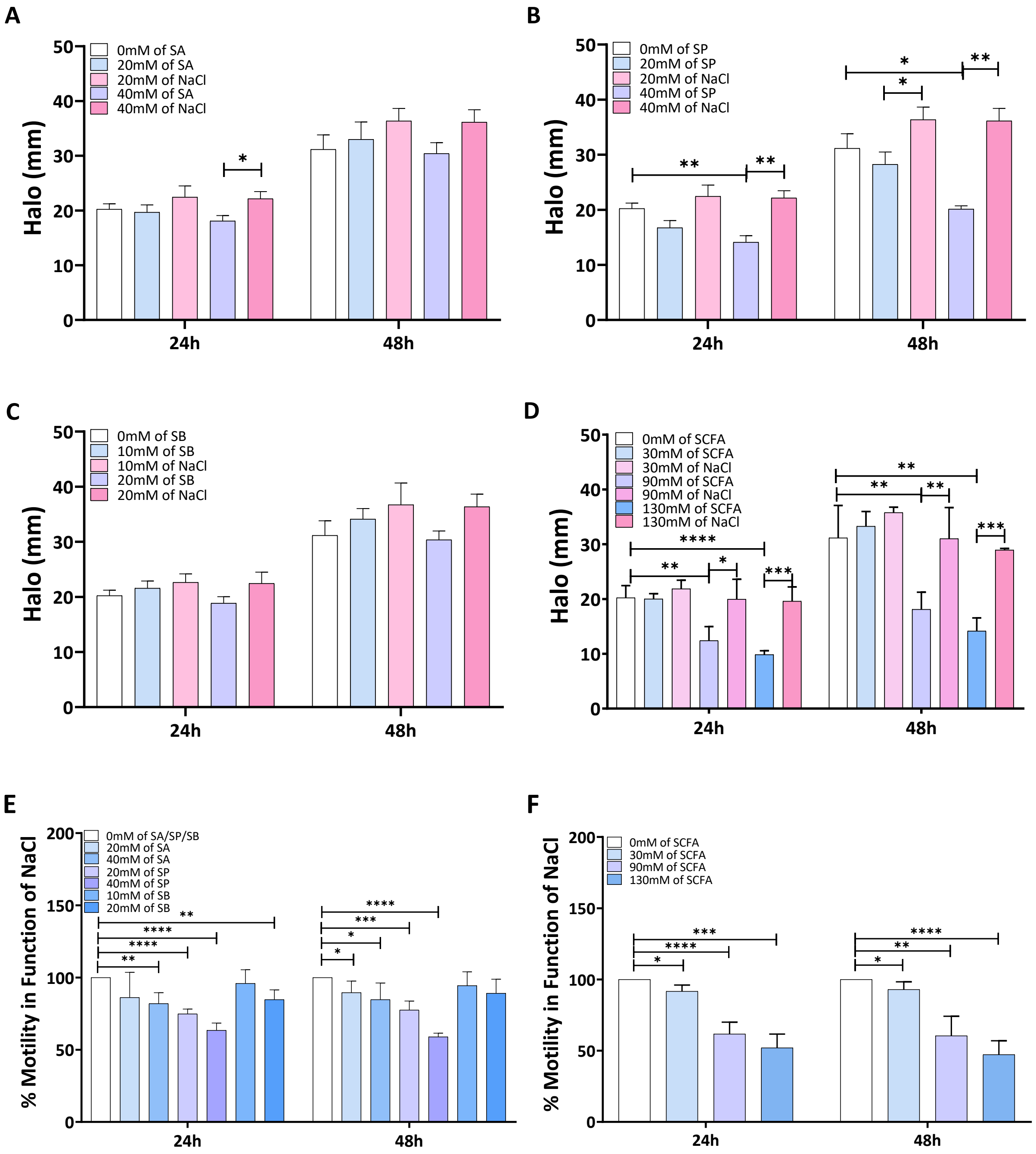


Figure 7. Effect of SCFA in the motility of *Aliarcobacter butzleri* DQ40A1 strain in the presence of different concentrations of SA (A), of SP (B); of SB (C); of SCFA mixtures (D). Percentage of motility with SA, SP and SB in function of the respective NaCl concentration (E). Percentage of motility with SCFA mixtures in function of the respective NaCl concentration (F). Data represents the mean ± the standard deviation of three independent results. Results were analysed using a Student's t- test against the absence of SCFA alone or in mixture and the respective NaCl osmotic controls. The $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<00001$.

Analysing the motility of the eight strains at 48 hours (Figure 8) and comparing by strains' origin, water strains A11-1 and W12-1 represented in Figures 8A and 8B, respectively, showed similar behaviours towards SCFA, except W12-1 was affected by 30 mM_SCFA. Strains from food processing responded similarly to high SCFA concentrations, both negatively affected (Figures 8C and 8D). Clinical isolates did not show clear origin correlation, with INSA3800, in Figure 8G showing a distinct behaviour compared to the other strains (Figures 8E, 8F and 8H). While there seems to be some origin-like behaviour among the strains, overall, the strains presented a similar response by higher SCFA concentrations. The complexity of the observed patterns suggests that factors beyond strain origin may contribute to the variations in motility among the strains.

The findings from the motility assay in this study align with some studies conducted on different microorganisms. For instance, research on *E. coli* has demonstrated susceptibility to SCFA, with a potentiation of motility at concentrations of 12 mM, and an inhibition of motility from 65 to 123 mM (Zhang *et al.*, 2020). Additionally, a study on *Salmonella enterica* have indicated a significantly reduction in motility by exposure to acetate, propionate and butyrate, in their acid form (Lamas *et al.*, 2019). Likewise, sodium butyrate at 5 and 20 mM has been shown to reduce motility in *C. jejuni* (Gunther *et al.*, 2023). These results do not correlate with the findings from this study, as SB showed no discernible effect on the motility of *A. butzleri*. However, the results showed that SP may be the reason behind the behaviour of the SCFA, as it is the compound that individually affected the most the motility of the bacterium.

The conclusions reached by these studies are in accordance with the present results, suggesting a general trend across different microorganisms, including *A. butzleri*, where there is an overall reduction in motility in response to multiple SCFA concentrations. The influence of SCFA in motility could suggest a modulation of this behaviour along different parts of the intestine, associated with a limitation to easily migrate to different locations and penetrate the mucus. This effect may have a role in stopping this pathogen from reaching its preferable niche and therefore slow the infection process.

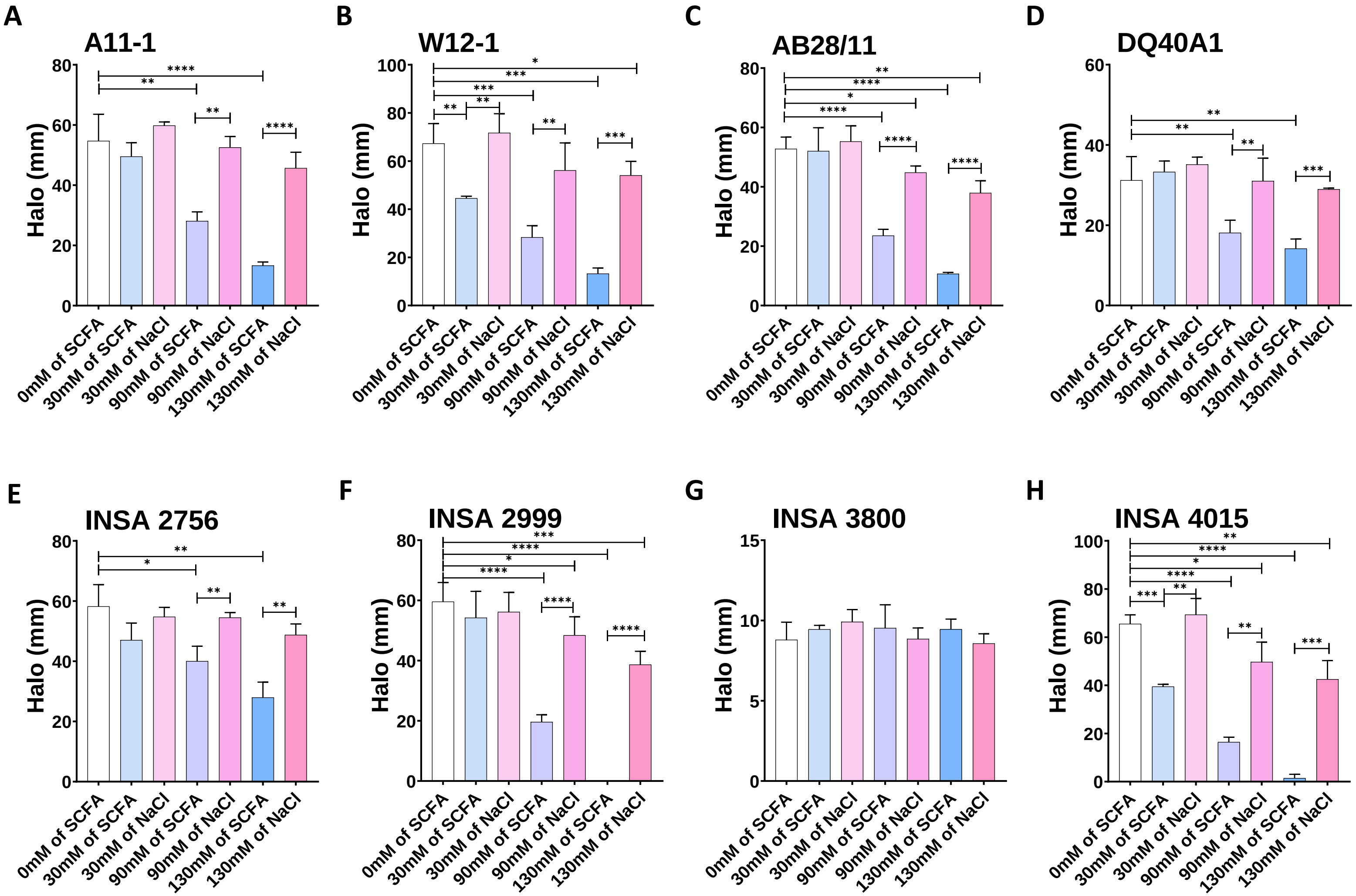


Figure 8. Bacterial motility of the SCFA mixtures and respective NaCl concentrations for the eight *Aliarcobacter butzleri* strains present in the study at 48 hours, A11-1- (A); W12-1 (B); AB28/11 (C); DQ40A1 (D); INSA 2756 (E); INSA 2999 (F); INSA 3800 (G); INSA 4015 (H). The data represents the mean ± the standard deviation of at least three independent results. Results were analysed using a Student's t- test against 0mM of SCFA. *p<0.05, **p<0.01, ***p<0.001, ****p<00001.

4.4. Effect of SCFA on the biofilm formation of *Aliarcobacter butzleri*

Biofilms represent complex structures formed by bacteria to provide protection against adverse conditions, exhibiting high resistance to eradication. They play an extremely important role in influencing the bacteria's pathogenicity, virulence, and survival (Salazar-Sánchez *et al.*, 2022).

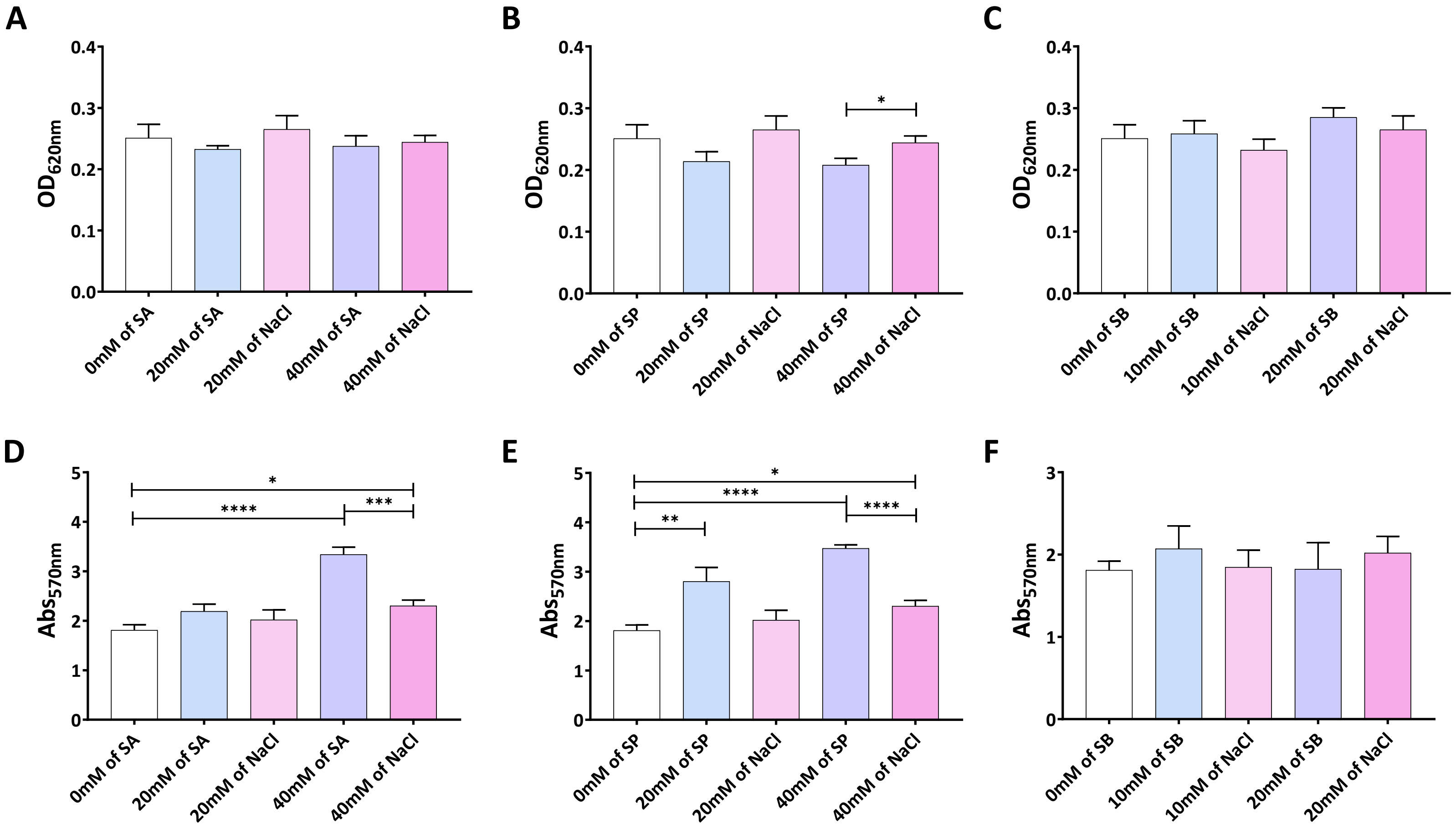
In this study, *A. butzleri*'s biofilm formation was evaluated in the presence of various compounds, including, SA, SP, SB, SCFA and NaCl. This aimed to unveil the impact of these substances on biofilm formation while excluding potential effects related to osmolarity.

To assess if the effect of SCFA in biofilm formation may relate to a growth reduction, the OD_{620nm} was measured previously to the staining of the biofilms (Figures 9A, 9B, 9C and 9G). Under the tested conditions, only 40 mM_SP reduced the growth comparing to the correspondent osmolarity control (40 mM_NaCl) (Figure 9B). Regarding the biofilm formation by *A. butzleri* DQ40A1, it can be observed that incubation with 40 mM_SA and 20 and 40 mM_SP led to an increase in biofilm formation, such as noticed with all the SCFA concentrations tested (Figure 9D, 9E, 9F and 9H). NaCl also displayed an effect on this strain's behaviour, as the 40 and 130 mM concentrations appeared to slightly potentiate the biofilm formation (Figures 9D, 9E and 9H), suggesting an influence by osmotic concentrations. Analysing the effect of the compounds on biofilm formation, the results suggest that SP is likely the main responsible for the effect observed with the mixtures, in line with the previously described for motility. SP was individually capable of altering the bacterium's behaviour towards the biofilm formation ability by increasing it, unlike the remaining compounds, and is therefore an important result to take into consideration.

Considering the classification of the strains regarding their ability to form biofilm in the absence and presence of SCFA, following the formula developed by Stepanović *et al.*, (2000), the strains may be classified as non-adherent, weakly adherent, moderately adherent and strongly adherent. In the absence of SCFA the strain INSA3800 is classified as non-adherent, A11-1 and AB28/11 as weakly adherent, INSA2999 and INSA4015 as moderately adherent and finally, the strains W12-1, DQ40A1 and INSA2756 are classified as strongly adherent. However, in the presence of concentrations of SCFA, five out of eight strains maintain their previous classification where, AB2811, INSA2999 and

INSA4015 were the exception. In the presence of 30 mM_SCFA both INSA2999 and INSA4015 strains become strongly adherent, but at a concentration of 90 mM_SCFA, while INSA2999 remains strongly adherent, INSA4015 converts to a classification as weakly adherent. The AB28/11 isolate when in the presence of 90 mM_SCFA becomes non-adherent, as well as INSA2999 and INSA4015 with 130 mM_SCFA.

Regarding the other seven strains' biofilm formation ability, W12-1 displayed a similar behaviour to the DQ40A1 strain, showing an increase in the biofilm formation with the three concentrations of SCFA (Figures S17B and 9H, respectively). The opposite can be notably seen with the INSA3800 and INSA4015 strains, displaying different behaviour from the other strains (Figures S21B and S22B). The results obtained for these strains, showed an overall decrease in the biofilm formation, which was not verified with the DQ40A1 strain, and may be correlated with each strain's natural ability to form biofilm. Furthermore, unlike the behaviour of most strains, AB28/11 displayed no significant results regarding biofilm formation. Comparing the effect of SCFA in *A. butzleri* DQ40A1 with the remaining seven strains presented in Figure 9L and Figures S16D to S22D, no discernible effect on biofilm percentage of strain INSA2756 in the presence of SCFA mixtures in function of the controls with NaCl was observed as well (Figure S19D). The other strains showed different behaviours from the DQ40A1, in Figure 9L, however, for both strains A11-1 and INSA3800, a positive effect regarding the biofilm percentage in the presence of 30 mM_SCFA was observed, contrasting with a negative impact by the 90 and 130 mM_SCFA (Figures S16D and S21D). For INSA4015, similarly to these previous strains, biofilm was also affected by the 90 and 130 mM_SCFA (Figure S22D). The results of INSA3800 presented in Figure S21D, unlike any other strain, exhibit 0% of biofilm formation with 90 and 130 mM_SCFA, when in comparison to the osmotic concentrations.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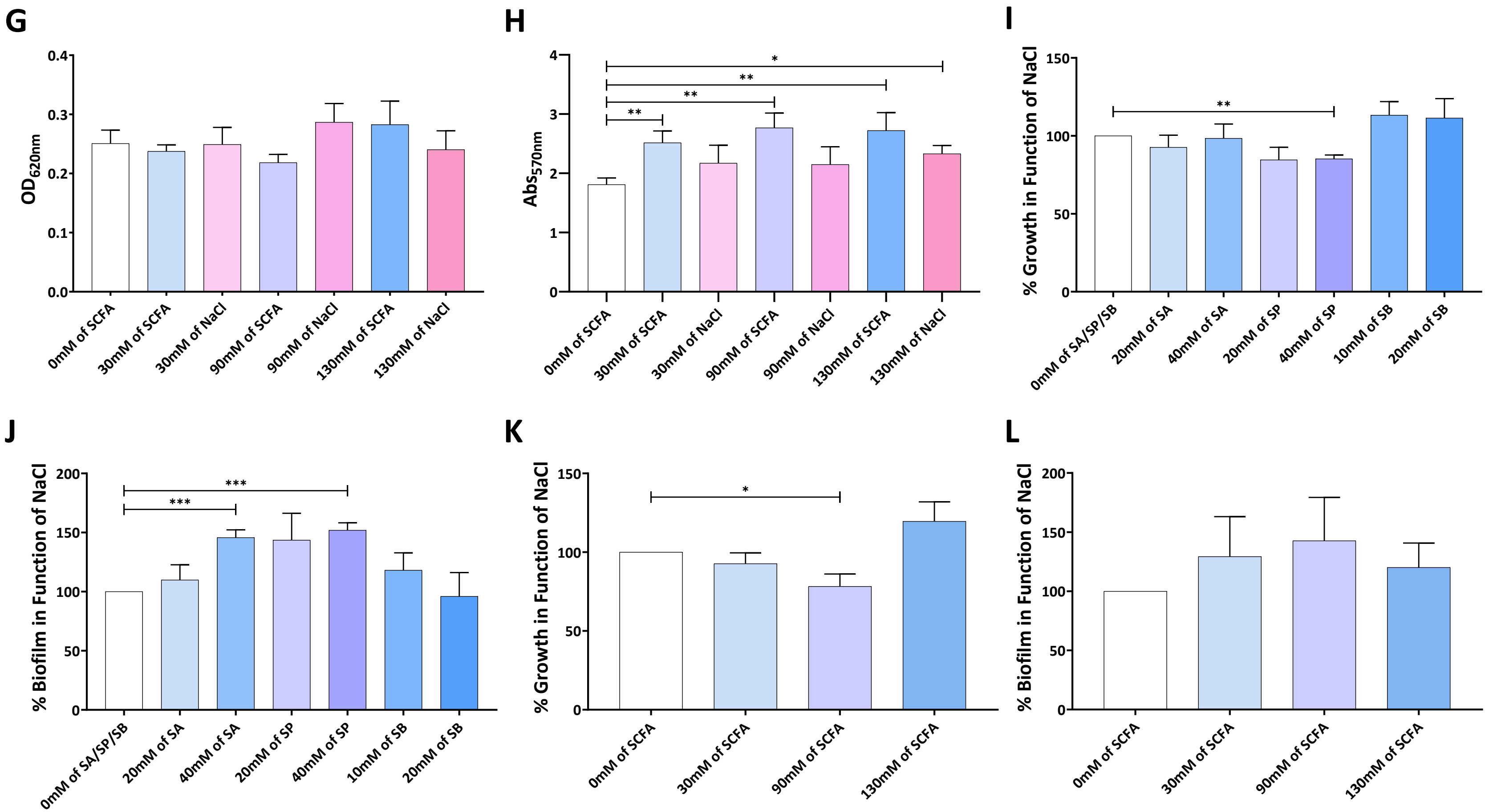

Figure 9. Bacterial growth of the *Aliarcobacter butzleri* DQ40A1 strain in the presence of SA and respective NaCl concentrations (A); SP and respective NaCl concentrations (B); SB and respective NaCl concentrations (C); Evaluation of biofilm formation by violet crystal staining in the presence of SA and respective NaCl concentrations (D); SP and respective NaCl concentrations (E); SB and respective NaCl concentrations (F); and growth and biofilm formation in presence SCFA mixtures and respective NaCl concentrations (G and H, respectively). Percentage of growth and biofilm in function of respective NaCl concentrations in the presence of SA, SP and SB (I and J, respectively). Percentage of growth and biofilm in function of respective NaCl concentrations in the presence of SCFA (K and L, respectively). The data represents the mean ± the standard error of the mean of at least three independent results. Results were analysed using a Student's t- test against absence of SCFA alone or in mixture and the respective NaCl osmotic controls. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<00001$.

Overall, each strain exhibits a unique behaviour, making it challenging to conclude any origin-related pattern. However, most strains displayed an increase in biofilm formation when incubated with 30 mM_SCFA, with the strains, W12-1, DQ40A1 and INSA2756 being also positively affected by the 90 mM_SCFA, while INSA3800 and INSA4015 were affected negatively (Figures 10B, 10D, 10E, 10G and 10H, respectively). Regarding the 130 mM concentration, with the exception of AB28/11 and INSA2756, most strains demonstrated a negative response to this mixture in comparison to the absence of SCFA (Figures 10C and 10E). These findings underscore the strain-specific responses to SCFA and their complexity of biofilm formation in different strains of *A. butzleri*. Additionally, these differences may imply that the strains behave differently in *in vivo* conditions, underscoring the complexity of *A. butzleri*'s responses.

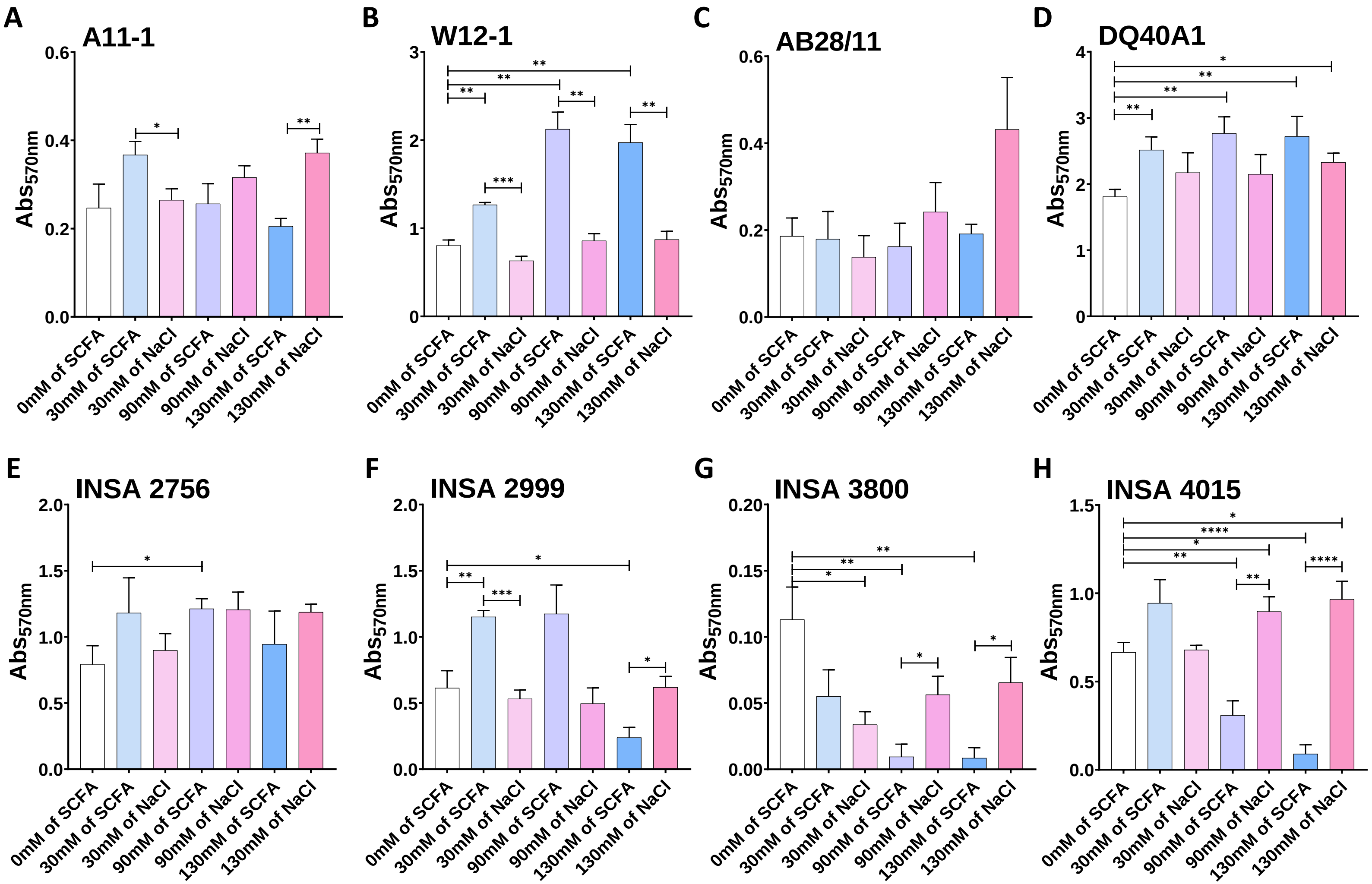

Figure 10. Biofilm formation in the presence of SCFA mixtures and respective NaCl concentrations for the eight strains present in the study at 570nm, A11-1- isolate (A); W12-1 isolate (B); AB28/11 isolate (C); DQ40A isolate (D); INSA 2756 isolate (E); INSA 2999 isolate (F); INSA 3800 isolate (G); INSA 4015 isolate (H). The data represents the mean ± the standard error of the mean of at least three independent results. Results were analysed using a Student's t- test against 0mM of SCFA and respective NaCl osmotic controls. *p<0.05, **p<0.01, ***p<0.001, ****p<00001.

Considering the effect of SCFA on the regulation of putative biofilm-related genes in Figure 6B, it can be seen that, in *A. butzleri* DQ40A1, *luxS* was overexpressed with all the concentrations of SCFA, while *flaA* was overexpressed with the 30 and 90 mM concentrations. Both putative virulence genes may play a role in the formation of biofilm by *A. butzleri,* since *luxS* has been related to Quorum Sensing, as it is the signalling system responsible for cell-to-cell communication, which is used by the bacteria to regulate biofilms. The flagellum is also an important structure that takes part in biofilm formation, and is correlated to *flaA* (Salazar-Sánchez *et al.*, 2022). According to Fan *et al.* (2020), in order to form new biofilm, the genes related to the motility and flagella formation must be upregulated, so that bacterial cells get closer together. Thus, correlating with the increase displayed by these biofilm formation results.

There are studies reporting a significantly reduced biofilm formation in *Salmonella enterica* by SCFA in their acidic form (Lamas *et al.*, 2019), as well as in *C. jejuni* with sodium butyrate at 5 and 20 mM (Gunther *et al.*, 2023). This observation does not align with the results obtained in this study, where it was registered an increase in this virulence factor's production by most strains in the presence of SCFA. Furthermore, Lamas *et al.* (2019) suggested that the effect the SCFA on biofilm formation might be attributed to acetic acid (acetate). Acetate is believed to inhibit the production of extracellular polysaccharides and exert anti-quorum sensing activity in foodborne pathogens like *E. coli* and *S.* Typhimurium.

However, as previously mentioned, propionate may also be the main cause to the results observed, where an enhancement of biofilm formation was verified, given that this compound is able to, individually, potentiate biofilm formation (Figure 9E). These observations do not correspond to the results found by Liu *et al.*, (2022) with *S.* Typhimurium*,* as they concluded that both propionate and butyrate, in concentrations between 5-21 mM and 4.5-18 mM, respectively, are able to reduce biofilm formation. This information may provide insight into the negative results observed in some *A. butzleri*'s strains, regarding biofilm formation when exposed to SCFA.

Overall, the obtained results suggest that SCFA influence biofilm formation in a strain-dependent form. Considering the features associated with biofilm formation, this increase can be seen as a protection mechanism by the bacterium to the adverse condition (Lebeaux, Ghigo and Beloin, 2014). This emphasizes the complex nature of bacterial responses.

4.5. Adhesion and Invasion of *Aliarcobacter butzleri* to Caco-2 cells

Aliarcobacter's ability to adhere and invade Caco-2 cells, a human intestinal cell line, has already been studied, and *A. butzleri* is considered one of the most invasive species tested (Levican *et al.*, 2013). Adhesion has been considered an important first step to the bacterial colonization of the host (Haiko and Westerlund-Wikström, 2013), which is facilitated by the presence of the flagellum in this pathogenic bacterium (Santos, 2023). Invasion is also an infection-related mechanism, whose studies in *A. butzleri* showing strain-specific pathogenic mechanisms, as different strains presented different adhesion and invasion abilities (Baztarrika *et al.*, 2023).

Given the information above, these are important virulence factors to be taken into consideration in this study, to better perceive these mentioned stages that lead to infection. Thus, this assay has been focused on the ability of *A. butzleri* to adhere to and invade Caco-2 cells in the presence of SCFA mixtures.

Firstly, it was important to understand the effect of these mixtures on the Caco-2 cell line in order to assess any potential harm caused to the cells. For this, a cell viability assay was conducted with MTT (Figure 11).

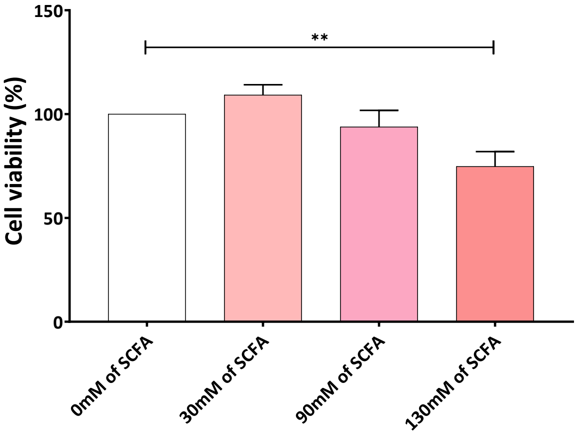

Figure 11. Cell viability of Caco-2 cells in the presence of SCFA mixtures for 24 h, determined by evaluation of cellular metabolic activity through the measurement of formazan crystals. The data represents the mean ± the standard error of the mean of at least three independent results. Results were analysed using a Student's t- test against the absence of SCFA. **p<0.01.

The results indicate that at 30 and 90 mM_SCFA, there was no significant harmful effect on cell viability. However, at 130 mM concentration, there seems to have been a slight impact, reducing viability to approximately 75% after 24 h of incubation with SCFA. Despite this reduction, the viability percentage remained relatively high, and thus, the mixtures created were considered safe for use with the Caco-2 cell line. Therefore, the adhesion and invasion assays in Caco-2 cells were carried out with the *A. butzleri* DQ40A1 strain exposed to all three mixtures (Figure 12).

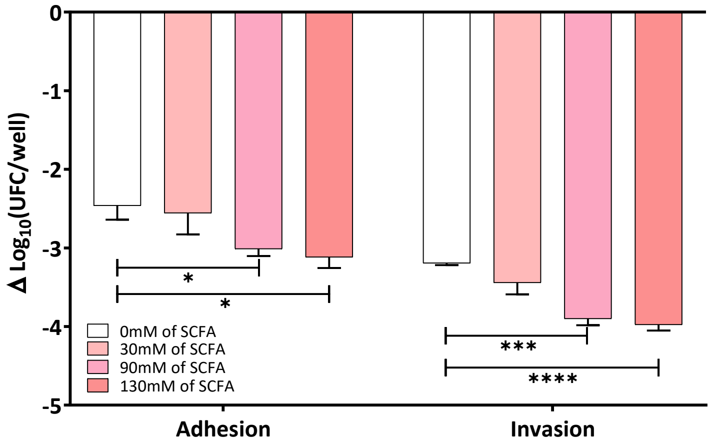

Figure 12. Adhesion and Invasion of *Aliarcobacter butzleri* DQ40A1 in Caco-2 cells in the presence of SCFA mixtures. The data represents the mean of ΔLog_{10}(UFC/well) ± the standard error of the mean of at least three independent results. Results were analysed using a Student's t- test against absence of SCFA. *$p<0.05$, ***$p<0.001$, ****$p<00001$.

By analysing the results, it can be observed that both adhesion and invasion abilities were influenced by SCFA in this strain, being noticeable that 90 and 130 mM_SCFA were capable of reducing not only the adhesion to Caco-2 cells, but also the invasion *of A. butzleri* in this cell line. Adhesion is said to be related to the expression of several genes, such as the *cadF* and the *flaA* gene. When comparing these findings, in Figure 12, with the gene expression data, Figure 6A, it would be expected a potentiated effect of the adhesion ability, under the influence of the 90 mM_SCFA, due to an overexpression of the *flaA* gene. This expectation was not confirmed in Figure 12, with a decrease in the adhesion ability of the *A. butzleri* DQ40A1 strain occurring in the presence of 90 and 130 mM_SCFA. Regarding the invasion ability, despite the observed overexpression of the *ciaB* gene at 90 and 130 mM_ SCFA, and of *flaA* gene at 90 mM_SCFA (Figure 6A), all the mixture concentrations appeared to reduce the invasion of Caco-2 cells by the DQ40A1 strain.

The study of *A. butzleri* gene regulation in the infection process still remains relatively unexplored; however, Buzzanca et al. (2023) work showed that, in an *in vitro* cellular infection model, a strain-dependent upregulation of iron metabolism and organic acid metabolism-related genes occurs. Also, among the putative virulence genes studied by us, *cadF* gene was upregulated. Other study, focusing on *A. butzleri* as an endocytobiont of *Acanthamoeba castellanii*, showed an initial upregulation of *flaA*, *cadF* and *ciaB* genes; however, after the relationship was established between both organisms it was verified a decrease in the gene transcription (Medina *et al.*, 2019). Therefore, these results suggest that during the infection process, the gene expression of *A. butzleri* may vary along the infection period, which could be correlated or even explain the upregulation of the genes aforementioned, and the reduction observed in adhesion and invasion abilities in our assays.

Studies involving SCFA with other bacteria, such as *E. coli*, have been carried out and demonstrated varying effects on the adhesion and invasion abilities. For example, in *E. coli* , a concentration of 65 mM of a mixture of SCFA significantly reduced the adhesion and invasion of Caco-2 cells, with a more pronounced impact on the invasion ability (Zhang *et al.*, 2020). A study performed by Liu *et al.*, (2022) also concluded that both propionate (5 and 21 mM) and butyrate (4.5 and 18 mM) were able to reduce the adhesion and invasion ability of *S.* Typhimurium to Caco-2 cells, which correlates with the results displayed in Figure 12, where a reduction in adhesion and invasion in the presence of SCFA is observed. However, in another study, both 20 mM_SP and 20 mM_SB supplementation were able to increase the adherence and invasion of AIEC to Caco-2 cells, unlike 40 mM_SA (Pace *et al.*, 2021). The results showed by this study do not translate the previous results, suggesting possible differences in the composition of the supplementation with SCFA, whether it is in the presence of a mixture with all three SCFA or the SCFA individually.

C. jejuni's ability to invade a Caco-2 cell line has also been tested in the presence of SCFA and has yielded conflicting results. In one study, SB, which is known to induce apoptosis and reduce proliferation in Caco-2 cells, resulted in a decrease of invasion which could be related to the reduced cell viability in the monolayer, with concentrations of 2.5 and 5 mM of this compound after 72 h. Sodium butyrate also displayed a protection effect on the Caco-2 cells against this bacterium's invasion (van Deun *et al.*, 2008). Despite these findings, another study that incubated *C. jejuni* with 6.25 and 3.13 mM of SA, SP and SB concentrations, showed no alteration in the invasive and adherence abilities with Caco-2 cells (Van Deun *et al.*, 2008).

The reported reduction of invasion ability was also observed in *in vivo* studies, where a study conducted in mice to evaluate the infection and invasion ability of *S*. Typhimurium showed that in the presence of concentrations found in the mice's cecum (42 mM_SA, 8.5 mM_SP and 12.8 mM_SB), the bacterium had its invasion ability repressed. When the model of infection was treated with antibiotics, a reduction in SA, SP and mainly SB was verified, allowing *S*. Typhimurium to invade host cells (Garner *et al.*, 2009). These results suggest a possible reduction effect by SCFA, mainly butyrate, in the invasion of bacteria.

The observed decrease in cell viability in Caco-2 cells with the 130 mM concentration, might be explained through the increase in SB concentration, according to the previously mentioned studies, suggesting a reduction in the host cell numbers by this compound. A decline in adhesion and invasion of *A. butzleri,* was also notable in this study, which goes accordingly to the described by Zhang *et al.* (2020). The results from the present study suggest that the tested concentrations of SB may protect the host cells, resulting in lack of invasion, as previously described. These results strongly support that SCFA have, in fact, a protection role in the colonization of the host by pathogens. It also suggests a complexity of host-microbe interactions, and SCFA-dependent effects on bacteria and cell lines, which deserves further research.

Chapter V - Conclusions

Aliarcobacter butzleri is recognized as an emerging enteropathogen, ubiquitously distributed along the environment. It is also associated with gastrointestinal diseases, being responsible for symptoms such as abdominal cramps, diarrhoea, nausea and other additional physiological manifestations. The pathogenicity and virulence of *A. butzleri* have been studied over the years, however, little is known about the mechanisms involved in these factors, especially when it encounters the presence of SCFA along the intestine. Therefore, this work's focus was on the effect of SCFA on this bacterium's virulence and pathogenicity.

In order to understand the impact of SCFA on *A. butzleri*'s behaviour, the bacterium's survival and growth were evaluated in their presence. A variety of SCFA concentrations, corresponding to the ones that may be found along the intestine, were used in this assay. The results showed that overall, the *A. butzleri*'s strains used in this study are affected by high concentrations, with an increase of the lag phase or even growth inhibition.

Following the previous results, the virulence-associated genes expression was also performed to further the understanding of the *A. butzleri's* behaviour in presence of SCFA. Among the genes used for this assay, both *flaA* and *luxS* were the most affected by most concentrations of the SCFA mixtures, presenting an overexpression. The *ciaB* gene also displayed an overexpression in the presence of 130 mM_SCFA. These results suggest a potential effect by these compounds on the virulence features associated with these genes.

Consequently, the bacterial motility was evaluated with the eight *A. butzleri* strains used in this study, in the presence of various SCFA concentrations. *A. butzleri* showed a reduction in motility by the higher concentrations of SCFA, which was verified for most strains, being affected by the compounds at concentrations similar to those found in the large intestine. However, this result does not correlate with the prevision taken from the *flaA* gene overexpression, suggesting that other factors may be behind this virulence mechanism.

Regarding the biofilm formation ability, the previous trend was not established with this virulence factor. This study showed a strain-dependent behaviour, as different effects were observed, such as a biofilm formation potentiation for three out of eight *A. butzleri* isolates, while two out of seven isolates had their biofilm formation ability reduced by SCFA. By comparing the mentioned results with those obtained from the evaluation of gene expression, it is possible to understand that the ability of the DQ40A1 isolate to form biofilm and the genes that are believed to be linked to this mechanism, *flaA* and *luxS*, are overexpressed by the SCFA, leading to a possible link between the two features. Regardless, this interaction still needs to be further explored with different strains.

The last aim of this study was to understand the effects of SCFA on host cells and how they may affect the interaction between the bacterium and the host cells, and for that, a Caco-2 cell line was used. Results obtained from the cell viability assay displayed no concerning effect on the viability of Caco-2 cells. Therefore, the adhesion and invasion assays were conducted in the presence of SCFA, where the bacterium's ability to adhere and invade the Caco-2 cell line was reduced by 90 and 130 mM_SCFA. Although the invasion appeared to be reduced by high concentrations of SCFA, this result collides with the expectations taken from the overexpression of the *ciaB* gene.

These results bring to light a lot of information which allows for new questions to arise regarding the virulence mechanisms used by *A. butzleri*. As observed, different strains possess different virulence behaviours and pathogenicity, depending on the intestinal location it is found in, which will induce different effects on the host.

To conclude, this work has allowed to further understand the virulence and survival of *A. butzleri* when faced with adverse conditions that this bacterium may find in the gastrointestinal tract, the SCFA. Throughout this study, the evidence provided suggests that SCFA have a potential modulatory effect on the mechanisms associated with the virulence factors of this bacterium, although a genetic variability is suggested for this species due to its strain-dependent behaviour.

Chapter VI - Future Perspectives

Given the vast prevalence of *A. butzleri* and its pertinent virulence mechanisms, a deeper comprehension of its virulence becomes imperative. While this study marginally enhances understanding of the effect of SCFA on *A. butzleri* survival and virulence, further investigation is required to better understand how this colonization resistance mechanism may influence the pathogenicity profile of *A. butzleri*. Therefore, more studies are needed to complement this work such as:

- To perform in *vivo* assays in order to correlate to the *in vitro* assays;
- To expand the number of isolates used to broaden the results;
- To perform adhesion and invasion assays also in the presence of NaCl to understand the osmolarity effects on the cell line;
- To perform adhesion and invasion assays with more cell lines, in the presence of SCFA to evaluate possible different behaviours;
- To evaluate gene expression during the infection of Caco-2 cells in the presence of SCFA, to better understand the possible interaction;
- To evaluate the correlation of phenotypic and transcriptomic data to identify different genetic determinants involved in the *A. butzleri*'s response to SCFA.

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Waite, D. W., Vanwonterghem, I., Rinke, C., Parks, D. H., Zhang, Y., Takai, K., Sievert, S. M., Simon, J., Campbell, B. J., Hanson, T. E., Woyke, T., Klotz, M. G. and Hugenholtz, P. (2018) ‘Addendum: Comparative genomic analysis of the class *Epsilonproteobacteria* and proposed reclassification to *Epsilonbacteraeota* (phyl. nov.) [Front. Microbiol., 8, (2017), (682)] doi: 10.3389/fmicb.2017.00682’, *Frontiers in Microbiology*, 9, p. 369739. doi: 10.3389/fmicb.2018.00772.

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Annex I

Growth curves of seven *A. butzleri* isolates

A11-1

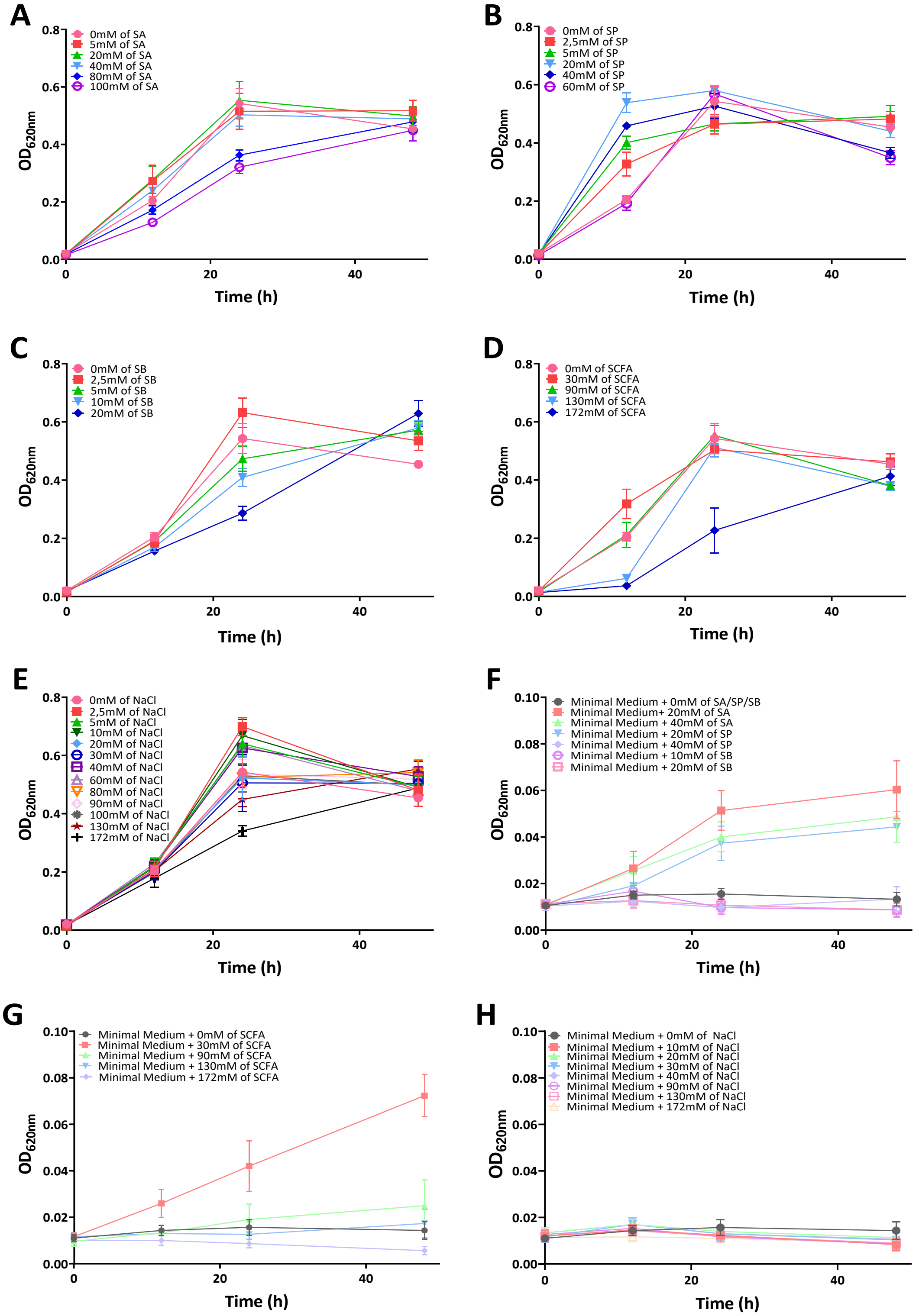


Figure S1. Growth curves for the *Aliarcobacter butzleri* A11-1 strain in the presence of different concentrations of SCFA, such as SA (A), SP (B), SB (C), Mixtures of the three SCFA (D), Different concentrations of NaCl, to exclude the effects of the osmolarity (E), SA, SP and SB in Minimal Medium (F), Mixtures of the SCFA in Minimal Medium (G) and NaCl in Minimal Medium (H). The data represents the mean ± the standard error of the means, considering at least three independent assays. Results were analysed using a two-way ANOVA test. (statistical analysis is presented in table S1 of the Annex I).

W12-1

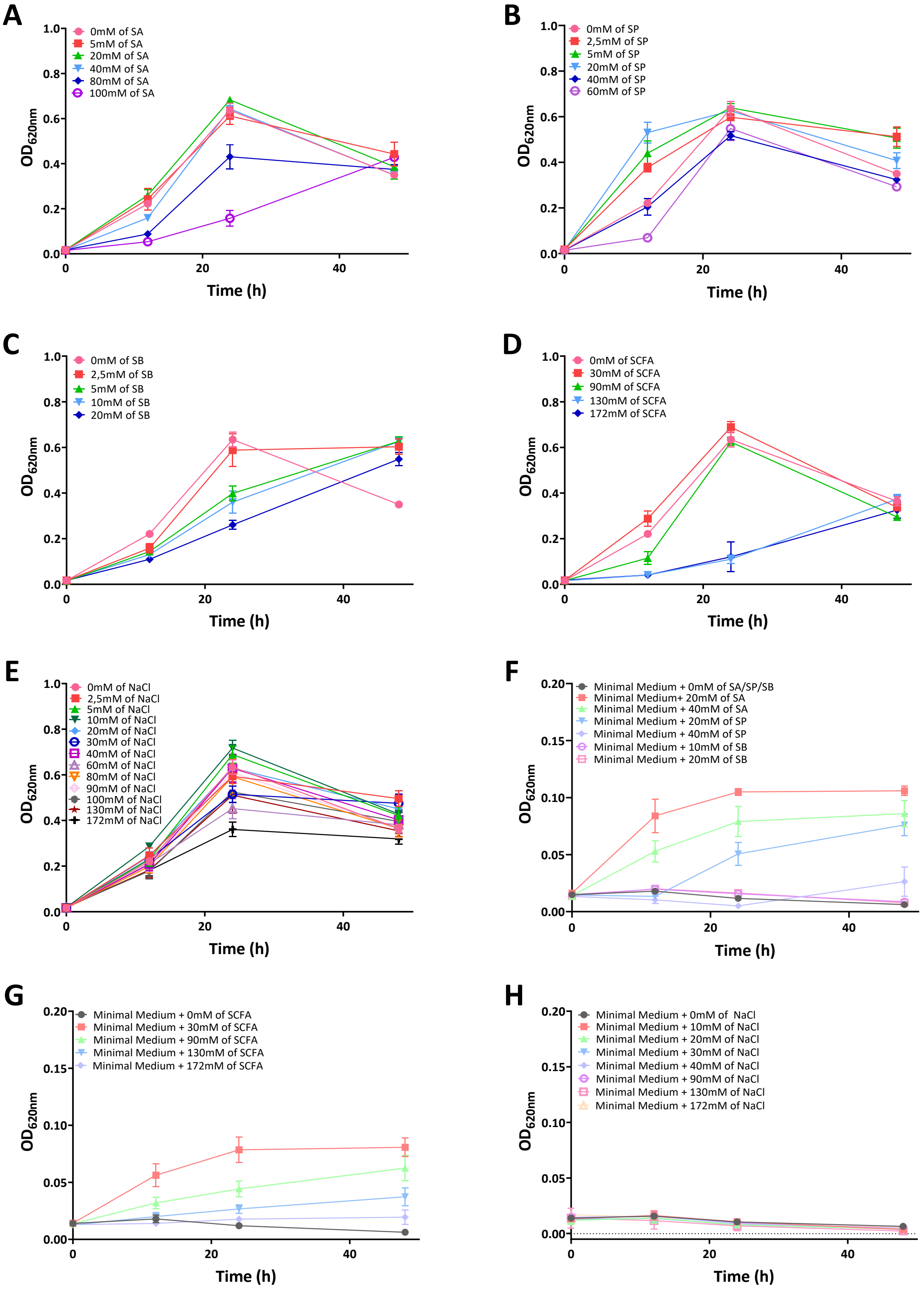


Figure S2. Growth curves for the *Aliarcobacter butzleri* W12-1 strain in the presence of different concentrations of SCFA, such as SA (A), SP (B), SB (C), Mixtures of the three SCFA (D), Different concentrations of NaCl, to exclude the effects of the osmolarity (E), SA, SP and SB in Minimal Medium (F), Mixtures of the SCFA in Minimal Medium (G) and NaCl in Minimal Medium (H). The data represents the mean ± the standard error of the means, considering at least three independent assays. Results were analysed using a two-way ANOVA test. (statistical analysis is presented in table S1 of the Annex I).

AB28/11

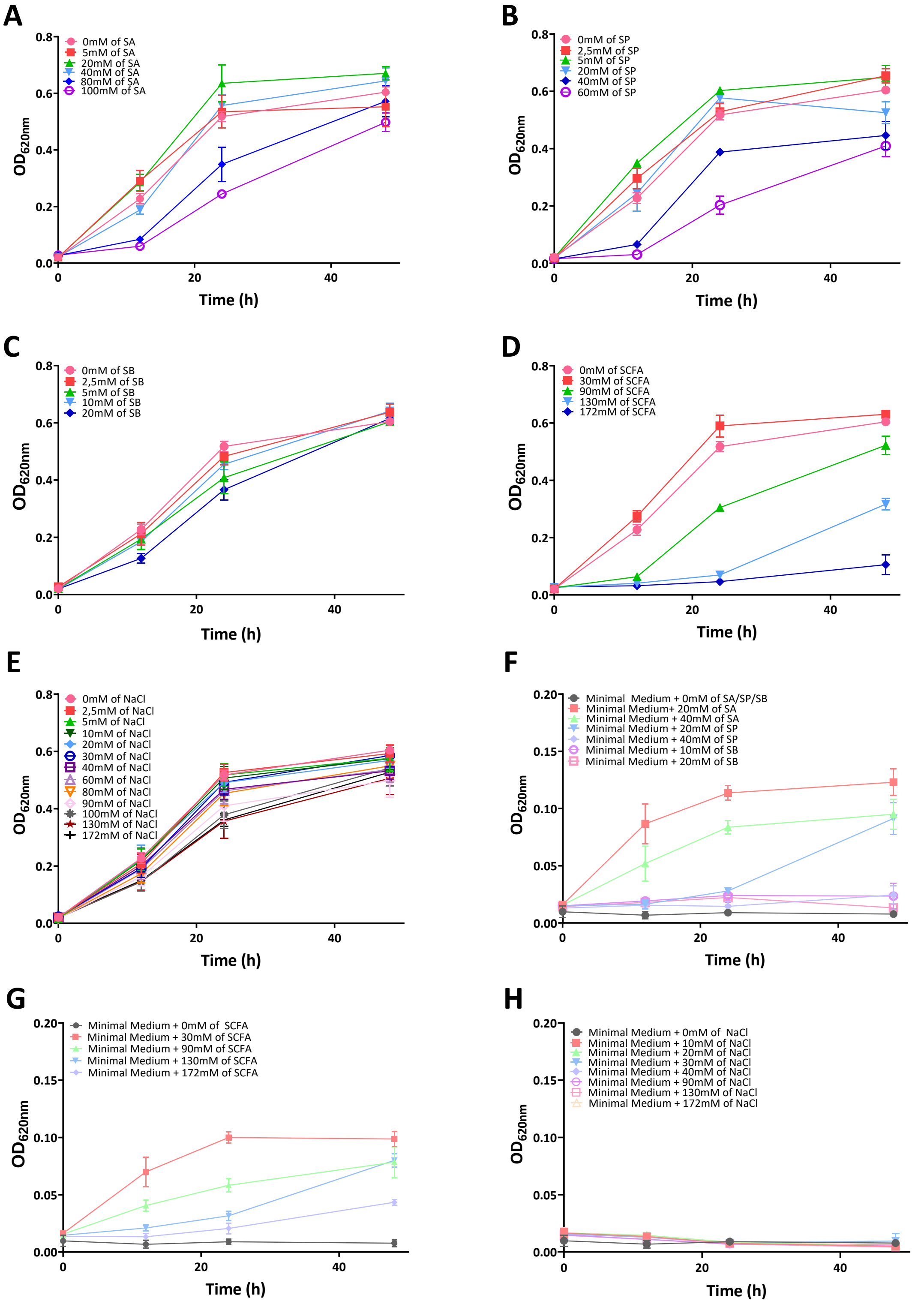


Figure S3. Growth curves for the *Aliarcobacter butzleri* AB28/11 strain in the presence of different concentrations of SCFA, such as SA (A), SP (B), SB (C), Mixtures of the three SCFA (D), Different concentrations of NaCl, to exclude the effects of the osmolarity (E), SA, SP and SB in Minimal Medium (F), Mixtures of the SCFA in Minimal Medium (G) and NaCl in Minimal Medium (H). The data represents the mean ± the standard error of the means, considering at least three independent assays. Results were analysed using a two-way ANOVA test. (statistical analysis is presented in table S1 of the Annex I).

INSA2756

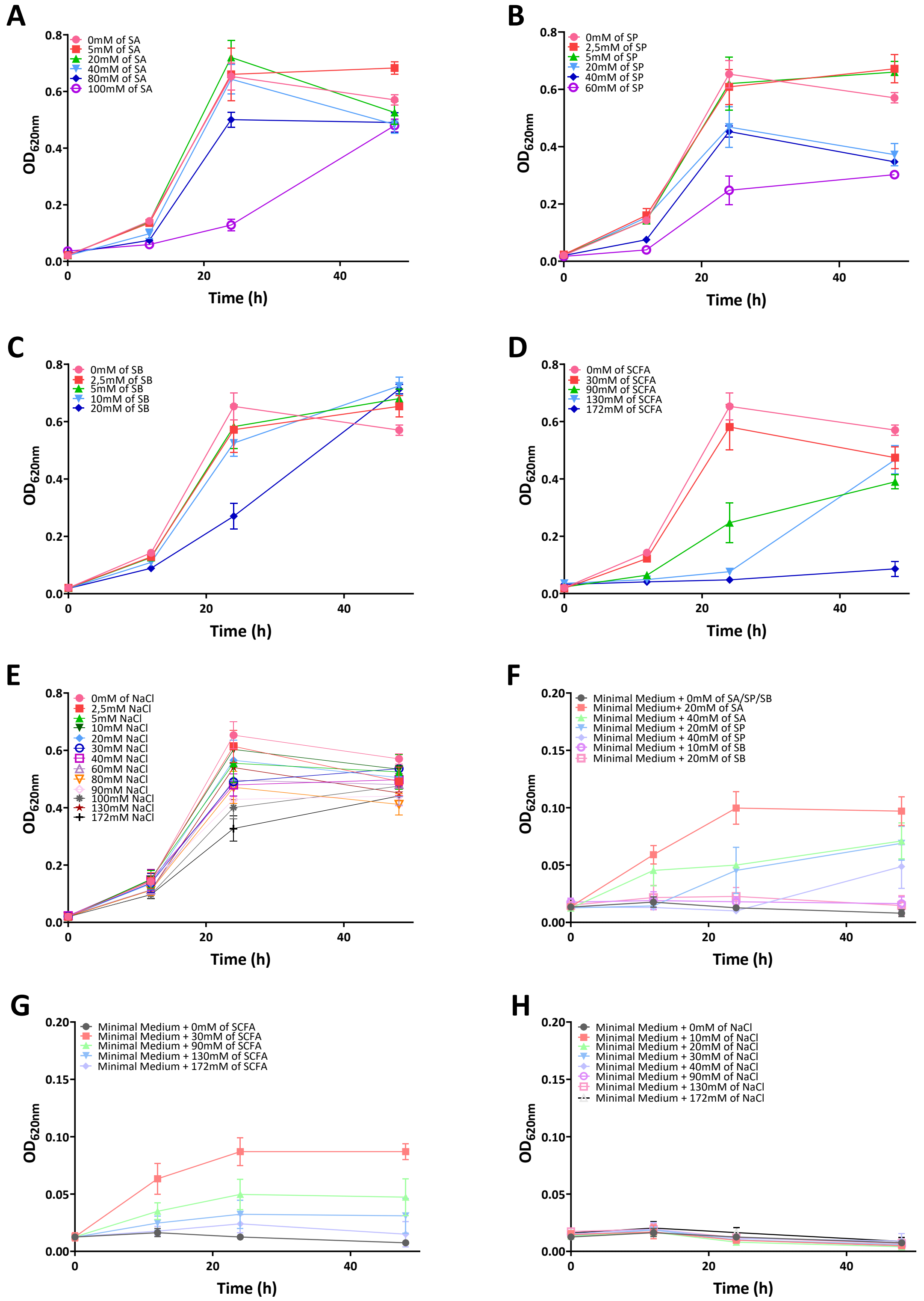


Figure S4. Growth curves for the *Aliarcobacter butzleri* INSA2756 strain in the presence of different concentrations of SCFA, such as SA (A), SP (B), SB (C), Mixtures of the three SCFA (D), Different concentrations of NaCl, to exclude the effects of the osmolarity (E), SA, SP and SB in Minimal Medium (F), Mixtures of the SCFA in Minimal Medium (G) and NaCl in Minimal Medium (H). The data represents the mean ± the standard error of the means, considering at least three independent assays. Results were analysed using a two-way ANOVA test. (statistical analysis is presented in table S1 of the Annex I).

INSA2999

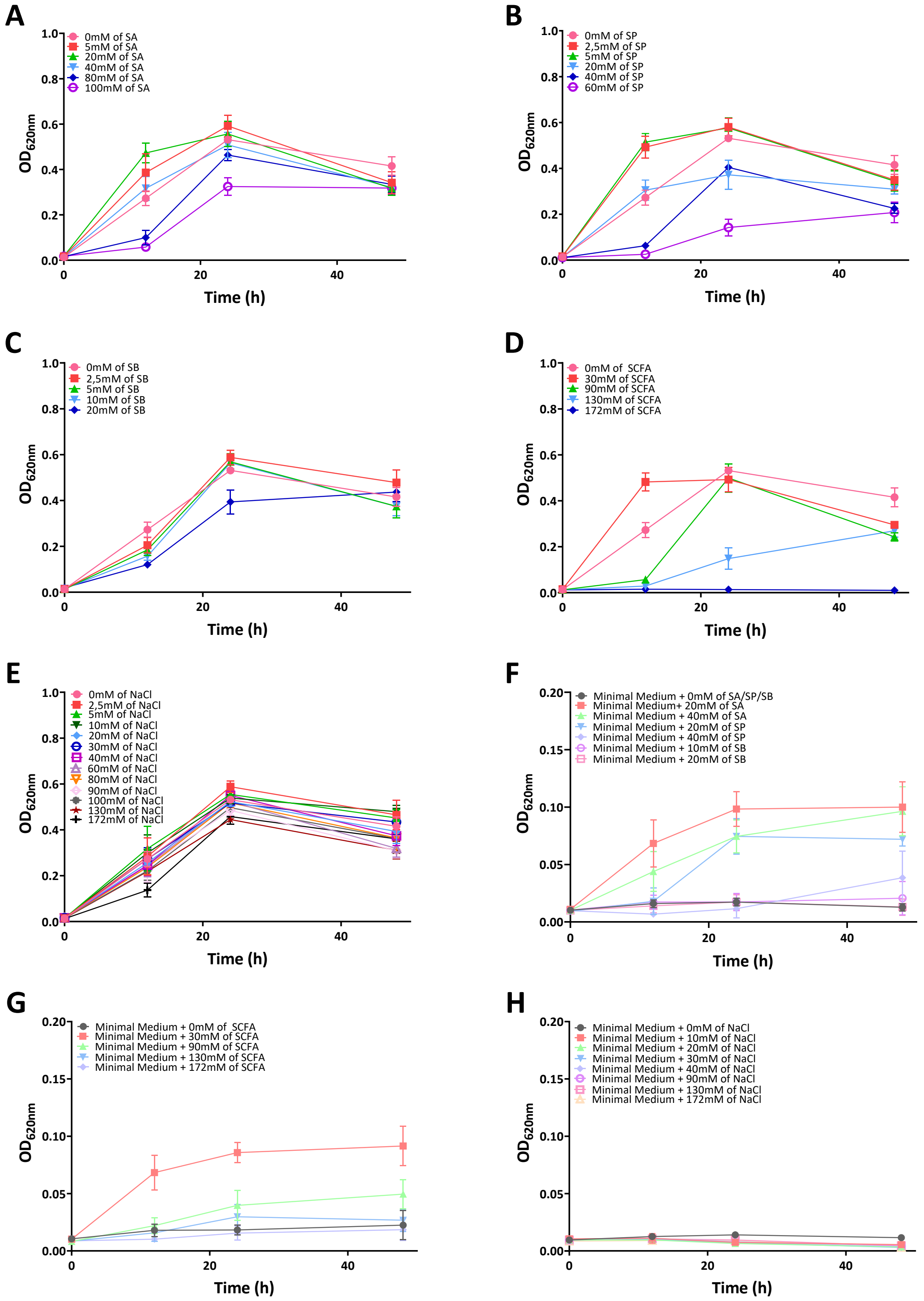


Figure S5. Growth curves for the *Aliarcobacter butzleri* INSA2999 strain in the presence of different concentrations of SCFA, such as SA (A), SP (B), SB (C), Mixtures of the three SCFA (D), Different concentrations of NaCl, to exclude the effects of the osmolarity (E), SA, SP and SB in Minimal Medium (F), Mixtures of the SCFA in Minimal Medium (G) and NaCl in Minimal Medium (H). The data represents the mean ± the standard error of the means, considering at least three independent assays. Results were analysed using a two-way ANOVA test. (statistical analysis is presented in table S1 of the Annex I).

INSA3800

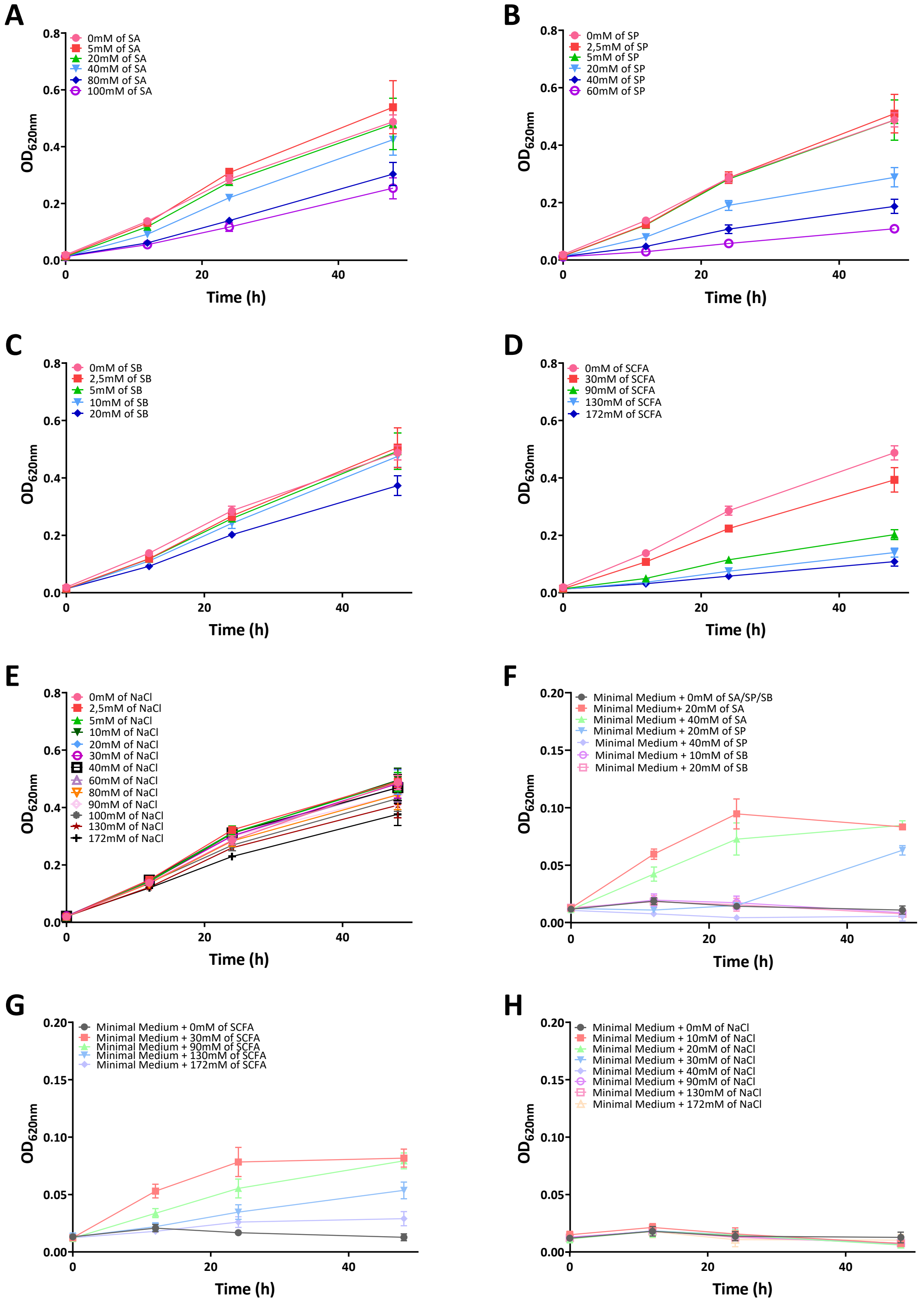


Figure S6. Growth curves for the *Aliarcobacter butzleri* INSA3800 strain in the presence of different concentrations of SCFA, such as SA (A), SP (B), SB (C), Mixtures of the three SCFA (D), Different concentrations of NaCl, to exclude the effects of the osmolarity (E), SA, SP and SB in Minimal Medium (F), Mixtures of the SCFA in Minimal Medium (G) and NaCl in Minimal Medium (H). The data represents the mean ± the standard error of the means, considering at least three independent assays. Results were analysed using a two-way ANOVA test. (statistical analysis is presented in table S1 of the Annex I).

INSA4015

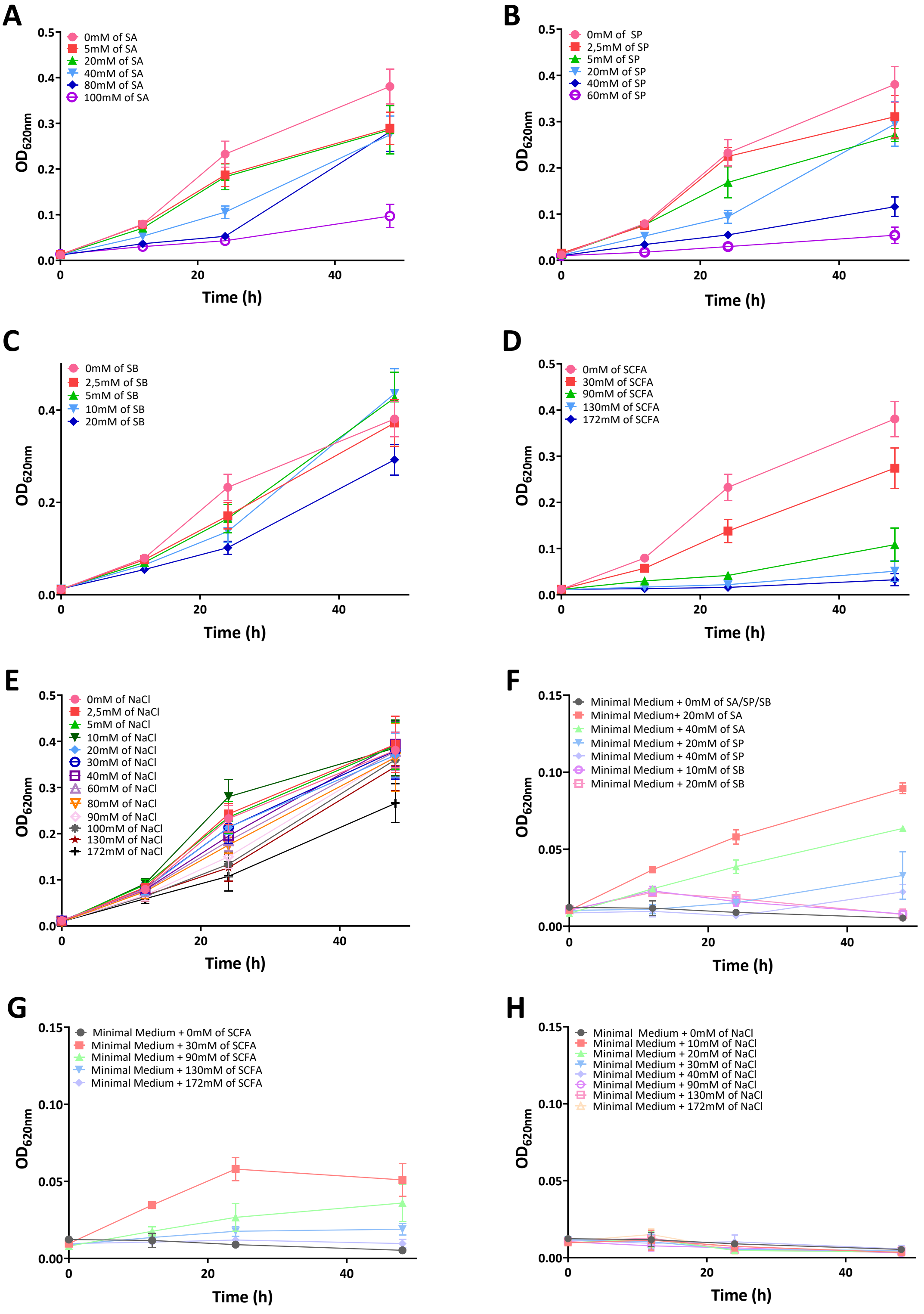
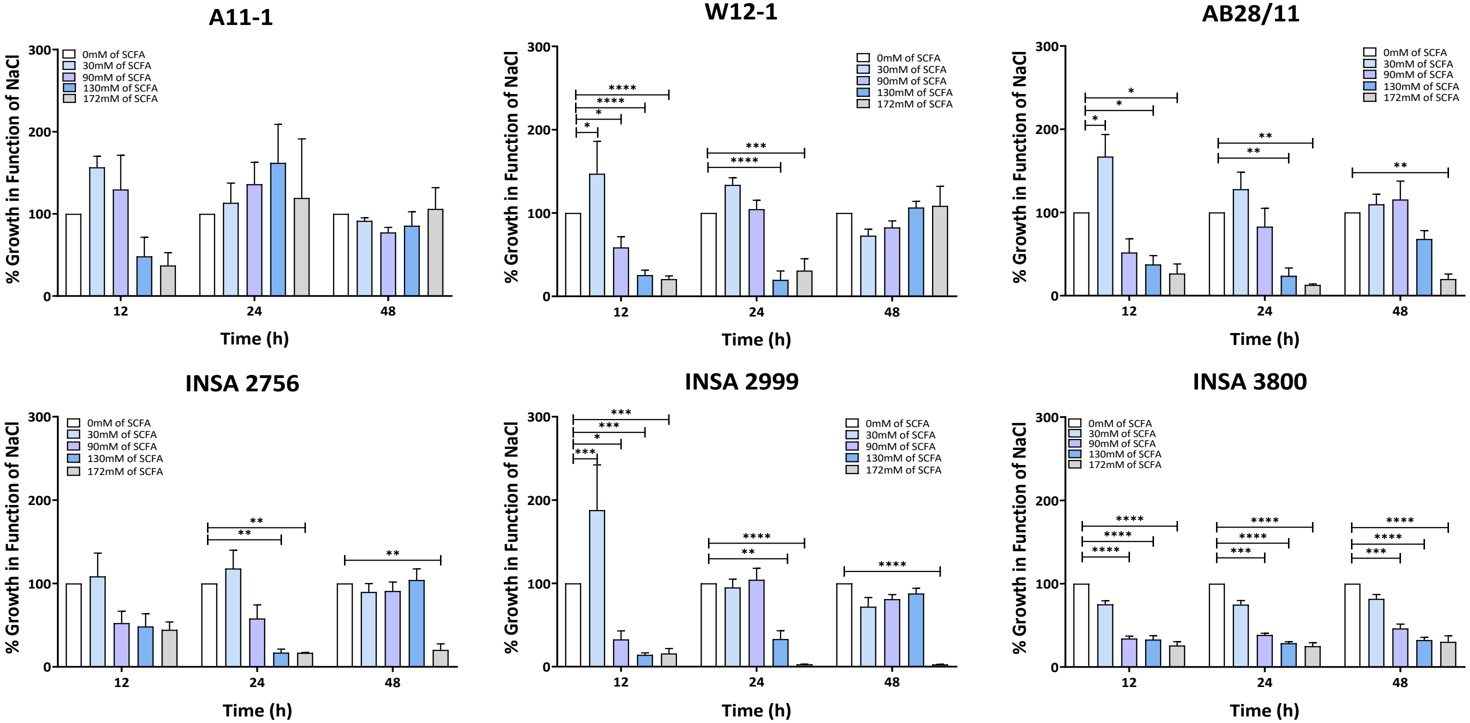


Figure S7. Growth curves for the *Aliarcobacter butzleri* INSA4015 strain in the presence of different concentrations of SCFA, such as SA (A), SP (B), SB (C), Mixtures of the three SCFA (D), Different concentrations of NaCl, to exclude the effects of the osmolarity (E), SA, SP and SB in Minimal Medium (F), Mixtures of the SCFA in Minimal Medium (G) and NaCl in Minimal Medium (H). The data represents the mean ± the standard error of the means, considering at least three independent assays. Results were analysed using a two-way ANOVA test. (statistical analysis is presented in table S1 of the Annex I).

Growth percentage in function of NaCl for the remaining seven strains in this study



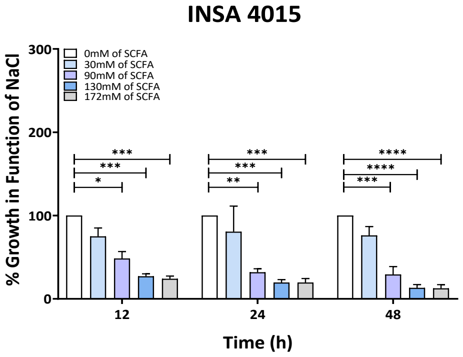


Figure S8. Percentage of growth of the *Aliarcobacter butzleri* strains in the presence of different concentrations of SCFA mixtures. Results were analysed using two-way ANOVA test. The data represents the mean ± the standard error of the mean of at least three independent results. *p<0.05, **p<0.01, ***p<0.001, ****p<00001.

Motility evaluation of the remaining seven strains:

A11-1

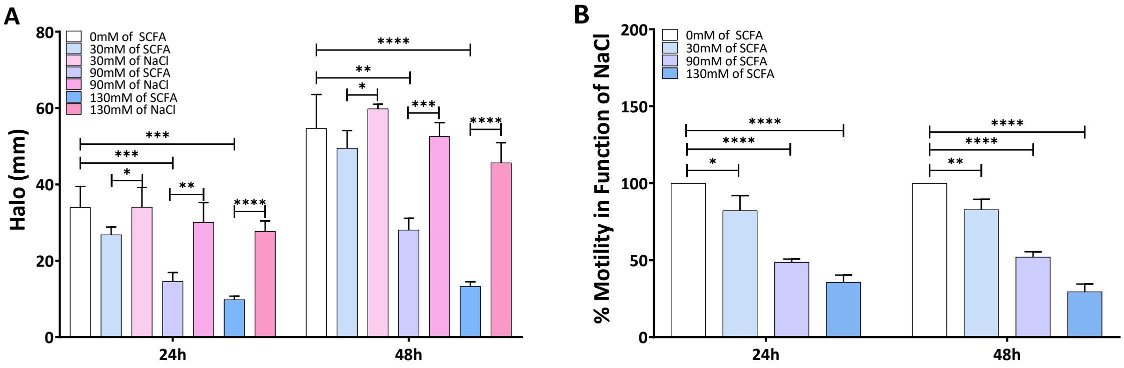


Figure S9. Effect of SCFA mixtures in the motility of *Aliarcobacter butzleri* A11-1 (A). Percentage of motility in function of the SCFA mixtures' respective NaCl concentrations (B). The data represents the mean ± the standard deviation of at least three independent results. Results were analysed using a Student's t- test against 0mM of SCFA and respective NaCl concentrations. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<00001$.

W12-1

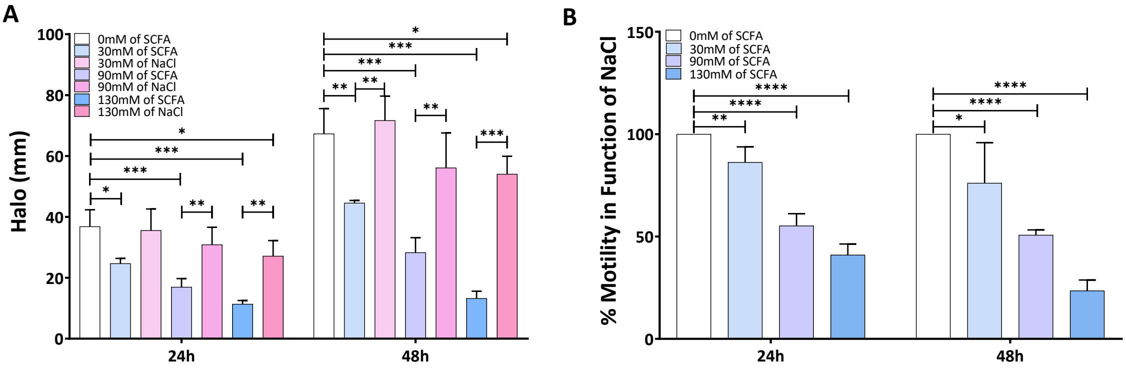


Figure S10. Effect of SCFA mixtures in the motility of *Aliarcobacter butzleri* W12-1 (A). Percentage of motility in function of the SCFA mixtures' respective NaCl concentrations (B). The data represents the mean ± the standard deviation of at least three independent results. Results were analysed using a Student's t- test against 0mM of SCFA and respective NaCl concentrations. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<00001$.

AB28/11

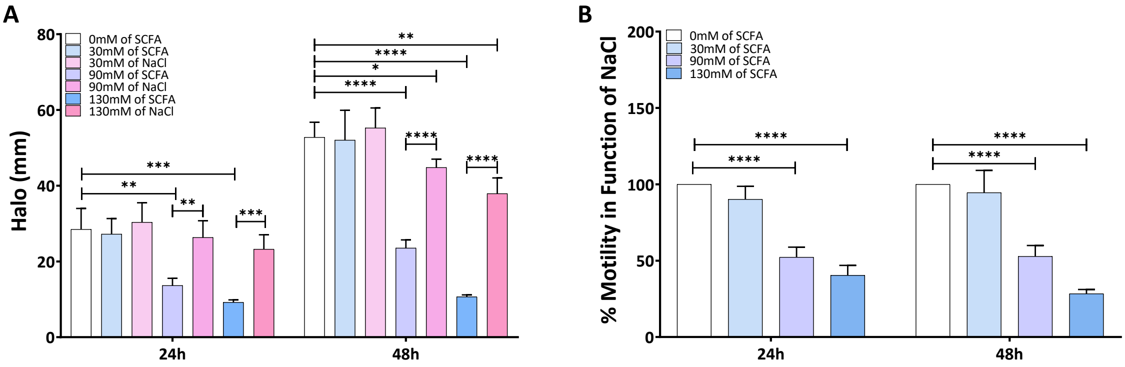


Figure S11. Effect of SCFA mixtures in the motility of *Aliarcobacter butzleri* AB28/11 (A). Percentage of motility in function of the SCFA mixtures' respective NaCl concentrations (B). The data represents the mean ± the standard deviation of at least three independent results. Results were analysed using a Student's t- test against 0mM of SCFA and respective NaCl concentrations. *p<0.05, **p<0.01, ***p<0.001, ****p<00001.

INSA2756

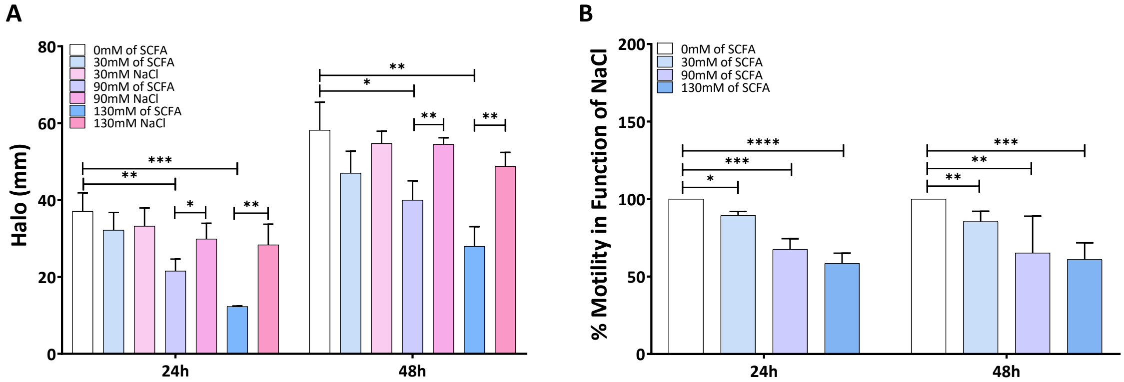


Figure S12. Effect of SCFA mixtures in the motility of *Aliarcobacter butzleri* INSA2756 (A). Percentage of motility in function of the SCFA mixtures' respective NaCl concentrations (B). The data represents the mean ± the standard deviation of at least three independent results. Results were analysed using a Student's t- test against 0mM of SCFA and respective NaCl concentrations. *p<0.05, **p<0.01, ***p<0.001, ****p<00001.

INSA2999

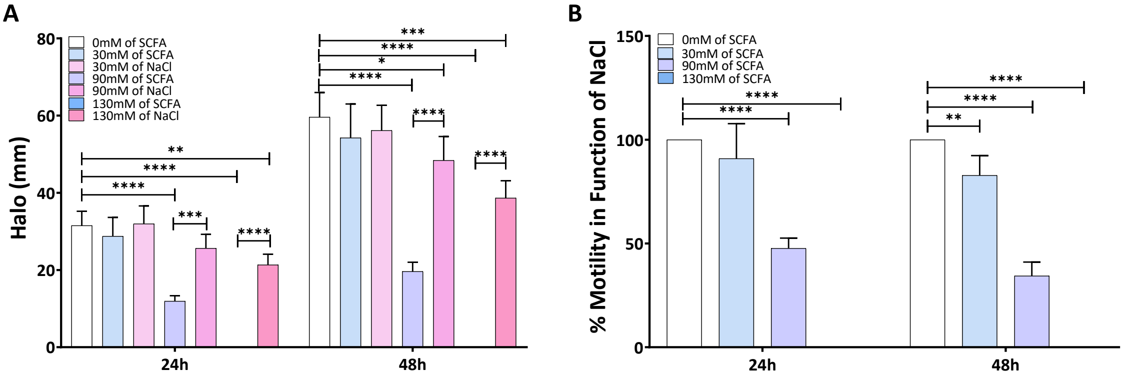


Figure S13. Effect of SCFA mixtures in the motility of *Aliarcobacter butzleri* INSA2999 (A). Percentage of motility in function of the SCFA mixtures' respective NaCl concentrations (B). The data represents the mean ± the standard deviation of at least three independent results. Results were analysed using a Student's t- test against 0mM of SCFA and respective NaCl concentrations. *p<0.05, **p<0.01, ***p<0.001, ****p<00001.

INSA3800

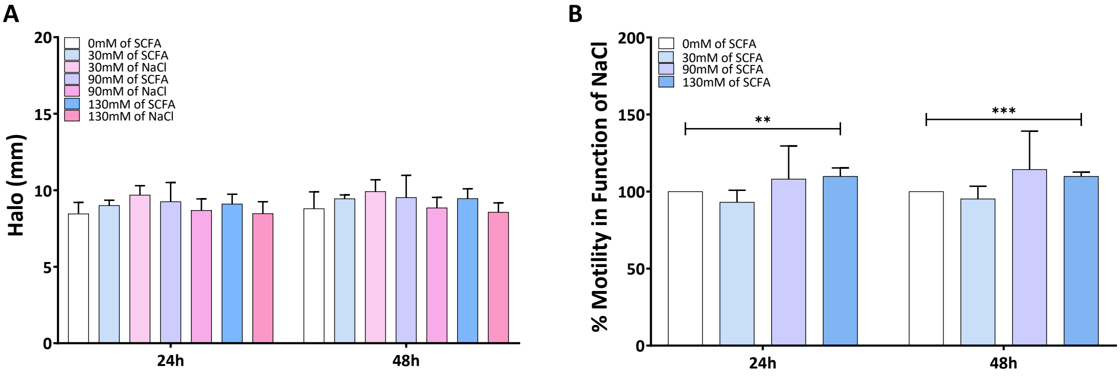


Figure S14. Effect of SCFA mixtures in the motility of *Aliarcobacter butzleri* INSA3800 (A). Percentage of motility in function of the SCFA mixtures' respective NaCl concentrations (B). The data represents the mean ± the standard deviation of at least three independent results. Results were analysed using a Student's t- test against 0mM of SCFA and respective NaCl concentrations. *p<0.05, **p<0.01, ***p<0.001, ****p<00001.

INSA4015

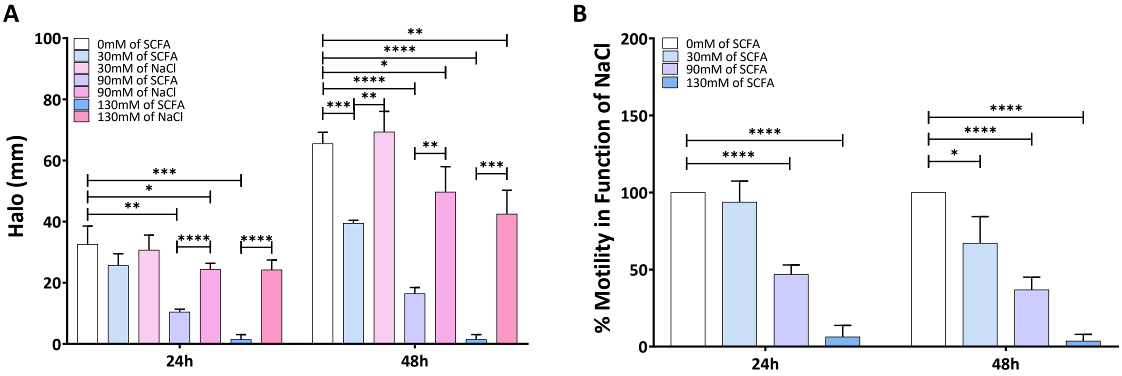


Figure S15. Effect of SCFA mixtures in the motility of *Aliarcobacter butzleri* INSA4015 (A). Percentage of motility in function of the SCFA mixtures' respective NaCl concentrations (B). The data represents the mean ± the standard deviation of at least three independent results. Results were analysed using a Student's t- test against 0mM of SCFA and respective NaCl concentrations. *p<0.05, **p<0.01, ***p<0.001, ****p<00001.

Biofilm formation evaluation of the remaining seven strains:

A11-1

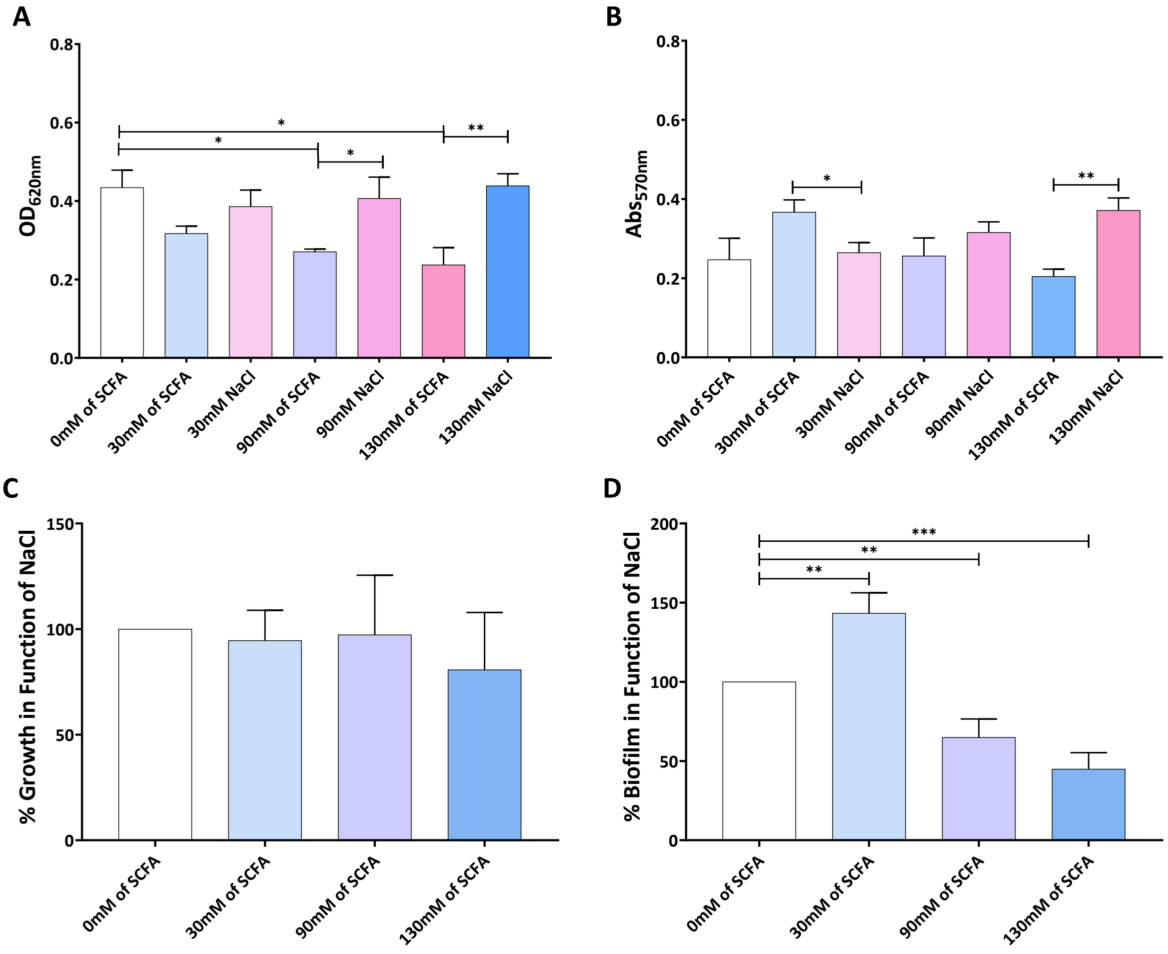


Figure S16. Bacterial growth of the *Aliarcobacter butzleri* A11-1 strain in the presence of different concentrations of SCFA mixtures and NaCl concentrations (A). Biofilm formation in the presence of SCFA mixtures and respective NaCl concentrations (B). Percentage of growth with SCFA mixtures in function of the respective NaCl concentrations (C). Percentage of biofilm formation in function of the SCFA's NaCl concentrations (D). The data represents the mean ± the standard error of the mean of at least three independent results. Results were analysed using a Student's t- test against 0mM of SCFA. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

W12-1

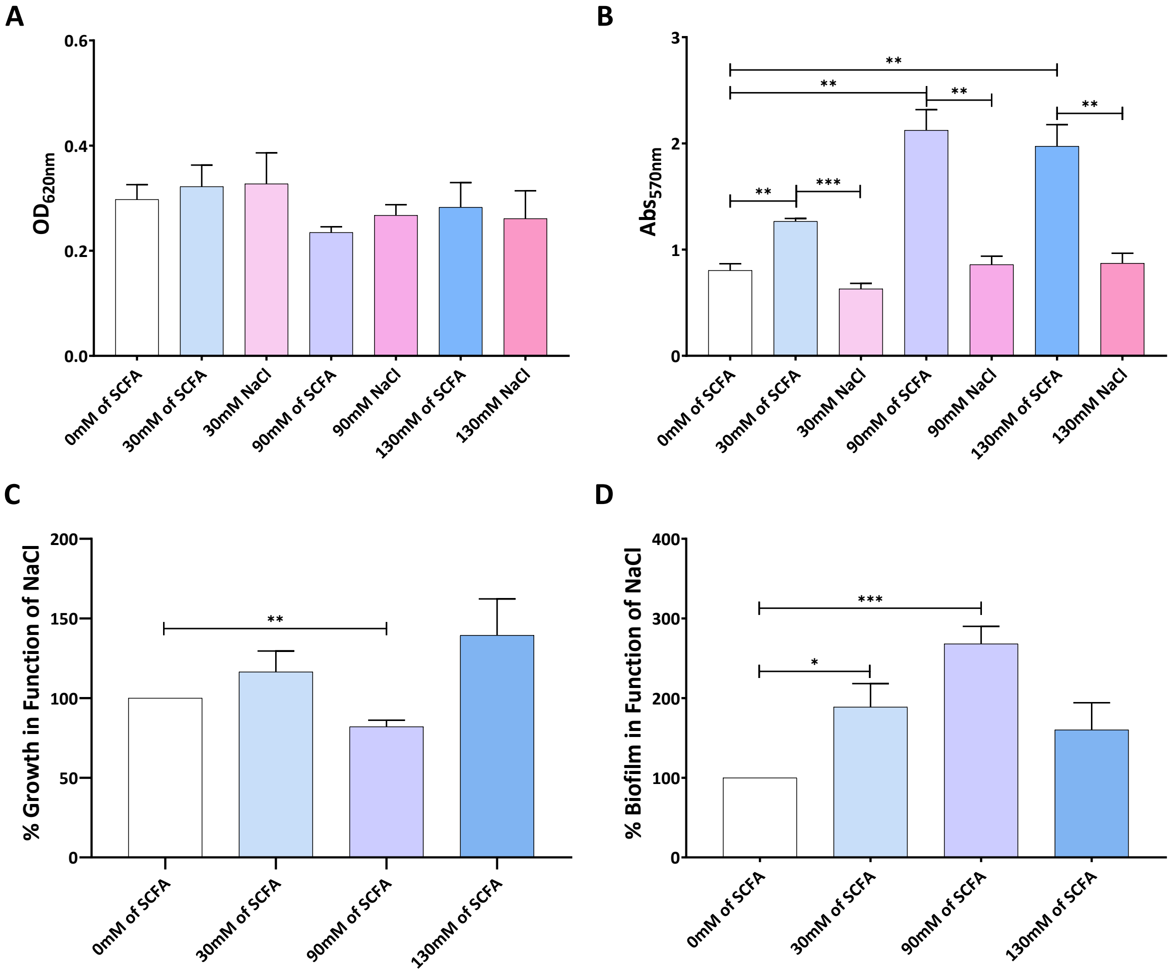


Figure S17. Bacterial growth of the *Aliarcobacter butzleri* W12-1 strain in the presence of different concentrations of SCFA mixtures and NaCl concentrations (A). Biofilm formation in the presence of SCFA mixtures and respective NaCl concentrations (B). Percentage of growth with SCFA mixtures in function of the respective NaCl concentrations (C). Percentage of biofilm formation in function of the SCFA's NaCl concentrations (D). The data represents the mean ± the standard error of the mean of at least three independent results. Results were analysed using a Student's t- test against 0mM of SCFA. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

AB28/11

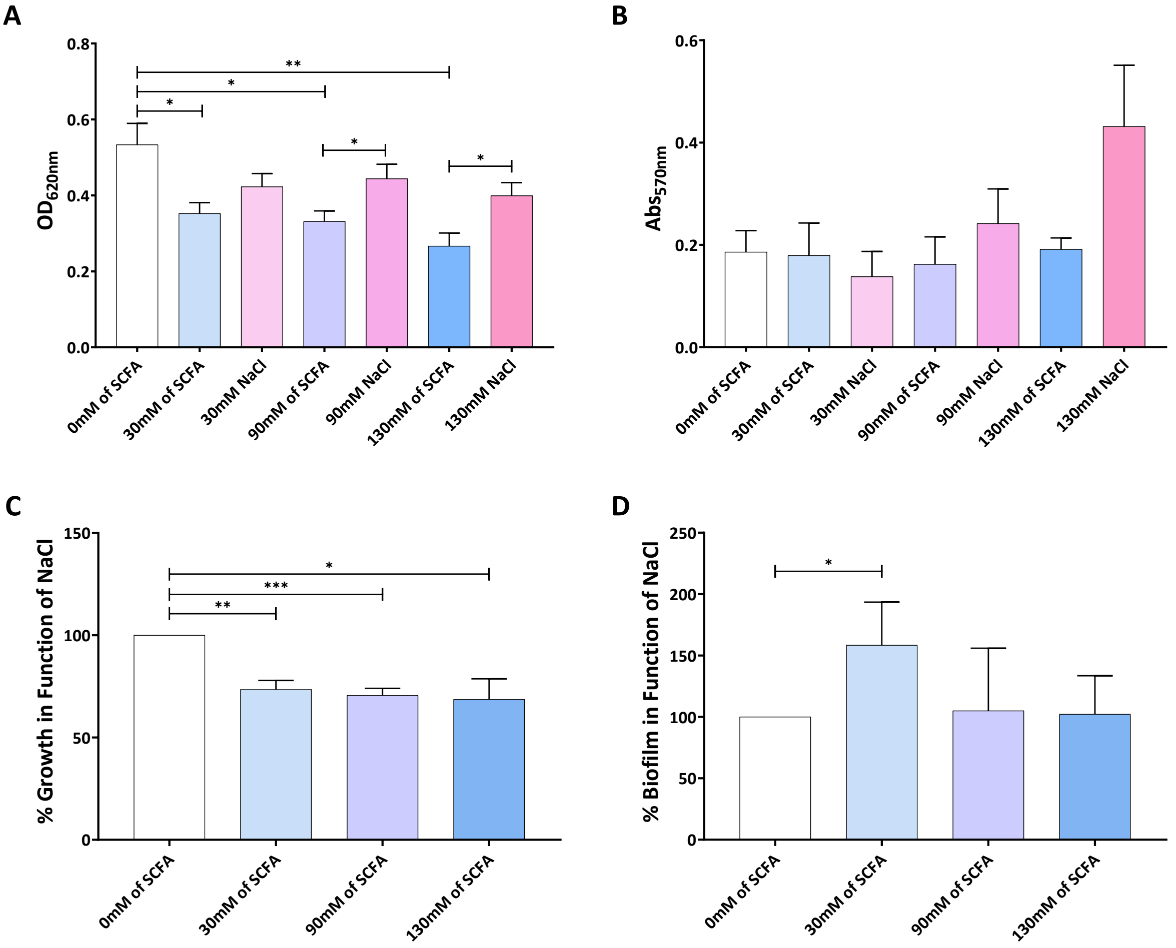


Figure S18. Bacterial growth of the *Aliarcobacter butzleri* AB28/11 strain in the presence of different concentrations of SCFA mixtures and NaCl concentrations (A). Biofilm formation in the presence of SCFA mixtures and respective NaCl concentrations (B). Percentage of growth with SCFA mixtures in function of the respective NaCl concentrations (C). Percentage of biofilm formation in function of the SCFA's NaCl concentrations (D). The data represents the mean ± the standard error of the mean of at least three independent results. Results were analysed using a Student's t- test against 0mM of SCFA. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

INSA2756

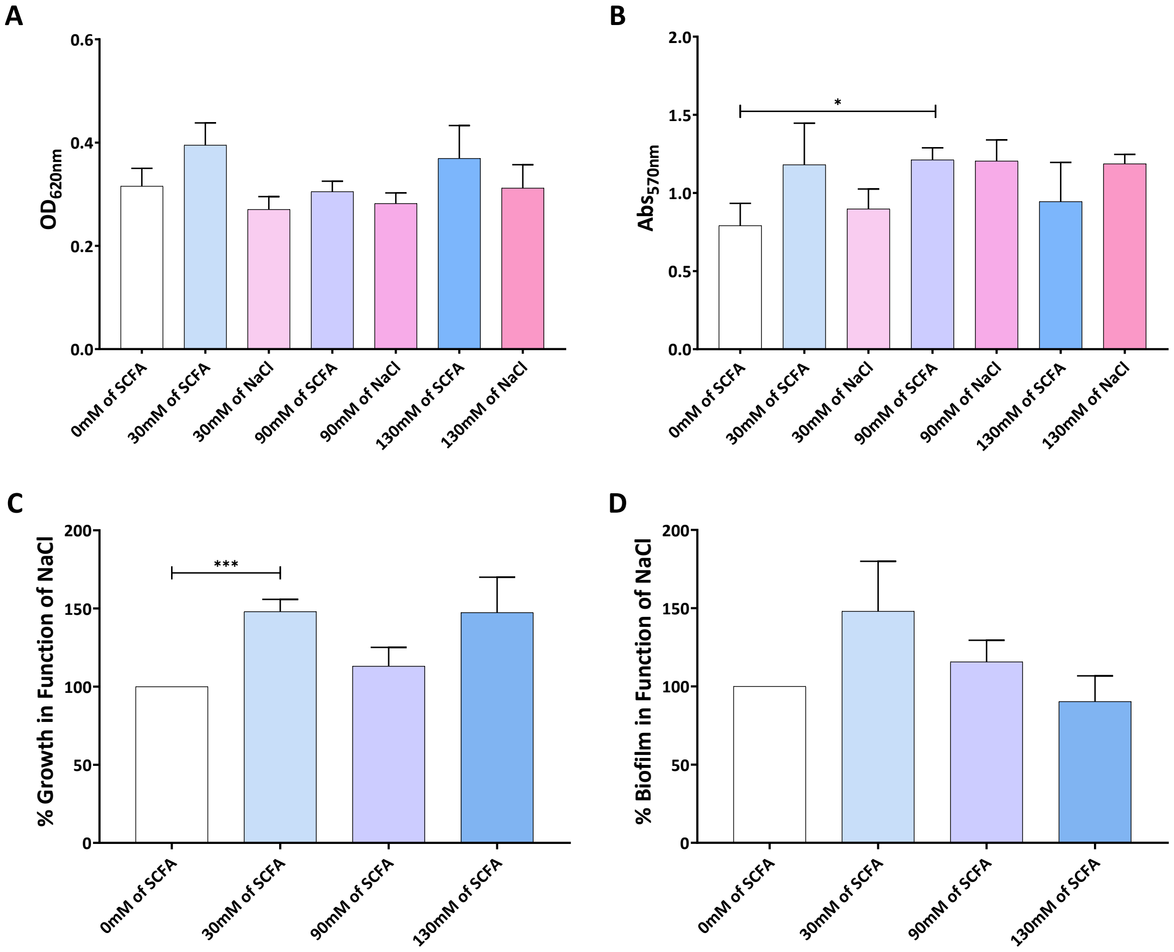


Figure S19. Bacterial growth of the *Aliarcobacter butzleri* INSA2756 strain in the presence of different concentrations of SCFA mixtures and NaCl concentrations (A). Biofilm formation in the presence of SCFA mixtures and respective NaCl concentrations (B). Percentage of growth with SCFA mixtures in function of the respective NaCl concentrations (C). Percentage of biofilm formation in function of the SCFA's NaCl concentrations (D). The data represents the mean ± the standard error of the mean of at least three independent results. Results were analysed using a Student's t- test against 0mM of SCFA. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

INSA2999

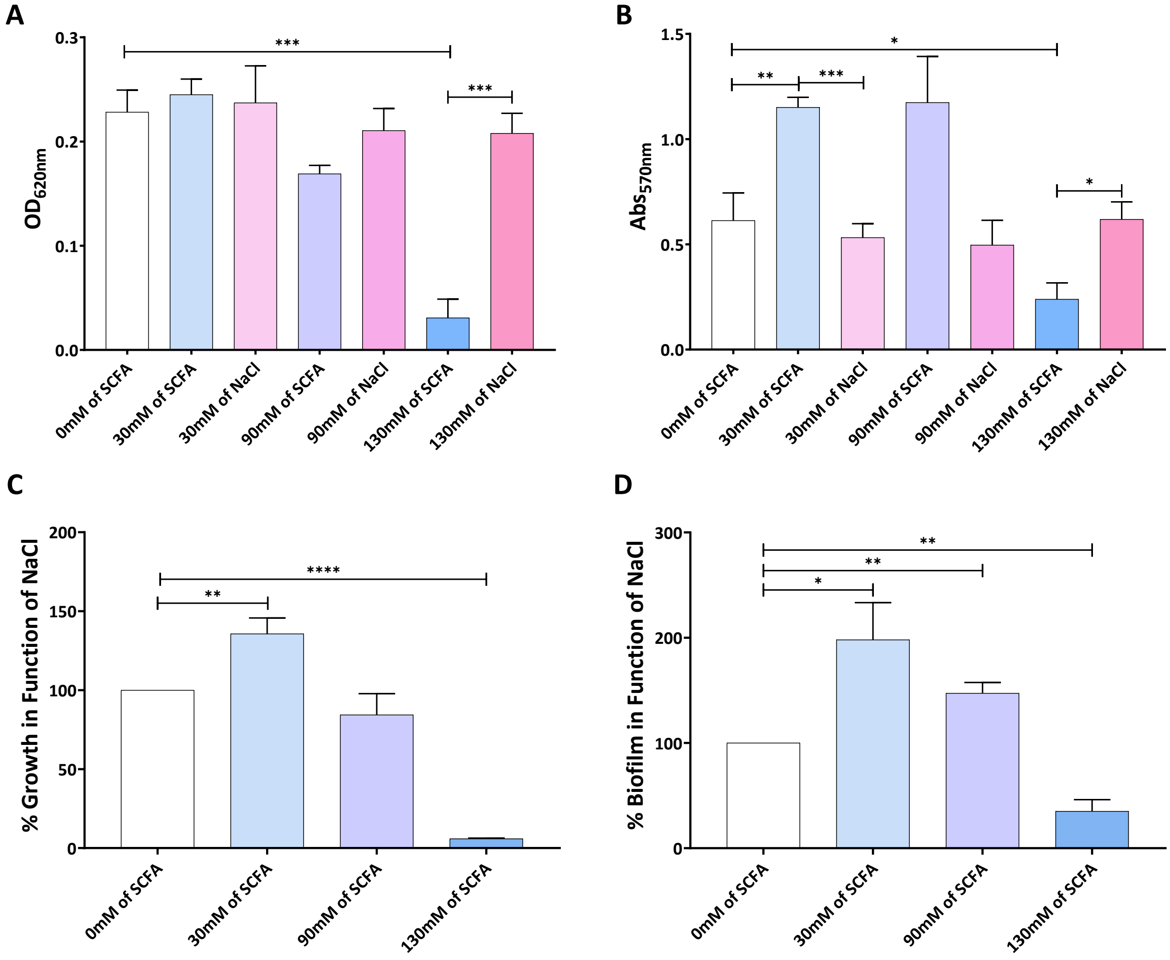


Figure S20. Bacterial growth of the *Aliarcobacter butzleri* INSA2999 strain in the presence of different concentrations of SCFA mixtures and NaCl concentrations (A). Biofilm formation in the presence of SCFA mixtures and respective NaCl concentrations (B). Percentage of growth with SCFA mixtures in function of the respective NaCl concentrations (C). Percentage of biofilm formation in function of the SCFA's NaCl concentrations (D). The data represents the mean ± the standard error of the mean of at least three independent results. Results were analysed using a Student's t- test against 0mM of SCFA. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

INSA3800

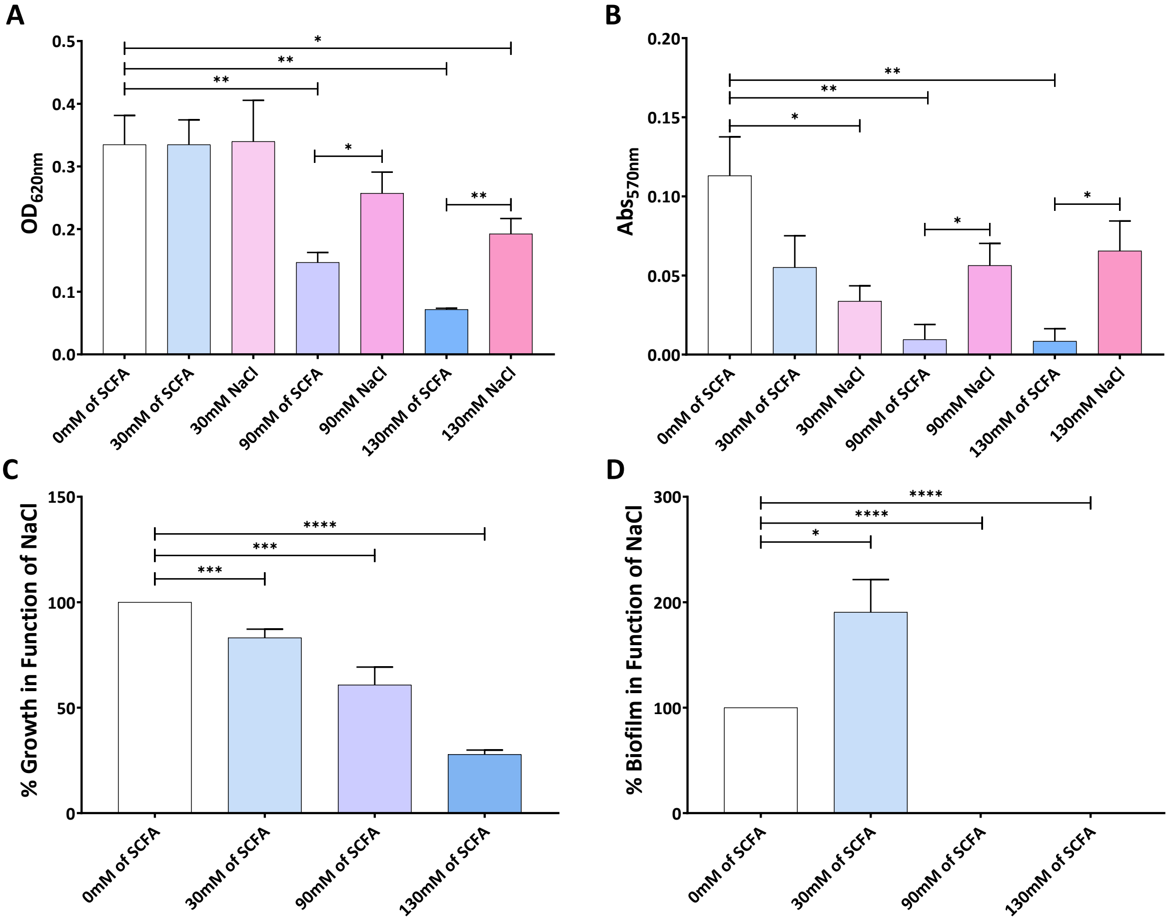


Figure S21. Bacterial growth of the *Aliarcobacter butzleri* INSA3800 strain in the presence of different concentrations of SCFA mixtures and NaCl concentrations (A). Biofilm formation in the presence of SCFA mixtures and respective NaCl concentrations (B). Percentage of growth with SCFA mixtures in function of the respective NaCl concentrations (C). Percentage of biofilm formation in function of the SCFA's NaCl concentrations (D). The data represents the mean ± the standard error of the mean of at least three independent results. Results were analysed using a Student's t- test against 0mM of SCFA. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

INSA4015

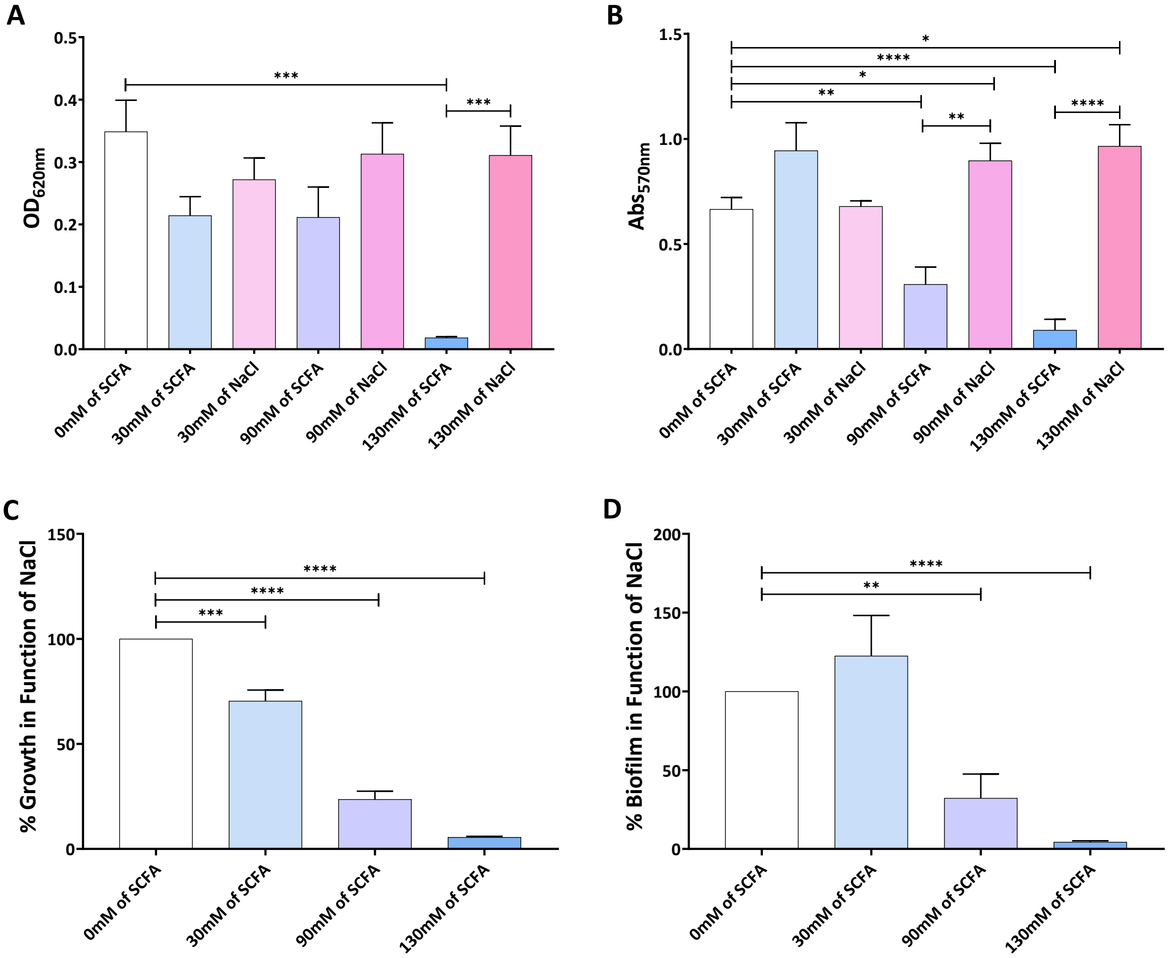


Figure S22. Bacterial growth of the *Aliarcobacter butzleri* INSA4015 strain in the presence of different concentrations of SCFA mixtures and NaCl concentrations (A). Biofilm formation in the presence of SCFA mixtures and respective NaCl concentrations (B). Percentage of growth with SCFA mixtures in function of the respective NaCl concentrations (C). Percentage of biofilm formation in function of the SCFA's NaCl concentrations (D). The data represents the mean ± the standard error of the mean of at least three independent results. Results were analysed using a Student's t- test against 0mM of SCFA. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Table S1. Statistical analysis of the growth curves for the eight strains in study. The values presented represent the p-value from 0h to 48h. Results were analysed using a two-way ANOVA test against TSB or Minimal Medium growth curves. *P* values<0.05 are marked in bold.

Compounds	**Strains**							
	A11-1	W12-1	AB28/11	DQ40A1	INSA			
					2756	2999	3800	4015
5mM SA	>0.9999; 0.3645; 0.9613; 0.4324	>0.9999; 0.9709; 0.9308; **0.0337**	>0.9999; 0.3826; 0.9936; 0.5862	>0.9999; >0.9999; >0.9999; 0.6069; **0.0016**; **0.0014**; **0.0026**; **0.0450**; 0.1839; 0.7806; 0.9967; >0.9999; 0.9491; 0.9251; 0.9251	>0.9999; 0.9998; 0.9998; **0.0353**	0.9999; 0.1014; 0.5413; 0.3534	0.9999; 0.9998; 0.9780; 0.6130	>0.9999; >0.9999; 0.5930; **0.0167**
20mM SA	>0.9999; 0.3079; 0.9996; 0.7584	>0.9999; 0.7370; 0.5578; 0.7733	>0.9999; 0.4675; **0.0144**; 0.3317	>0.9999;0.9999; 0.9986; 0.9950; **0.0019; <0.0001; <0.0001; <0.0001; 0.0011**; 0.1481; 0.7158; 0.9959; 0.9997; 0.2972; 0.3772	>0.9999; >0.9999; 0.3866; 0.6829	0.9999; 0.0005; 0.9757; 0.1146	0.9998; 0.9877; 0.9996; 0.9997	>0.9999; 0.9985; 0.4071; **0.0118**
40mM SA	>0.9999; 0.8984; 0.8456; 0.8910	>0.9999; 0.2428; 0.9997; >0.9999	>0.9999; 0.8015; 0.8060; 0.7991	>0.9999; 0.9998; 0.9827; 0.9963; 0.6477; **<0.0001; <0.0001; <0.0001; 0.0013**; 0.2014; 0.8181; >0.9999; >0.9999; 0.1667; 0.7572	>0.9999; 0.6684; 0.9997; 0.0999	>0.9999; 0.8255; 0.9854; 0.1423	0.9998; 0.7014; 0.3662; 0.4038	>0.9999; 0.8500; **0.0001; 0.0038**
80mM SA	>0.9999; 0.9064;**0.0003**; 0.9732	>0.9999; **0.0002; <0.0001**; 0.9248	0.9998; **0.0019; 0.0002**; 0.9011	>0.9999; 0.9997; 0.8751; 0.4919; 0.4529; 0.9853; 0.1663; **0.0044; 0.0030**; 0.0683; 0.3324; 0.8528; 0.9768; 0.7391; 0.9846	0.9997; 0.3494; **0.0092**; 0.2153	>0.9999; **0.0011**; 0.4297; 0.2599	0.9999; 0.2191; **0.0019; <0.0001**	>0.9999; 0.4461; **<0.0001; 0.0204**
100mM SA	0.9999; 0.2514; **<0.0001**; 0.9999	>0.9999; **<0.0001; <0.0001**; 0.1010	0.9997; **0.0002; <0.0001**; 0.0320	>0.9999; 0.9997; 0.8404; 0.3467; 0.0826; 0.0579; 0.1516; 0.9122; 0.5173; 0.4968; 0.5852; 0.9332; 0.9985; 0.9885; 0.9941	0.9952; 0.1729; **<0.0001**; 0.1256	0.9999; **<0.0001; <0.0001**; 0.1223	0.9998; 0.2367; **0.0010; <0.0001**	>0.9999; 0.3875; **<0.0001; <0.0001**
2.5mM SP	>0.9999; **0.0017**; 0.1085; 0.8986	>0.9999; **0.0003**; 0.7653; **<0.0001**	>0.9999; 0.1458; 0.9979; 0.4263	>0.9999; >0.9999; 0.9967; 0.9997; **0.0029; 0.0010; 0.0013; 0.0076;** 0.0615; 0.6867; 0.9630; 0.9799; 0.9457; >0.9999; 0.3457	>0.9999; 0.9947; 0.8050; 0.1024	0.9999; **<0.0001**; 0.6670; 0.3958	0.9998; 0.9896; >0.9999; 0.9351	0.9999; 0.9998; 0.9996; 0.0919
5mM SP	>0.9999; **<0.0001**; 0.1157; 0.7373	>0.9999; **<0.0001**; 0.9998; **<0.0001**	>0.9999; **0.0014; 0.0424**; 0.5488	>0.9999; >0.9999; 0.9863; 0.9999; **0.0142; <0.0001; <0.0001; <0.0001; 0.0011**; 0.19146; 0.7036; 0.5112; 0.9943; 0.9999; 0.0641	>0.9999; >0.9999; 0.9511; 0.2573	0.9999; **<0.0001**; 0.7369	0.9998; 0.9843; 0.9999; >0.9999	>0.9999; 0.9999; 0.2371; 0.0019

20mM SP	>0.9999; **<0.0001**; 0.7498; 0.9942	>0.9999; **<0.0001**; 0.9996; 0.3570	0.9999; 0.9839; 0.2618; 0.0681	>0.9999; 0.9996; 0.7180; 0.1959; 0.2109; 0.8894; **0.0009; <0.0001; 0.0034**; 0.7165; 0.9916; 0.5229; 0.0229; 0.0358; 0.5090	>0.9999; 0.9997; **0.0004; 0.0001**	>0.9999; 0.9485; **0.0025;** 0.0861	0.9997; 0.2093; **0.0084; <0.0001**	>0.9999; 0.8685; **<0.0001; 0.0227**
40mM SP	>0.9999; **<0.0001**; 0.9904; **0.0397**	>0.9999; 0.9913; **0.0034;** 0.9314	0.9998; **<0.0001; 0.0006; <0.0001**	>0.9999; 0.9983; 0.6330; 0.1025; **0.0022; 0.0002; <0.0001; <0.0001;** 0.9943; >0.9999; 0.9997; 0.9889; 0.2605; 0.0680; 0.6535	>0.9999; 0.5237; **0.0004; <0.0001**	>0.9999; **<0.0001; 0.0239; 0.0003**	0.9997; **0.0138; <0.0001; <0.0001**	>0.9999; 0.4523; **<0.0001; <0.0001**
60mM SP	0.9997; 0.9978; 0.9497; **0.0233**	>0.9999; **<0.0001;** 0.0528; 0.3582	0.9998; **<0.0001; <0.0001; <0.0001**	>0.9999; 0.9974; 0.5428; 0.0619; **0.0006; <0.0001; <0.0001; <0.0001; <0.0001; <.0001; <0.0001; 0.0001; 0.0139**; 0.0640; 0.0760	>0.9999; 0.1348; **<0.0001; <0.0001**	>0.9999; **<0.0001; <0.0001; <0.0001**	0.9996; **0.0021; <0.0001; <0.0001**	>0.9999; 0.1583; **<0.0001; <0.0001**
2.5mM SB	0.9993; 0.9861; 0.0627; 0.0538	>0.9999; 0.1859; 0.5469; **<0.0001**	0.9987; 0.9716; 0.6400; 0.6939	>0.9999; >0.9999; 0.9919; >0.9999; >0.9999; >0.9999; 0.9998; 0.9948; 0.8639; 0.9889; 0.9956; 0.9870; 0.7121; 0.9999; 0.2762	>0.9999; 0.9922; 0.1721; 0.1556	>0.9999; 0.4090; 0.4419; 0.4617	0.9997; 0.9471; 0.9579; 0.9572	>0.9999; 0.9999; 0.2776; 0.9980
5mM SB	0.9998; 0.9768; 0.2012; **0.0020**	>0.9999; 0.1193; **<0.0001; <0.0001**	>0.9999; 0.6666; **0.0022;** >0.9999	>0.9999; >0.9999; 0.9846; 0.9979; >0.9999; 0.9980; 0.8655; 0.5560; 0.9998; >0.9999; 0.9885; 0.8770; 0.4270; 0.6900; 0.6411	>0.9999; 0.9871; 0.3657; **0.0342**	>0.9999; 0.1819; 0.7819; 0.7354	0.9997; 0.9397; 0.8395; 0.9993	>0.9999; 0.9963; 0.2017; 0.5461
10mM SB	0.9999; 0.6827; **0.0051; 0.0009**	>0.9999; **0.0477; <0.0001; <0.0001**	>0.9999; 0.4591; 0.1402; 0.6011	0.9999; >0.9999; 0.9280; 0.8181; 0.8292; 0.4622; **0.0248**; 0.0510; 0.0686; **0.0227**; 0.1071; 0.4497; 0.0507; 0.4224; 0.6589	>0.9999; 0.8458; **0.0223; 0.0015**	>0.9999; 0.0450; 0.8452; 0.7302	0.9992; 0.8395; 0.4919; 0.9873	>0.9999; 0.9795; **0.0320**; 0.3728
20mM SB	>0.9999; 0.4004; **<0.0001; <0.0001**	>0.9999; **0.0096; <0.0001; <0.0001**	>0.9999; **0.0050; <0.0001;** 0.9913	>0.9999; 0.9991; 0.5835; 0.1643; **0.0451; 0.0044; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001;** 0.9705; **0.0001**	>0.9999; 0.5049; **<0.0001; 0.0035**	0.9998; **0.0043; 0.0087;** 0.9760	0.9997; 0.4558; **0.0410; 0.0026**	>0.9999; 0.8798; **0.0016; 0.0485**
30mM SCFA	>0.9999; 0.0244; 0.7895; 0.9990	>0.9999; 0.1143; 0.2690; 0.8378	>0.9999; 0.1680; **0.0112;** 0.6665	>0.9999; 0.9999; 0.9667; 0.6202; **<0.0001; <0.0001; <0.0001; <0.0001; <0.0001;** 0.1267; 0.9554;	>0.9999; 0.9723; 0.2776; 0.0858	>0.9999; **<0.0001;** 0.7243; **0.0079**	0.9988; 0.4876; **0.0233; 0.0003**	>0.9999; 0.8868; **0.0075; 0.0016**

				0.8601; 0.8905; **0.0473;** 0.0616				
30mM SCFA pH 6.5				>0.9999; 0.9997; 0.6869; 0.2231; 0.7314; **<0.0001; 0.0012**; >0.9999; **0.0384; 0.0003; <0.0001; <0.0001; 0.0016;** 0.8519				
90mM SCFA	0.9999; 0.9997; 0.9977; 0.2383	>0.9999; **0.0039;** 0.9954; 0.1023	0.9990; **<0.0001; <0.0001; 0.0034**	>0.9999; 0.9981; 0.8243; 0.2194; 0.1015; 0.9756; **<0.0001; <0.0001; <0.0001; 0.0007; 0.0205**; 0.3878; 0.8988; 0.2060; 0.9776	>0.9999; 0.1928; **<0.0001; 0.0002**	>0.9999; **<0.0001;** 0.8844; **0.0004**	0.9979; **0.0007; <0.0001; <0.0001**	>0.9999; 0.3522; **<0.0001; <0.0001**
90mM SCFA pH 6.5				>0.9999; 0.9970; 0.3564; 0.0095; **<0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001**; 0.7673				
130mM SCFA	0.9998; **0.0026;** 0.8926; 0.2215	>0.9999; **<0.0001; <0.0001;** 0.9954	0.9976; **<0.0001; <0.0001; <0.0001**	>0.9999; 0.9952; 0.5824; 0.0699; **0.0010; <0.0001; <0.0001; <0.0001; <0.0001**; 0.1372; 0.9846; 0.9042; 0.9118; 0.6593; 0.9999	0.9932; 0.1376, **<0.0001;** 0.0945	>0.9999; **<0.0001; <0.0001; 0.0009**	0.9955; **<0.0001; <0.0001; <0.0001**	>0.9999; 0.1561; **<0.0001; <0.0001**
130mM SCFA pH 6.5				>0.9999; 0.9948;0.3265; **0.0058; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001**				
172mM SCFA	0.9991; **<0.0001; <0.0001;** 0.6862	>0.9999; **<0.0001; <0.0001;** 0.6693	0.9952; **<0.0001; <0.0001; <0.0001**	>0.9999; 0.9971; 0.7283; 0.1502; **0.0036; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001**; 0.5442; 0.4153; 0.9959	0.9975; 0.0929; **<0.0001; <0.0001**	>0.9999; **<0.0001; <0.0001; <0.0001**	0.9962; **<0.0001; <0.0001; <0.0001**	>0.9999; 0.1220; **<0.0001; <0.0001**
172mM SCFA pH 6.5				>0.9999; 0.9965; 0.3382; **0.0062; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001;**				

				<0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001				
2.5mM NaCl	0.9998; 0.9999; **0.0093;** 0.9991	>0.9999; 0.9993; 0.9867; **0.0086**	>0.9999; 0.9994; 0.9997; 0.9997	>0.9999; >0.9999; 0.997; 0.9993; 0.9994; 0.9994; 0.9990; 0.9800; 0.5227; 0.9801; 0.9996; 0.9996; 0.9998; 0.9702; 0.9957	>0.9999; >0.9999; 0.9908; 0.5620	>0.9999; 0.9997; 0.8595; 0.9429	>0.9999; 0.9996; 0.8700; 0.9999	>0.9999; 0.9999; 0.9997; 0.9996
5mM NaCl	0.9998; 0.9996; 0.2852; 0.9873	>0.9999; 0.9998; 0.8168; 0.7568	0.9999; 0.9999; >0.9999; 0.9991	>0.9999; >0.9999; 0.9998; 0.9995; 0.9995; 0.9996; 0.9994; 0.9990; 0.9570; 0.9995; >0.9999; 0.9997; >0.9999; 0.9707; 0.9990	>0.9999; 0.9998; 0.3948; 0.9723	>0.9999; 0.9880; 0.9993; 0.9989	>0.9999; 0.9998; 0.9870; >0.9999	>0.9999; 0.9997; >0.9999; 0.9997
10mM NaCl	0.9998; 0.9996; 0.0694; 0.9887	>0.9999; 0.7285; 0.4158; 0.7476	>0.9999; 0.9997; 0.9997; 0.9990	>0.9999; >0.9999; 0.9998; 0.9996; 0.9995; 0.9995; 0.9991; 0.9990; 0.9893; 0.9997; 0.9999; 0.9998; >0.9999; 0.9175; 0.9761	>0.9999; 0.9998; 0.9734; 0.9925	>0.9999; 0.9993; 0.9998; 0.8581	>0.9999; 0.9999; 0.9914; 0.9997	>0.9999; 0.9996; 0.9430; 0.9999
20mM NaCl	0.9999; 0.9999; 0.9995; 0.9489	>0.9999; 0.9994; 0.9999; 0.2271	>0.9999; >0.9999; 0.9991; 0.9909	>0.9999; 0.9999; 0.9999; 0.9996; 0.9995; 0.9993; 0.9918; 0.9886; 0.9891; >0.9999; 0.9999; 0.9995; 0.9997; 0.6609; 0.6753	>0.9999; 0.9998; 0.5425; 0.7770	>0.9999; 0.9996; 0.9998; 0.9993	>0.9999; 0.9998; 0.9870; >0.9999	>0.9999; >0.9999; 0.9994; 0.9997
30mM NaCl	>0.9999; >0.9999; 0.9899; 0.8952	>0.9999; 0.9997; 0.0590; **0.0459**	>0.9999; 0.9907; 0.9992; 0.9994	>0.9999; >0.9999; >0.9999; >0.9999; 0.9998; 0.9998; 0.9997; 0.9993; 0.9992; >0.9999; 0.9996; 0.9921; 0.9919; 0.1422; 0.9849	>0.9999; 0.9999; **0.0176;** 0.9958	>0.9999; >0.9999; 0.9996; 0.9995	0.9999; 0.9998; 0.9993; 0.9999	>0.9999; >0.9999; 0.9994; >0.9999
40mM NaCl	>0.9999; 0.9996; 0.4976; 0.6223	>0.9999; 0.9997; 0.9999; 0.9787	>0.9999; 0.9991; 0.9151; 0.5581	>0.9999; >0.9999; 0.9999; 0.9997; 0.9998; 0.9997; 0.9998; 0.9998; 0.9997; 0.9914; 0.9995; 0.9999; >0.9999; 0.5864; 0.6753	>0.9999; 0.9999; **0.0029;** 0.6604	>0.9999; 0.9993; 0.9995; 0.9849	>0.9999; 0.9996; 0.9880; 0.9991	>0.9999; >0.9999; 0.9895; 0.9996
60mM NaCl	>0.9999; 0.9994; 0.3689; 0.9993	>0.9999; 0.9997; **0.0004**; 0.9996	>0.9999; 0.9867; 0.8488; 0.6508	>0.9999; >0.9999; 0.9999; 0.9999; >0.9999; >0.9999; 0.9999; 0.9998; 0.9999; 0.9651; 0.9823; 0.9994; 0.9995; 0.2850; 0.6020	>0.9999; 0.9991; **0.0098**; 0.3860	0.9999; 0.9993; 0.9998; 0.2386	>0.9999; 0.9997; 0.9993; 0.9992	>0.9999; >0.9999; 0.9353; >0.9999
80mM NaCl	>0.9999; >0.9999; 0.9995; 0.3885	>0.9999; 0.9993; 0.9802; >0.9999	>0.9999; 0.8642; 0.7114; 0.8581	>0.9999; >0.9999; >0.9999; >0.9999; 0.9997; 0.9997; 0.9894; 0.9448; 0.9993;	>0.9999; 0.9990; **0.0015; 0.0086**	>0.9999; 0.9990; 0.9996; 0.9662	>0.9999; >0.9999; >0.9999; 0.7038	>0.9999; 0.9999; 0.9297; 0.9996

				0.7048; 0.3985; 0.8224; 0.9861; 0.3904; 0.9344				
90mM NaCl	>0.9999; >0.9999; 0.9999; 0.7812	>0.9999; 0.9995; 0.9993; 0.9999	0.9999; 0.7048; 0.1082; 0.1176	>0.9999; >0.9999; 0.9996; 0.9996; 0.9995; 0.9991; 0.8613; 0.5413; 0.6003; 0.3374; 0.5891; 0.9990; 0.9996; **0.0062**; 0.9995	>0.9999; >0.9999; <0.0001; 0.0572	>0.9999; 0.9991; 0.9602; 0.1448	>0.9999; 0.9997; 0.9994; 0.7128	>0.9999; 0.9996; 0.3726; 0.9996
100mM NaCl	>0.9999; 0.9998; 0.9997; 0.9792	0.9999; 0.9595; 0.1099; 0.9927	>0.9999; 0.3719; **0.0131;** 0.7453	>0.9999; >0.9999; 0.9998; 0.9998; 0.9997; 0.9996; 0.9821; 0.8563; 0.9990; 0.4702; 0.5483; 0.4063; 0.7741; 0.3589; 0.9912	>0.9999; 0.9712; <0.0001; 0.3150	>0.9999; 0.9694; 0.9930; 0.9124	>0.9999; >0.9999; 0.9992; 0.3618	>0.9999; 0.9995; 0.1614; 0.9993
130mM NaCl	>0.9999; >0.9999; 0.3365; 0.2249	>0.9999; 0.9830; **0.0462**; 0.9997	>0.9999; 0.3916; **0.0021**; 0.1829	>0.9999; >0.9999; 0.9995; 0.9993; 0.9920; 0.9207; 0.1614; **0.0199**; 0.0293; **0.0111**; 0.1265; 0.8667; 0.9993; **0.0155**; 0.9991	>0.9999; 0.9990; 0.1412; 0.1736	>0.9999; 0.9142; 0.3567; 0.1634	>0.9999; 0.9993; 0.9866; **0.0496**	>0.9999; 0.9996; 0.1018; 0.9818
172mM NaCl	>0.9999; 0.9992; **0.0003**; 0.9959	0.9999; 0.9752; **<0.0001;** 0.9399	>0.9999; 0.5808; **0.0097**; 0.6025	>0.9999; >0.9999; 0.9994; 0.9880; 0.9185; 0.4478; **0.0088; <0.0001; <0.0001; <0.0001; <0.0001; 0.0040;** 0.2908; 0.9993; 0.9997	>0.9999; 0.9663; **<0.0001**; 0.1082	>0.9999; 0.0288; 0.5842; 0.8905	>0.9999; 0.9991; 0.3509; **0.0012**	>0.9999; 0.9993; **0.0282; 0.0196**
Minimal Medium								
20mM SA	>0.9999; 0.2911; **<0.0001; <0.0001**	0.9997; **<0.0001; <0.0001; <0.0001**	0.9747; **<0.0001; <0.0001; <0.0001**	>0.9999; 0.9914; 0.8453; 0.9997; 0.9908; 0.2342; 0.0005; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001	>0.9999; 0.0296; <0.0001; <0.0001	>0.9999; 0.0054;<0.0001; <0.0001	0.9998; <0.0001; <0.0001; <0.0001	0.9980; 0.0003; <0.0001; <0.0001
40mM SA	0.9999; 0.3799; **0.0035; <0.0001**	0.9999; **<0.0001; <0.0001; <0.0001**	0.9806; **0.0001; <0.0001; <0.0001**	>0.9999; 0.9525; 0.7417; 0.9981; >0.9999; 0.8729; 0.1221; **0.0042; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001**	0.9999; 0.1573; 0.0589; **<0.0001**	>0.9999; 0.2874; **0.0020; <0.0001**	>0.9999; **0.0028; <0.0001; <0.0001**	0.9539; 0.1263; **<0.0001; <0.0001**
20mM SP	0.9999; 0.9752; **0.0114; 0.0001**	>0.9999; 0.9597; **<0.0001; <0.0001**	0.9896; 0.8459; 0.2358; **<0.0001**	>0.9999; 0.9499; 0.4534; 0.7807; 0.7949; 0.6633; 0.5945; 0.6587; 0.6971; 0.6655; 0.8123; 0.8143; 0.8365; 0.8815; 0.8815	>0.9999; 0.9996; 0.0673; **0.0001**	>0.9999; 0.9998; **0.0078; 0.0055**	0.9999; 0.7077; 0.9999; **<0.0001**	0.9980; 0.9999; 0.7417; **<0.0001**
40mM SP	>0.9999; 0.9977;	0.9997; 0.8204;	0.9981; 0.8534;	>0.9999; 0.8996; 0.3975; 0.7485; 0.7872; 0.6631;	>0.9999; 0.9980;	>0.9999; 0.9715;	0.9998; 0.3528;	0.9689; 0.9980;

	0.9135; >0.9999	0.9013; **0.0384**	0.9802; 0.2793	0.6662; 0.7285; 0.7542; 0.7343; 0.8030; 0.8107; 0.8307; 0.7485; 0.7485	0.9997; **0.0136**	0.9976; 0.2918	0.4678; 0.9229	0.9962; **0.0205**
10mM SB	>0.9999; 0.9997; 0.9135; 0.9702	>0.9999; 0.9997; 0.9900; 0.9995	0.9842; 0.5735; 0.3870; 0.3382	>0.9999; 0.9952; 0.8996; 0.9996; 0.9996; 0.9980; 0.9996; 0.9997; 0.9999; 0.9999; >0.9999; 0.9999; 0.9999; 0.9995; 0.9997	0.9982; 0.9999; 0.9965; 0.9697	>0.9999; 0.9999; >0.9999; 0.9918	>0.9999; 0.9998; 0.9944; 0.9981	0.9980; 0.2031; 0.6584; 0.9942
20mM SB	0.9999; 0.9983; 0.9618; 0.9702	0.9998; 0.9996; 0.9778; 0.9997	0.5735; 0.6731; 0.5146; 0.9802	>0.9999; 0.8583; 0.9927; 0.9139; 0.9056; 0.9139; 0.9289; 0.9538; 0.9590; 0.9639; 0.9927; 0.9821; 0.9947; 0.9913; 0.9975	0.9998; 0.9995; 0.9347; 0.9902	>0.9999; 0.9998; >0.9999; >0.9999	0.9997; >0.9999; 0.9997; 0.9920	0.9996; 0.2809; 0.4133; 0.9968
30mM SCFA	0.9999; 0.3095; **0.0025; <0.0001**	>0.9999; **<0.0001; <0.0001; <0.0001**	0.7578; **<0.0001; <0.0001; <0.0001**	0.9999; >0.9999; 0.9999; >0.9999; 0.9606; 0.2911; **0.0008; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001**	>0.9999; **0.0005; <0.0001; <0.0001**	>0.9999; **0.0002; <0.0001; <0.0001**	0.9998; **0.0002; <0.0001; <0.0001**	0.9858; **0.0069; <0.0001; <0.0001**
90mM SCFA	0.9990; 0.9998; 0.9719; 0.3859	>0.9999; 0.2588; **0.0008; <0.0001**	0.8179; **<0.0001; <0.0001; <0.0001**	>0.9999; 0.7685; 0.6228; 0.5148; 0.6644; 0.9186; >0.9999; 0.9997; 0.7216; 0.3557; **0.0425**; 0.0666; **0.0084; <0.0001; <0.0001**	>0.9999; 0.2933; **0.0063; 0.0032**	0.9992; 0.9637; 0.1932; **0.0113**	0.9992; 0.2389; **<0.0001; <0.0001**	0.9237; 0.8000; **0.0490; 0.0003**
130mM SCFA	0.9999; 0.9990; 0.9807; 0.9807	0.9997; 0.9980; 0.2835; **0.0028**	0.8945; 0.1422; **0.0073; <0.0001**	>0.9999; 0.9186; 0.5559; 0.3626; 0.4057; 0.5393; 0.6892; 0.8397; 0.9509; 0.9970; 0.9999; >0.9999; 0.9999; 0.7759; <0.0001	>0.9999; 0.8755; 0.2475; 0.1312	0.9997; >0.9999; 0.6997; 0.5910	>0.9999; 0.9992; 0.0558; **<0.0001**	0.9685; 0.9953; 0.5384; 0.1678
172mM SCFA	0.9998; 0.9313; 0.9807; 0.5659	0.9998; 0.9666; 0.8892; 0.3039	0.9496; 0.7578; 0.2870; **0.0002**	0.9991; 0.9767; 0.6760; 0.5220; 0.7401; 0.5878; 0.6760; 0.8001; 0.9251; 0.9966; 0.9997; 0.9998; 0.9927; **0.0047; <0.0001**	>0.9999; 0.9998; 0.7082; 0.9129	0.9998; 0.9687; 0.9997; 0.9687	0.9998; 0.9878; 0.5303; 0.0944	0.9858; 0.9997; 0.9784; 0.9237
10mM NaCl	0.9996; 0.9999; 0.8548; 0.3194	0.9998;0.9998; >0.9999; 0.9802	0.4675; 0.4675; 0.9995; 0.9838	0.9941; 0.4731; 0.8188; 0.9974; 0.9999; 0.9994; 0.9972; 0.9997; 0.9996; 0.9930; 0.9936; 0.9734; 0.8777; 0.2403; 0.6748	0.9954; 0.9997; 0.9832; 0.9919	>0.9999; 0.4766; 0.0168; 0.1239	0.9941; 0.9907; 0.9995; 0.8973	0.9813; 0.9999; 0.9978; 0.9954
20mM NaCl	0.9713; 0.9445;	0.9972; 0.9979;	0.4287; 0.3228;	0.9998; 0.9997; 0.9997; 0.9842; 0.9979; 0.8843;	0.9997; 0.9999;	0.9997; 0.2056;	0.9999; >0.9999;	0.9813; 0.9999;

	0.9947; 0.9056	0.9906; 0.9906	0.9999; 0.9838	0.8023; 0.8968; 0.8188; 0.7853; 0.8345; 0.4824; 0.5679; 0.1602; 0.6748	0.8478; 0.9496	**0.0068**; 0.059	0.9997; 0.7624	0.8257; 0.9978
30mM NaCl	0.9997; 0.9445; 0.9445; 0.7257	0.9997; 0.9957; 0.9906; 0.9906	0.6347; 0.5496; 0.9997; 0.9979	0.9995; 0.9996; 0.9996; 0.9842; 0.9997; 0.8843; 0.7590; 0.8968; 0.7766; 0.7408; 0.7500; 0.4824; 0.4282; 0.1025; 0.5776	0.9996; 0.9932; 0.9919; 0.9995	0.9997; 0.2798; **0.0107;** **0.0168**	>0.9999; >0.9999; 0.9997; 0.8685	0.9998; 0.9995; 0.9110; 0.9998
40mM NaCl	0.9713; 0.9445; 0.9445; 0.7939	>0.9999; >0.9999; 0.9995; 0.9948	0.5496; 0.5920; 0.9995; 0.9838	0.9997; 0.9995; 0.9996; 0.9914; 0.9996; 0.9141; 0.8422; 0.8968; 0.8188; 0.7408; 0.7939; 0.4370; 0.3450; 0.0630; 0.3147	0.9832; 0.9735; 0.9996; 0.9995	>0.9999; 0.4766; **0.0259;** **0.0259**	>0.9999; >0.9999; 0.9997; 0.8360	0.9997; 0.9914; 0.9995; 0.9999
90mM NaCl	0.9947; 0.9996; 0.7257; 0.3770	0.9998; >0.9999; 0.9997; 0.9976	0.7194; 0.8678; 0.9941; 0.9979	0.9995; 0.9979; 0.9997; 0.9842; 0.9998; 0.8843; 0.8023; 0.8641; 0.7766; 0.6941; 0.9029; 0.9997; 0.9997; 0.9822; 0.9997	0.9919; 0.9999; 0.9919; 0.9832	0.9997; 0.2798; **0.0168;** **0.0392**	0.9997; >0.9999; >0.9999; 0.8360	0.9954; 0.8719; 0.9813; 0.9914
130mM NaCl	0.9996; >0.9999; 0.7939; 0.4403	>0.9999; 0.8841; 0.9419; 0.8741	0.7988; 0.8678; 0.9972; 0.9436	0.9995; 0.9997; >0.9999; 0.9974; 0.9998; 0.9842; 0.9087; 0.9246; 0.8907; 0.7853; 0.7939; 0.5293; 0.5198; **0.0309**; 0.5776	0.8008; 0.9735; 0.9999; 0.9999	0.9997; 0.3706; 0.0860; 0.0586	>0.9999; >0.9999; 0.9997; 0.9224	0.9978; 0.9997; 0.8719; 0.9997
172mM NaCl	0.9999; 0.9445; 0.5802; 0.4403	0.9732; 0.9996; 0.9419; 0.9999	0.5759; 0.4256; >0.9999; 0.9996	0.9971; 0.9856; 0.9327; 0.7380; 0.9996; 0.9486; 0.9073; 0.9436; 0.7602; 0.5496; 0.5732; 0.3438; 0.2410; **0.0445**; 0.9486	0.9496; 0.8978; 0.9227; 0.9996	0.9938; 0.1475; 0.0586; **0.0168**	>0.9999; >0.9999; 0.9941; 0.9979	0.9978; 0.9421; 0.9110; 0.9995

Annex II

Poster presentations:

Fonseca I., Mateus C., Domingues F., Oleastro M., Ferreira S. (2023, March 2) Efeito da exposição de ácidos gordos de cadeia curta na virulência de *Aliarcobacter butzleri* [Poster presentation]. VII Jornadas de Educação e Investigação em Saúde, Guarda, Portugal, 2023.

VII JORNADAS
EDUCAÇÃO E INVESTIGAÇÃO EM SAÚDE

POLI TÉCNICO GUARDA

CERTIFICADO

Certifica-se que

Inês Fonseca, Cristiana Mateus, Fernanda Domingues, Mónica Oleastro, Susana Ferreira

foram autores do Poster apresentado com o título: **Efeito da exposição a ácidos gordos de cadeia curta na virulência de *Aliarcobacter Butzleri***, nas VII Jornadas de Educação e Investigação em Saúde, que decorreram no dia 02 de março de 2023, organizadas pela Escola Superior de Saúde do Politécnico da Guarda.

Guarda, 01 de março de 2023

A Diretora da Escola Superior de Saúde	A Presidente do Conselho Técnico-Científico	O Presidente do Conselho Pedagógico
(Profª. Maria Hermínia Nunes Barbosa)	(Profª. Doutora Ermelinda Marques)	(Prof. Luís António Videira)

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Avenida Dr. Francisco Sá Carneiro, n.º 50 | 6300-559 Guarda

Tel. 271 220 100 | Fax. 271 222 690

Fonseca I., Mateus C., Domingues F., Oleastro M., Ferreira S. (2023, December 7-9) Effects of Short Chain Fatty Acids on *Aliarcobacter butzleri*'s Virulence [Poster presentation]. Congress of Microbiology and Biotechnology, Covilhã, Portugal, 2023.

MICRO BIOTEC 23

December 7th – 9th | 2023

UBI, Covilhã

CONGRESS OF MICROBIOLOGY AND BIOTECHNOLOGY 2023

Certificate

We hereby certify that,

Susana Ferreira

presented the **Poster**, entitled:**"Effects of Short Chain Fatty Acids on Aliarcobacter butzleri's Virulence"** at the **Congress of Microbiology and Biotechnology 2023**, held from **07 to 09 December 2023**, in Covilhã, Portugal.

Were co authors of this work: **Inês Fonseca, Cristiana Mateus, Fernanda Domingues, Mónica Oleastro, Susana Ferreira**

Covilhã, 09th of December 2023

Ana Cristina Dias Cabral
Congress Chair

Susana Ferreira
Congress Chair

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Oral presentations:

Fonseca I., Mateus C., Domingues F., Oleastro M., Ferreira S. (2023, July 10-11) Modulation of *Aliarcobacter butzleri'* survival and virulence [Oral presentation]. XVIII International CICS-UBI Symposium, Covilhã, Portugal, 2023.

CICSUBI
Centro de Investigação em Ciências da Saúde
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XVIII International CICS-UBI Symposium

CERTIFICATE

I herewith certify that Inês Fonseca has participated as speaker in the XVIII International CICS-UBI Symposium, which was held the 10th and 11th July 2023.

The Organizing Committee

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