



CATÓLICA

ESCOLA SUPERIOR DE BIOTECNOLOGIA

PORTO

**SKIN MICROBIAL COMPETITION STUDIES AND MECHANISMS OF MICROBIAL
ADHESION TO KERATINOCYTES**

Thesis presented to Escola Superior de Biotecnologia of the Universidade Católica Portuguesa
to fulfill the requirements of Master of Science degree in Applied Microbiology

by

Catarina Filipa Branco Pereira Ribeiro Camilo

[Maio 2020]



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ADHESION TO KERATINOCYTES**

ESTUDOS DE COMPETIÇÃO MICROBIANA NA PELE E MECANISMOS DE ADESÃO
MICROBIANA A QUERATINÓCITOS

Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa*
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Place: Escola Superior de Biotecnologia da Universidade Católica Portuguesa

Supervision: Freni Kekhasharú Tavaría

[May 2020]

Resumo

A pele é um órgão importante nos mamíferos e desempenha um papel essencial na defesa do organismo humano, sendo a sua primeira barreira face ao ambiente externo. Os principais filos que fazem parte da microbiota de uma pele saudável são: Actinobacteria (51.8%), Firmicutes (24.4%), Proteobacteria (16.5%), and Bacteroidetes (6.3%), sendo os géneros predominantes o *Corynebacterium*, *Propionibacterium* e o *Staphylococcus*. Quando ocorre disbiose (desequilíbrio entre a microbiota comensal e os microrganismos oportunistas), podem-se originar patologias da pele. A maioria dos distúrbios da pele, são principalmente inflamatórios e são tradicionalmente tratados com corticosteróides. No entanto, existem alguns efeitos colaterais relacionados ao seu uso, como efeitos cutâneos deletérios, anormalidades eletrolíticas, hipertensão, hiperglicemia, efeitos imunológicos e neuropsicológicos, entre outros. Uma prescrição a longo prazo também está associada à osteoporose. Na tentativa de reduzir esses efeitos colaterais, os probióticos foram sugeridos como coadjuvantes na terapia, via aplicação tópica.

Em vista disso, o presente trabalho teve como objetivo realizar estudos de competição entre probióticos selecionados e bactérias patogénicas nas seguintes combinações: (*Lactobacillus paracasei* / *Staphylococcus aureus*; *L. paracasei* / *Staphylococcus epidermidis*; *L. paracasei* / *Escherichia coli*; *L. paracasei* / *Pseudomonas aeruginosa*); (*Propionifera* *innocua* / *E. coli*; *P. innocua* / *P. aeruginosa*); (*Bifidobacterium longum* spp. *infantis* / *S. aureus*; *B. longum* spp. *infantis* / *S. epidermidis*; *B. longum* spp. *infantis* / *E. coli*; *B. longum* spp. *infantis* / *P. aeruginosa*). O probiótico que apresenta a melhor vantagem competitiva foi posteriormente testado em ensaios de cultura celular usando células HaCat (linha celular de queratinócitos da pele humana) para entender-se o probiótico (*L. paracasei*) foi capaz de impedir a adesão das bactérias patogénicas (*S. aureus*, *S. epidermidis* e *P. aeruginosa*). Os melhores resultados foram obtidos pela técnica de deslocamento face ao *S. aureus*, onde foi observada uma redução de 3.8 unidades logarítmicas. Estes resultados não coincidem com os obtidos pelos ensaios de competição de células no seu estado livre. As principais razões provavelmente são a acessibilidade das células no meio, a auto/coagregação das células e a estimulação pelo Quorum Sensing. Com o intuito de se compreender alguns dos mecanismos utilizados para impedir essa adesão, também foi realizado um ensaio de adesão das proteínas. A

partir dos resultados obtidos, pode-se inferir que parte do processo de adesão implica a utilização de proteínas, embora não seja um mecanismo exclusivo.

Palavras-chave: pele; células livres; adesão; patologias na pele; probióticos

Abstract

As major organ in mammals, and first barrier of defense against external environment, the skin plays an essential role in the defense of the human organism, harboring a characteristic microflora. The most commonly found phyla of bacteria in healthy skin are Actinobacteria (51.8%), Firmicutes (24.4%), Proteobacteria (16.5%), and Bacteroidetes (6.3%) in which the most prevalent genera are *Corynebacterium*, *Propionibacterium* and *Staphylococcus*. Dysbiosis occurs when there is an unbalance between the commensal microbiota and opportunistic microorganisms., which can lead to skin pathologies. The majority of skin disorders, which are mainly inflammatory, are traditionally treated with corticosteroids. However, there are some side effects linked to their use such as deleterious cutaneous effects, electrolyte abnormalities, hypertension, hyperglycemia, immunologic, and neuropsychologic effects, among others. The long-term prescription is also associated with osteoporosis. In an attempt to reduce those side effects, probiotics have been suggested as co-adjuvants in the therapy, via topical application.

In this context, the present work was aimed at performing competition studies between selected probiotics and pathogenic bacteria in the following combinations: (*Lactobacillus paracasei*/*Staphylococcus aureus*; *L. paracasei*/*Staphylococcus epidermidis*; *L. paracasei*/*Escherichia coli*; *L. paracasei*/*Pseudomonas aeruginosa*); (*Propioniferax innocua*/*E. coli*; *P. innocua*/*P. aeruginosa*); (*Bifidobacterium longum* spp. *infantis*/*S. aureus*; *B. longum* spp. *infantis*/*S. epidermidis*; *B. longum* spp. *infantis*/*E. coli*; *B. longum* spp. *infantis*/*P. aeruginosa*). The probiotic presenting the best competitive advantage was then further tested in cell culture assays using HaCat cells (keratinocyte cell line from human skin) to assess the probiotic (*L. paracasei*) preventive role in the adhesion of the pathogenic bacteria (*S. aureus*, *S. epidermidis* and *P. aeruginosa*). The best results were achieved with the displacement technique towards *S. aureus*, in which a reduction of 3.75 logarithmic units was observed. These results did not match those obtained by the planktonic cell competition assays. The main reasons probably being cell accessibility in the media, cell (s) self / coaggregation and the empowerment by quorum sensing. In order to gain some insight on the mechanisms used to prevent this adhesion, a protein adhesion assay was also conducted. From the results obtained it can be inferred that part of the adhesion process uses proteins, although not being an exclusive mechanism.

Keywords: skin; planktonic cells; adhesion; skin disorders; probiotics

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“My goal is simple. It is a complete understanding of the universe, why it is as it is and why it exists at all.” By Stephen Hawking

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Table 1 - Selected organisms that are used as probiotic agents.

List of Abbreviations

AD	Atopic Dermatitis
AggLb	Auto-aggregation-promoting protein
AHLs	Acyl-homoserine-lactones
AMPs	Antimicrobial peptides
AtlE	Autolysin/adhesin
<i>B. longum</i> spp. <i>infantis</i>	<i>Bifidobacterium longum</i> spp. <i>infantis</i>
<i>B. bifidum</i>	<i>Bifidobacterium bifidum longum</i>
BM	Basement membrane
<i>C. albicans</i>	<i>Candida albicans</i>
CD8 + T	transmembrane glycoprotein, co-receptor for the T cell receptor (TCR)
CFU	Colony Forming Unit
CGRP	Calcitonin gene-related peptide
CoNS	Coagulase-negative Staphylococci
CSH	cell surface hydrophobicity
DCs	Dendritic cells
DETCs	dendritic epidermal T cells
DHNA	dihydroxy-2-naphtoic acid
<i>E. coli</i>	<i>Escherichia coli</i>
Eap	Extracellular adherence protein
ECM	Extracellular matrix
Embp	Extracellular matrix binding protein
EPS	Extracellular polymeric substance
EPSs	<i>exopolysaccharides</i>
FLG	filaggrin gene
Fn	fibronectin
FnBPA	fibronecting binding protein A
FnBPB	fibronecting binding protein B
FnBPs	fibronecting binding proteins
FOS	fructooligosaccharide
GPs	glycoproteins
HA	hyaluronic acid
HaCaT	spontaneously immortalized keratinocyte cell line
HePS	heteropolysaccharides
IFN- γ	interferon gamma
IFNs	interferon
IL-17A	interleukin 17A
Integrin $\alpha 5\beta 1$	Fibronectin receptor
<i>L. acidophilus</i>	<i>Lactobacillus acidophilus</i>
<i>L. bulgaricus</i>	<i>Lactobacillus bulgaricus</i>
<i>L. casei</i>	<i>Lactobacillus casei</i>
<i>L. fermentum</i>	<i>Lactobacillus fermentum</i>
<i>L. paracasei</i>	<i>Lactobacillus paracasei</i>
<i>L. reuteri</i>	<i>Lactobacillus reuteri</i>
<i>L. rhamnosus</i>	<i>Lactobacillus rhamnosus</i>
<i>L. salivarius</i>	<i>Lactobacillus salivarius</i>
<i>L. plantarum</i>	<i>Lactobacillus plantarum</i>
LAB	Lactic Acid Bacteria

LPS	lipopolysaccharides
logs	Logarithmic units
MHC	major histocompatibility complex
MSCRAMMs	adhesive matrix molecules
NCC	neural crest cells
NHEK	Normal Human Epidermal Keratinocyte
NO	Nitric oxide
NPPCs	neural progenitor cells
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
<i>P. innocua</i>	<i>Propioniferax innocua</i>
PACT	photodynamic antimicrobial chemotherapy
PBS	Phosphate buffer solution
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. epidermidis</i>	<i>Staphylococcus epidermidis</i>
<i>S. salivarius</i>	<i>Streptococcus salivarius</i>
SC	stratum corneum
SCFAs	short chain fatty acids
SCORAD	SCORing Atopic Dermatitis
SE	staphylococcal enterotoxins
SP	substance P
spp	strains of same species
SSTI	skin and soft tissue infection
Th1	Type 1 T helper cells
Th2	Type 2 T helper cells
TLRs	Toll-like receptors
TNFs	tumor necrosis factor
ZnPc-(Lys) ₅	zinc-Pentalysine β-Carbonylphthalocyanine
ZnPc	zinc phthalocyanine

1. Introduction

1.1 Skin

The skin is the largest organ in mammals, playing a pivotal role in the defense of the human organism. It is the first barrier to the external environment that can resist a wide range of opportunistic organisms. The presence of bacteria, viruses and fungi in skin is largely documented (Kong & Segre, 2012; Pappas, 2009; Schommer & Gallo, 2016). The skin has a normal microbiota with the main groups being Actinobacteria (51.8%), Firmicutes (24.4%), Proteobacteria (16.5%), and Bacteroidetes (6.3%). The majority of the identified genera are *Corynebacterium*, *Propionibacterium* and *Staphylococcus* (Schommer & Gallo, 2016). The abundance of each group is strongly dependent on the characteristics of the appropriate niche. (Hancock *et al.*, 2010; Ouwehand *et al.*, 2003). The skin is a unique and variable ecosystem that provides many niches, in which large populations of microbes are subject to variable ecological variables such as humidity, temperature, pH, and the composition of antimicrobial peptides and lipids (Krutmann, 2009). The normal pH of human skin is 5.4–5.9 (Nguyen & Soulika, 2019).

The challenge for the skin's immune system is that it is charged with resisting infections but must do so under normal conditions in the absence of cell recruitment and inflammation (Hirobe, 2014; Pandey, 2010; Sanford & Gallo, 2014). Main skin-resident immune cells, Langerhans cells (LCs) together with melanocytes, occupy epidermis, whereas the other types of immune cells such as various dendritic cell (DCs) subpopulations, macrophages, and several T cell types reside in deeper layer—dermis. The success of the response of the skin immunity depends on the flexibility of dermal vessels and the lymph nodes that drain the skin (Matejuk, 2018). Keratinocytes constitute a major structural element of outer layer of the skin and recent studies found unexpected role for keratinocytes in innate and adaptive immunity (Nestle *et al.*, 2009). Keratinocytes, neutrophils and epithelial cells create a major source of antimicrobial peptides (AMPs), small cationic and amphipathic molecules, acting as a first line of defense (Harder *et al.*, 1997). Abnormal AMPs expression leads to the development of inflammatory skin diseases and susceptibility to infections. According to (Wollenberg *et al.*, 2011), malfunctioning of some AMPs such as cathelicidin and b-defensins may play a role in atopic dermatitis lesions. One of AMPs, a member of cathelicidin family

(LL37), produced by keratinocytes has an essential role in promoting angiogenesis and wound healing (Zanetti, 2004). Keratinocytes express on the surface and within, endosomes Toll-like receptors (TLRs). Activation of TLRs on keratinocytes promotes Th1 (Type 1 T helper cells) responses and production of interferons (IFNs) (Miller, 2008). Keratinocytes are able to generate the production of classic pro-inflammatory cytokines such as IL-1b and IL18 via inflammasome signaling pathway (Tschopp et al., 2009). IL-1 produced by keratinocytes can upregulate expression of intercellular adhesion molecule (ICAM)-1. Upregulation of adhesion molecules on dermal endothelial cells and MHC class II (major histocompatibility complex) on keratinocytes and LCs facilitate leukocyte trafficking into the skin. Besides IL-1 and IL-18, keratinocytes are able to produce IL-6, IL-10, and tumor necrosis factors (TNFs) (Albanesi *et al.*, 2005). Keratinocytes own the ability to induce T cells activation or antigen-specific tolerization. Keratinocytes are not able to prime T cells; however, they can stimulate antigen experienced CD4 and CD8 cells (Black *et al.*, 2007).

1.2 Skin properties/ structure

The skin consists of two major components: the epithelium and connective tissue (Pasparakis *et al.*, 2014), with three main layers: the surface epidermis, the subjacent dermis, and the subcutaneous tissue (hypodermis, the lowest layer) (Graham *et al.*, 2019; Hirobe, 2014). According to Tabassum & Hamdani (2014), Epidermis, the outer most layer of the skin, varies in thickness in different regions of the body: on the eyelids (0.05 mm), the palms and soles (1.5 mm). The dermis also varies in thickness depending on the location of the skin. It is 0.3 mm on the eyelid and 3.0 mm on the back of the body. The dermis is attached to an underlying hypodermis or subcutaneous connective tissue. The subcutaneous tissue is a layer of fat and connective tissue that houses larger blood vessels and nerves (Tabassum & Hamdani, 2014).

(Baroni *et al.*, 2012), affirms that epidermis is a continually renewing epithelium, usually subdivided into several layers or strata, starting with the basal layer (or stratum basale) just above the dermis and proceeding upward through the spinous and granular layers to the top layer, the stratum corneum. The physical barrier mainly consists of the stratum corneum, although the cell–cell junctions and associated cytoskeletal proteins in the lower layers provide further important components. The predominant cell type at epidermis is the keratinocyte, they synthesize and express several structural proteins and

lipids during their maturation. Keratinocytes compose the bulk of the epithelium, undergo keratinization, a form the dead superficial layer of the skin. Their role is to produce keratin and filaggrin, which are involved in regulating the barrier function (Baroni *et al.*, 2012). The renewal of the epidermis / epithelium is supported by the proliferation and differentiation of keratinocytes (Hirobe, 2014). This characteristic of producing keratin and filaggrin may be a deciding factor in the adhesion / entry of pathogens to the human organism. The epidermis is predominantly composed by three cell types: keratinocytes, melanocytes, and fibroblasts (Hirobe, 2014). In the human body, the presence of melanocytes is typical of epidermis, hair and iris where they give color to these structures (Cichorek *et al.*, 2013). Recent evidence shows that melanocytes are able to secrete a wide range of signal molecules, including cytokines, POMC peptides (proopiomelanocortin), catecholamines, NO (nitric oxide) in response to UV (ultraviolet) irradiation and other stimuli (Papadimitriou *et al.*, 2015; Tsatmali *et al.*, 2002).

According to Nafisi & Maibach (2018), the dermis supports the epidermis that grows, the rate and direction of which depend on its position and perhaps on the way it is loaded, the dermal thickening (Nafisi & Maibach, 2018). The dermal thickening develops into a dermal papilla and the surrounding area forms the hair bulb. The lower area of the hair bulb is designated by the matrix that has numerous of melanocytes (Hirobe, 2014). Dermis also includes cutaneous invaginations and appendages, including sweat glands (eccrine and apocrine) sebaceous glands and hair follicles, associated with their own microbiota (Grice & Segre, 2011). The dermis consists on fibroblasts, beneath the epidermis. The melanocytes have the ability to produce and distribute melanin, originate from embryonic cells named neural crest cells (NCC) Nafisi & Maibach (2018). The dermis is rich in extracellular matrix and contains stromal cells such as fibroblasts, fibrocytes and structural cells of the blood and lymph vessels (Pasparakis *et al.*, 2014). Eccrine glands, present in dermis as well, are more abundant and are present on all skin surfaces continuously bathing it with their secretion, which is composed by water and salt. The main function is the thermoregulation through water evaporation. Those glands excrete water and electrolytes as well, leading to the acidification of skin, which prevents colonization by microorganisms. The apocrine glands are located in the armpit, nipple and genitoanal regions, which, in response to adrenaline, produce odorless secretions. The sebaceous glands are connected to the hair follicle, forming the pilosebaceous unit, and secrete the

lipid rich substance designated by sebum. This has the function of protecting and lubricating the skin and providing antibacterial defense (Grice *et al.*, 2008; Grice & Segre, 2011). A layer of lipids, which is both sebaceous and keratinocyte in origin, covers the surface of the skin. Lipids produced by the epidermal cells are an insignificant fraction of the total extractable surface lipid on areas rich in sebaceous glands. Due to the holocrine activity of the sebaceous gland, its product of secretion (sebum) is eventually released to the surface of the skin and coats the fur as well. The sebaceous lipids are primarily non-polar lipids such as triglycerides, wax esters and squalene, while epidermal lipids are a mixture of ceramides, free fatty acids and cholesterol. Recent studies have elucidated the roles that epidermal surface lipids have on normal skin functions and acne (Pappas, 2009).

In figure 1, it can be observed how epidermal can differentiate.

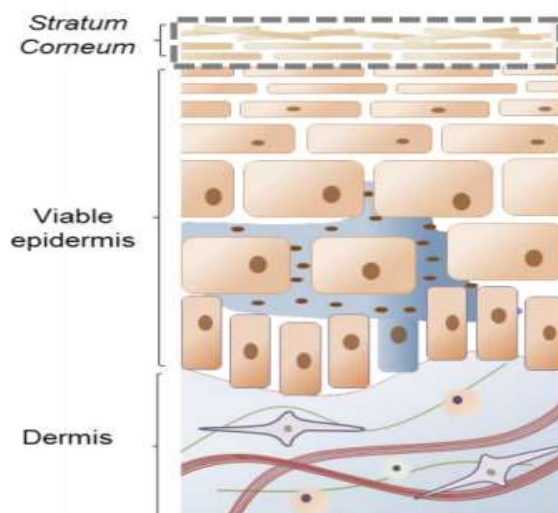


Figure 1- Epidermal differentiation (Adapted from (Florine *et al.*, 2018))

The epidermal lipids of keratinocyte origin play an essential role in the skin's barrier function. These lipids provide a barrier against the movement of water and electrolytes as well as a barrier against microbial invasion. The stratum corneum (SC) works as a permeability barrier, which limits the entrance of water and minerals, and is localized in the outer layers of the epidermis. The SC consists of the upper layers of corneocytes, which are terminally differentiated keratinocytes. These cells are imbedded in a lipophilic extra cellular medium composed of equal proportions of ceramides, cholesterol and free fatty acids. The above lipid mixture originates the lamellar bodies found in the epidermis (Pappas, 2009). Keratinocytes cells continuously desquamate from the surface and are replaced by cells derived from the lowest layer of the epidermis,

the basal layer (Hirobe, 2014). However, all the epithelial zones, at epidermidis, are also colonized by non-epithelial immune cells, such as Langerhans cells and dendritic epidermal T cells (DETCs) (Pasparakis *et al.*, 2014). Dendritic cells (DC) are a heterogeneous population of leukocytes that are critical in immunological response; and they arise from a bone marrow HSC- derived lineage dependent on the receptor tyrosine kinase FLT3 (Haniffa *et al.*, 2015).

(Sandilands *et al.*, 2009) refer that nowadays, loss-of-function mutations in FLG (filaggrin gen), the human gene encoding profilaggrin and filaggrin, have been identified as the cause of the common skin condition Itchyosis vulgaris (characterized by dry, scaly skin). These mutations represent a strong predisposition factor for atopic eczema, asthma and allergies. Profilaggrin is the major component of the keratohyalin granules within epidermal granular cells. During epidermal terminal differentiation, the profilaggrin polyprotein is dephosphorylated and rapidly cleaved by serine proteases to form monomeric filaggrin, which binds to and condenses the keratin cytoskeleton and thereby contributes to the cell compaction process that is required for squame biogenesis. Within the squames, filaggrin is citrullinated, which promotes its unfolding and further degradation into hygroscopic amino acids, which constitute one element of natural moisturising factor. Filaggrin is therefore in the frontline of defense and protects the body from the entry of foreign environmental substances that can otherwise trigger aberrant immune responses (Sandilands *et al.*, 2009).

1.3 Skin microbiota

The normal skin flora influences the anatomy, physiology, susceptibility to pathogens, and morbidity of the host. The human body, which contains about 10¹³ cells, routinely harbors about 10¹⁴ bacteria, that is designated by normal microbial flora (Baron, 1996).

As mentioned before, microorganism's abundance is dependent on body site characteristics, as shown in Figure 2.

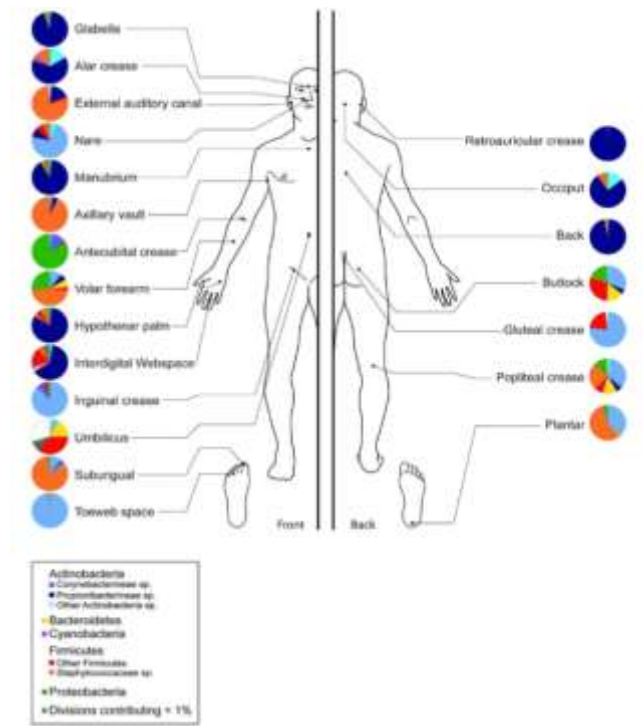


Figure 2- The skin microbiome. Sebaceous sites are labeled in blue, moist sites are labeled in green and dry surfaces are labeled in red. Family-level classification of bacteria colonizing an individual subject is shown (Adapted from (Kong & Segre, 2012).

The skin microbiota is categorized in two groups: (1) Resident microbes belonging to a relatively fixed group of microorganisms normally found on skin. Resident microorganisms are usually commensal, not being harmful to the host and the majority provide benefit; and (2) transient microbes that do not establish themselves permanently on the epidermis, arise from the environment and persist for hours or days (Kong & Segre, 2012).

Despite this equilibrium, intrinsic factors such as age, genetic component, sex and immune reactivity influence the composition of skin microorganism communities. Additionally, environmental factors such as climate and extrinsic factors like occupation, lifestyle, geographical location, use of antibiotics, or cosmetics and hygiene may also have a big impact on microbial resident flora (Grice *et al.*, 2008; Zeeuwen *et al.*, 2013). Overall, bacterial diversity is the lowest in sebaceous sites, since just a few microorganisms are able to tolerate these conditions. For example, sebaceous sites on the face are predominately populated by *Propionibacterium* spp. and *Staphylococcus* spp. In moist places such as the axilla, *Corynebacterium* spp. prevail, although *Staphylococcus* spp. are present as well. These microorganisms are correlated with the malodor produced by the sweat by apocrine glands. In contrast, in dry places mixed

populations of bacterial species of β -Proteobacteria, Flavobacteria and phyla of Actinobacteria, Firmicutes and Bacteroidetes are part of the skin microbiota, too (Grice *et al.*, 2008; Schommer & Gallo, 2016). Cool, acidic and desiccated skin is an inhospitable environment for microbial growth (Kong & Segre, 2012) The Gram-negative microbial abundance in skin is normally associated by contamination from the gastrointestinal tract (Grice *et al.*, 2008).

1.4 The bacterial role in skin disease

Skin is a continuously self-renewing organ that dynamically manages the outside-inside-outside relationships of the human body and actively participates in the host defenses (Baroni *et al.*, 2012). Some dermatological conditions can lead to an abnormal skin barrier, changing the homeostasis (Seo *et al.*, 2012). Skin diseases contributed 1.79% to the global burden of disease measured in disability-adjusted life years (DALYs). Skin diseases arranged in order of decreasing global DALYs are as follows: dermatitis (atopic, contact, seborrheic), acne vulgaris, urticaria, psoriasis, viral skin diseases, fungal skin diseases, scabies, melanoma, pyoderma, cellulitis, keratinocyte carcinoma, decubitus ulcer, and alopecia areata (Karimkhani *et al.*, 2017). The abnormal conditions lead to ecological dysbiosis such as humidity, temperature, pH, and the composition of antimicrobial peptides and lipids (Krutmann, 2009).

Indeed, keratinocytes represent 95% of the epidermal skin cells. Primarily, they play the structural and barrier function of the epidermis, but their role in the initiation and perpetuation of skin inflammatory and immunological responses, and wound repair, is also well recognized (Wikramanayake *et al.*, 2014).

1.4.1 - Adhesion Mechanisms

There are many factors that enhance bacterial ability to remain in host organism thereby increasing pathogenicity (Ribet & Cossart 2015). An essential requirement for colonizing the skin surface is adherence to keratinocytes (Coates *et al.*, 2014). Bacterial adhesion is an extremely complicated process that depends on the expression of a repertoire of surface proteins called adhesins, notably microbial surface components and recognizing adhesive matrix molecules (MSCRAMMs), the receptors (Josse *et al.*, 2017). Is also affected by the environmental factors, such as the presence of serum proteins or antibiotics, the bacterial properties and the material surface characteristics

(Yuehwei & Friedman, 2002). Koziel and Potempa (2013) claim that the skin constitutes a great barrier against commensal and pathogenic bacteria, which colonize this organ and bacteria that do not adhere quickly to the surfaces are rapidly killed by the immune system (Katsikogianni & Missirlis, 2004; O'Toole *et al.*, 2000). One important physicochemical phenomenon that characterizes the process of bacterial adhesion is bacterial surface charge. Bacteria attach quickly and tightly to positively charged surfaces, and electrostatic repulsion destabilizes cell contact with negatively charged surfaces. The layer of the bacteria cell wall that is in contact with the extracellular environment is complex and exposes many different functional groups (carboxylate, hydroxyl, phosphate, and amine moieties) that may interact with substrates (Renner and Weibel, 2011).

The adhesion abilities of bacteria have been linked with various different surface components including (lipo) teichoic acids, polysaccharides, and proteins (Wang *et al.*, 2018). Probiotic microorganisms' express cell-surface adhesins that mediate microbial adhesion to the ECM (extracellular matrix proteins) components of host tissue such as mucin, fibronectin, collagen, laminin or fibrinogen. The ability to bind to collagens is expressed by 70% of *Lactobacillus* isolates, and it seems that *Lactobacilli* express multiple adhesin types interacting with these abundant tissue proteins (Yadav *et al.*, 2013). Through the action of cell-surface adhesins, pathogens successfully interact with ECM, preserving peristalsis and enabling colonization of the tissue and infection (Dubreuil *et al.*, 2002). It is demonstrated that a group of proteins exposed on the pathogen's cell surface termed "adhesins" has been identified as the molecular basis for bacterial adherence to certain host molecules (Vaca *et al.*, 2019). Laminin and collagen are multifunctional glycoproteins that play an important role in cellular morphogenesis, cell signaling, tissue repair and cell migration. These proteins are present in tissues in the form of polymeric sheets that also contain nidogen, perlecan and agrin cross-linked proteins involved in various functions, and form a part of the basement membrane (BM), establish a protective layer around blood capillaries and are included in the extracellular matrix (ECM). The ECM is the acellular proteinaceous part of the animal connective tissues - consisting of collagen, elastin, fibrillin, laminin, fibronectin, vitronectin, thrombospondin, proteoglycans and hyaluronic acid (HA) (Hynes, 2009) - and is involved in building structural scaffolds, regulation of physiological processes, cellular signaling, migration and transport of solutes across the body tissues and cellular barriers (Mouw *et al.*, 2015). More specifically, adherence coordination can be

mediated by the fibronectin molecules which provide important human protein–protein and protein–oligosaccharide interactions during the formation of the ECM (Vaca *et al.*, 2019). Invasive pathogens break the basal lamina and degrade ECM proteins of interstitial spaces and connective tissues using various ECM-degrading proteases or surface-bound plasminogen and matrix metalloproteinases recruited from the host (Singh *et al.*, 2012). Collagens, the major glycoproteins (GPs) of connective tissues, account for 30% of the total protein in the human body, where they are involved in maintaining tissue architecture, cell adhesion, angiogenesis and development (Myllyharju & Kivirikko, 2004).

In fact, a study performed by Piwat (2015) demonstrated that there is a correlation between the total adhesion ability and the internalization of all *Lactobacillus* strains and surface charges. This information was however, only clearly observed in *L. fermentum* and *L. paracasei* strains. The individual strain with high surface charges was significant in internalization and in aggregation, given that the coefficient correlation was much higher. Cellular aggregation is defined as ability of cells to form precipitates. Auto-aggregation involves cells of the same bacterial strain, while genetically distant cells co-aggregate (Schachtsiek *et al.*, 2004). According to (Boris *et al.*, 1997) and (Nikolic *et al.*, 2010), in *Lactobacilli*, the two types of proteins responsible for manifestation of the aggregation phenomenon are the soluble proteins and the cell-surface proteins. In this study the decrease of *Lactobacillus paracasei* is probably to inactivation of intrinsic and extrinsic proteins, through the chemical compound trypsin, since its adherence is mainly due to the protein complex AggLb that plays an important role in colonization of host tissue and prevention of pathogen colonization (Miljkovic *et al.*, 2015). Another essential point is that bacteria contribute to skin infections, and are strongly associated with skin atopy, through number of bacterial adhesins that allows the microbe to adhere to and invade eukaryotic cells. One of these adhesive molecules is the multifunctional extracellular adherence protein (Eap) which is overexpressed in situ in human wounds, and shown to delay wound healing and strongly enhancing the internalization (Bur *et al.*, 2013). Commensal and pathogenic species residing in skin both express proteases that when secreted by commensals, contribute to homeostatic bacterial coexistence on skin (De Veer *et al.*, 2014). Is explained by Richmond & Harris (2014), that innate cells form rapid, but nonspecific, responses to infection. They generally recognize non-self-molecular patterns on pathogens or pathogen-associated molecular patterns (PAMPs), through receptors called pattern-recognition receptors

(PRRs are intracellular or cell surface receptors activated by DAMPs (Damage-associated molecular patterns are endogenous danger molecules that are released from damaged or dying cells and activate the innate immune system by interacting with pattern recognition) to induce inflammation) (Roh & Shon, 2018). Adaptive immune populations form slower, but pathogen-specific, responses to infection through specialized and unique antigen-specific receptors formed via genetic rearrangement (Richmond & Harris, 2014).

As reported by Vegandesan & Narayana (2011), the structural biology of Gram-positive cell surface adhesins is an emerging field of research, whereas Gram-negative pilus assembly and anchoring have been extensively investigated and are well understood. Gram-positive surface proteins known as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) and individual proteins that assemble into long, hair-like organelles known as pili have similar features at the primary sequence level as well as at the tertiary structural level (Vegandesan & Narayana, 2011). It is identified that *Staphylococcus aureus* proteases are essential for changing the bacterial phenotype from adhesive to invasive by degrading adhesins on the bacterial cell surface. Secreted staphylococcal proteases mediate pathogen penetration by degrading collagen and elastin, essential components of connective tissue in the dermis. Those proteases contribute to an inflammatory reaction manifested by edema, redness and pain. The inflammatory reaction can also be fueled by the activation of protease-activated receptors on keratinocyte (Kozziel & Potempa, 2013). Attachment to horizontal surfaces stimulates bacterial growth (particularly in nutrient-poor environments) because it increases the local concentration of nutrients. Also, increasing the substrate surface area, provides more area on which nutrients can adsorb, allowing cells to grow at nutrient concentrations that would normally be too low to support growth (Tuson & Weibel, 2014). Additionally, *S. aureus* has at least 29 surface proteins (Gill *et al.*, 2005) but some of those have other, at least primary, functions in *S. aureus* physiology, such as the immune evasion factor protein A (Forsgren & Nordström, 1974). Equally important, the pathogen *S. aureus* uses its fibronectin binding proteins (FnBPs) to invade host cells and it has been hypothesized that this provides a protected niche from host antimicrobial defenses. FnBPs contain multiple tandem fibronectin-binding repeats (FnBRs) which bind fibronectin with varying affinity, but it is unclear what selects for this configuration (Edwards *et al.*, 2011). In contrast, the capacity of *S. epidermidis* to produce adhesins and specially to secrete extracellular enzymes and

toxins is much less pronounced than *S. aureus* (Heilmann, 2011). Also, in a study of Fn binding in *S. epidermidis* was a good fibronectin (Fn) binder, too (Josse *et al.*, 2017). *S. epidermidis* has at least 18 genes for such proteins and the corresponding protein products show considerable functional redundancy for the human adhesion (Bowden *et al.*, 2005; Gill *et al.*, 2005). This is likely due to the substantial change in hydrophobicity that accompanies the expression of this highly abundant surface protein. For example, changes in teichoic acid structure mediated by D-alanylation impact binding of autolysin to their surface, exemplifying how secondary effects may contribute to the impact of polymeric macromolecules to surface attachment (Peschel *et al.*, 2000). Staphylococci express an extensive range of surface proteins involved in their adhesion to extracellular matrix (ECM), plasma proteins or directly to host cells. The most predominant of these proteins are the microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) (Patti *et al.*, 1994). All MSCRAMMs share a similar structure, with two adjacent IgG-folded domains mediating their attachment to components of the host ECM such as collagen, fibrinogen, or Fn (Becker *et al.*, 2014; Foster *et al.*, 2014). Host cell adhesion mainly involves Fn forming a bridge between $\alpha 5\beta 1$ integrin on the cellular side and FnBPs (which are MSCRAMMs) on the bacteria *S. aureus* (Hussain *et al.*, 2002). Rather, Eap seems to promote the adhesion and internalization of *S. aureus* and other pathogenic bacteria encountered in the context of polymicrobial skin infection (Bur *et al.*, 2013). *S. aureus* adhered to cells via interactions between FnBPs, Fn, and $\alpha 5\beta 1$ integrins. The resulting clustering of integrins may then be enough to trigger the signaling cascade, and later the cellular invasion, the internalization process (Josse *et al.*, 2017). *S. epidermidis* has capacity for biofilm formation, has resistance to antimicrobial peptides, and the presence of surface adhesins that facilitate adherence to different host tissue molecules. Many of these staphylococcal adhesins are part of a family of structurally related proteins referred to as *S. epidermidis* surface (SES) proteins. These cell wall-anchored proteins interact with host matrix molecules, such as fibronectin, collagen, and fibrinogen. *S. epidermidis* unfinished genomic sequence (Bowden *et al.*, 2005). In earlier studies, they found that SdrF, one of these SES proteins, is present in both colonizing and clinical (eg, bacteremia) isolates (approximately 54%–67%) and that it binds collagen (Bowden *et al.*, 2005; Trivedi *et al.*, 2017). The SES protein SdrF contributes to colonization of skin and mucosal surfaces. Was investigated the SdrF adherence to keratin and to cells that express this ligand on their surface. Keratins are

related structural proteins that are found in abundance on epithelial surfaces (Fuchs, 1995). *S. epidermidis* doesn't produce many toxins and tissue damaging exoenzymes, as does *S. aureus* but the success of *S. epidermidis* as a pathogen has to be attributed to its ability to adhere to surfaces and to remain there, under the cover of a protecting extracellular material, forming a biofilm (Rupp & Archer, 1994; Vuong & Otto, 2002). *S. epidermidis* and other CoNS species found that *S. epidermidis* was a good Fn binder (Switalski *et al.*, 1983). However a study realized by (Josse *et al.*, 2017) revealed huge variations in binding activity between *S. epidermidis* strains and between other CoNS species. In *S. epidermidis*, the giant extracellular matrix binding protein (Embp) has been shown to bind Fn. The internalization of *S. epidermidis* (and that of other CoNS) by NPPCs (neural progenitor cells) is a more controversial issue. While several studies have reported that *S. epidermidis* is internalized by different types of NPPCs, namely endothelial cells. One tempting hypothesis is that the internalization of *S. epidermidis* by NPPCs occurs through a tripartite Embp-Fn α 5 β 1 system analogous to the FnBP-Fn- α 5 β 1 integrin process for *S. aureus*. Concluding, *S. epidermidis* adhere as well by FnBPs, Fn, and α 5 β 1 (Josse *et al.*, 2017).

On the other hand, Gram-negative bacteria express variable outer membrane proteins (adhesins) to attach to the host and to initiate the process of infection. Adhesins described in Gram-negative bacteria as structures like pili or fimbriae, are filamentous surface proteins that comprise a scaffold-like domain anchored to the bacterial membrane with strong binding specificities that allow bacterial attachment to the host (Cossart & Pizarro-Cerdá, 2006). Indeed, once adhesion is established to host cells, pathogens are skilled to spread within the host and express and/or release further virulence factors leading to infection. Such virulence factors include, e.g., bacterial toxins (modulating host cell functions), cell surface carbohydrates or proteins (protecting the bacterium from host defense), and exoenzymes (contributing to bacterial dissemination) (Vaca *et al.*, 2019). Additionally, many Gram-negative bacteria, particularly species of the family Enterobacteriaceae, have been shown to possess pili or fimbrial structures (Al-Ghazzewi *et al.*, 2014). Moreover, *Pseudomonas aeruginosa* produces a multitude of pathogenicity factors during infections. Some are structural constituents and others are secreted or directly injected into host cells. Among structural constituents, *P. aeruginosa* flagellum and pili are responsible for motility and bacterial adhesion to host cells. Additionally, lipopolysaccharide (LPS), a complex glycolipid, and lectins (LecA and LecB) are present in the outer membrane of *P.*

aeruginosa also contribute to its pathogenicity (Ruffin & Brochiero, 2019). To achieve permanent adhesion under such variable conditions, *P. aeruginosa*, bacterial cells have developed a series of adhesins able to facilitate adhesion under various environmental conditions. *P. aeruginosa* secretes outer membrane vesicles (OMVs) which contain virulence factors such as: pro-elastase, hemolysin, phospholipase C, protease, alkaline phosphatase and β -lactamase that can damage the cells of host and also other bacteria (Kadurugamuwa & Beveridge, 1997). It has been reported that the bacteria adhere to epithelial cells via a variety of adhesins, e.g., type IV pili, lipopolysaccharides (LPS), several exoenzymes, and exopolysaccharides (Esen *et al.*, 2001; Wu *et al.*, 2015). *P. aeruginosa* has two distinct modes of motility: I) flagella mediated motility, whereby bacteria swim, and II) a twitching motility system. Both of them plays an important virulence factor, both flagella and pili have been shown to mediate the adherence to epithelial cells (Goldberg, 2007). Both pili and flagella are bacterial filamentous protein structures that helps in adhesion and host invasion (Gerven *et al.*, 2011). According to the author (Porrás-gómez *et al.*, 2012) there is adhesion by flagellin and pili and have an important role in the host infection. The flagellum of *P. aeruginosa* plays an indirect role in membrane permeabilization and surfactant protein-mediated bacterial clearance. Likewise, pili is involved during inflammation due to glycosylation in the interface between pili and host cells. Pili, flagella, exoenzyme S, and mucoid exopolysaccharide are recognized as major adhesins in *P. aeruginosa*. Invading pathogens are recognized by TLRs on epithelial cells and innate immunocytes, both of which are then activated to express inflammatory mediators. It is analysed that lectins are sugar-binding proteins and contribute to adhesion by interacting, for example, with the carbohydrate moiety glycosphingolipids or to mucin (Tillotson & Tillotson, 2009). Curutiu *et al.*, (2018) claim that the regulation of the virulence factors expression is coordinated by quorum sensing (QS), an intercellular communication system based on cell density dependent molecules with autoinductory properties that play a pivotal role in the pathogenesis of various infections (Curutiu *et al.*, 2018).

In line with Mikkelsen study, the three most common ways of growing bacteria *in vitro* are as planktonic cultures, colonies on agar plates, and biofilms in continuous-flow systems. Biofilms are known to express genes different from those of planktonic cells, and biofilm cells are generally believed to closely resemble planktonic cells in stationary phase (Mikkelsen *et al.*, 2004). It is described that biofilms are aggregation of bacteria which are organized into structural communities and produce

exopolysaccharide matrix as a major component for their stability. Basic structure of biofilm consists of micro colonies framed in extracellular polymeric substance. Formation of biofilm is multi-step process which involves attachment, growth and expansion (Zubair *et al.*, 2014). The biofilm formation is considered to be one of the main characteristics of a probiotic strain, because it is thought that the longer the strain remains adhered to the surface of the host cells, the more benefits it can confer (Nadell *et al.*, 2009). According to the Center for Disease Control and Prevention about 65% of bacterial infections in humans are connected with the formation of bacterial biofilms (Cvitkovitch *et al.*, 2003). These, biofilms often exhibit resistance to antibiotic treatment with several mechanisms contributing to this resistance, including (1) the barrier function of the biofilm matrix; (2) the presence of dormant cells and highly resistant small colony variants; (3) and upregulation of several biofilm-specific antibiotic resistance genes (Tuson & Weibel, 2014). Also, bacteria attached to surfaces often exist as biofilms, which play several protective roles. Nevertheless, some of the planktonic bacteria may recognize binding proteins in acquired pellicle, i.e., α -amylase and proline-rich glycoproteins/proteins and bind to the pellicle (Katsikogianni & Missirlis, 2004). Interestingly, aggregation is the foundation of bacterial interactions in biofilm formation. Generally, probiotics can inhibit the adherence of pathogenic bacteria to mucosa either by forming a barrier via auto-aggregation or by direct coaggregation with the pathogens (Huang *et al.*, 2011). In a previous study, in addition to displaying antimicrobial and anti-adhesive, *Lactobacillus paracasei* exhibited a strong auto-aggregating phenotype, which was maintained after washing and resuspending the cells in PBS (phosphate buffer solution), which could suggest that this attribute can be related to cell surface components and not to excreted factors (Gudinã *et al.*, 2010). Moreover, it was suggested that the auto-aggregative phenotype in *Lactobacillus paracasei* spp. *paracasei* BGNJ1-64 was promoted by the expression of the gene encoding the auto-aggregation-promoting factor (AggLb) that contributes to its high aggregation capacity, as well as a strong, specific interaction with host cell collagen, indicating that there is a direct relationship between cell aggregation, hydrophobicity, and collagen binding (Melgaço *et al.*, 2018). Indeed, AggLb is the largest known cell-surface protein in Lactobacilli and belongs to the collagen-binding superfamily. It was demonstrated that AggLb protein has a useful probiotic function in effective colonization of host tissue and prevention of pathogen colonization and, its deletion AggLb causes a loss of the capacity to form cell aggregates, whereas

overexpression increases cellular aggregation, hydrophobicity and collagen-binding potential (Miljkovic *et al.*, 2015). Complementary to this, sugars in the form of monosaccharides, polysaccharides oligosaccharides, and glycoconjugates (glycoproteins, glycolipids) are essential components of infecting microbes and host cells and are involved in cell signaling associated with modulation of inflammation in all integumental structures. They play an important role in microbial adherence, colonization and biofilm formation, and in virulence (Lloyd *et al.*, 2007).

As explained by Tuson & Weibel, most of the interactions of bacteria with surfaces produce changes in the expression of genes that influence cell morphology and behavior, including genes essential for motility and surface attachment (Tuson & Weibel, 2014). Bacterial adhesion to biomaterial surfaces is the essential step in the pathogenesis of these infections, both specific and non-specific interactions may play an important role in the ability of the cell to attach to (or to resist detachment from) the biomaterial surface. However, bacterial attachment to cell surface induce the expression of adhesins, such as mucin, fibronectin, collagen, laminin, and fibrinogen, which mediate adhesion (Katsikogianni & Missirlis, 2004).

1.5 - Dysbiosis-associated skin disorders and common bacterial pathogens

The disturbance of skin homeostasis (dysbiosis) can lead to several pathologies, namely, wounds, atopic dermatitis (AD), rosacea, psoriasis and acne, on immunosuppressed individuals (Kong & Segre, 2012). These are the examples of the breakdown of homeostasis, that can overcome the skin barrier and become pathogenic when resident and/or transient bacterial populations colonize producing disease (Kong & Segre, 2012). According to Ardura & Koh (2018), bacterial skin infections usually are caused by staphylococci or streptococci. In immunocompromised patients, both gram-positive and gram-negative bacteria, including enteric organisms and *Pseudomonas* spp..

Is demonstrated that many of the skin cells that are eliminated daily contain bacteria (Zulkowski,2013). Wounds that derive from diabetes or burn infections are most commonly caused by *Streptococcus pyogenes*, *Enterococcus* spp., fungi and/or viruses. The species *Staphylococcus epidermidis*, comprises more than 90 percent of the resident aerobic flora, is a common commensal microorganism in skin, but sometimes can cause infection or disease, most frequently in hospital devices. After the entry, biofilms are formed, which protect them from the host immune system and from

antimicrobial treatments, increasing the levels of antibiotic resistance (Baron, 1996; Grice *et al.*, 2008; Grice & Segre, 2011). Recent findings indicate that chronic wound pathology may be caused by changes in skin microbiota; specifically, *S. aureus* and *Pseudomonas aeruginosa* are the two bacterial species mainly involved in biofilm-based wound infections (Wong *et al.*, 2013), with the latter being difficult to eradicate mainly due to acquired antibiotic resistance (Hancock, 1998). Among the previous bacteria's the *Staphylococcus aureus* is related with atopic dermatitis and the loss-of-function mutations in the filaggrin gene and reduced levels of filaggrin breakdown products on skin, and other diseases. This microorganism produces a variety of secreted virulence factors that enhance an inflammatory reaction and prevent healing of skin in atopic dermatitis. It is responsible for a wide range of superficial and invasive infections ranging in severity from mild to fatal. Treatment of *S. aureus* infections is often complicated by the high prevalence of these antibiotic resistant strains (Edwards *et al.*, 2011). Also, the genus *Corynebacterium* spp. are associated with skin diseases, and are diphtheroid bacteria responsible for pitted keratolysis, a common plantar infection confined to the thick stratum corneum. Some illnesses like erythrasma, and trichobacteriosis are known that arise from the presence of these bacteria (Miajlovic *et al.*, 2010). Other microorganisms like *Escherichia coli* are often isolated from skin and soft tissue infections (SSTI), as well. These bacteria can lead to cellulitis localized in the lower or upper limbs, necrotizing fasciitis, surgical site infections, infections after burn injuries, and others. Over the years, this microorganism has become resistant to some antibiotics such as quinolones, chloramphenicol, tetracycline, and others (Petkovšek *et al.*, 2009). To illustrate I will describe next some of the most relevant pathogenic species on the skin.

1.5.1 – *Staphylococcus aureus*

Staphylococcus aureus is a pathogenic bacteria and a common colonizer of the infant gut (Persson *et al.*, 2011). *S. aureus* is a potent immune activator as superantigenic staphylococcal enterotoxins (SE) are able to engage large numbers of conventional T cells via MHC-mediated binding to the variable domain of the T cell receptor (TCR) (Johansson *et al.*, 2016). Studies performed by (Miller *et al.*, 2015), affirm that the *S. aureus* is a common cause of infections in community-dwelling persons, especially among those who have contact with the healthcare system and hospitalized patients (Mccaig *et al.*, 2006). *S. aureus* is a commensal bacterium in the

respiratory tract mucosa of most people and infects the skin of AD patients (Gould *et al.*, 2007). Skin disorders like impetigo, cellulitis, erysipela, boils, carbuncles, folliculitis, and necrotizing fasciitis are also caused by this pathogen (Sukumaran & Senanayake, 2016). According to (Ryu *et al.*, 2014), the incidence of skin infections by *S. aureus* reflects in part the competition between host cutaneous immune defenses and pathogen virulence factors. The author states that *S. aureus* was a key contributor to SSTIs. SSTIs occur after 2 to 5% of all surgeries, although there is considerable heterogeneity depending on the type of procedure, population studied, comorbid illnesses, experience of the surgeon, setting, and antimicrobial prophylaxis utilized (Tong *et al.*, 2015). *Staphylococcus species* are the dominant bacteria present on skin and are divided into two main groups, based on coagulase activity. Coagulase triggers the coagulation of soluble fibrinogen-forming insoluble fibrin, resulting in the formation of a clot (Sullivan *et al.*, 2019). The production of lantibiotics is abundant within commensal coagulase negative staphylococci; for example *Staphylococcus gallinarum*, *Staphylococcus epidermidis* and *Staphylococcus hominis* that produce the lantibiotics, gallidermin, epidermin and hominacin, respectively (Götz *et al.*, 2014). The majority of our commensal or resident skin microbiota are beneficial to skin health, however, even the most abundant of *Staphylococcus sp.* on human skin, *S. epidermidis*, can become pathogenic if the conditions are suitable, and occasionally the microbes that inhabit our skin can develop into opportunistic pathogens (Cogen *et al.*, 2009). The disturbances on the microbial composition of the skin are responsible for skin diseases such as psoriasis, AD, impetigo and acne, and sometimes these imbalances can also prevent healing of chronic wounds (Grice, 2014). The release of bioactive peptides and/or bacteriocins which, as mentioned above, are important antimicrobial metabolites produced by LAB, and are proteinaceous in nature, may have contributed for the decrease in the numbers of the *S. epidermidis*. Such compounds inhibit specific microorganisms, particularly Gram-positive bacteria (Tan *et al.*, 2014), exhibiting a broad range of activity against some species of the genera *Bacillus*, *Enterococcus*, *E. coli*, *Salmonella*, *Clostridium*, *Shigella*, *Staphylococcus* and *Streptococcus* (Sarkar & Mandal, 2016). *Staphylococcus aureus* is Gram-positive cocci that has been identified as the most virulent among all staphylococcal species that cause skin and soft tissue infections, surgical site infections, and hospital-acquired bloodstream infections. In some infections, survival, dissemination, and pathogenesis of staphylococci are supported by the formation of a biofilm (Hor & Liang, 2014).

1.5.2 – *Staphylococcus epidermidis*

Coagulase-negative staphylococcus organisms they are part of normal flora of human skin, however these bacteria can also be pathogens in skin and soft tissue infections. According to Natsis & Cohen (2018), skin and soft tissue infections have been observed to be caused by many coagulase-negative staphylococcus organisms: *S. epidermidis*, *Staphylococcus capitis*, *Staphylococcus haemolyticus*, *Staphylococcus hominis*, *Staphylococcus saprophyticus*, among others. Coagulase-negative staphylococcus skin infections predominantly present as abscesses and paronychia. They are most common in elderly patients or those individuals who are immunosuppressed and tend to be broadly susceptible to antibiotic treatment (Natsis & Cohen, 2018). Specifically, *S. epidermidis* has been frequently implicated in endocarditis and infections of surgical implants with reference to its biofilm production as a virulence factor. Thus, this bacteria is now recognized as the most frequent cause of nosocomial sepsis. The immune response triggered by biofilm create a physical bacterial adherence with diminished penetration capacity and by prompting a paradoxical anti-inflammatory cytokine response (Nguyen *et al.*, 2017; Spiliopoulou *et al.*, 2012).

1.5.3 – *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is responsible for complications on serious illnesses such as hospital acquired infections and sepsis syndromes. This organism causes severe and often fatal hospital-acquired infections, especially in immunocompromised hosts, but can also be present in the otherwise healthy patient (Bassetti *et al.*, 2018; Defeza *et al.*, 2004; Wu *et al.*, 2011). *P. aeruginosa* remains one of the most common serious bacterial infections to present in burn patient populations (Santucci *et al.*, 2003). The incidence of multi-drug-resistant strains of *P. aeruginosa* has been steadily increasing, and has had particularly devastating effects on burn units (Wu *et al.*, 2011). Several factors are involved in the acute infection: on the surface of *P. aeruginosa*, the pili allow adherence to the epithelium and the exoenzyme S reinforces the adherence to epithelial cells. The exotoxin A is responsible for tissue necrosis. Phospholipase C is a thermolabile haemolysin, that plays a role in cellular lysis (Elogne *et al.*, 2018). *P. aeruginosa* produces at least four proteases causing bleeding and tissue necrosis (Ben *et al.*, 2011). These are the factors that lead to the prevalence of this pathogen.

According to (Arqués *et al.*, 2015) determining the antagonistic effect of probiotics on the growth of *P. aeruginosa* and the effectiveness of various bacteriocins of probiotics may be hindered by the proteolytic activity of microbial enzymes that are secreted only during active fermentation. While *Lactobacillus* spp. produce lactic and acetic acids that inhibit the growth of many bacteria through their undissociated forms at low pH, thus affecting the sensibility of *P. aeruginosa*. *P. aeruginosa* is a Gram-negative bacillus that is most frequently associated with opportunistic infection, but which can also present in the otherwise healthy patient. The cutaneous manifestations of *P. aeruginosa* infection range from superficial to deep and can occur in both immunocompromised and healthy individuals. In the case of the immunocompromised host, however, more significant morbidity and mortality can result from untreated *P. aeruginosa* infection (Wu *et al.*, 2011). Also, *Propionibacterium* sp. adhesion to intestinal cells leads to exclusion of invasive pathogenic bacteria by competitive adhesion or co-aggregation mechanisms so it might be inferred that the presence of *Propioniferax innocua* can in a certain way prevent or delay the internalization of the pathogen *P. aeruginosa* (Rabah *et al.*, 2017). *P. aeruginosa* strains are known to be sensitive to organic acids (Alakomi *et al.*, 2005).

1.5.4 – *Escherichia coli*

Escherichia coli, a member of the bacterial family of Enterobacteriaceae, is the most prevalent commensal inhabitant of the gastrointestinal tracts of humans and warm-blooded animals, as well as one of the most important pathogens (Allocati *et al.*, 2013). In agreement with Jayanthi study, *E. coli* have been implicated to cause urinary tract infections, wound infections, septicemia and neonatal meningitis. Extra-intestinal pathogenic *Escherichia coli* (ExPEC), the specialized strains of *E. coli* that cause most extra-intestinal *E. coli* infections, represent a major but little appreciated health threat (Jayanthi & Soumya, 2017). Additionally, *E. coli* strains, frequently are isolated enterobacteria from SSTI (Petkovšek *et al.*, 2009). *E. coli* was found to be the causative agent of neonatal omphalitis (Fraser *et al.*, 2006), cellulitis localized to lower or upper limbs, necrotizing fasciitis (Grimaldi *et al.*, 2010), surgical site infections infections after burn injuries, and others (Tourmousoglou *et al.*, 2008). This pathogen is an important causative agent in infections, since it was the third-most prevalent isolated species, preceded solely by *S. aureus* and *Pseudomonas aeruginosa* (Petkovšek *et al.*, 2009). Is demonstrated by Petkovšek and partners (2009), that resistance of pathogens to antimicrobial agents is a global health care problem. A large number of studies have

reported a meaningfully reduced virulence potential among *E. coli* isolates that are resistant to certain antibiotics, such as quinolones, chloramphenicol, tetracycline, and others (Petkovšek *et al.*, 2009).

1.6 - Host immunity responses to skin pathogens: an overview

Deregulation of immune responses often leads to impaired healing and poor tissue restoration and function (Nguyen & Soulika, 2019). Susceptibility to some infections normally are higher in young, old or immunosuppressed patients (Baron, 1996). According to Kwan & Tredget (2018), keratinocytes are an important element at wound healing. Moreover, in remodeling phase of wound healing begins once re-epithelialization of the wound is complete, and wounds taking longer than 2 weeks to re-epithelialize.

The skin immune system harbors a complex network of dendritic cells (DCs). In addition to creating cellular and humoral immunity against pathogens, skin DCs are involved in tolerogenic mechanisms to ensure the maintenance of immune homeostasis, as well as in pathogenesis of chronic inflammation in the skin when excessive immune responses are initiated and uncontrolled (Chu *et al.*, 2011). As for the DC subpopulation, Langerhans cells also support epidermal immunity, by displaying a potent cross-priming activity and initiating the CD8⁺ T cell responses in an IL-15-dependent manner. Conversely, they have been shown to contribute to the expansion of TReg cells, which suggests a role for Langerhans cells in tissue homeostasis (Clayton *et al.*, 2017). As antigen presentation specialists, Langerhans cells induce the production of Th2 cells (Type 2 T helper), but when reaches the epidermis, it induces the production of Th1 cells and, migrating to the draining lymph nodes, it induces a potent immune response with a strong Th2 interleukins 4,5 and 13 production, which are associated with the promotion of IgE and eosinophilic responses in atopy (caused by allergen penetration through the skin), and also interleukin-10, which has more of an anti-inflammatory response. In excess, Th2 responses will counteract the Th1 mediated microbicidal action (Kim & Kim, 2018). In contrast, antigen delivery into the derma induces predominantly Th1-type immune responses (Caramia *et al.*, 2008; Berger, 2000). Is evaluated in the dermis, myeloid dendritic cells are located more superficially than macrophages, which are present deeper and primarily perivascular in distribution. The main dendritic cell subsets that are present in the human dermis include: CD14⁺CD1a⁻ DCs, CD14⁻CD1a⁺ DCs and 6-Sulpho LacNAc⁺ DCs26, 27.

Expression of CD141 by CD14⁺ dermal DCs characterizes a subpopulation that produces the regulatory cytokine IL-10 and has potent immunoregulatory functions *in vitro* and *in vivo*. In contrast, CD14⁻CD141⁺ dermal DCs have been shown to be potent activators of CD8⁺ T cells (Pasparakis *et al.*, 2014b). Although inflammatory skin lesions contain a high number of dendritic cells, their origin is not clear. In animals this dendritic cells derive from monocytes (Ly6Chi) that are similar to CD14 in human (Haniffa *et al.*, 2015). Hence, mast cells take an important role in the immunological response too, are mostly located in the upper dermal part of the skin, where they can easily encounter, respond, protect from infections and stress caused by wound healing. During the flow of monocytes to the inflamed tissue, the homeostasis mechanisms are stressed and may lead to alterations in the resident populations (Matejuk, 2018). Furthermore, membranes antimicrobial peptides (AMPs) are also important in the skin defense, they are lipids amphipathics and are expressed constitutively or induced after cell activation in response to inflammatory or homeostatic stimulation. The most meticulously studied AMP families in human skin are the defensins and the cathelicidins (LL-37), which are produced by a variety of cells in the skin such as keratinocytes, fibroblasts, dendritic cells, monocytes, and macrophages, and sweat and sebaceous glands. Interestingly, AMPs have roles in modulating host immune responses. Human LL-37 was shown to induce differentiation of monocyte-derived dendritic cells, subsequent cytokine production, and expression of the co-stimulatory molecule CD86. LL-37 and β -defensins can also serve as alarmins for keratinocytes by inducing their proliferation and migration. Furthermore, human LL-37 exerts its alarmin effects on immune cells in a synergistic manner with other inflammatory mediators, such as IL-1 β (Nguyen & Soulika, 2019).

As mentioned before skin is the largest organ and is in permanent contact with the environment. Besides to physical, microbiological, and chemical barriers, the skin contains resident immune cells that serve guard functions and contribute to tissue homeostasis. In the event of an outrage, these cells act locally to initiate inflammatory responses, that leads to primary adaptive immunity response (Nguyen & Soulika, 2019). Present knowledge on immune-competent cells in the skin highlights the importance of the skin as a part of lymphatic system (Matejuk, 2018). Understanding the cellular and molecular interactions between immune cells and skin-resident cells is necessary to delineate the dynamics of wound repair (Nguyen & Soulika, 2019).

1.7 - Common treatments for skin diseases

Skin disorders, which are mainly of inflammatory nature, being characterized by the increased expression of multiple inflammatory genes (Barnes, 2006). It is demonstrated that corticosteroids are the most effective for anti-inflammatory and anti-proliferative purposes (Barnes, 2006; Pandey, 2010; Scott & Fong, 2016), although they have side effects based on age, site involved and types of skin disorder with subsequent short- and long-term effects on human health (Coondoo *et al.*, 2014). Also, short-term corticosteroid use is generally associated with mild side effects, including cutaneous effects, electrolyte abnormalities, hypertension, hyperglycemia, pancreatitis, hematologic, immunologic, and neuropsychologic effects, although occasionally, clinically significant side effects may occur (Abraham & Roga, 2014). Long-term corticosteroid use may be associated with more serious sequels, including osteoporosis, aseptic joint necrosis, adrenal insufficiency, gastrointestinal, hepatic, and ophthalmologic effects, such as glaucoma and cataracts, hyperlipidemia, growth suppression, and possible congenital malformations (Buchman, 2001; Daniel & Orchard, 2015). However, corticosteroid topical application has other disadvantages, like it can often cause skin irritability including thinning of the epidermis, which limits its use for a long period of time and can cause the recurrence of the disease (Stery, 1992). Mechanistically, the topical steroid usage causes skin to undergo through three pathogenesis of skin atrophy due to topical steroids: pre-atrophy, atrophy and tachyphylaxis. In the atrophy phase the steroids have an inhibitory effect on keratinocyte proliferation in the epidermis; leads to inhibition of collagen 1 and 3 synthesis in the dermis; and inhibition of fibroblasts and hyaluronan synthase 3 enzyme resulting in the reduction of HA in the extracellular matrix leading to dermal atrophy. Factors such as age, body site e.g. intertriginous areas, high-potency topical steroid, occlusion and moisture, can increase chances of atrophy (Abraham & Roga, 2014).

As discussed briefly in section 1.5.1 many skin disorders are often caused by pathogenic bacteria and therefore many treatments revolve around the compound's antimicrobial properties. Empirical evidences supported by its physicochemical nature have long established honey as one of the oldest wound and burn treatments, since it's able to moisturize injured tissue, decreased microbial infections, sooth inflammation, and prevent gauze sticking to wounds, making it an ideal wound dressing. Indeed, the wound healing properties of honey has been focused on antiseptic effects (Yaghoobi *et*

al., 2013). Different studies have shown that honey is able to promote angiogenesis, granulation, and epithelialization, stimulate lymphocytes and phagocytes, induce the expression of tissue repair molecular markers, and trigger epithelial-mesenchymal transition in keratinocytes (Burlando & Cornara, 2013). In vitro studies have shed light upon the mechanisms of action of honey on skin cells and its biocompatibility has been assessed, since the toxicity of honey on keratinocytes and fibroblasts is extremely low (Ranzato *et al.*, 2012). Then, essential oils extracted from medicinal aromatic plants, also represent therapeutic alternative to standard corticosteroid treatment, in which the major associated advantages lie on the diminished side effects, high successful treatment rate by the synergetic interaction between oils, they are easily absorbed and decrease of recurrence of infection and of resistances (Hadji-Minaglou & Bolcato, 2005; Li *et al.*, 2015; Nascimento *et al.*, 2017; Pauli & Schilcher, 2004; Pivetta *et al.*, 2018; Orchard, & Viljoen, 2018; Svendsen & Scheffer, 1985; Van Vuuren *et al.*, 2018). Furthermore, natural drugs from the plants have several advantages such as having fewer side-effects, better patient tolerance, being relatively less expensive and acceptable due to a long history of use. Plants like e. g., *Allium savatium*; *Barbados aloe* (Aloe vera); *Achyranthes aspera* (Devil's horsewhip); *Azadirachta indica*; *Camellia sinensis* (Green tea); *Eucalyptus globulus* (Blue gum); *Euphorbia walachii* (Wallich spurge); *Lavendula officinalis* (Lavender) and *Rosmarinus officinalis* (Rosemary) are the most utilized among others (Tabassum & Hamnida, 2014). Additionally, other natural polymers from animal and plant origin, that provide antimicrobial activity against skin infections can also be used. Chitosan, from animal origin, is safe and has an effective absorption as enhancer to improve mucosal, nasal, peroral drug delivery of hydrophilic macromolecules such as peptide and protein drugs and heparins; Chitosan nanoparticles are also appropriate for controlled drug release (Kaushik *et al.*, 2016). In this way, natural products represent alternative antimicrobials against dermatological infections from animal origin, marine origin and bacterial origin (Kon & Rai, 2017). Nevertheless, novel therapies are required to combat the skin disorders incidence, mostly due to the lack of specificity of natural treatments and the emergence of antibiotic resistance. For example, based on naturally-occurring bacterial viruses (phages), phage-therapy is used to infect and lyse bacteria at the site of infection (Lin *et al.*, 2017). Most phages are infectious only to the bacteria that carry their complementary receptor, which effectively determines lytic phage host range (Rakhuba *et al.*, 2010). Host specificity varies among phages, some of which are strain-specific,

whereas others have demonstrated the capability of infection across a range of bacterial strains and even genera (Koskella & Meaden, 2013; Motlagh *et al.*, 2016), although most bacteriophages demonstrate high specificity towards both Gram-positive and Gram-negative bacteria, and they are also highly efficient and relatively cost-effective (Nogueira *et al.*, 2017). In the same way photodynamic antimicrobial chemotherapy (PACT) is a strategy that utilizes photosensitizers and visible or ultraviolet light in order to give a phototoxic response (normally via oxidative damage) is now being considered a promising alternative to conventional antibiotic approach, given the increasing problematic of multiantibiotic resistance (Ullah *et al.*, 2018). Chen and coworkers (2016), as reported PACT's effect on bacteria involved on skin infections using a zinc phthalocyanine derivative, pentalysine β -carbonylphthalocyanine zinc (ZnPc-(Lys)₅). Compared with its anionic ZnPc counterpart, ZnPc-(Lys)₅ showed an enhanced antibacterial efficacy in vitro in an animal model of localized infection. Meanwhile, ZnPc-(Lys)₅ was observed to significantly reduce the wound skin blood flow during wound healing, indicating an anti-inflammation activity. This study provides new insight on the mechanisms of PACT in bacterial skin infection (Chen *et al.*, 2016). PACT is proposed as a potential, low-cost approach to the treatment of locally occurring infection and has been proposed as an alternative approach for the inactivation of bacteria, also (Mai *et al.*, 2017; Wainwright, 1998).

According to the study by (Zhou *et al.*, 2015) antimicrobial agents target a range of extra- and/or intracellular loci from cytoplasmic wall to membrane, intracellular enzymes and genetic materials. Based on their spatially distinct sites of action and distribution of location, antimicrobial resistance mechanisms of bacteria are categorized into three groups, coined the three lines of bacterial defense. In addition, some other bacteria employ the second line of defense, the cell wall, cell membrane, and encased efflux pumps. When antimicrobial agents permeate the first two lines of defense and finally reach the cytoplasm, many bacteria will make use of the third line of defense, including alterations of intracellular materials and gene regulation to protect themselves from harm by bactericides. It is important then, to employ therapies that target the main bacterial components and in parallel don't allow the gain of resistance to those approaches. As such, lately, an alternative and complementary therapy that has been growing in consideration to circumvent skin disorders and alleviate symptoms associated with them, is the topical application of probiotics, whose main action focuses

on the protection through antimicrobial action and skin reparation with proper hydration (Lopes *et al.*, 2017).

1.8 - Probiotics in Human Health

As previously described, the relationship of the microorganisms and the human/ host cells are crucial to a healthy skin, and are defined in three categories: One in which one species is benefited and the other is unaffected (commensalism), another in which both are favored (mutualism) and finally the last one where one is favored and the other damaged (predation). Indeed, the modification of the gut microbiota has been shown to be harmful when the gut ecosystem undergoes severe abnormal changes. Thus, alteration of the gut microbiota composition (dysbiosis) can lead to multiple diseases in humans and animals (Daillère *et al.*, 2016; Lye *et al.*, 2017; Nakamoto *et al.*, 2017). The relationship between health and gut microbiota has raised interest in the modulation of the gut microbiota by administration of probiotic species for the prevention of some diseases with several studies reporting positive interactions between the commensal microbiota and the human body (Azad *et al.*, 2018; Goudarzi *et al.*, 2014; Kitazawa *et al.*, 2015; Lebeer *et al.*, 2010).

Is described by the World Health Organization (WHO) that probiotic bacteria as “live microorganisms which when administrated in adequate amounts confer a health benefit on the host” (Gillor *et al.*, 2008). The strains most frequently used as probiotics include lactic acid bacteria and bifidobacteria (Bermudez-Brito *et al.*, 2012; Hemaiswarya *et al.*, 2013). Potential probiotic bacteria must be nonpathogenic and non-toxic bacteria species and, most differ in terms of their bioavailability, metabolic activity, and mode of action (Gillor *et al.*, 2008). Most probiotic products today are developed with bifidobacteria, lactobacilli, and other lactic acid bacteria, such as lactococci and streptococci. Other emergent skin probiotic strains include the bacterial genera *Bacillus*, *Escherichia*, and *Propionibacterium* and some other yeast genera, mainly *Saccharomyces*. Several species and strains of Lactobacilli, including *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, and *Lactobacillus helveticus*, have been widely studied in the prevention of human and animal diseases. Emphasised that the probiotic strains ingestion can be used to modulate the population of microorganisms in the gut microbiota and control the functioning of the ecosystem of gut microbiota, having different effects on the host cell that can help prevent and treat a wide range of disorders (Sanders *et al.*, 2013). The gut microbiota composition is likely

to affect many organ systems, including the cardiovascular, neural, immune, and metabolic systems (Azad *et al.*, 2018). In addition probiotics influence positively host healthy, direct or indirectly, mainly by improving barrier function, modulating the mucosal immune system, increasing adhesion to intestinal mucosa and simultaneous inhibition of pathogen adhesion; producing of antimicrobial agents and enhancing of digestion and absorption (Hemaiswarya *et al.*, 2013). Also, human gastrointestinal physiology also influences probiotic colonization since the low pH (due to gastric acid production) favors bacteria that are well adapted to acidic environments and is known to inhibit the growth of non-acid-tolerant bacteria, reinforcing the barrier function that helps in the prevention of nosocomial infections (Forestier *et al.*, 2008). Is identified that the expression of pathogenicity during bacterial infections is mediated by a cell density dependent phenomenon known as quorum sensing (QS) (Kalia *et al.*, 2013). Then, bacteria have the ability to evolve resistance to all known antimicrobials. Hence, although inhibition of quorum sensing (QS) has been hailed to reduce virulence in a manner that is impervious to bacterial resistance mechanisms (Contreras *et al.*, 2013). According to Miller & Bassler (2001), QS is the regulation of gene expression in response to fluctuations in cell-population density. QS in bacteria produce and release chemical signal molecules called autoinducers that increase in concentration as a function of cell density. There are effects at the gastrointestinal level which leads us to conclude that in a similar way, these mechanisms of potentiation, adhesion, exclusion, production of anti-microbial substances and modulation of the immune response might be present in the same way in different niches, such as the skin.

Below in Table 1, we can see some examples of probiotic microorganisms.

Table 1 - Selected organisms that are used as probiotic agents.

Bacteria strains	Model	Main results	References
<i>Lactobacillus acidophilus</i>	Eight-week-old male C57BL/mice	↑IL-10, Treg ↓ IL-6, IL-1 β , IL-17	(Park <i>et al.</i> , 2018)
<i>L. acidophilus</i>	BALB/c mice	↑ Lactobacilli, Bifidobacteria; ↓ S. aureus;	(Chen <i>et al.</i> , 2013)
<i>L. fermentum FTDC 812</i>	Eight week-old BALB/c mice	↑Lactobacillus	(Lye <i>et al.</i> , 2017)
<i>L. plantarum CCFM10, RS15-3</i>	58 week BALB/c mice	↑ Bacteroidetes, Firmicutes	(Zhao <i>et al.</i> , 2018)
<i>B. breve IPLA20004</i>	Human colon	↑ IL-8, IL-10, IL-12	(Sánchez <i>et al.</i> , 2015)

Bacteria strains	Model	Main results	References
<i>Saccharomyces boulardii</i>	Six week C57BL/6 mice	↑ Firmicutes, Proteobacteria, and Fibrobacteria	(Everard <i>et al.</i> , 2014)
<i>L. rhamnosus</i> , <i>B. bifidum</i>	Eight week C57BL/6 mice	↑ Firmicutes, Actinobacteria ↓ Bacteroidetes	(Bagarolli <i>et al.</i> , 2017)
<i>Lactobacillus paracasei</i> NCC2461 (ST11)	Germ-free mice	↑ Th1; ↑ IgG2a	(Benyacoub <i>et al.</i> , 2014)
<i>Lactobacillus rhamnosus</i> GG combined with <i>Bifidobacterium lactis</i>	Bacterial infections (Humans)	↓ Recurrent respiratory infections; ↓ Recurrent acute otitis	(Rautava <i>et al.</i> , 2009)
<i>Lactococcus lactis</i>	Crohn's disease (Humans)	↑ IL-10	(Hosseinidoust <i>et al.</i> , 2013)

Subsequently, probiotic bacteria must survive the transition to the target niche and then persist, serving to protect the host against infection by pathogenic microorganisms (Gillor *et al.*, 2008). Also, probiotic selection is correlated with the protection against microbial pathogens and has been associated with the stimulation of antibody secretion, as well as cell-mediated immune responses (Papadimitriou *et al.*, 2015). These microorganisms can produce antimicrobial compounds such as organic acids and specialized inhibitory agents, like bacteriocins, antimicrobial peptides and others (May *et al.*, 2001; Papadimitriou *et al.*, 2015). The bacteriocins produced by Gram-positive are generally cationic, amphiphilic, membrane permeabilizing peptides, and range in size from 2 to 6 kDa (Gillor *et al.*, 2008). These characteristics are important in the modulation of the immune response of the host. It is important to emphasise that probiotics have been traditionally used in cases of intestinal distress (Collado *et al.*, 2011). As explained by Azad (2018), probiotics are beneficial to health, as referred above, and their potential led to a significant increase in research interest in their use to modulate the gut microbiota (Azad *et al.*, 2008). The animal gut is a complex ecosystem of host cells, microbiota, and available nutrients, and the microbiota prevents several degenerative diseases in humans and animals via immunomodulation. Gut microbiota composition alterations may precede the development and manifestation of atopic episodes, while early colonization with *Escherichia coli* has been associated with a higher risk of developing eczema, and *Clostridium difficile* with eczema, recurrent wheeze, and allergic sensitization (Penders *et al.*, 2007).

1.8.1 Probiotics in skin health

Views on selected strains of probiotics have been successfully used to promote a 'healthy' microbiota pattern, antagonize pathogens and stimulate immune defense mechanisms in multiple preclinical and clinical studies (Gareau *et al.*, 2010; Reid *et al.*, 2019). In summary in association, topical or systemic antimicrobial therapy can be incorporated to correct skin conditions (Chase & Armstrong, 2012). A study reported by Lew & Liong (2013) showed that probiotics were able to alleviate lactose intolerance, suppress diarrhea, reduce irritable bowel symptoms, prevent inflammatory bowel disease and exhibit anti-colorectal cancer activities (Lew & Liong, 2013). In addition, probiotics have also been documented to exert dermal potentials such as improving atopic eczema, AD, healing of burn and scars, skin-rejuvenating properties and also improving skin's innate immunity (Lew & Liong, 2013). Another essential point, the gut-brain-skin axis concept, as proposed by Arck *et al.* (2010) suggests that modulation of the microbiome by deployment of probiotics can exert profound beneficial effects, for example, on skin inflammation and skin homeostasis (Arck *et al.*, 2010). Indeed, topical compositions containing *L. plantarum* extract are shown to reduce the incidence of both inflamed and noninflamed acne lesions when used regularly over a period of 2 months. The extracts had further been proposed as a preservative in cosmetic of pharmaceutical products. (Al-Ghazzewi & Tester, 2010). According to Sorokulova (2008), testing the probiotic potential of various microorganisms starts at the preclinical level and includes evaluations, requirements for the set of tests can vary depending on the bacterial species and the expected mechanism of action in the organism. Common procedures of probiotic strains include strain identification (i.e., determination of phenotypic and genotypic properties), safety evaluation (i.e., characterization of history of use (safety contact), assessment of resistance to antibiotics, and evaluation of pathogenic properties in vitro and in animal models), and efficacy testing (i.e., functional characterization) (Sorokulova, 2008). In addition, studies demonstrated that various *Lactobacillus* and *Bifidobacterium* strains were able to exert antioxidant action in vitro. Both intact cells and cell-free extracts were able to inhibit ascorbate autoxidation, to exert metal-chelating ability, to scavenge superoxide anion and other ROS (reactive oxygen species). Thus, probiotics may represent a useful therapeutic tool for the prevention of epidermal oxidative stress either via the topical route or via ingestion (Lin & Chang, 2000; Lin & Yen, 1999). In another study, *Lactobacillus* facial application by patients with mild-to-moderate acne symptoms was able to reduce

inflammatory lesions and comedone formation. This was associated with a temporary modulation of the skin microbiome, including a reduction in relative abundance of staphylococci and an increase in lactobacilli (Lebeer *et al.*, 2018). In vivo studies, with application of Bifidobacterium fermented soy milk for six weeks significantly restored changes in the elasticity and viscoelasticity of mouse skin, increased the HA content, and hydrated and thickened mouse skin (Miyazaki *et al.*, 2004).

1.8.2 Promising probiotics for skin disorders treatment

Is described that commensal skin microbiota plays an important role in both influencing the immune response of the skin and acting as a barrier against colonization of potentially pathogenic microorganisms and overgrowth of opportunistic pathogens (Holz *et al.*, 2017). Are explored specific strains of probiotic LAB and bifidobacteria have been shown to beneficially influence the composition and/or metabolic activity of endogenous microbiota (Langhendries *et al.*, 1995; Mohan *et al.*, 2006). Besides, the competition for essential nutrients, aggregation with pathogenic micro-organisms (Rolfe, 2018), competition for receptor sites and production of anti-microbial metabolites have all been reported to play a role (Monteagudo-Mera *et al.*, 2019). In addition, the production of organic acids by multiple probiotic strains, belonging both to LAB and Bifidobacterium are mainly responsible for antimicrobial activity against Gram negative pathogens. Although the health benefits of probiotics have been confirmed, the specific effects of these established Gram-positive (G+) and Gram-negative (G-) (Kandasamy *et al.*, 2017; Lukic *et al.*, 2017) is the production of antimicrobials compounds, which include organic acids, hydrogen peroxide, diacetyl, reuterin, and bacteriocins (Vieco-saiz *et al.*, 2019). Other low molecular mass compounds with antimicrobial activity against Gram-positive and Gram-negative bacteria, molds and yeasts have been described, including antifungal cyclic dipeptides, phenyl-lactic acid, 4-hydroxyphenyllactic acid and 3-hydroxy fatty acids (Suskovic *et al.*, 2013).

1.8.2.1 Lactic acid bacteria (LAB)

Is briefly outlined that lactic acid bacteria (LAB) constitute part of the autochthonous microbiota of many types of foods. They are defined as a cluster of lactic-acid-producing, low G + C%, non-spore-forming, Gram-positive rods and cocci,

catalase-negative bacteria which share many biochemical, physiological, and genetic properties (Gómez *et al.*, 2016).

Is described that lactobacilli are commensal, lactic acid-producing bacteria with proven beneficial effects when used as dietary supplements. Lactobacilli appear to protect against certain immune-mediated diseases and have been suggested to act as important immune modulators, especially during early life. To elaborate, certain probiotic *Lactobacillus* strains display bactericidal activity against Gram-negative or Gram-positive gastric or enterovirulent bacteria after direct contact in vitro (Moal & Servin, 2014). Several strains of LAB, such as, *Lactobacillus casei* DN-114 001, *Lactobacillus casei* DN-114 056, *L. casei* sp. *casei* ATCC-334, *L. paracasei* NCC2461, *Lactobacillus reuteri* DSM12246, *L. casei* CHCC3139 and *L. bulgaricus* LB-10 were shown to modulate cytokines and growth factor production in vitro and in vivo (Gueniche *et al.*, 2010). Is demonstrated that soft tissue infection in immunocompromised hosts can be challenging as they can be caused by unusual and diverse organisms (Eggimann, 1998). Such infections may progress rapidly to become life threatening and are difficult to eradicate with antibiotics alone in the absence of an intact immune system (Dryden, 2010). Is briefly outlined in studies performed by Etareri and Dolan (2017), that lactic acid bacteria can play a decisive role in human health care and *Lactobacillus* strains are the primary species implicated in these reduced risk outcomes towards eczema disease. Besides eczema further research in probiotics and dermatological pathologies have examined conditions such as acne, that they may be caused by infection with the pathogenic bacteria *Propionibacterium acnes* in acne (Dolan *et al.*, 2017; Etareri *et al.*, 2017). Eczema is also known as atopic dermatitis, is a chronic, relapsing, and itchy inflammatory skin condition (Schmitt *et al.*, 2011).

1.8.2.2 *Bifidobacterium* spp.

Is explored that probiotic agents as *Bifidobacterium* have been studied for their efficacy in the prevention and treatment of a broad spectrum of animal and/or human gastrointestinal disorders (Picard *et al.*, 2005). Along with numerous bifidobacterial strains have also been found useful in treating different clinical conditions (reduction of risks of preterm delivery, obesity and stress management, treatment of oral plaque, improvement of symptoms related to depressive disorders and schizophrenia and alleviating symptoms of allergic asthma). Therefore it is likely that different strains operate through a combination of more than one pathway instead of a single

mechanistic route (Sarkar & Mandal, 2016). Bifidobacteria have the ability to produce organic acids and other antimicrobial compounds such as proteinaceous compounds called bacteriocins (Mostafa *et al.*, 2015). Regardless of the potential of bifidobacteria to suppress the growth of both Gram-negative and Gram-positive bacteria, their ability to produce bacteriocins has so far been undervalued, being their antimicrobial activity often ascribed to the inhibitory action of organic acids and the related pH decrease (Martinez *et al.*, 2013). In recent studies, beneficial effects of LAB and bifidobacteria that extend beyond the gut were uncovered, as these bacteria also demonstrated their potential in promoting dermal health and exerting cellular immunity response required for skin defence. (Tan *et al.*, 2014).

1.8.2.3 *Propioniferax* spp.

Are presented other examples of probiotic bacteria is the genus, *Propioniferax*, a group of Gram-positive bacteria, included on the Propionibacteriaceae family with high percent G+C, belonging to the phylum Actinobacteria and order Actinomycetales. Strains like of *Propioniferax innocua* can be isolated from human skin (Gortz *et al.*, 2006). Several Propionibacterium species are human-associated microorganisms present in skin (Bhatia *et al.*, 2004). Propionibacterium organisms are found in acne, prosthetic joints, cerebrospinal fluid shunts, endocarditis, and osteomyelitis (Meredith & Ulrich, 2013). *Propioniferax innocua* bacteria received the generally recognized as safe (GRAS) status, and was considered nonpathogenic (Rabah *et al.*, 2017). Propionibacterium may have beneficial effects, such as adjuvant and anti-tumor activities. The principal metabolite produced by this bacteria is derived from glucose and is propionic acid (Patrick & Mcdowell, 2012). Hence these attributes may contribute to more efficient immunological responses to infections (Al-Ghazzewi & Tester, 2014). Propionibacterium are described as producers of nutraceuticals and beneficial metabolites that are responsible for their versatile probiotic attributes which include short chain fatty acids (SCFAs), conjugated fatty acids, surface proteins, and 1,4-dihydroxy-2-naphtoic acid (DHNA). These metabolites possess beneficial properties and their production depends on the strain and on the growth medium. In addition, Propionibacterium produce other peptides and organic acids (2-pyrrolidone-5-carboxylic acid, 3-phenyllactic acid, hydroxyphenyl lactic acid 3-phenyllactic acid with antiviral, anti-yeast and anti-fungal activities, and these bacteriocins are also active

against *P. aeruginosa* (Schwenninger *et al.*, 2008). Due to the slow growth, late bacteriocin synthesis and low production represent limitations for the practical application of bacteriocin-producing Propionibacteria (Zárate, 2012).

1.9 - Probiotics as topical application agents

As explored by Jones (2017) affirms that there are three mechanisms by which probiotic bacteria have positive effects when applied topically: 1) acting as protective shield, the microorganisms on the skin are recognized as foreign by the body's immune system, on which probiotics springs into action to deal with the inflammation; 2) when applied in a soap or cream on skin surface, helps the skin cells to detect the microbes that can cause damage and the immune response (known as 'bacterial interference', due to probiotics protection of the skin and interference with the ability of bacteria to elicit an immune response); 3) as an antimicrobial agent, as mentioned before; 4) evoking a calming effect, since when in contact with skin cells, probiotics block the skin cells signals for the immune system result in flares of acne or rosacea (Jones, 2017). Indeed, topical probiotic formulations are becoming increasingly available, providing defense against pathogenic bacteria, the reduction of inflammation, showcasing antiaging benefits and treating diseases caused by pathogenic microorganisms, representing an "emerging area" for skin health (Cinque *et al.*, 2011). The potential benefits of skin probiotics could strongly depend on how each species or strain is selected through the specific mechanisms underlying a specific effect on the healthy or disturbed skin (Hippe & Berit, 2011). More specifically, recent studies have demonstrated that some probiotic strains display potent immune-modulatory properties at the skin level, with the ability to modulate the host gene expression and immune system cellular differentiation, facilitated by their interaction with host receptors (Plaza-Diaz *et al.*, 2019). This enables priming of both the innate and adaptive immune systems, through a communication network between epithelium, macrophages, dendritic cells results in T-cell differentiation, thereby achieving immune homeostasis and tolerance for commensal microbiota (Llewellyn & Foey, 2017). Recently, the ability of *L. paracasei* CNCM-I 2116 (ST11) to modulate reactive skin-associated inflammatory mechanisms has been evaluated. To sum up that ST11 was able to abrogate vasodilation, edema, mast cell degranulation and TNF-alpha release induced by substance P, compared to control (Gueniche *et al.*, 2010). Moreover, studies hinting at the value of topical probiotics in acne include recent reports that strains of

Bifidobacterium longum and *Lactobacillus paracasei* NCC2461 can attenuate substance-P–induced skin inflammation, as measured by reduction of vasodilation, edema, mast cell degranulation, and tumor necrosis factor alpha (TNF- α) release (Bowe & Logan, 2011). This is of relevance because substance-P may be a primary mediator of stress-induced inflammation and sebum production, and therefore the reduction on the inflammatory cascade presents as a therapeutic application against the pathogenesis of acne (Kober & Bowe, 2015). It also seems that the *Bifidobacterium* plays an important role, had a significant decrease in skin sensitivity after treatment. In an experiment the topical application, that has the both bacteria, containing the 10% bacterial extract, for 29 days decreases skin dryness, as well (Gueniche *et al.*, 2010). In another experiment, subjects aged 1 to 13 years with severe chronic eczema took either a combination of 2 *Lactobacillus* strains (*L. rhamnosus* GG 19070-2 and *L. reuteri* DSM 122460) or placebo for 6 weeks. In the study 56% of probiotic-treated patients experienced subjective symptom improvement compared with 15% of placebo-group patients and eczema extent decreased. Also, serum eosinophilic cationic protein values used to monitor disease activity in AD decreased with probiotic therapy. Probiotic therapy was accompanied by only moderate changes in production of the cytokines IL-4, IFN- γ , IL-10. The modest influence of the probiotics on the improvement of AD could be attributed to the older age of the subjects and the severity of the eczema (Rosenfeldt *et al.*, 2003). Besides, studies in immunocompromised patients, including burn patients, use probiotics to decrease the rate of infection and accelerate wound healing. In a study, a topical application of *Lactobacillus plantarum* reduced *Pseudomonas aeruginosa* skin infections in a mouse model of burn wounds (Valdéz *et al.*, 2005). Another study performed by Barzegari *et al.* (2017), looking to evaluate the impact of topical application of *Lactobacillus acidophilus* on second-degree burn wounds, performed in vivo in rats, showed that the percent of wound healing in the 3th and 7th days of experiments, was significantly higher in rats receiving the probiotic. Despite precautionary measures, incidence of burns is still one of the important medical problems, especially in nosocomial infections. In addition to prevention of the burn infection, the bacteria had beneficial effects on wound healing processes like reducing the inflammatory response and accelerating re-epithelialization (Barzegari *et al.*, 2017). In this case, rodent cages can become easily contaminated with *S. aureus* and *P. aeruginosa* as in nosocomial infections where there is this incidence (Ahirrao *et al.*, 2017; Barzegari *et al.*, 2017). Skin commensals are also great candidates as a topical

strategy given their inherent adaptation to the skin environment. For example, *Propioniferax innocua*, a skin commensal, has been found to degrade established biofilms (Yu *et al.*, 2019). Is analyzed the adhesion of pathogenic bacteria to human keratin showed a high variability among micro-organisms. For *Propionibacterium acnes*, only the probiotic *P. innocua* had the ability to significantly reduce its adherence, 17.4% (Lopes *et al.*, 2017). Additionally, a coinfection study using pathogenic *Staphylococcus aureus* suggested that the topical application of probiotic bacteria *Lactobacillus reuteri* ATCC 55730 and *Lactobacillus rhamnosus* AC413 can improve skin health or combat disease. Keratinocyte survival was significantly higher when probiotic application occurs before the infection with *S. aureus* and effectively reduces the concentration (pathogen). This suggests that the protective mechanism for *L. reuteri* mediated protection of keratinocytes was by competitive exclusion of the pathogen from its binding sites on the cells. Their results suggested that use of a topical probiotic prophylactically could inhibit the colonization of skin by *S. aureus* and prevention of infection. (Prince *et al.*, 2012). Additionally, the inhibitory effects of probiotics on biofilm formation by skin pathogens were evaluated in (i) competition (ii) exclusion and (iii) displacement, in this study. The bacterial cell surface charge and hydrophobicity have been shown to influence the strength of bacterial adhesion (Harty & Knox, 1991; Piette & Idziak, 1992).

Overall, probiotics modulate the development of the immune system, often shifting the immune response toward regulatory and anti-inflammatory conditions. This ability of probiotics to modify chronic inflammatory states suggests that probiotics may have a role in treating chronic inflammatory conditions (Kober & Bowe, 2015). Topical drug delivery is a valuable and painless route of drug administration, offering an easy way for patients, and prevents the initial hepatic metabolic route of the drug (Mehdi-Alamdarloo *et al.*, 2016).

1.10 Significance and Impact of study

In recent years, many researchers have been focused on discovering new and efficient topical agents with natural origin and fewer sides and effects (Mehdi-Alamdarloo *et al.*, 2016). The topical use probiotics may be an alternative, co-adjuvant approach to a targeted treatment of several skin illnesses and a complement to conventional therapies which present many undesirable side effects.

1.11 Work objectives

The objectives of this thesis were:

1- To address the competition between probiotics (*Lactobacillus paracasei*, *Propioniferax innocua*, *Bifidobacterium longum* spp. *infantis*) and the pathogenic microorganisms (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*) and observe the impact of probiotics on pathogens.

2- Evaluate the adhesion capacity to keratinocytes of these probiotics in the presence of pathogenic bacteria in vitro.

3- The mechanism of protein adhesion was studied.

2. Materials and Methods

2.1 Microorganisms and culture conditions

The microbial strains used in the current study, their origin and culture conditions are summarized in Table 1. All strains were preserved at - 80 °C in the appropriate media with 30 % (v/v) of glycerol in sterile cryovials, of 1 mL, until further analysis.

Microorganisms	Origin	Media	Incubation conditions
<i>Bifidobacterium longum</i> spp. <i>infantis</i>	DSMZ 20088	MRS	Anaerobic, 37°C
<i>Lactobacillus paracasei</i> L26	Delvo Pro LAFTI	MRS	Aerobic, 37°C
<i>Propioniferax innocua</i>	ATCC 8251	MRS	Aerobic, 37°C
<i>Staphylococcus aureus</i>	Internal Collection CINATE	BHI	Aerobic, 37°C
<i>Staphylococcus epidermidis</i>	ATCC 155	BHI	Aerobic, 37°C
<i>Escherichia coli</i>	Internal Collection CINATE	BHI	Aerobic, 37°C
<i>Pseudomonas aeruginosa</i>	Internal Collection CINATE	BHI	Aerobic, 37°C

For each assay, the studied bacterial were reactivated from frozen state in Man, Rogosa and Sharp (MRS, Biokar Diagnostics, Beauvais, France) media and Brain Heart Infusion (BHI, Biokar Diagnostics, Beauvais, France) media, for probiotic and pathogenic bacteria, respectively. Bacterial suspensions were incubated at 37°C with one propagation step at (10% v/v).

2.2 Competitive inhibition assay

To assess the response of the pathogenic bacteria to exposure to probiotic bacteria, competition studies among several bacteria (*L. paracasei* with *S. aureus*, with *S. epidermidis*, with *E. coli* and *P. aeruginosa*; *Propioniferax innocua* with *E. coli* and *P. aeruginosa*; *Bifidobacterium longum* spp. *infantis* with *E. coli* and *P. aeruginosa*) were carried out specifically upon the growth pattern/curve of all bacteria.

After the growth of bacteria, the cells were harvested by centrifugation (5,000 × rpm, 10 min, 4°C), washed twice with phosphate-buffered saline PBS pH 7.4 (10 mM Na₂HPO₄, 1 mM KH₂PO₄, 140 mM NaCl, 3 mM KCl) and suspended in Brain Heart Infusion (Li *et al.*, 2015). Bacteria such as *Bifidobacterium longum* spp. *infantis*, *Lactobacillus paracasei* and *Propioniferax innocua* were grown for 48 hours on MRS and pathogenic bacteria, *Staphylococcus aureus*, *Staphylococcus epidermidis*,

Escherichia coli and *Pseudomonas aeruginosa* on BHI for 24 hours, both at 37°C (Siddiquee *et al.*, 2012).

Afterwards, in a 100 mL volume flask, with BHI medium, the study was carried out, in which the pathogen infectious load-probiotic ration, based on literature research was at a concentration of 2,5% (v/v) and the probiotic at 3,5% (v/v). Experimentally, the inoculation of 3,5% of *Bifidobacterium longum* spp. *infantis*, *Lactobacillus paracasei* and *Propioniferax innocua* favours the competition against the pathogens that it was 2,5% of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and *Pseudomonas aeruginosa*. Sampling was carried out over a period of 6 hours, respectively at 0, 30 minutes, 1, 3 and 6 hours. At each sampling time, decimal dilutions (100 fold) were made until 10^{-6} and plated on MRS for *Lactobacillus paracasei* (*L. paracasei*), *Bifidobacterium longum* spp. *infantis* (*B. longum* spp. *infantis*), *Propioniferax innocua* (*P. innocua*), MacConkey Agar and Baird Parker Agar, Cetrimide Agar for *E. coli*, *S. aureus* and *S. epidermidis* and *P. aeruginosa*, respectively. The plates were incubated for 6 h at 37°C (Siddiquee *et al.*, 2012).

2.3 Cell culture

2.3.1 Adhesion protocol

Adhesion of probiotic/pathogenic strains to human keratin was carried out following the methodology described by Ouwehand (2003) slightly modified with the protocol described by Laparra (2011).

The immortalized human keratinocytes (HaCaT) cells (CLS Cell Line, Germany) were grown in a 24-well microtiter plate until confluence with DMEM medium (Biowest, Riverside, USA) supplemented with 10% fetal bovine serum (FBS), (Biowest, Riverside, USA) and 1% of Antibiotic/ antimycotic (AB/AM) (Biowest, Riverside, USA), in an incubator with 95% (v/v) humidified air and 5% (v/v) CO₂ at 37°C. The keratinocytes were seeded into 24 well tissue culture plates (Thermofisher) at a density of 5×10^4 cells per well (80% confluence). In the first step, the cells were washed twice with PBS (pH 7.4) to remove the excess free cells (unattached), re-suspended in DMEM (antibiotic-free, fetal bovine serum-free) before the adhesion assay, for 30 minutes and left in contact to clean the antibiotic influence (Li *et al.*, 2015). The bacterial cells were harvested by centrifugation at 5,000 rpm for 10 min at 4°C and washed twice with PBS to remove residues from the growth medium (pH 7.4).

To test the bacterial adhesion, three methods were evaluated: displacement, competition and exclusion. The displacement assay has the objective of evaluating the adhesion and displacement capacity of bacteria (Al-Saedi *et al.*, 2016). Competition tests the competition of the microorganisms through diverse mechanisms by which bacterial species can coexist with, or dominate, other organisms competing for the same pool of resources. Finally, exclusion, between species results in the elimination of one species from a given habitat or region (Hibbing *et al.*, 2010).

The three trials were performed as follows: For the displacement assay, the cells were infected with 100 μ L of pathogen and one hour later the probiotic (*L. paracasei*/ *B. longum* spp. *infantis*, /*P. innocua*) bacteria were added (100 μ L) and one hour more allowed for adherence; for the exclusion assay, first the cells were infected with 100 μ L of the probiotic (*L. paracasei*/*B. longum* spp. *infantis*, /*P. innocua*) and one hour later the pathogenic bacteria were added (100 μ L) and one hour allowed for adherence; for competition assays, cells were infected at the same time with 100 μ L of pathogen and 100 μ L of the probiotic (*L. paracasei*/ *B. longum* spp. *infantis*, /*P. innocua*) bacteria were added, and two hours allowed for adherence (Balaban *et al.*, 2003). After these periods, the non-adherent cells were removed, and the well was washed with 200 μ L of PBS (twice) and afterwards 500 μ L of triton X-100 (Sigma-Aldrich, St. Louis, Missouri, EUA) was added to each well. This step promotes the detachment of cells and the microorganisms that adhered to the cells. Finally, the cells were harvested, and the total viable cell numbers of the microorganisms were determined by plating 20 μ L of each serial decimal dilution in three replicates in MRS and the respective selective media plates. Results were expressed as log CFU/mL (Li *et al.*, 2015; Lopes *et al.*, 2017)

Mechanisms of adhesion

2.3.2 Protein adhesion protocol

To assess the bacterial adhesion, the HaCaT cells were grown as described previously. The cells were washed twice with PBS (pH 7.4) to remove the excess free cells and antibiotic before the adhesion assay, for 30 minutes and left in contact to clean the antibiotic influence (Li *et al.*, 2015). The bacterial cells were harvested by centrifugation 5,000 rpm for 10 min at 4°C and washed twice with PBS (pH 7.4) to remove the residues of the growth medium, and then re-suspended in trypsin (0.4%) for

15 minutes at 37°C. After the exposition the cells were centrifuged and washed twice with the trypsin neutralizer, PBS, and resuspended in keratinocyte medium, DMEM, prior to use in adhesion assays; The bacterial strains were incubated with HaCat cells at the same time, L26/SA; L26/SE; L26/PA, 100 µL of each for 2 hours at 37°C. After this, the non-adherent cells were removed, and the well was washed with 200 µL of PBS (twice) and afterwards 500 µL of triton X-100 added to each well to detach the cells and the microorganism adhered to the cells. After this, cells were harvested and plate-counted after serial decimal dilutions (Li *et al.*, 2015).

Pre-tests were performed before the experiment, to verify the most suitable time of exposition to the chemical compound and trypsin resistance, with *L. paracasei*, *S. aureus* and *P. aeruginosa*, for which the bacteria were exposed 15 minutes, 30 minutes, 1 hour and 2 hours in a singular way, after washing with PBS (twice). Cells were counted by spread plating after serial decimal dilutions.

2.3.3. Carbohydrate analysis

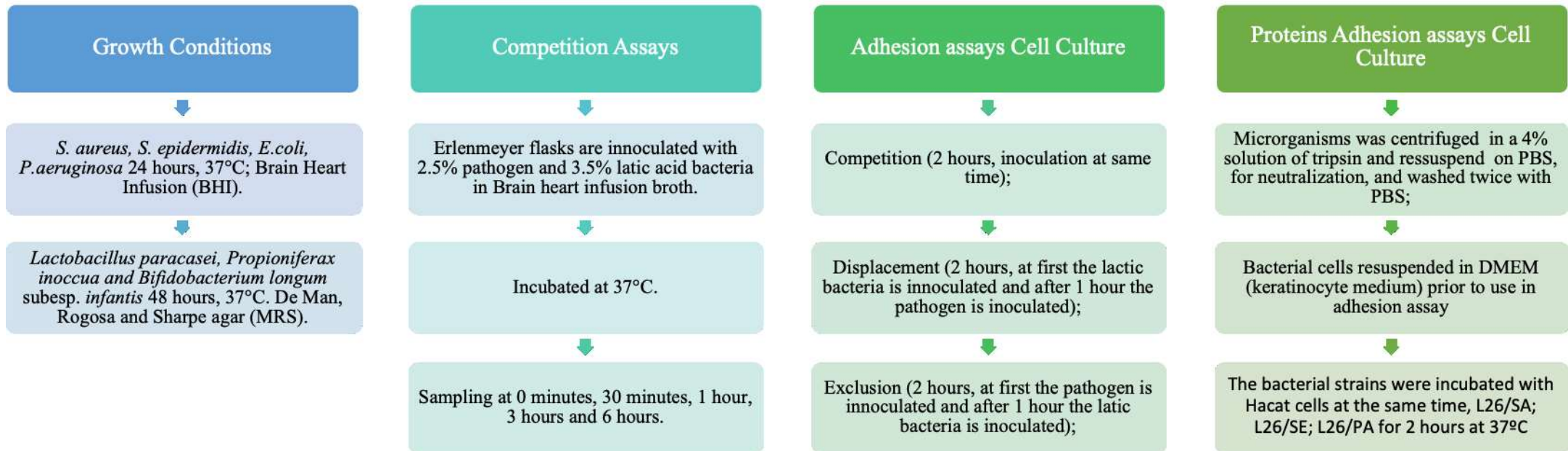
To assess the bacteria adhesion through carbohydrates, HaCaT cells were grown in a 24-well plate until confluence with DMEM medium supplemented with 10% FBS and 1% of AB/AM, and incubated with 95% (v/v) humidified air and 5% (v/v) CO₂ at 37°C. The keratinocytes were seeded at a density of 5x10⁴ cells per well and allowed to grow until 80% confluence. The cells were washed twice with PBS (pH 7.4) to remove the excess free cells and antibiotic before the adhesion assay, and left for 30 minutes to clean the antibiotic influence (Li *et al.*, 2015). The bacterial cells were harvested by centrifugation at 5,000 rpm for 10 min at 4°C and washed twice with PBS (pH 7.4) to remove the residues of growth medium. Bacteria were treated with 50 mM sodium meta-periodate (Sigma-Aldrich, Gillingham, UK) in 0.1 M citrate phosphate buffer (pH 4.5). Cells were incubated for 30 minutes at 37°C and washed twice before resuspending with DMEM for infection. Serial dilution counts were performed to check if bacterial viability was affected by the sodium meta-periodate (Li *et al.*, 2015; Lopes *et al.*, 2017).

Pre-tests were performed with *L. paracasei*, *S. aureus* and *P. aeruginosa* to verify the resistance to sodium meta-periodate, for which bacteria were exposed for 5, 15, 30 and 60 min, after being washed with PBS (twice). Cells were counted by spread plating after serial decimal dilutions.

Statistical analysis

The normality of the data distribution was determined through the Shapiro-Wilk test. To analyse the differences between sample groups, when a normal distribution was observed, analyses of variance (ANOVA) one-way (Repeated Measures) test was used in association with Tuckey's test, for all the analysis. For the competition, displacement and exclusion assays, the independent t-test was performed. For the protein adhesion assay, the t-test for paired samples was applied. The differences were considered statistically significant at a 5% confidence degree level. All statistical analysis was performed using IBM SPSS Statistics v.19.0.0 (New York, USA) software.

Methodology Summary



4. Results and Discussion

4.1 Competition studies

Competitions studies were carried out to reveal the interaction of selected pathogenic bacteria with probiotics. In competition studies the assays were performed by one probiotic and one pathogen, i. e., *Lactobacillus paracasei* (L26) with *S. aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa*; *Propioniferax innocua* with *E. coli* and *P. aeruginosa*; *Bifidobacterium longum* spp. *infantis* with *S. aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa*.

4.1.1 *Lactobacillus paracasei* / *Staphylococcus aureus*

Figure 4 shows the competition assay results of *Lactobacillus paracasei* (L26) – *Staphylococcus aureus* in liquid media.

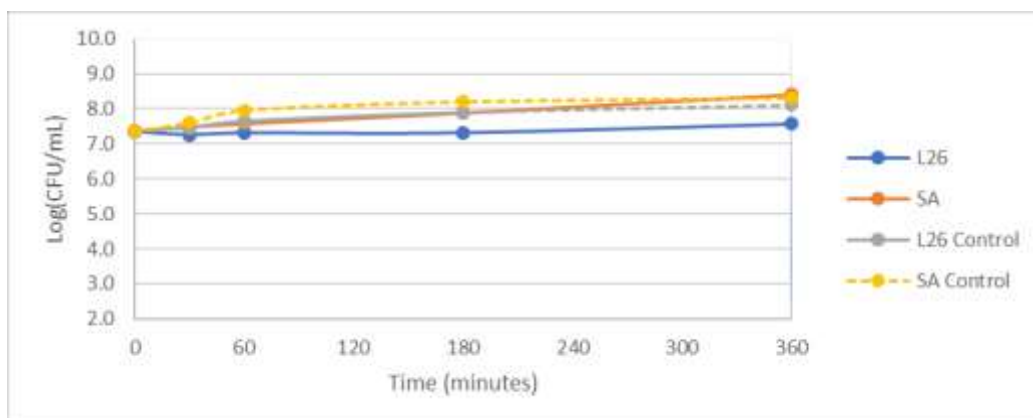


Figure 3- Variation of viability (log CFU/mL) of probiotic *L. paracasei* and pathogen *S. aureus* and respective controls in the competition study, during 360 minutes of the assay.

Figure 3 shows that when co-occurring, *L. paracasei* remains consistently 7.45 logarithmic units (logs) without major alterations. On the contrary, *Lactobacillus paracasei* decreased slightly in the beginning, approximately 0.2 logs and increased since the third sampling point until the end, approximately 1.0 logs. Previously performed studies indicate that lactic acid bacteria are capable of inhibiting the pathogenic *S. aureus* which was not observed in this experiment (Alp *et al.*, 2019; Gan *et al.*, 2001; Vuotto *et al.*, 2014).

According to a previous study by Alp (2019), the constant growth observed of *S. aureus* over the course of the experiment is probably due to the duration of exposition

of the two cultures (co-culture) of the two bacteria. There is a significant inhibition from 48 to 72 hours of *S. aureus* towards *L. rhamnosus*, which might be due to the inhibition of the coagulase enzyme as a result of the probiotic presence. During the experiment there were produced metabolites by the Lactobacilli, such as the acids originated from the fermentative activity, that led to a decline in the pH values; bacteriocins release and competition for nutrients, which were capable to inhibit *S. aureus* growth (Alp *et al.* 2019). However, such evidences were not observed in our experiments due possibly the lack of ability of *L. paracasei* to inhibit the coagulase production. Bacteriocins have their synthesis mediated by genetic mechanisms, as well. These molecules present characteristics such as heat stability, tolerance to low pHs, refrigeration and freezing, and resistance to weak organics solvents, salts and enzymes. They are, however, very sensitive to proteolytic action (Souza *et al.*, 2005). Another study realized by Bendali *et al.* (2011) concluded that the *L. paracasei* strain was able to produce a bacteriocin-like substance active against staphylococcal strains. Therefore, a 2-log reduction in *S. aureus* cell numbers was registered when in co-culture with *L. paracasei* (Bendali *et al.*, 2011). Moreover, a study performed with *L. paracasei* spp. *paracasei* BGBUK2-16 exhibited considerable antagonistic activity against *S. aureus* in culture. The *L. paracasei* spp. *paracasei* BGBUK2-16 effect is dependent on the initial concentration of the pathogen. The initial number of viable *S. aureus* cells was 10² CFU/mL, but all cells were killed after 24-h. The decrease in pathogen growth correlated with maximum Bac217 (bacteriocin) production. Although a complete inhibition of the pathogen's growth was not achieved in the culture containing an inoculum of 10⁷ CFU/ ml, the number of viable cells significantly decreased with 24 h of incubation. The *L. paracasei* spp. *paracasei* BGBUK2-16 growth was not affected in mixed cultures (Lozo *et al.*, 2004). Equally important study by Misaghi *et al.* (2017), claim that co-cultures (LAB/*S. aureus*) with LAB strains had a significantly inhibitory effect on *S. aureus* growth. Through an experiment, *S. aureus* grew during the 24 h incubation period to 9.4 logs CFU/mL and bacterial counts remained the same over the following 48 h. Co-cultures with LAB strains however had a significantly inhibitory effect on *S. aureus* growth since the cell densities of *S. aureus* co-cultured with *L. fermentum*, *L. paracasei* and *L. acidophilus* rose to only 6.3, 6.2 and 6.4 log after 24 h LAB strains reduced the cell densities of *S. aureus* by 2-3 log compared to the control at all evaluation times (Misaghi *et al.*, 2017).

The results obtained in this study do not match the ones found in the literature as there should have been a more evident inhibition or decrease in the numbers of viable cells of the pathogen. A plausible explanation for the maintenance in viability in the pathogenic bacteria might be due to the cell disposition in the liquid medium, as well as their bacteriocins dissemination. According to Blom *et al.* (1997), other intrinsic factors that may influence bacteriocin diffusion could be pH, salt concentration, nitrite and nitrate, aqueous phase available for diffusion, fat content and fat surface available for solubilization. Moreover, the distance the bacteriocin molecule must diffuse to reach the target cell, and amount of target cells and/or cells capable of adsorbing the bacteriocin will be of importance (Benmouna *et al.*, 2018; Blom *et al.*, 1997; Mills *et al.*, 2011). Indeed, evidence suggests that biofilm and planktonic cells exhibit singular biological properties, and one of the characteristic features of biofilms is their intrinsic tolerance to antimicrobials and immune attack (Camarillo-Márquez *et al.*, 2018). Microorganisms are not solitary entities and often grow in multispecies biofilm communities (Stoodley *et al.*, 2002). In a study by Ochieng'Olwal *et al.* (2018) it was concluded that *S. epidermidis* biofilms were less susceptible to physico-chemical stress than the analogous planktonic cells. The species colonize these communities by interacting with one another through physical (e.g. aggregative) and chemical (e.g. cell–cell signaling) interactions. One type of cell–cell interaction, is designed coaggregation and is characterized by the highly specific recognition and adherence of different species of microorganisms to one another (Hojo *et al.*, 2009; Rickard *et al.*, 2003). Adherence through co-aggregation may be critical for the temporary retention of bacteria on surfaces and may simplify bacterial colonization. It is likely that metabolic communication, genetic exchange, production of inhibitory factors (e.g., bacteriocins, hydrogen peroxide, etc.), and quorum-sensing are crucial regulatory factors that determine the bacterial composition and/or metabolism (Rickard *et al.*, 2003). The coaggregation occurs between different species of bacteria that is distinct from autoaggregation, which is defined to be the adherence of genetically identical bacteria to one another (Stevens *et al.*, 2015). Autoaggregation-dependent microcolony formation could result in an effective increase in concentration of secreted effectors at or near the host cells that modulates virulence. Autoaggregation, by definition, can only take place between close bacteria. This view is supported by the recent work showing that, under high competition with single cells, cells positioned at the top of aggregates enjoy a competitive advantage (Trunk *et al.*, 2018). Since the bacteria rapidly adhere to polymer

material, they start to proliferate to form multi-layered cell clusters on the polymer surface, which are embedded in extracellular material (Katsikogianni & Missirlis, 2004). An accumulated biomass of bacteria and their extracellular material (slime) on a solid surface is called biofilm (O'Toole *et al.*, 2000). According to the author (Katsikogianni & Missirlis, 2004) after biofilm establishment, non-adherent and some adherent daughter cells escape from the slime layer, either by switching off slime production through a mechanism of phenotypic modulation, or by exhaustion conditions that support slime production, and are then free to drift to new colonization sites to repeat the colonization process.

4.1.2 *Lactobacillus paracasei* / *Staphylococcus epidermidis*

Figure 4 shows the competition between *L. paracasei* (L26) and the pathogenic bacteria *S. epidermidis*.

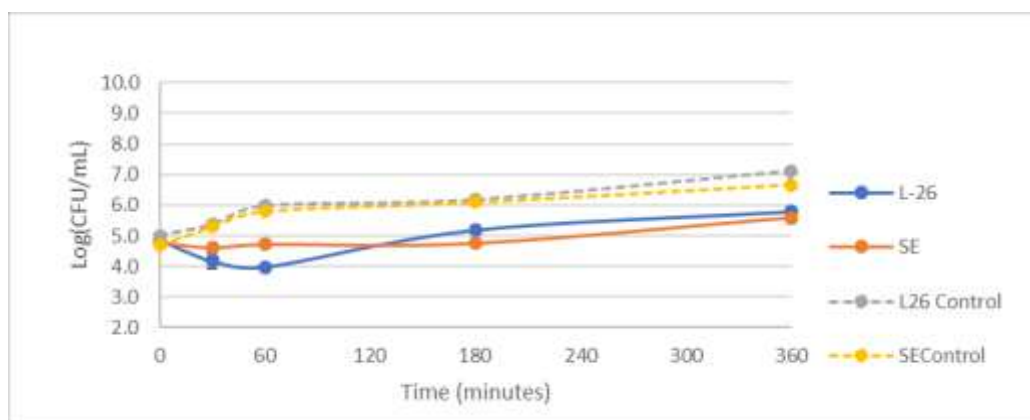


Figure 4- Variation of viability (log CFU/mL) of probiotic *L. paracasei* and pathogen *S. epidermidis* and respective controls in the competition study, during 360 minutes of the assay.

At figure 4, in the beginning of the assay until the 130 minutes the *L. paracasei* is observed to decreased slightly, 2.0 logs and *S. epidermidis* remains constant. After 130 minutes approximately, a reversal in the growth pattern can be verified, when the *L. paracasei* grew more than *S. epidermidis*, approximately 0.5 logs, whereas up to this time an overlap of the growth of *S. epidermidis* with *L. paracasei* is observed. These results corroborate the findings by other authors (Alp et al. 2019) where *Lactobacillus* reduced the growth of *S. epidermidis*, through pH reduction resulting from the fermentative activity and production of antimicrobial substances (bacteriocins). Many LAB strains also EPSs into their extracellular environment which contribute to cell

protection and the production of heteropolysaccharides mainly by *Lactobacillus*, lactococci and streptococci (Etareri *et al.*, 2017).

4.1.3 *Lactobacillus paracasei* / *Escherichia coli*

Figure 5 displays the evolution of viability over the course of the competition assay between *Lactobacillus paracasei* and *Escherichia coli*.

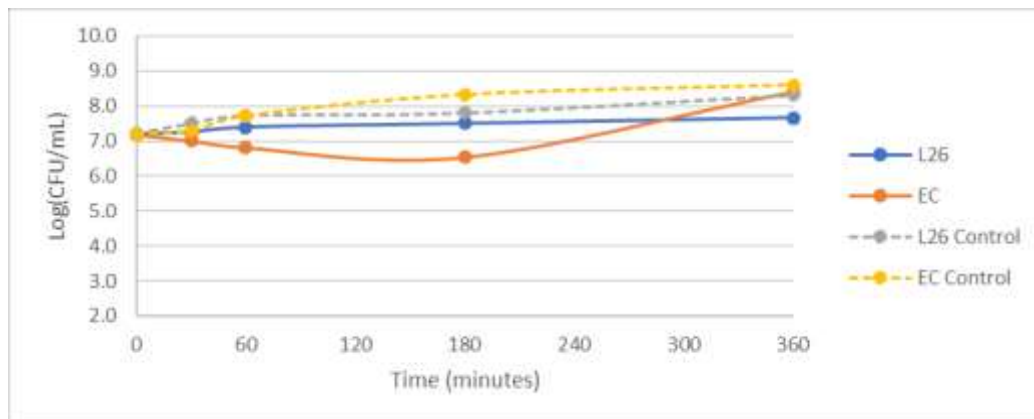


Figure 5 - Variation of viability (log CFU/mL) of probiotic *L. paracasei* and pathogen *E. coli* and respective controls in the competition study, during 360 minutes of the assay.

In figure 6, we observe that *E. coli* growth decreased slightly by 180 minutes of sampling (from 7.2 to 6.5 logs), but till the end it grew sharply to 8.4 logs. Throughout the experiment *L. paracasei* viable numbers remained constant, increasing its cellular concentration smoothly from 7.3 to 7.7 logs, from about 30 minutes until the end of the experiment. Therefore, *L. paracasei* has the ability to remain stable throughout sampling. The decay of *E. coli* is probably due to the release of antimicrobial substances, including organic acids, hydrogen peroxide, and bacteriocin by *L. paracasei*. *L. paracasei* bacteria is able to produce bacteriocins effective against *E. coli* (Caridi, 2002). Plus, it has been demonstrated, in a study performed by Deng *et al.* (2015), that certain strains of *L. paracasei* exhibit important characteristics, for example good survival at low pH. Previous studies have indicated that the lactic acid bacteria inhibit the pathogen *E. coli* which was not observed in this study. This was probably because the study focused on evaluating growth inhibition by measuring the halo diameter method (disk diffusion). The inhibition zones observed in *E. coli* by LAB were in the range of 9.8-11.1 mm (Pyar *et al.*, 2011). In this study, the antibacterial effects of *L. paracasei* were investigated by disc diffusion method and the growth was conducted

in molasses of soy-whey (planktonic cells). This outcome of inhibition could be explained by a different response by the probiotic bacteria, *L. paracasei* to planktonic cells, to biofilm formation or auto-aggregation/coaggregation by the *E. coli*. In Pyar *et al.* study (2011)., the response is through biofilm formation. The results obtained by Pyar *et al.* study on disc diffusion method, the inhibition of *E. coli* was higher. Comparison with the present study, it demonstrates the different inhibition response in free cells and biofilm. In disk diffusion, the inhibition could be explained by a good diffusion of metabolites that resulted in the growth inhibition of the pathogenic microorganisms. Other gastrointestinal study demonstrates a decrease, as well, in the pathogen numbers, but with oral intake of probiotics, (used to attenuate skin diseases, such as acne, seborrheic dermatitis, or rosacea). The group receiving probiotics showed an 89% improvement in their facial dermatoses compared to 56% improvement achieved with diet and standard therapy in the control group (Porubsky *et al.*, 2017). That is, indirectly through the absorption of LAB in the intestine we can infer that it has positive effects on the treatment of skin diseases, mitigating the side effects of its adhesion and internalization. The results from in this experience, suggest that *E. coli* agglomeration occurs (auto-aggregation) and with another bacteria (coaggregation), because inhibition by *L. paracasei* bacteria becomes easier when the cells are in free form/ planktonic cells, because there is a greater exposition to bacteriocins than that found when they are in clusters.

4.1.4 *Lactobacillus paracasei* / *Pseudomonas aeruginosa*

The following graph, figure 6, show the competition between *Lactobacillus paracasei* and *Pseudomonas aeruginosa*.

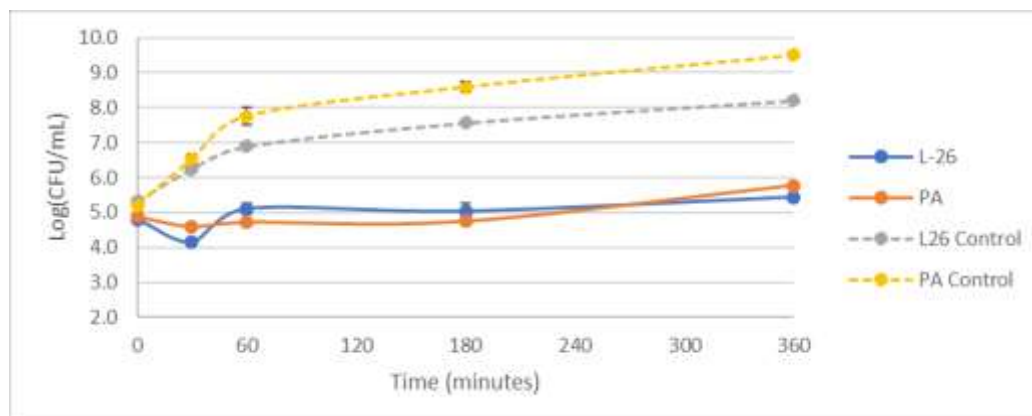


Figure 6 – Variation of viability (log CFU/mL) of probiotic *L. paracasei* and pathogen *P. aeruginosa* and respective controls in the competition study, during 360 minutes of the assay

We can observe in figure 6, that the viable numbers of *Pseudomonas aeruginosa* and the LAB decreased approximately 1.0 logs up to 30 min of contact. Sensibly around 45 minutes into the assay, LAB numbers overlap with those of *P. aeruginosa* up to 270 minutes after this time *P. aeruginosa* numbers increased beyond those of L26. After 270 minutes, an increase in the cell concentration of the pathogen is verified, 1.0 logarithmic unit, probably due to the active production of its virulence factors (Youenn *et al.*, 2014). Although, one mechanism proposed for the reduced anti-pseudomonas activity is the resistance of *P. aeruginosa* to the antimicrobial substances in the cell free supernatants (CFS) of the lactobacilli (hydrogen peroxide, lactic acid, and bacteriocin-like molecules (El-Mokhtar *et al.*, 2020). In the present study, as shown in the graph, it is observed that the numbers of *P. aeruginosa* are always higher when compared to those of *L. paracasei*, which contradicts previous results. The reason behind this behavior might be that bacteriocin dispersion is not optimal in the liquid medium, due to the ability to cells to self-aggregate within the same bacterial species or coaggregate between different species (Stevens *et al.*, 2015; Trunk *et al.*, 2018). The decrease in pathogenicity in the first 30 minutes is probably due to its sensitivity to lactic and acetic acids produced by LAB (Alexandre *et al.*, 2014; Jalilsood *et al.*, 2015) reported the ability of a new Lactobacillus strain to form biofilms, which provides the strong inhibitory effect against some spoilage and pathogenic bacteria. According to previous studies performed with *L. fermentum*, there are compounds secreted, that inhibit the growth, cytotoxicity, and biofilm formation of several *S. aureus* and *P. aeruginosa* strains; however, these studies were performed by the disc diffusion method (Varma *et al.*, 2011). Is described that the disc diffusion method is an antimicrobial susceptibility test. Nevertheless, disc diffusion has advantages as simplicity, low cost, the ability to test high number of microorganisms and antimicrobial agents, and the ease to interpret results provided (Balouiri *et al.*, 2016). In this disc diffusion method, the results provided are qualitative, classifying bacteria as susceptible, intermediate and resistant strains, depending on the size of the inhibitory zone. Furthermore, this test is dependent on appropriate diffusion. As such, the molecular weight of the drug molecules is a crucial factor when using this method. In addition, imperfections and flaws in the agar plates can affect diffusion and lead to false results (Schumacher *et al.*, 2017). In a different study, also using a different LAB strain, it was demonstrated that Lactobacillus plantarum had potent inhibition effect on the production of quorum-sensing signal molecules of *P. aeruginosa*, acyl-homoserine-lactones (AHLs), with two virulence factors being

influenced by these molecules: elastase production and biofilm formation. At infected burn wounds, in vivo, samples were taken after 5, 10 and 15 days demonstrated inhibition of *P. aeruginosa* colonization by *L. plantarum*. There was also an improvement in tissue repair, enhanced phagocytosis of *P. aeruginosa* by tissue phagocytes (Valdéz *et al.*, 2005). Indeed, in a previous study by Coman *et al.* (2014) suggested that overall, Lactobacillus strains possess inhibition effects over pathogens, specifically *L. paracasei* against *P. aeruginosa*.

4.2.1 *Propioniferax innocua* / *Escherichia coli*

In Below we can see the graphs of competition of *Propioniferax innocua* with *Escherichia coli* (figure 7).

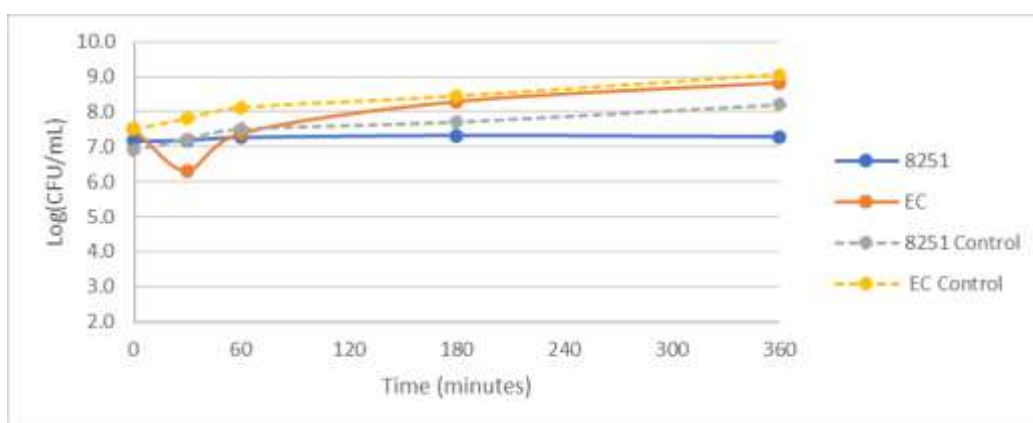


Figure 7 - Variation of viability (log CFU/mL) of probiotic *P. innocua* and pathogen *E. coli* and respective controls in the competition study, during 360 minutes of the assay

In the *P. innocua*/ *E. coli* competition assays, depicted in Figure 8, in the first sampling point at time zero minutes to 30 minutes there is a decrease in the pathogen's viable numbers, of about 1.2 logarithmic units. From 30 min onwards, the numbers of *E. coli* grew abruptly until the end, close to 2.5 logs. However, *P. innocua* remained constant throughout the sampling time until the end, around 4.5 logs. Previous studies showed that the production of propionic acid from glucose by *P. innocua* leads the decrease of pH that resulted in the significant reduction of number of *E. coli* (Malicki *et al.*, 2004; Yokota *et al.*, 1994). Since acid production by probiotics inhibits the growth of pathogenic bacteria, a reduction in pH may be one of the causes of the decrease in the pathogen's cell concentration. (Miyazaki *et al.*, 2010). In a study performed by Lopes *et al.* (2017), among several antibiofilms assays *P. innocua* was the only one that showed antimicrobial activity and ability to break down matures *E. coli* biofilms while most of them prevented biofilm formation only. Hence, the inhibitions of biofilm formation

(antibiofilm) and virulence characteristics provide other means of addressing infections (Raorane *et al.*, 2019).

The results obtained herein do not match those found in the literature; the reason again may focus on the planktonic state of the cells. Because in biofilm, many bacteria are known to regulate their cooperative activities and physiological processes through a mechanism called quorum sensing (QS), in which bacterial cells communicate with each other by releasing, sensing and responding to small diffusible signal molecules. Generally, many QS-controlled activities have been involved in the virulence and pathogenic potential of bacteria (Li & Tian, 2012). High cell density and close proximity of diverse species of microorganisms are typical of life in natural biofilms, where organisms are involved in complex social interactions that occur both within and between species and can be either competitive or cooperative (Davey & George, 2000; Kolenbrander *et al.*, 2002). The cell density and the numerous signaling molecules such as acyl homoserine lactones, peptides, autoinducer-2, diffusion signaling factors, and α -hydroxyketones have been studied in bacteria. Most biofilm systems have demonstrated enhanced resistance to external factors such as antibiotics, shear force, and the host immune system (Irie & Parsek, 2008). In biofilms, EPS also play a vital role in the formation of physical and social interactions, an enhanced rate of gene exchange, and antimicrobials tolerance (Gebreyohannes *et al.*, 2019). Moreover, studies also demonstrate that biofilm cells undergo a higher rate of mutation than their planktonic counterparts resulting in a 10-fold increase in the efficiency of transfer of plasmid having antibiotic resistance gene, when biofilm is exposed to a sub-lethal concentration of that antibiotic. To the best of our knowledge, there is a lack of information on competition tests with *P. innocua*. The existing studies refer to biofilms, which in no way resemble cell in the planktonic state. As mentioned above, in biofilms the mechanism of action is much more enhanced than in the planktonic cell state.

4.2.2 *Propioniferax innocua* / *Pseudomonas aeruginosa*

Below we can see the graphs of competition of *Propioniferax innocua* with *Pseudomonas aeruginosa* (figure 8).

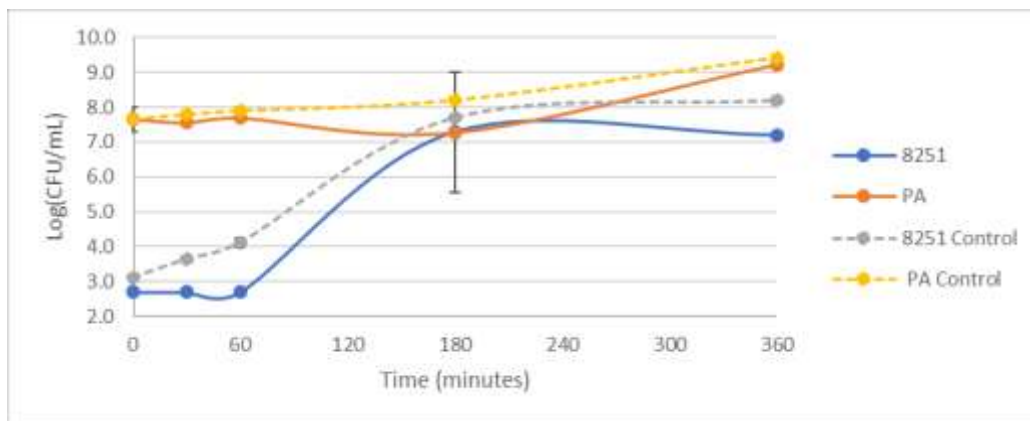


Figure 8 – Variation of viability (log CFU/mL) of probiotic *P. innocua* and pathogen *P. aeruginosa* and respective controls in the competition study, during 360 minutes of the assay.

In figure 8, between 60 and 180 min, a decrease in the cell concentration of the pathogenic *P. aeruginosa* is visualized, approximately 0.5 logs, while *P. innocua* remains constant. Between 180 minutes and 240 minutes there is a slight overlap of *P. innocua* against the pathogen. From the 240 minutes the pathogen grows, close to 2.0 logs, instead of the *P. innocua* that decreases slightly its cell concentration, about 0.10 logs. The slight decrease in *P. aeruginosa* is probably due to the production peptides and organic acids (2-pyrrolidone-5-carboxylic acid, 3-phenyllactic acid, hydroxyphenyl lactic acid 3-phenyllactic acid) and these bacteriocins (Camarillo-Márquez *et al.*, 2018).

In this study an extra stimulus was required to exclude the *P. aeruginosa* bacteria biofilm. In another study performed by Lopes *et al.* (2017), *P. innocua* was able to destroy preformed biofilms of *P. aeruginosa*. Preformed-biofilm destruction was of 12%, compared to the initial concentration.

The data obtained do not match those described above, as the reasons may be that bacteriocin dispersibility in the planktonic cells, is not the most effective, because self-aggregation and coaggregation of the bacterial cells can potentiate the response to other microorganisms. Later in the experiment the pH increase is an extrinsic factor for *P. aeruginosa* can restore and recover its biomass (Camarillo-Márquez *et al.*, 2018; Kouya *et al.*, 2007; Stevens *et al.*, 2015; Trunk *et al.*, 2018).

4.3.1 *Bifidobacterium longum* spp. *infantis* / *Staphylococcus epidermidis*

Below we can see the graphs of competition of *Bifidobacterium longum* spp. *infantis* with *Staphylococcus epidermidis*.

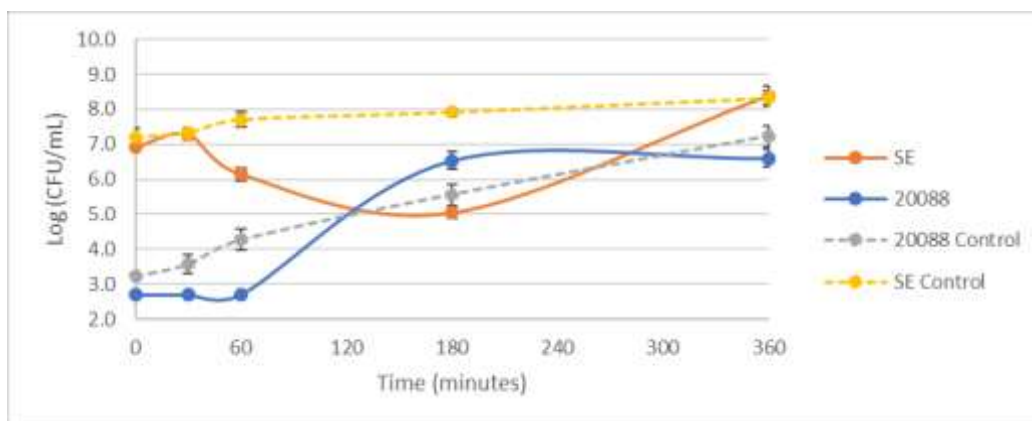


Figure 9 – Variation of viability (log CFU/mL) of probiotic *B. longum* spp. *infantis* and pathogen *S. epidermidis* and respective controls in the competition study, during 360 minutes of the assay.

Figure 9 shows the growth pattern of *B. longum* spp. *infantis* in the presence of *S. epidermidis*. In this competition graph, can be observed a considerable difference at cellular concentration between both bacteria, about 4.22 logs at the beginning of the experience. We hypothesize that O₂ presence might be a significant factor for the observed difference at cellular concentration both bacteria, specially the *B. longum* spp. *infantis*, which can also be accountable for the loss in probiotic viability, which goes in line with previously demonstrated bifidobacteria high susceptibility to oxygen (Talwalkar & Kailasapathy, 2003). Up to 120 minutes of assay the pathogen bacteria decreased the cellular biomass around about 2.0 logs. Although the probiotic bacteria grew until the end of the assay. Surely, it has previously been reported that Bifidobacterium spp. can actually inhibit the growth of pathogen bacteria by decreasing the pH by producing short-chain fatty acids (SCFA), lactic acid and acetic acid (Barba-Vidal *et al.*, 2017; So *et al.*, 2002). Between 120 and 300 minutes, approximately, bifidobacteria was in higher cell concentration. After the 300 minutes until the end the *S. epidermidis* grew to a cellular concentration of 8.37 logs. Moreover, studies by Lau and Liong (2014) reveal that LAB and bifidobacteria are able to produce antimicrobial compounds that inhibit opportunistic wound skin pathogens, with high percentage of inhibition (73.7% to 88.2%), when compared to the control. Upon neutralization, the antimicrobial activity showed a drastic drop in the percentage of inhibition. The study was performed by the spectrophotometric analysis of the cell-free supernatant (CFS). This was not found in the current study, quite possibly due to its disposal and aggregation in the liquid medium, and coaggregation capacity. This view is supported

by recent work (Trunk *et al.*, 2018) showing that, under high competition with single cells, cells positioned at the top of aggregates benefited from the competitive advantage.

4.3.2 *Bifidobacterium longum* spp. *infantis* / *Staphylococcus aureus*

Below we can see the graphs of competition of *Bifidobacterium longum* spp. *infantis* with *Staphylococcus aureus*.

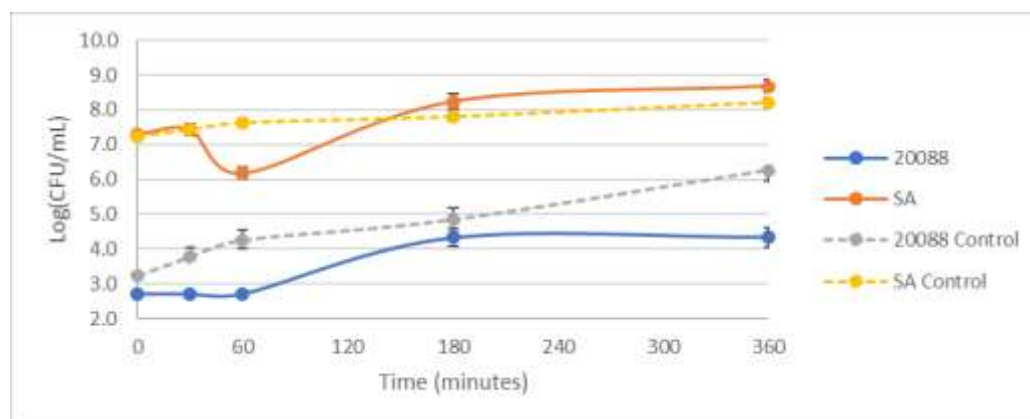


Figure 10 - Variation of viability (log CFU/mL) of probiotic *B. longum* spp. *infantis* and pathogen *S. aureus* and respective controls in the competition study, during 360 minutes of the assay.

Figure 10 shows the growth pattern of *B. longum* spp. *infantis* in the presence of *S. aureus*. In this competition graph, we cannot infer any conclusion inherent to the action of the probiotic *B. longum* spp. *infantis* on the pathogenic *S. aureus*, because there is an abysmal distinction in the initial biomass of the bacteria, around 4.6 logs. *S. aureus* from 30 to 60 minutes decreased about 1.25 logs. The pathogen *S. aureus* increased the biomass concentration about 2.49 logs until the end. The bifidobacteria numbers remained constant up to 60 minutes, increasing until 250 minutes, about 1.6 logs, and remaining constant thereafter until the end of the experiment. On the other hand, the pathogen decreased in its cell concentration from 30 to 60 minutes, after which the bacterium gradually grew up to 180 minutes. The decreased of *S. aureus* cellular concentration can be due to the release bioactive compounds. These bioactive compounds were found to be useful in dermatological applications including hyaluronic acid, peptidoglycan, lipoteichoic acid, and sphingomyelinase. These compounds are produced by LAB at an effective concentration to inhibit pathogens causing dermal illness (Tan *et al.*, 2014). Besides, the therapeutic effects exhibited by these microorganisms are due to the secretion of various inhibitory compounds, particularly

LAB, which can produce growth-inhibitive compounds such as lactic acid, acetic acid, bacteriocin, hydrogen peroxide, and diacetyl (Suskovic *et al.*, 2013). Additionally, a study provided by (Lahtinen *et al.*, 2007) concluded that Bifidobacterium supernatants inhibited the growth of *S. aureus*. The ability of bifidobacteria to produce hydrogen peroxide has been reported earlier, and maybe responsible for this inhibition. These findings again contradict the results in this experiment; in the performed studies, the results were obtained using the disk diffusion method and this experiments were performed in liquid media and cells in a liquid media have different behaviors and different modes of action, whether in aggregation/flocculation, auto-aggregation or coaggregation (Christensen & Brüggemann, 2014; Trunk *et al.*, 2018).

4.3.3 *Bifidobacterium longum* spp. *infantis* / *Escherichia coli*

Below we can see the graphs of competition of *Bifidobacterium longum* spp. *infantis* with *Escherichia coli*.

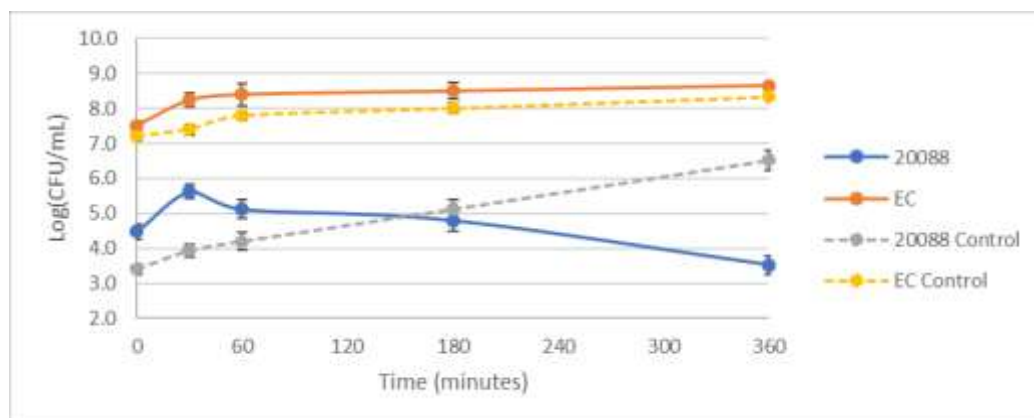


Figure 11 – Variation of viability (log CFU/mL) of probiotic *B. longum* spp. *infantis* and pathogen *E. coli* and respective controls in the competition study, during 360 minutes of the assay.

From the beginning and throughout the experiment (Figure 11) *E. coli* showed a higher cellular concentration than *B. longum* spp. *infantis*, in the first sampling point, 7.51 logs and 4.48 logs, respectively; the discrepancy is probably due to the oxygen demands of both bacteria. Bifidobacterium is aerotolerant, designated as a facultative anaerobe (González *et al.*, 2004; Underwood *et al.*, 2015) while *E. coli* is aerobic. Thus, bifidobacteria as stress sources too, including heating, exposure to low water activities, osmotic shock and presence of oxygen (Ruiz *et al.*, 2011). At zero sampling time until up to the sampling time of 30 min the bifidobacteria grows 1.14 logs, from this sampling point to 180 minutes steadily decreases approximately 1.0 log, decreasing the cellular concentration until the end of the experiment 2.0 logs. *E. coli*, on the other side,

increased its numbers up to 30 min, around to 1.0 Log, remaining constant thereafter. previously, in antimicrobial analysis of supernatants with the disc diffusion method, concluded that there was a significant reduction in the growth; inhibition values between 75-95% of their log CFU in monoculture, are reported. This inhibition is due to the release of organic acids such as acetic acid and lactic acid in a ratio of 3:2, which drops the pH probably enough to antagonize pathogenic bacteria (Cheikhoussef *et al.*, 2007). In addition, studies performed by (Abdelhamid *et al.*, 2018) also exhibited a strong antibacterial activity of Bifidobacterium (inhibition zones of 11.8–23.1 mm) against all *E. coli* isolates. *B. longum* spp. *infantis* caused the highest inhibition (57.9%) of *E. coli* biofilms. Bifidobacterium species were able to reduce the growth of drug-resistant *E. coli* when investigated using the agar well diffusion method. Another study corroborating these results is from Barba-Vidal *et al.* (2017). Oral ingestion of *B. longum* spp. *infantis* probiotic resulted in the reduction of pathogen excretion or ileal colonization (33% reduction of animals with countable coliforms), on day 3; Increased intraepithelial lymphocytes on day 8; and improved the fermentation profile by increasing butyric acid. In conclusion, this probiotic demonstrated potential to reduce the intestinal colonization by pathogens and to stimulate local immune response. Organic acids that are released in response to stress of pathogenic invasion, play a key role in reducing intestinal pH, preventing the growth and colonization by acid sensitive and putrefactive pathogens. Murine studies based in in vivo experiments demonstrated the protective role of bifidobacterial acetate against enterohaemorrhagic *Escherichia coli* (Sarkar & Mandal, 2016). Organic acids' release also occurs in the skin where there is a drop in pH thus hindering colonization by the pathogenic bacteria (Lau & Liong, 2014). Besides, in a study by Gueniche *et al.* (2010) when a *B. longum* sp. lysate extract was applied onto the skin, SP (Substance P) induced vasodilation significantly decreased [97.4 ± 60.7 vs $144 \pm 71.2 \mu\text{m}^2$] ($p = 0.0003$). Adding SP to culture medium raises edema score to 1.8 ± 0.7 compared with 1.1 ± 0.6 in unstimulated skin samples ($p = 0.02$). When bacterial extract was applied to stimulated skin samples, the edema score significantly decreased to 1.2 ± 0.7 ($p = 0.009$). Using nerve cell cultures in vitro, after 6 h of incubation with BL *Bifidobacterium longum* sp. extract, no significant stimulation of CGRP (Calcitonin gene-related peptide) spontaneously released was noticed (34.0 ± 5.2 compared with $38.2 \pm 11.8 \text{ pg/ml}$ in the control group). After 6 h of pre-incubation with BL bacterial extract (0.3% and 1%) followed by neurone stimulation by capsaicin, a significant decrease in CGRP release was observed ($p <$

0.01) (37.5 ± 12.2 pg/ml after 0.3% of BL compared to 91.0 ± 16.4 pg/ml in the control group treated with capsaicine alone (decrease of 36%) and 32.6 ± 5.3 pg/ml after 1% of BL compared with 91.0 ± 16.4 pg/ml in the control group (decrease of 41%), $p < 0.01$). The results showed a significant decrease in skin sensitivity in volunteers who applied the cream containing BL bacterial extract for 2 months (day 57, $p = 0.0024$). Thus, the BL Cream application increased skin resistance to physical aggression. One of the reasons why the results are not in agreement with the literature is because the assays performed are evaluated in biofilm and in these experiments through planktonic cells. The hidden mechanisms controlling the response of attached or immobilized cells (biofilm) as compared to that of planktonic ones are not fully elucidated, except for a few studies, which have focused on differential expression of proteins in attached and suspended cells (Azeredo *et al.*, 2017). Equally important, Chen and Wen (2011) demonstrated that the bacterial biofilm in persistent infections and control strategies is more resistant to drug treatments than defective biofilms. The biofilm cells defenses are different from the ones adopted by planktonic cells, such as activation of efflux pumps, acquisition of new enzymes and mutations of the drug targets. So, studies indicate that diverse model species – including *P. aeruginosa*, *V. cholerae*, *Escherichia coli*, and *Staphylococcus aureus* – differentially express as much as 10% of their genomes when in biofilm vs. planktonic growth conditions. However, gene expression studies also illustrate that biofilms of different strains or species may be as different from one another as they are from a planktonic population (Nadell *et al.*, 2009).

4.3.6 *Bifidobacterium longum* spp. *infantis* / *Pseudomonas aeruginosa*

Below we can see the graphs of competition of *Bifidobacterium longum* spp. *infantis* with *Pseudomonas aeruginosa*.

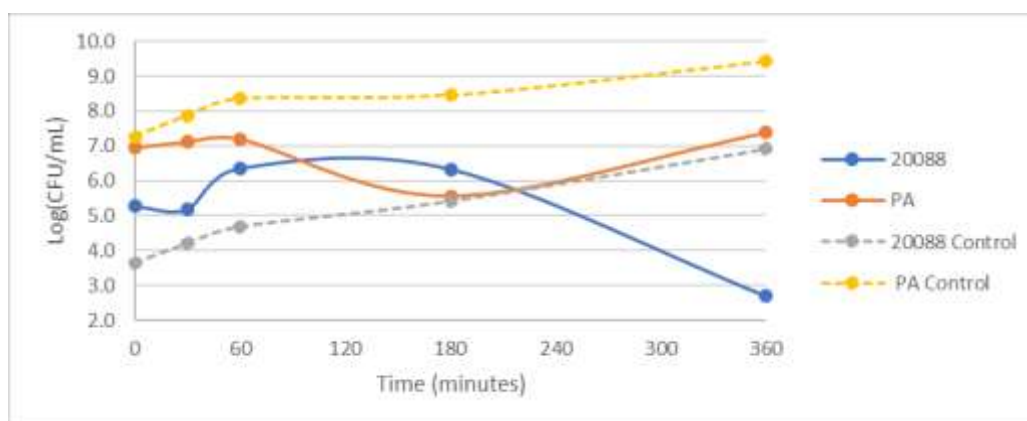


Figure 12 – Variation of viability (log CFU/mL) of probiotic *B. longum* spp. *infantis* and pathogen *P. aeruginosa* and respective controls in the competition study, during 360 minutes of the assay.

Figure 12 shows the prevalence of *P. aeruginosa* against *Bifidobacterium longum* spp *infantis* throughout the experiment. The *Bifidobacterium* increased its concentration up to 60 min, about 1.0 logs, and thereafter to 180 min increased slightly its cellular concentration (0.021 logs). A plausible reason for this slight increase is due to the decrease in pH. Similarly, bifidobacteria ferment carbohydrates and produce organic acids (acetic acid, lactic acid, propionic acid), exopolysaccharides and short-chain fatty acids (SCFAs), whose antifungal efficiency is directly proportional to chain length stated by Inturri *et al.* (2019). Particularly, bifidobacteria produce acetate and lactate as well as vitamins, antioxidants, polyphenols, and conjugated linoleic acids, whereas lactobacilli produce lactate and small proteins (Inturri *et al.*, 2019). Several mechanisms have been suggested for the inhibitory response of bifidobacteria towards gram positive and negative pathogens, including a decrease of the local pH via the production of organic acids, the inhibitory action of undissociated organic acid molecules, the competition for nutrients, the competition for adhesion sites, the stimulation of the host's immunity, and the production of specific antibacterial substances (Cheikhoussef *et al.*, 2008). The *Bifidobacterium longum* spp *infantis* decreased its concentration from 180 min onwards 3.6 logs and continue until the end of the experience. Throughout the experiment the bacteria *P. aeruginosa* increased its biomass, about 1.8 logarithmic unit, most noticeably from 180 minutes to the last sampling point without a considerable interference in its growth, except from 60 to 180 minutes, which decreased smoothly about 1.6 logs. On other hand, a study performed with oral ingestion of bifidobacteria by mice proves, as well, that the administration of *B. longum* spp. *infantis* significantly decreased viable counts of *P. aeruginosa* in the liver and blood compared with other

groups. Although this study is performed with another aim, the gut, we can transpose to the direct action of competition on the skin, because the *B. longum* spp. *infantis* possessed the capacity to suppress the adherence and enhance the exclusion of *P. aeruginosa* (Matsumoto *et al.*, 2008). Such as a study on fermented milk and kefir, a decrease (3 logs) in the viable numbers of *P. aeruginosa* in the presence of *Bifidobacterium longum* subsp *infantis* is also reported (Fijan, 2016). Although the study was performed on fermented milk and kefir, and not on epithelial skin cells, and the matrix /substrate is distinct, the bacteria are the same, from which we can infer that *Bifidobacterium longum* spp. *infantis* inhibited the pathogen. Another study showed that *Bifidobacterium longum* BB536 has been shown to exclude *P. aeruginosa* from epithelial cells, by competitive exclusion in vitro. Oral administration of this probiotic at a 1.0×10^9 CFU concentration, taken for 10 days, decreased viable cells of *P. aeruginosa* in the liver, blood and intestinal contents (Wong *et al.*, 2019). Also, a study with the bacteria *Bifidobacterium infantis* G4 demonstrated inhibition towards *P. aeruginosa* (Shuhaimi *et al.*, 1999). In contrast that observed above, it has been reported (Mostafa *et al.*, 2015) that several strains of Bifidobacterium have a broad spectrum of antagonistic activity against both gram-positive and gram-negative bacteria. The expected action of bacteriocins against gram-negative bacteria can be attributed to compounds (acetic and lactic acids) and possibly other antimicrobial substances produced during culturing. Various species of bifidobacteria, *B. lactis*, *B. infantis* and *B. longum* have the ability to prevent the adhesion of the pathogenic bacteria such as *P. aeruginosa*, therefore, reducing the risk of infection (Acton, 2013).

These results do not agree with the results found in this study possibly due to their form of interaction as free cells/planktonic cells rather than biofilm such as happened previously with *E. coli*. Probiotic and pathogenic *P. aeruginosa*, forms of action and vice versa may not be as expected because the expression is distinct in the form of biofilm and free cells (Azeredo *et al.*, 2017; Chen & Wen, 2011; Nadell *et al.*, 2009).

Analyzing all microorganisms over time, there are significant differences ($\alpha=0,005$) at each sampling point for *Lactobacillus paracasei*, *Propioniferax innocua* and *Bifidobacterium longum* spp. *infantis* with the respective pathogenic bacteria *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and *Pseudomonas aeruginosa* combination.

In this method the rejection of H_0 (Similar means for each combination of bacteria) occurs, because α is the probability (P value) rejection of H_0 being this true;

and the acceptance of H_1 occurs (There exist differences in the means) because of sufficient evidence in the sample.

Weaknesses and strengths of the method used:

Almost all cultivation-based methods being simple to practice, command enormous significance and applications in bacteriology. Further, CFU-based techniques provide information on the most abundant populations among the cultivable community (Thomas *et al.*, 2015). The method used in these experiments, serial dilution method and plating method have advantages and disadvantages. The dilution and plating method are a quick and simple, detects only the viable cells are detected and counted it allows for the selective counting (through selective media) of the microbial population (Elliott *et al.*, 2007). Moreover, the purpose of the serial dilution method is to estimate the concentration (number of colonies, organisms, bacteria) of an unknown sample by counting the number of colonies cultured from serial dilutions of the sample, and then back track the measured counts to the unknown concentration (CFU/mL) (Ben-David & Davidson, 2014). Spread-plating offers several advantages over pour-plating such as more flexibility in handling, less interfering effects on temperature sensitive organisms, the avoidance of aerobic organisms getting trapped inside agar medium, the surface enumeration of CFU and the easy selection of distinct colony types (Thomas *et al.*, 2015).

4.4.1 *Lactobacillus paracasei* adherence to HaCat cells ‘in vitro’

The epidermis has been studied both as a model of tissue differentiation and for its medical importance in wounds, oncogenesis, congenital and acquired skin dysbiosis, and infections. As a differentiation model system, human keratinocytes are attractive because primary, immortalized, or transformed cells are all readily available for comparison (Wilson, 2013). As well as studies carried out in the literature with the specie *L. paracasei* demonstrate its effectiveness at competition, displacement and exclusion against the pathogens. The following graphs show the adherence of *Lactobacillus paracasei* in the presence of *S. aureus*, *S. epidermidis* and *Pseudomonas aeruginosa*. Firstly, in competition trials, the wells are simultaneously inoculated with both probiotic and pathogen at the same time (Singh *et al.*, 2016). Secondly, in the displacement assay, the addition is first pathogenic planktonic cells on probiotic biofilm cells. Finally, in exclusion assay, the cells were first exposed to probiotic and later the

pathogen. In the displacement assay, the cells were first exposed to pathogen and later to the probiotic (Prince *et al.*, 2012).

At Figures 13, 14 and 15 can be observed the results of protein adhesion assays.

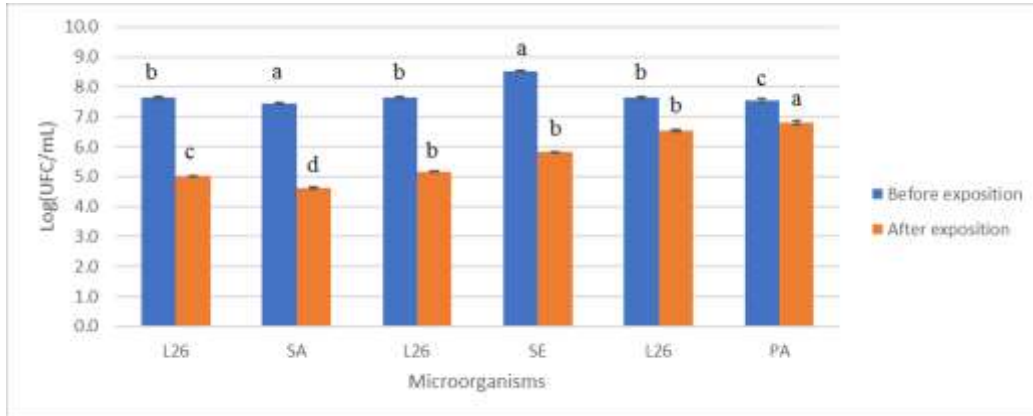


Figure 13 - Interaction of probiotics *L. paracasei* (L26) with pathogenic (*S. aureus*, *S. epidermidis* and *P. aeruginosa*) in HaCat cells, competition assay. Columns with different letters are significantly ($p \leq 0.05$). The column on the left (blue) represents the control and, on the right, (orange) we have the representation of the respective cocultures after 2 hours of exposure.

In Figure 13 we can observe the behavior of pathogenic bacteria against the probiotic, in the competition technique. All pathogenic bacteria decreased their concentration after 2 hours of contact time. In the competition method, the one that stood out for its sharp decrease was *S. aureus* (2.8 logarithmic units). In the exclusion method, the most marked reduction was observed for *S. epidermidis* (1.8 logarithmic units) and in the displacement method the largest reduction was in *S. aureus* (3.8 logarithmic units).

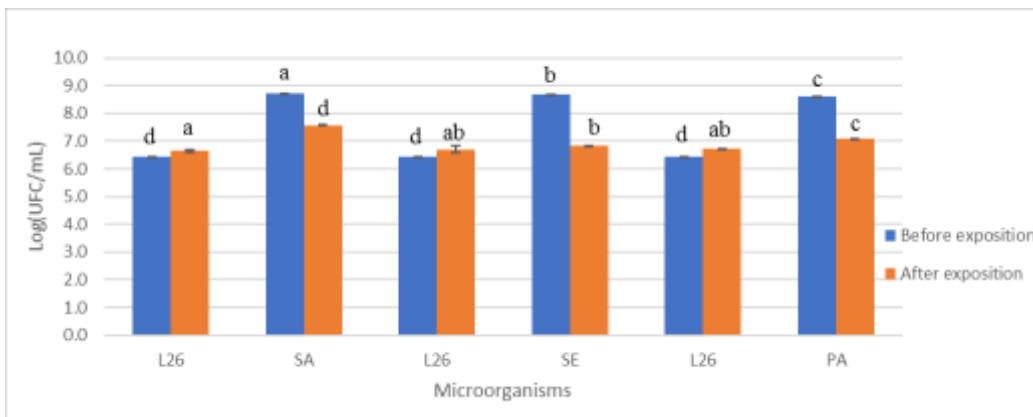


Figure 14 - Interaction of probiotics *L. paracasei* (L26) with pathogenic (*S. aureus*, *S. epidermidis* and *P. aeruginosa*) in HaCat cells, exclusion assay. Columns with different letters are significantly ($p \leq 0.05$). The column on the left (blue) represents the control and, on the right, (orange) we have the representation of the respective cocultures after 2 hours of exposure.

Figure 14 shows the behavior of pathogenic bacteria against the probiotic, in the exclusion technique. All pathogenic bacteria decreased their concentration when in contact up to 2 hours. In the exclusion method *S. aureus* decreased 1.1 logs; *S. epidermidis* decreased 1.8 logs and *P. aeruginosa* decreased 1.5 logs. At displacement method *S. aureus* decreased 3.8 logs; *S. epidermidis* decreased 2.2 logs and *P. aeruginosa* decreased 1.7 logs after 2 hours contact time.

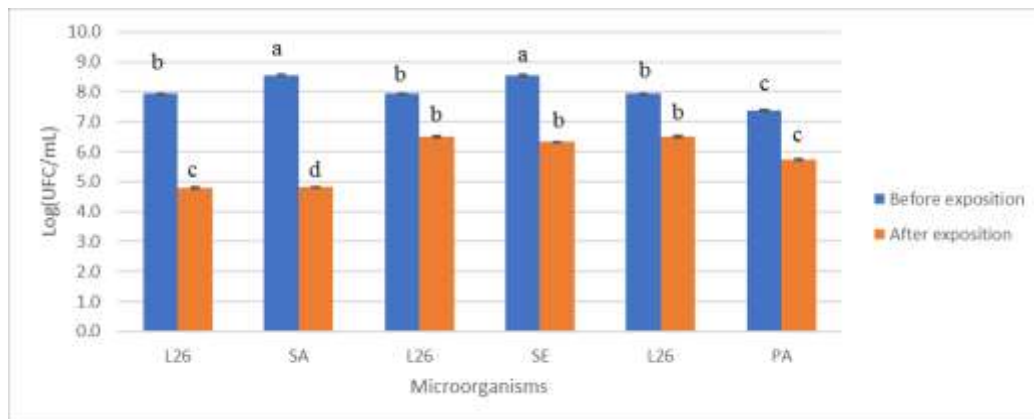


Figure 15 - Interaction of probiotics *L. paracasei* (L26) with pathogenic (*S. aureus*, *S. epidermidis* and *P. aeruginosa*) in HaCat cells, displacement assay. Columns with different letters are significantly ($p \leq 0.05$). The column on the left (blue) represents the control and, on the right, (orange) we have the representation of the respective cocultures after 2 hours of exposure.

In the displacement technique (Figure 15), the same trend is observed. A decrease in the viable numbers of the three tested pathogens was observed, either by competition, exclusion or displacement. In the competition method, *S. aureus* decreased 2.8 logs; *S. epidermidis* decreased 2.7 logs and *P. aeruginosa* decreased almost 0.8 logs after 2 hours contact time.

Many authors have demonstrated that certain bacterial probiotic extracts have anti-adhesion and anti-microbial properties when applied to cutaneous and mucous surfaces. The potential use of probiotic microorganisms capable of producing antimicrobial toxins (bacteriocins, bacteriocin-like substances, organic acids, and H₂O₂ (Roudsari *et al.*, 2015). In the same way, the antimicrobial properties (the biosurfactant) might exhibited a considerable antiadhesive activity against some microorganisms. In microbial adhesion and desorption has been widely described, and adsorption of biosurfactants isolated from lactobacilli to solid surfaces might constitute an effective strategy to reduce microbial adhesion and combating colonization by pathogenic microorganisms (Gudinã *et al.*, 2010). Is explained by previous studies that *Lactobacillus* strains, *L. fermentum* and *S. salivarius*, have the ability to inhibit the *S. aureus* growth, including three MRSA (methicillin-resistant *Staphylococcus aureus*) strains. However,

Lactobacillus salivarius has a higher inhibition capacity (Kang *et al.*, 2017). In the same way, (Zárate & Nader-Macias, 2006) affirm that Lactobacilli are predominantly micro-organisms of the vaginal microbiota, that play a major role in the maintenance of a healthy urogenital tract by preventing the colonization of pathogenic bacteria. *S. aureus* attachment to the skin, is the precursor to colonization and infection. These authors conclude that four Lactobacillus strains, including *L. paracasei*, were able to exclude, compete with, and displace *S. aureus* to different degrees. These differences in the capacity of adhesion could be due to differences in the composition of the cell wall of Gram-negative and Gram-positive bacteria (e.g. lipopolysaccharide in *P. aeruginosa* and teichoic acids in *S. aureus*) and their content of specific adhesion factors (proteinaceous adhesins, polysaccharides, lipoteichoic acids, etc.). *Pseudomonas aeruginosa* is a recurrent opportunistic pathogen at urogenital tract and burn wounds (Zárate & Nader-Macias, 2006). Views on the adherence of pathogens to Hacat cells, in the absence of Lactobacilli, varied among the strains studied. These differences in the capacity of adhesion could be due to differences in the composition of the cell wall of Gram-negative and Gram-positive bacteria (e.g. lipopolysaccharide in *P. aeruginosa* and teichoic acids in *S. aureus*) and their content of specific adhesion factors (proteinaceous adhesins, polysaccharides, lipoteichoic acids, etc.). *S. aureus* attachment to the skin, is the precursor to colonization and infection. Further, three types of assays were performed in a study by Zárate and Nader-Macias (2006), in order to determine the inhibitory effect of Lactobacilli on adhesion of urogenital pathogens. Similar adhesion levels were observed for the four Lactobacilli, *L. acidophilus* CRL 1259, *L. crispatus* CRL 1266; *L. paracasei* CRL 1289 and *Lactobacillus salivarius* CRL. For instance, *L. acidophilus* CRL 1259 blocked by exclusion 37.7% of *S. aureus* adherence, whereas *L. salivarius* CRL 1328 inhibited these pathogens by the same mechanism in a 78.7%. The highest inhibition of *S. aureus* adhesion was produced by *L. salivarius* CRL 1328 (53.1–78.7%). Then, in another study performed by Edwards *et al.* (2011) with the aim of determine the kinetics of keratinocyte - *S. aureus* interactions, as well. The results of keratinocyte adhesion show that the cellular concentration of *S. aureus* did not change significantly 3.0×10^5 CFU ($\cong 3\%$ inoculum) over time (up to 90 mins), and the *S. aureus* attached after 15 minutes in exposure, suggesting that all available binding sites on the host cells were occupied. In contrast, in invasion after 15 mins, 10^3 CFU *S. aureus* had internalized, regardless of the high number of adherent bacteria and there was no significant increase up to 30 mins, indicating that the invasion process includes a

lag-phase. Furthermore, to compare how invasion of keratinocytes compared to endothelial cells we also examined the adhesion to and invasion of EA (endothelial adhesion) *S. aureus* adhesion to endothelial cells was identical to that of keratinocytes after 15 minutes. Is explored this difference between the invasion efficiency of keratinocytes and endothelial cells may be due to differences in the density of the host cell ligand, the cell surface $\alpha 5\beta 1$ integrin, mentioned above. In my study, the results reported refer only to one sampling time, focusing on the initial sampling and the final, after exposure of the lactic acid bacteria to the pathogenic bacteria. One of the reasons why the results are different from mine is possible due the time of internalization of *S. aureus*. In addition, in another study performed by Miljkovic *et al.* (2015) we have the confirmation of the previously obtained results in which exposure of the two bacteria, *L. paracasei* and the pathogenic *S. aureus* ATCC25923, leads to an exclusion of *S. aureus* ATCC25923, too. The BGKP1 bacteria, a *L. paracasei* spp. *paracasei*, inhibits adhesion of *S. aureus* ATCC25923 by 57.8%, while BGKP1-20/pALb35 (*Lactococcus lactis* spp. *lactis*) strain inhibits adhesion of *S. aureus* ATCC25923 by 62.9%. To sum up, in this study was concluded that the keratinocyte survival was significantly higher when the probiotic was applied prior (exclusion technique) to or simultaneously (competition technique) with *S. aureus* infection, there better results of inhibition, but not when it was added after infection had started (displacement technique). Is briefly outlined by Chen *et al.* (2018), depending on the bacterial cell concentration, a positive outcome at normal concentration may occur, as well as, a less positive outcome. Such change can lead to abnormal cell multiplication, leading to inflammation of the cell tissue. Frequently, during acute inflammatory responses, cellular and molecular events and interactions efficiently minimize depending on injury or infection. This mitigation process contributes to restoration of tissue homeostasis and resolution of the acute inflammation. However, uncontrolled acute inflammation may become chronic, contributing to a variety of chronic inflammatory diseases (Chen *et al.* 2018). Is demonstrated by the following studies below are an orally supplementation of proBiotik to pregnant women. The SCORAD (SCORing Atopic Dermatitis) reduction by single or coculture uptake demonstrate the importance and correlation of different microbiomes. The supplementation of symbiotic formulation that contains seven probiotic strains and FOS (Fructooligosaccharide) to infants, supplementation with *L. rhamnosus* GG showed a reduction in SCORAD score suggest that disturbance in skin and intestinal microbiota is majorly linked to skin diseases. These studies focused on supplementation

and the results are derived from the absorption in the gut that is reflected in the control of cell tissue adhesion and inflammation, and it is possible to compare with topical application since the purpose of the studies was to control the disease and symptoms. The supplementation of proBiotik (a mixture of *L. salivarius*, *Lactobacillus casei*, *Lactobacillus acidophilus*, and *Bifidobacterium bifidum*) to 1-13 years old children showed the predominant reduction in SCORAD (SCORing Atopic Dermatitis), IgE, IL-6, IL-5, and IFN- γ levels while TNF- α (tumor necrosis factor alfa), IL-10, IL-2, and IL-4 levels were not affected. The results claimed that the proBiotik® pur was effective against AD (Sivamaruthi *et al.*, 2018). Besides another report suggested that the supplementation of *L. fermentum* VRI-033 PCC, for eight weeks significantly reduced the SCORAD, and the severity of AD in infants of 6-18 months old (Weston *et al.*, 2005). The different intervention of *Lactobacillus sakei* KCTC 10755BP (2-10 years old children; 5.0×10^9 CFU twice a day), and *Lactobacillus plantarum* CJLP133 (1-13 years old children; 0.5×10^{10} CFU per day) for 12 weeks significantly reduced the SCORAD, disease activity, and improved the symptoms of atopic eczema-dermatitis syndrome (Han *et al.*, 2012; Patrick & McDowell, 2012; Woo *et al.*, 2010). In a study was conducted on 220 children aged 1-18 years with moderate-to-severe. The children were randomized to receive LP (*L. plantarum*), LF (*L. fermentum*), LP (*L. paracasei*) + LF (*L. fermentum*), mixture, and placebo for 3 months displayed changes in severity scoring of atopic dermatitis (SCORAD) (Wang & Wang, 2015). Subsequently, in a study with two sets of probiotic formulations (*Bifidobacterium longum* BL999 and *L. rhamnosus* LPR; *Bifidobacterium longum* BL999 and *Lactobacillus paracasei* ST11), given orally to pregnant women during two months before the delivery and two months after giving birth resulted in reduced risk of eczema development in infants (Kalliomäki *et al.*, 2001; Kalliomäki *et al.*, 2003). Some of the studies suggested that the supplementation of *L. rhamnosus* GG at different concentrations to infants does not have protective effects against AD while supplementation (for four weeks) of *L. rhamnosus* GG showed a reduction in SCORAD score, and the symptoms of AD syndrome in IgE-sensitized infants. While, the cocktail with a probiotic mixture containing *L. rhamnosus* GG, *Propionibacterium freudenreichii* ssp. *shermanii* JS, *Bifidobacterium breve*, and *L. rhamnosus* LC705 along with galactooligosaccharides displayed no impact on the incidence of allergic diseases, and no allergy- preventive effect, reduced atopic eczema in infants at high risk for allergy was reported (Kuitunen *et al.*, 2009; Kukkonen *et al.*, 2007; Sivamaruthi *et al.*,

2018). However, not all the probiotic formulations and intervention studies are successful regarding health benefits to human subjects. For example, the supplementation of symbiotic formulation that contains seven probiotic strains and FOS to infants (1-36 months old), and the intervention of *Lactobacillus paracasei* CNCM I-2116 or *B. lactis* CNCM I-3446 to 3-6 months old infants showed no statistical significance in SCORAD scores and other assessed parameters between treated and placebo groups. Infants (postnatal period: 48 h) were supplemented with *Lactobacillus acidophilus* LAVRI-A1 in maltodextrin and found that the intervention did not prevent the development of AD, significantly (Gore *et al.*, 2012; Grüber *et al.*, 2007; Meneghin *et al.*, 2012; Shafiei *et al.*, 2011; Taylor *et al.*, 2007). The key aspect discussed according to the literature Buriti & Saad (2007), the species *L. casei* and *L. paracasei* have the same function but are from different strains. The species *L. casei*, *L. paracasei* and *L. rhamnosus* are part of “*Lactobacillus casei* Group”. They have very similar physiological behavior and nutritional needs, multiplying in very similar environmental conditions. Thus, the paracasei strain has the same action as the casei probiotic, only the morphological structure is different. *Lactobacillus casei*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus* are phenotypically and genotypically closely related (Huang *et al.*, 2018). However, is not clear, they have same designation but have similarities between strains of the same bacterial species with regard to their physicochemical, such as, the Electrophoretic Mobility (EM) was variable between strains and species and depended on the pH value. Lactobacilli in this study must be regarded as having surfaces with a slightly negative charge at alkaline pH (Pelletier *et al.*, 1997). In demonstrated an antiadhesive activity of, *L. casei*, biosurfactant was evaluated and determined using the microdilution method in 96-well culture plates against a variety of bacteria strains. The biosurfactant showed antiadhesive activity against most of the micro-organisms tested, but the antiadhesive effect depends on the concentration and the micro-organism tested, as well. For all the microorganisms studied, the antimicrobial activity was observed even at low biosurfactant concentrations, and a complete growth inhibition was achieved for 12 of the 18 micro-organisms at the highest biosurfactant concentration assayed (50 mg mL⁻¹). Besides, even when minimum inhibitory concentration (MIC) and (MBC) were not reached, a high growth inhibition was observed (from 71.6 to 91.5%) with the highest biosurfactant concentration assayed (50 mg mL⁻¹). Then against *L. casei* strains (56.5%–63.8% inhibition) for a biosurfactant concentration of 50 mg mL⁻¹. Regarding the pathogenic

bacteria, high antiadhesive percentages were obtained for *S. aureus* (76.8%), *S. epidermidis* (72.9%). On the contrary, low activity was obtained for *P. aeruginosa* (21.2%) (Gudinã *et al.*, 2010). Is explored in experiments on burn wounds (after contamination with *P. aeruginosa* and then treatment with kefir) showed a reduction of their size and a reduced healing time when kefir was administered, as supplementation, alone than in the co-presence of silver sulfadiazine (a common topical antibiotic used for the treatment of *P. aeruginosa* on burn wounds) (Huseini *et al.*, 2012). Moreover in a study where burn wounds were contaminated with 8 different pathogens (e.g., *S. aureus*, *S. salivarius*, *S. pyogenes*, *P. aeruginosa*, *C. albicans*, *Streptococcus typhimurium*, *Listeria monocytogenes* and *E. coli*) and kefir was applied to the subject's infected areas the growth of these pathogens was considerably reduced (Lolou & Panayiotidis, 2019; Rodrigues *et al.*, 2005). Additionally in a study on human skin wounds, using the three-dimensional tissue-engineered models it has been shown that *P. aeruginosa* colonized the upper epidermal layers before invasion into the dermis, causing a loss of epidermis and de-keratinization of the skin constructs, as well as partial loss of basement membrane (Shepherd *et al.*, 2009). The virulence of *P. aeruginosa* was tested toward cultured mammalian cells and a decrease in the viability of human keratinocytes when infected (Hosseinidoust *et al.*, 2013).

Analyzing the microorganisms' combination, *Lactobacillus paracasei*/*Staphylococcus aureus*, *Lactobacillus paracasei*/*Staphylococcus epidermidis* and *Lactobacillus paracasei*/*Pseudomonas aeruginosa* combination at 0 and 2 hrs, there are significant differences ($\alpha=0,005$) at both sampling point for each combination, in all assays. A significant, and sharply difference in the numbers of viable cells in competition and displacement assay concentrations was observed, especially in the logarithmic concentrations at competition and displacement. The results were at competition a decrease of *S. aureus* (4 logarithmic units), *S. epidermidis* (2.6 logarithmic units) and *P. aeruginosa* (2.9 logarithmic units); at displacement *S. aureus* decreased (3.7 logarithmic units), such as, *S. epidermidis* (2.3 logarithmic units) and *P. aeruginosa* (1.7 logarithmic units). This demonstrates that *L. paracasei* has an inhibitory role in relation to these pathogenic bacteria in the three assays (competition, displacement and exclusion). At the literature don't exist a lot of information about these trials, specially towards *S. epidermidis* and *P. aeruginosa*. As presented above the studies are essential about the supplementation and the gut absorption, that have their reflection on the skin's immune response. Studies with topical application

with probiotics are still occasional. Many experimental studies have shown that probiotics exert specific influences in the intestinal on epithelial cells and immune cells with antiallergic potential (Caramia *et al.*, 2008). Although there is still a potential role for probiotics in preventing skin diseases, there are many unanswered questions, including species/strain selection, dosing and timing of probiotic administration and the population or populations most likely to benefit (Kopp & Salfeld, 2009). Application of the probiotic bacteria in skin might afford a protective shield, similar to a physical barrier. This so-called bacterial interference, through competitive inhibition of binding sites, is thought to prevent colonization by other, potentially pathogenic, bacterial strains (Roudsari *et al.*, 2015).

For statistical analysis through Independent Test and ANOVA (Statistical Test – The Analysis of Variance), the hypothesis were, the rejection of H_0 (Similar means for each combination of bacteria) occurs, because α is the probability (P value) rejection of H_0 being this true; and the acceptance of H_1 occurs (There exist differences in the means) because of sufficient evidence in the sample.

4.5.1 Protein Adhesion

Adhesion of bacteria to host surfaces is a crucial aspect of host colonization as the mechanical clearing of pathogens and confers a selective advantage towards bacteria of the endogenous flora (Ribet & Cossart, 2015). According to Krasowska & Sigler (2014), cell surface hydrophobicity (CSH) plays a crucial role in the attachment or detachment from the surfaces. The influence of CSH on adhesion of microorganisms to biotic and abiotic surfaces in medicine. The hydrophobic properties of microbial surfaces are conducive to adhesion to abiotic and biotic surfaces and to penetration of host tissues (Krasowska & Sigler, 2014). Another important tenet is that microorganisms can switch between hydrophobic and hydrophilic phenotypes in response to changes in environmental conditions (temperature, composition of nutrients, etc.) and growth phases (Bujdaková *et al.*, 2013).

Figure 16 shows protein adhesion between *Lactobacillus paracasei* and *Staphylococcus aureus*, *Lactobacillus paracasei* and *Staphylococcus epidermidis* and *Lactobacillus paracasei* with *Pseudomonas aeruginosa*.

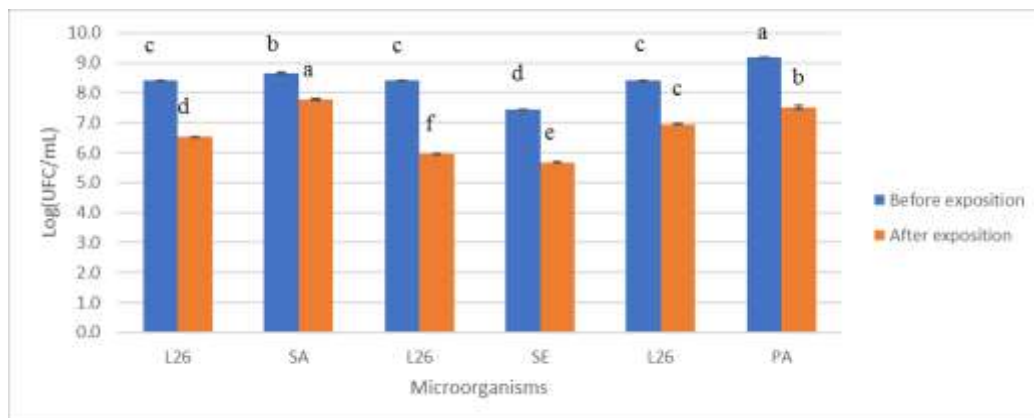


Figure 16 – Protein adhesion interaction of probiotics *L. paracasei* (L26) with pathogenic (*S. aureus*, *S. epidermidis* and *P. aeruginosa*) in HaCat cells. Columns with different letters are significantly ($p \leq 0.05$). The column on the left (blue) represents the control and, on the right, (orange) we have the representation of the respective cocultures after 15 minutes of exposure to trypsin.

Analyzing the microorganisms' combination, *Lactobacillus paracasei*/*Staphylococcus aureus*, *Lactobacillus paracasei*/*Staphylococcus epidermidis* and *Lactobacillus paracasei*/*Pseudomonas aeruginosa* combination at 0 hrs and 15 minutes, there are significant differences ($\alpha=0,005$) at both sampling points for each combination. A significant difference was observed in protein adhesion assay, this means that part of the adhesion to epidermal cells is partially due to proteins, because beyond to the competition of bacteria by inactivating protein binding through trypsin, there was a significant decrease in cell concentration for every bacteria. Adhesion through membrane proteins is evident. After inactivation of the proteins, by the trypsin, at the end of the experiment there were a decrease in the number of adhered bacteria when compared with the control, particularly for *S. epidermidis*, reduction of 1.8 log units, for *P. aeruginosa* 1.7 log units and for *S. aureus* 1.0 log units. The adherence ability is important for successful colonization and accomplishment of auspicious effect over an extended period of time (Miljkovic *et al.*, 2015). In adhesion study trypsin was used as a cleavage agent for peptide bonds, although it is possible to use proteinase k as it has the same function (Baird & Craik, 2013; Saaenger, 2013).

Is analysed by Banar *et al.* (2016), it was concluded that trypsin enzymes reduced the ODs of the *P. aeruginosa* biofilms. OD reduction from 1.5 to approximately 0.3 log units, measured at a length of 550 nm at a concentration of 1.5 $\mu\text{g} / \text{ml}$ trypsin. And according with their results, trypsin destroys *P. aeruginosa* biofilms. However, there is some contradictory information, as an accomplished experiment by Hazlett *et al.* (1992) because in a study, trypsin significantly enhanced the binding of *P. aeruginosa* to eye

tissue. Possibly due to in my study the target cells being different and certainly with another receptors and the reagent in my study is trypsin and not be the proteinase K. Finally, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of corneal epithelium obtained from trypsin versus proteinase K-treated scarified corneas, revealed that trypsin treatment (20 $\mu\text{g/ml}$ for 10 min) generally enhanced corneal epithelial proteins, while similar treatment with proteinase K, but for 30 min, generally decreased corneal epithelial proteins and consequently the bacterial adhesion (Hazlett *et al.*, 1992). Additionally, in a assay the trypsin significantly increased bacterial binding, as well, 1.96 bacteria per $\mu\text{m}^2/\text{minute}$, 2.2 bacteria per $\mu\text{m}^2/\text{minute}$ and 2.0 per $\mu\text{m}^2/\text{minute}$, respectively at times 15, 30 and 60 minutes (Singh *et al.*, 1991). Additionally, in study realized by Bar *et al.* (2013), experiments performed with 40 $\mu\text{g ml}^{-1}$ of Eap (protein) degraded with proteinase K showed no effect on adhesion or internalization. When compared with results on Eap-promoted adhesion and internalization of strain *S. aureus* (SA113), the 8325-4 WT (mutant) strain revealed a comparable promotion of adhesion and internalization upon preincubation with Eap. Also, preincubating keratinocytes with exogenous Eap increased the adhesion and internalization of the eap mutant to/into keratinocytes to the same extents as those observed with the parental strain. Thus, showing that an eap mutant adhered as efficiently to endothelial cells as did its parental strain, it can now be inferred that bacterial cell wall-bound Eap is of only minor importance for the binding of *S. aureus* to both endothelial and epithelial cells. These results illustrate again that the Eap-induced internalization rates cannot be explained by binding phenomena only. Moreover, they suggest that Eap in wound tissue of *S. aureus*-infected wounds not only promotes the internalization rates of microorganisms of the Eap-producing species (Bur *et al.*, 2013). Is evaluated in a study by Ridley *et al.* (2012), all *S. aureus* strains tested bound fibronectin and invaded epithelial cells to some degree; Mutant strains (NCTC 8325-4 Reference isolate cured of all prophages and plasmids (rsbU), Du5883 Mutant of 8325 (fnbA- fnbB-), Newman FnBA and FnBB wall-anchorage mutante, NCTC 6571 (Oxford) Reference lab strain, S-235 Local clinical isolate) but mutant strains Du5883 and Newman exhibited much lower levels of cell invasion than the other strains tested, which was expected and assumed to be due to their lack of fibronectin-binding proteins (FnBPs). Nevertheless, invasion by strain NCTC 8325-4 was 15- to 20-fold lower than that shown by strain NCTC 6571 (Oxford) and the clinical isolate S-235, despite the former binding fibronectin comparably and expressing both FnBPs. These data indicate

that the ability to invade epithelial cells does not rest solely with FnBP functionality (Ridley et al., 2012). It is briefly outlined, that SdrF bound human keratins-1 and -10 and adhered to keratinocytes and epithelial cells. Binding involved both the A and B domains. Anti-SdrF antibodies reduced adherence of *S. epidermidis* to keratin and keratinocytes (Arrecubieta et al., 2009). In the study performed by Trivedi et al. (2017), SdrF bound human keratins-1 and -10 and adhered to keratinocytes and epithelial cells. Binding involved both the A and B domains. Anti-SdrF antibodies reduced adherence of *S. epidermidis* to keratin and keratinocytes. It is defined, that RNA interference (RNAi) is a conserved biological response to double-stranded RNA that mediates resistance to both endogenous parasitic and exogenous pathogenic nucleic acids and regulates the expression of protein-coding genes. Quick progress in understanding of RNAi-based mechanisms has led to applications in studies of gene function as well as in therapeutic applications for the treatment of disease (Kim & Rossi, 2008). In Trivedi study, RNAi reduced keratin synthesis in keratinocytes and as result SdrF adherence. These studies demonstrate that SdrF mediates adherence to human keratin and suggest that SdrF may facilitate *S. epidermidis* colonization of the skin. The results show that SdrF facilitates adherence to both keratin types, NHEK (Normal Human Epidermal Keratinocyte) and desquamated human nasal epithelial cells, suggesting that SdrF may contribute to *S. epidermidis* colonization of these surfaces (Trivedi et al., 2017). However, it was reported that treatment of the bacterial cells with trypsin resulted in a significant decrease in hydrophobicity; nevertheless, the adhesion levels did not drop to the same extent. The discrepancies between the studies could be partly due to the fact that different substrates are used to test the adhesion abilities these are likely to have different physiochemical properties as well as surface receptors, in my study the media used was MRS and BHI; and this may be one reason for these contradictory results (Cunliffe et al., 1999). The results obtained in the experience are in agreement with the literature, as mentioned above. Hence, according to Kobiela & Boddupally (2014), skin perform as a sentinel, shaping how and when to respond against environmental insults throughout both homeostatic and dysbiosis states. Epidermal keratinocytes are regularly challenged by several stresses such as ultraviolet (UV) radiation, chemical, mechanical, and microbial insults. Functions between keratinocytes are needed to integrate environmental stimuli into the network of cellular interactions that control skin homeostasis, regulated anti-microbial and wound-healing responses. Epithelial barrier dysfunction and inflammation are main contributors to the pathogenesis of skin disease;

nonetheless, much remains unknown about how these two processes overlap and how they contribute independently to disease initiation (Kobielak & Boddupally, 2014).

In this method the rejection of H_0 (Similar means for each combination of bacteria) occurs, because α is the probability (P value) rejection of H_0 being this true; and the acceptance of H_1 occurs (There exist differences in the means) because of sufficient evidence in the sample.

5. Conclusion

In conclusion, in this work we have demonstrated the antimicrobial ability and adhesive properties of the microorganisms *L. paracasei*, *P. innocua* and *B. longum* spp. *infantis* against several pathogenic bacteria, such as, *S. aureus*, *S. epidermidis*, *E. coli* and *P. aeruginosa*. The results obtained with the *L. paracasei* demonstrates greater ability against the pathogenic bacterias, relatively to *P. innocua* and *B. longum* spp. *infantis*, yet the difference was not statistically significant ($p \leq 0,05$). This was one of the reasons *L. paracasei* was further used in the cell culture assays. This work focused on competition studies using planktonic cells and in keratinocyte adhesion/binding trials of competition, exclusion and displacement.

It cannot be said that competition with planktonic cells is a more effective approach when compared to the biofilm method, stated through the present studies found in the literature. The reasons for this might be the antimicrobial capacity, cell agglomeration (auto and coaggregation) inside the same species and opposite species, and diffusion of bacteriocins. Protein adhesion was also studied to infer on the mechanisms of this adhesion.

The cell culture part was carried out to verify whether adherence was partly achieved by proteins, it can be stated that part of the adhesion is by proteins, but not exclusively, because significant differences ($p \leq 0,05$) were found.

Previous studies show a good action of *L. paracasei* on biofilm exclusion of the pathogens, having this ability *in vitro*, possibly topical application, *in vivo*, could be a solution through the current treatment, in addition to supplementation, would be a way of eliminating pathogenic bacteria. Therefore, *L. paracasei* can be used as an alternative antimicrobial/therapeutic agent in the medical field for applications against pathogenic microorganisms in diseases, infections and wounds in the genital and gastrointestinal

tracts, as well as in the skin, making it a suitable alternative/co-adjuvant to conventional antibiotics.

6. Future Work

The present work labor an experimental approach toward the antimicrobial capacity assessment of some relevant microorganisms, such *Lactobacillus paracasei*, *Propioniferax innocua* and *Bifidobacterium longum* subsp *infantis*), their protein adhesion to HaCat's cells, with a possible biological property against pathogenic bacteria.

It would be important to understand how bacteria react in the presence of each other and whether there really is a decrease in pathogens, or perhaps inhibition through mechanistic approaches. Investigating the mechanisms of bacteriocin structure/function, genetic organization, ecology, and evolution, as well. Test new probiotics, in different concentrations, or bacteria that seems to have an inhibitory effect towards the pathogens, analyze their cellular organization while in competition (if they are aggregated or not). Understand how bacteria of the same specie bind to each other and to bacteria of the different species. Would also be important study the impact on bacteriocin production and release after those aggregation. It is important to understand the widen the range of beneficial microorganisms competing with the pathogens and test different concentrations of both. In future work it would be interesting to radioactively mark membrane proteins that play an important role on adhesion and to understand their real impact of colonization. Repeat carbohydrate adhesion assays to understand its impact on skin adhesion. There is a wide space for additional investigation on this topic, skin.

Their ability to prevent infections and the modulation of immunological response caused by pathogens. A way of trying to perceive interactions between bacteria more quickly can be trough PNA fish assay method. Allows faster identification, quantification, visualization of a microbial community and analysis of three-dimensional spatial distribution of cells. A new development in biotechnological tactics as a way of treatment, with more attenuated side effects and less expensive.

7. References

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