Isolation & characterization of Trace Amines (TAs) producing human skin commensals

And

Isolation and characterization of L-Forms in *Staphylococcus aureus* HG001 and JE2 (MRSA)

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Tübingen, den 12.03.2024

Title of Chapter one

Isolation & characterization of Trace Amines (TAs) producing human skin commensals

INDEX (Chapter one)

Title of Chapter two

Isolation and characterization of L-Forms in *Staphylococcus aureus* HG001 and JE2 (MRSA)

INDEX (Chapter two)

Chapter one

Isolation & characterization of Trace Amines (TAs) producing human skin commensals

List of Figures (Chapter one)

List of Table (Chapter one)

Abstract

Skin serves as the natural habitat for a diverse array of microorganisms, with approximately ten million microbes inhabiting each square centimeter of wet skin. Among these organisms, biogenic amines are produced through the decarboxylation of aromatic amino acids, a process carried out by microorganisms, plants, and mammals. Within this category, 'trace amines' constitute a group of monoamines characterized by their low abundance in brain tissue. Notable examples of trace amines found in mammals include tryptamine (TRY), tyramine (TYM), phenylethylamine (PEA), and octopamine (OCT), which function as neurotransmitters or neuromodulators. Interestingly, these trace amines are not solely produced by the host organism but are also synthesized by various bacterial species within the human microbiota. Studies have demonstrated that bacterial production of these trace amines can interact with adrenergic receptors, influencing bacterial invasion into host cells and wound healing processes. Notably, certain staphylococcal species harbor a staphylococcal aromatic amino acid decarboxylase (SadA) with unusual specificity.

In this study, we aimed to analyze the spectrum of trace amine-producing species residing on human skin. Utilizing samples from 30 subjects, we isolated approximately 1909 skin bacteria under aerobic conditions. The colony-forming unit (CFU) counts varied significantly among subjects, ranging from $19x10^1$ to $1381x10^3$ CFU/cm^2. Subsequently, we analyzed the culture supernatants of these skin isolates for trace amine production using RP-HPLC. Our findings revealed trace amines in the culture supernatant of 269 skin isolates (14%). Furthermore, we categorized the trace amine producers based on their capacity to produce one, two, or all three trace amines. Through 16S rRNA analysis, we classified the isolates into two major bacterial families: Staphylococcacae (80%) and Bacillacae (20%). Notably, staphylococcal species were found to predominantly produce all three trace amines, including DOPA, while bacteria from other genera exhibited greater diversity in trace amine production. Our research focused on trace amines shed light on the skin microbiome, differentiating trace amine producers from non-producers across various bacterial genera. Overall, our study sheds light on the spectrum of neuromodulator-producing skin bacteria and elucidates the enzymes responsible for their synthesis. This work contributes significantly to our understanding of the role of the skin microbiota in interacting with neuronal receptors.

1.Introduction

1.1 Human Skin

1.1.1 Skin Commensals

The term ‘’commensalism’’ reflects a relationship between host and organism where both can benefit. Human skin harbors a high number of diversified, innocuous commensals on their surface.

Microbial colonization starts at the beginning of life, and the delivery process affects the microbial diversity of the host (Grice and Segre 2011); (Chu et al., 2017). However, different intrinsic factors like skin site, intra– and interpersonal variability, ethnicity, gender, and age, as well as some extrinsic factors such as lifestyle, hygiene routine, cosmetic use, antibiotics, geographical location, climate, and seasonality also determine the microbial population on the host (Giacomoni, Mammone et al. 2009); (Ursell, Metcalf et al. 2012); (Sanford and Gallo 2013); (Pinto, Ciardiello et al. 2021). Despite the layered skin structure, different areas of the human body favor various microorganisms to grow, but this growth also may vary in pH, temperature, moisture, UV exposure, sebum content, and topography (Grice and Segre 2011).

The complex and interlinked structure of human skin makes it an ideal habitat for a diverse range of microbial organisms. With its three-dimensional features, the skin provides a vast terrestrial terrain for microbial life. As the largest organ in the human body, the skin covers an approximate surface area of 25 m^2, offering ample space for a symbiotic relationship between the host and microbes (Gallo 2017).

Different regions of the body exhibit variations in skin thickness, ranging from approximately 0.5 mm on the eyelids to 1.5-2.0 mm on the face and up to 4.0 mm on the heels of the feet. This diversity in skin thickness contributes to the unique microbial environment found on different body sites (Schroeder 2019). In contrast to the intestinal commensals, which are separated from body sites by an antimicrobial mucus layer, the skin allows direct contact between host cells, including immune cells, and the microbial community residing in the dermal layers.

Furthermore, the skin contains numerous hair follicles, sweat glands (eccrine and apocrine), and sebaceous glands. These structures, akin to the gut villi, provide additional niches for microbial existence and diversification. Different body sites can be categorized based on their suitability for microbial growth, including sebaceous or oily sites such as the forehead, scalp, chest, and back; moist sites such as the nose and mouth, underarms, elbow bend, abdominal area, lower buttocks, back of the knee, and foot; and dry sites like the volar forearm, buttocks, and palm (Cundell 2018).

The unique characteristics of the skin not only create favorable conditions for microbial growth but also contribute to temporal and interpersonal variations in the skin microbiome. These variations further enhance microscale diversity, leading to a wide range of host-microbe relationship patterns (Grice and Segre 2011).

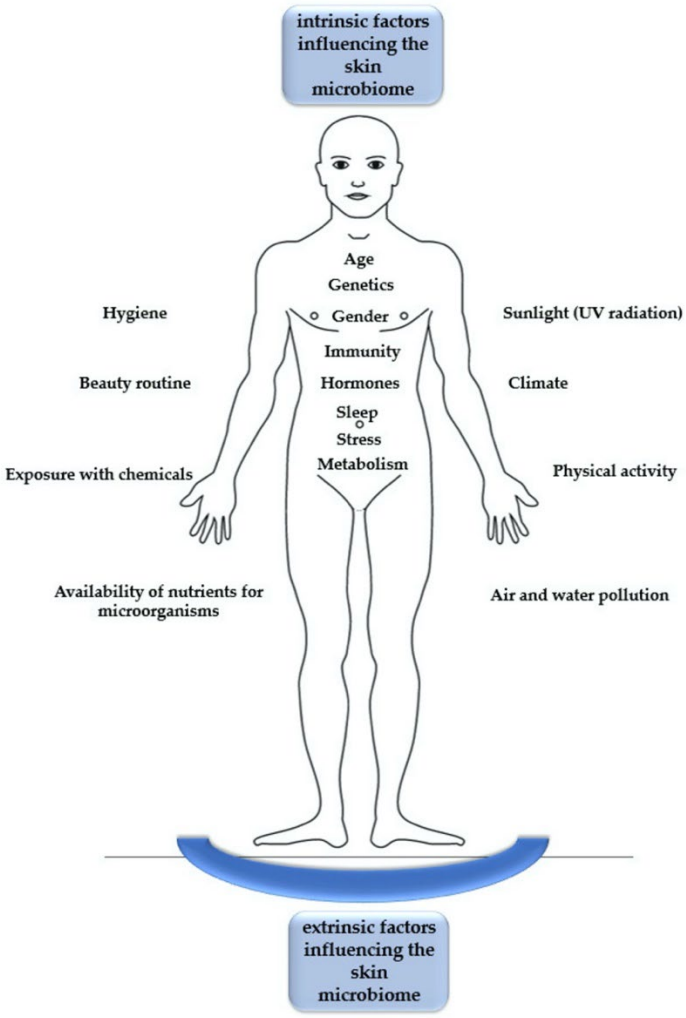


Figure 1. The intrinsic and extrinsic factors that influence the skin microbiome (Skowron, Bauza-Kaszewska et al. 2021).

Understanding the intricate relationship between the host and the skin microbiome is essential for comprehending the dynamic and complex ecosystem that exists on our skin.

1.1.3 Microbial community on skin

The skin harbors a diverse array of microbes, and its microbial composition is more distinct compared to other human organs. The average number of microbes isolated from the skin, as determined by traditional culturing methods, ranges from 10^3 to 10^4 colony-forming units (CFU) per square centimeter. However, this count varies across different body sites. In the most humid areas, such as the groin or underarms, the microbial count can exceed 10^6 CFU/cm^2. In drier areas like the scalp, forehead, and areas around the ears and head, the count is around 10^6 CFU/cm^2. On the upper back, chest, and arms, the count ranges from 10^4 to 10^5 CFU/cm^2 (Skowron, Bauza-Kaszewska et al. 2021). Notably, the microbial counts on hair and beards are also significant contributors to the skin microbiome (Byrd, Belkaid et al. 2018), (Andersen, B.M. 2018).

The majority (>90%) of bacteria in the skin microbiome can be classified into four main types: Actinobacteria (36-51%), Firmicutes, Proteobacteria, and Bacteroides (24-34%, 11-16%, and 6-9% respectively (Gallo 2017); (Byrd, Belkaid et al. 2018); (McLoughlin et al., 2021). Coagulase-negative Staphylococcus, particularly *Staphylococcus epidermidis* (10^3-10^4 CFU/cm^2), anaerobic *Cutibacterium acnes* (formerly Propionibacterium acnes), various species of Corynebacterium, Micrococcus, Streptococcus, and Acinetobacter are among the prominent bacteria found throughout the skin (Cundell 2018); (Andersen, B.M. 2018); (Murillo and Raoult 2013); (Buerger, S. 2020). In a study conducted by Samaras, S. and Hoptroff, M. in 2020, the skin microbiome of healthy individuals was analyzed, revealing that all areas of the skin can harbor various species of Cutibacterium, Staphylococcus, and Corynebacterium, which collectively make up 45-80% of the overall skin microbial community. Additionally, Archaea, including Thaumarchaeota, have been found on healthy human skin, as confirmed by several researchers (Cundell 2018); and (Buerger, S., 2020).

Various studies conducted over the years have demonstrated the diverse bacterial communities present in three main body sites on human skin, as illustrated in Figure 2 (Grice and Segre 2011); (Sanford and Gallo 2013); (Dreno, Araviiskaia et al. 2016); (Byrd, Belkaid et al. 2018); (Lunjani, Hlela et al. 2019); (McLoughlin et al., 2021); (Rozas, Hart de Ruijter et al. 2021).

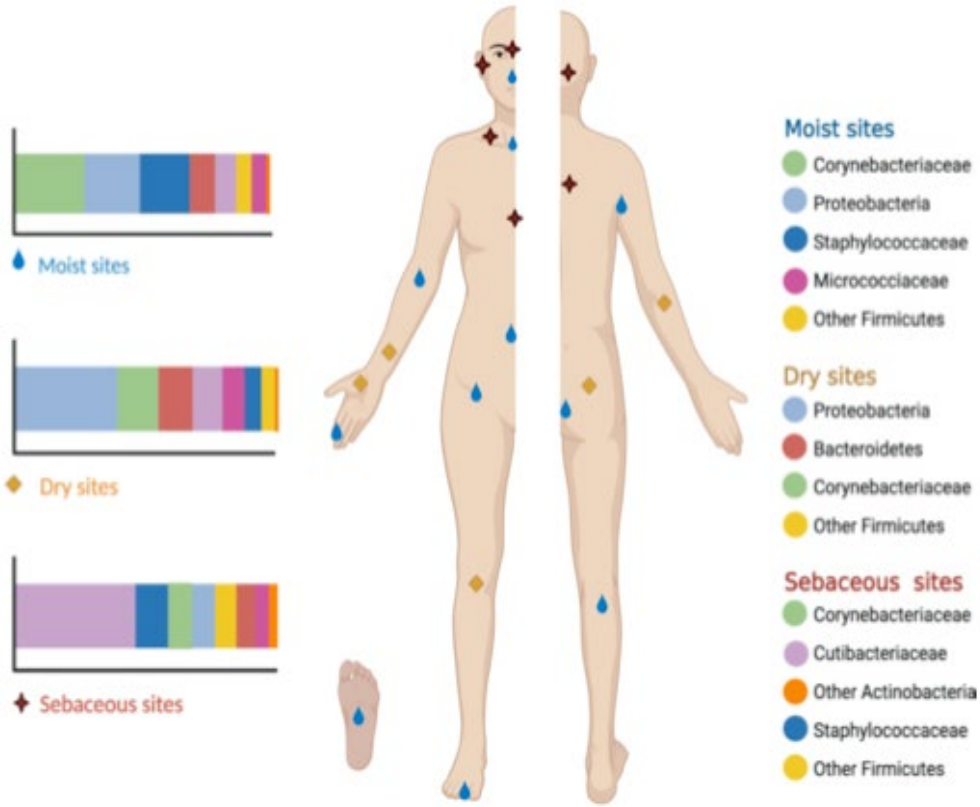


Figure 2: **Moist sites include the axilla, antecubital fossa, navel, groin, popliteal fossa and soles.** Oily sites include the forehead, alar creases, retro auricular creases and the back. Dry sites of the skin include the forearms, hands, buttocks and legs (Carmona-Cruz, Orozco-Covarrubias et al. 2022).

In addition to bacteria, the skin microbial community also includes fungi and viruses, although bacteria are the most abundant. A study by (Oh, Byrd et al. 2016) revealed that fungi and certain DNA viruses are present on the skin surface, albeit in lower numbers compared to bacteria. The composition of these microorganisms in healthy individuals is influenced by the nature of the skin sites, as highlighted in a review paper by Allyson L. Byrd, Yasmine Belkaid, and Julia A. Segre in 2018. For example, lipophilic Propionibacterium species are more prevalent in sebaceous sites, while Staphylococcus and Corynebacterium species are more commonly found in moist

areas. Fungal growth on the skin varies across different sites, with Malassezia species being abundant in core body and arm sites, and a mixed fungal population including *Malassezia spp., Aspergillus spp., Cryptococcus spp., Rhodotorula spp., Epicoccum spp.*, and others being present on the feet (Findley, Oh et al. 2013). Interestingly, while fungal presence is generally low, the foot region shows a higher availability of fungal populations.

The presence of DNA viruses on the skin is influenced more by the individual person rather than specific anatomical sites. However, due to the lack of shared marker genes among viruses, the overall presence of viruses can be observed through the purification of virus-like particles or shotgun metagenomics sequencing (Oh, Byrd et al. 2016); and (Hannigan, Meisel et al. 2015). It is important to note that there is currently no available data on RNA viruses isolated from healthy skin. For a visual representation of these microbial components, refer to Figure 3.

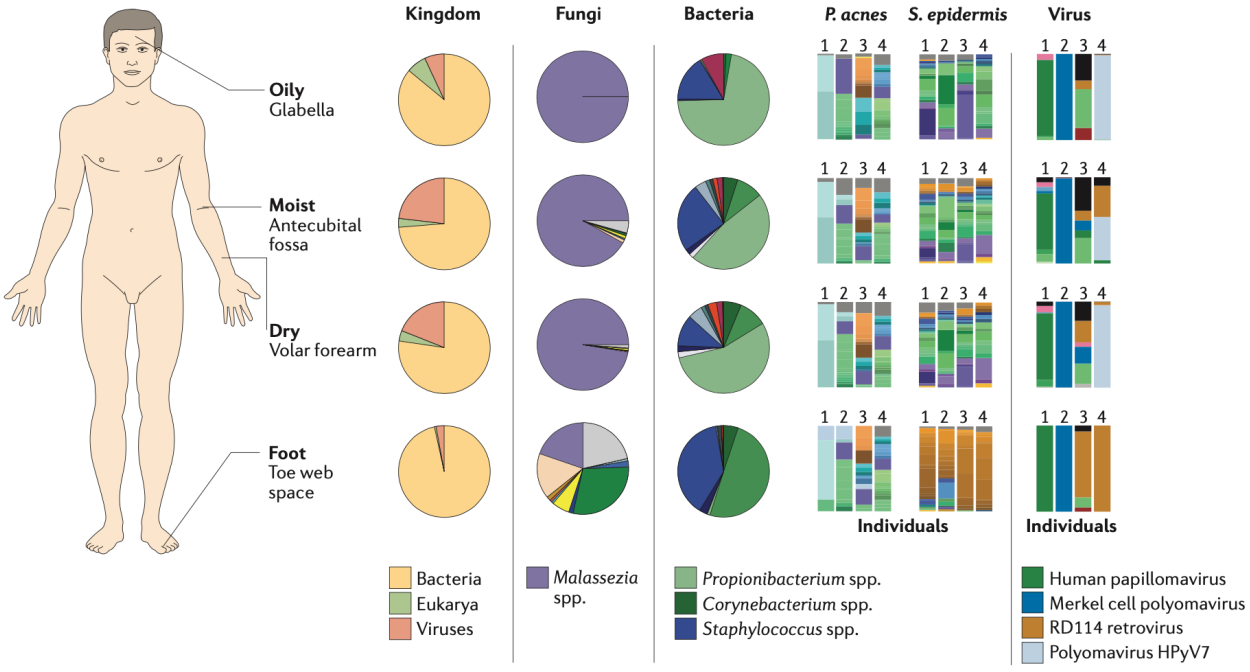


Figure 3: **Skin microbial communities are shaped by physiological characteristics and the individual.** Four sites are shown to represent major microenvironments of the skin: glabella (also known as the forehead) sebaceous (oily); antecubital fossa (moist); volar forearm (dry); and toe web space (foot). Pie charts represent consensus relative abundances of the kingdom, fungi and bacteria across

healthy adults[2]. The bacterial species *Propionibacterium acnes* and *Staphylococcus epidermidis* and eukaryotic DNA viruses are displayed as bar charts for four representative individuals to highlight how individuality shapes these communities[25]. For kingdom, fungi, bacteria and virus relative abundance plots, major taxa colours are identified in the legend. Unlabeled colours may be grouped as 'Other'. For the *P. acnes* and *S. epidermidis* bar charts, similar colours represent closely related strains (Byrd, Belkaid et al. 2018).

1.1.4 Role of skin microbes

The skin, being the largest organ in the human body, and its associated microbial community play various roles in human physiology and pathology. Skin microbiota can contribute to the development of pathological conditions, including nervous and psychological disorders (Dinan and Cryan 2015), metabolic disturbances (Sonnenburg and Backhed 2016); (Brunkwall and Orho-Melander 2017); and immunological problems (Honda and Littman 2016); (Thaiss, Zmora et al. 2016).

Moreover, for sound health conditions, skin microbiomes play some crucial roles like maintaining skin homeostasis, shielding against pathogens, and helping in the immune modulation process (Giacomoni, Mammone et al. 2009); (Ursell, Metcalf et al. 2012); (Sanford and Gallo 2013); (Pinto, Ciardiello et al. 2021). These functions are essential for preserving the integrity and functionality of the skin, promoting overall well-being and health.

Understanding the complex interactions between the skin and its microbial inhabitants is crucial for comprehending the role of the skin microbiota in both physiological and pathological conditions. Ongoing research in this field continues to shed light on the intricate relationship between the skin and its microbial community, opening up new avenues for therapeutic interventions and personalized approaches to skin health management.

1.2 Trace amines

1.2.1 Trace amines and their past days

The study of trace amines (TAs) in vertebrates has been a focus of research for approximately 15 years (Borowsky, Adham et al. 2001); (Bunzow, Sonders et al. 2001). However, the exploration of endogenous compounds known as trace amines dates back 150 years. In 1970, Alan Boulton and his colleagues introduced the term "trace amines" to differentiate them from other monoamines such as catecholamines and indoleamines. The term "trace amines" refers to their lower abundance in endogenous tissues, typically less than 10 ng/g or 100 nM, which is nearly 100 times lower than the corresponding neurotransmitters (Berry 2004).

Trace amines are structurally related to classical monoamines and exhibit neurotransmitter activity. This small group of trace amines includes β-phenylethylamine (PEA), p-tyramine (TYR), tryptamine (TRP), and p-octopamine (OCT). The structures of these trace amines and other structurally related monoamines can be seen in Figure 4.

The first reported trace amine, phenylethylamine, was discovered in the laboratory of Marceli Nencki in the late 1870s (Grandy 2007). It was initially identified in the context of bacterial putrefaction and fermentation, where PEA was produced through the anaerobic decarboxylation of L-phenylalanine during bacterial decomposition. While the initial focus was on trace amines found in foodstuffs, subsequent research has shown increasing interest in the microbial production of these trace amines.

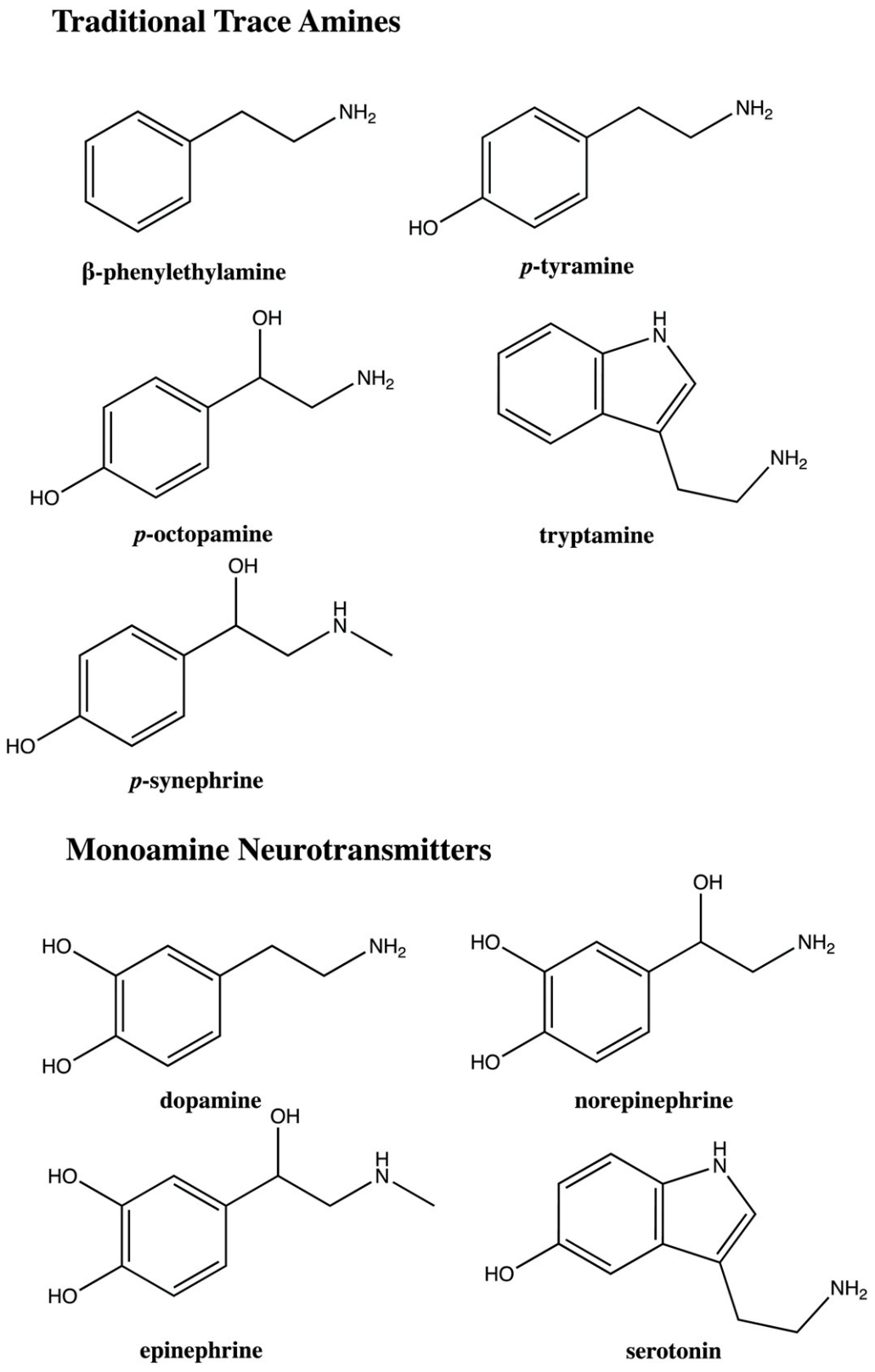


Figure 4: **Relationship of archetypal trace amines to the monoamine neurotransmitters** (Gainetdinov, Hoener et al. 2018).

1.2.2 Trace amines, their receptors and storage

The study of trace amines gained significant attention in 2001 with the discovery of a new family of G protein-coupled receptors (GPCRs) belonging to the rhodopsin-like family. This family of receptors displayed strong selectivity for trace amines such as PEA, TYR, and OCT (Borowsky, Adham et al. 2001); (Bunzow, Sonders et al. 2001). However, prior to the identification of these receptors, the trace amine-associated receptor (TAAR)1 was already known to researchers (Grandy 2007); (Sotnikova, Zorina et al. 2008); (Lam, Espinoza et al. 2015); (Liberles 2015) for its involvement in trace amine activity. It is interesting to note that TAAR is only found in vertebrates, while invertebrates possess selective receptors for TYR and OCT. Roeder (Roeder 2005) in 2005 and Lange (Lange 2009) in 2009 revealed that invertebrates have adrenergic receptor-like systems that facilitate the activity of TYR and OCT, in addition to TAAR. The concentrations of TYR and OCT are likely higher in invertebrates compared to vertebrates (Roeder 2005). Furthermore, there have been discussions about the relevance of different endogenous ligands of TAARs in relation to microbiota and their production.

When TAAs interact with TAAR1, particularly PEA and TYM, they can regulate the synaptic activity of dopaminergic neurons and alter the activity of the dopamine D2 receptor (D2R) (Borowsky, Adham et al. 2001)

There were also shreds of evidence about the wide distribution of TAAR family and their expressions in different peripheral tissues as well as in brain tissues of mammals. This TAARs distribution and expression is illustrated by Rutigliano (Rutigliano, Accorroni et al. 2017) in 2017 by the following figure.

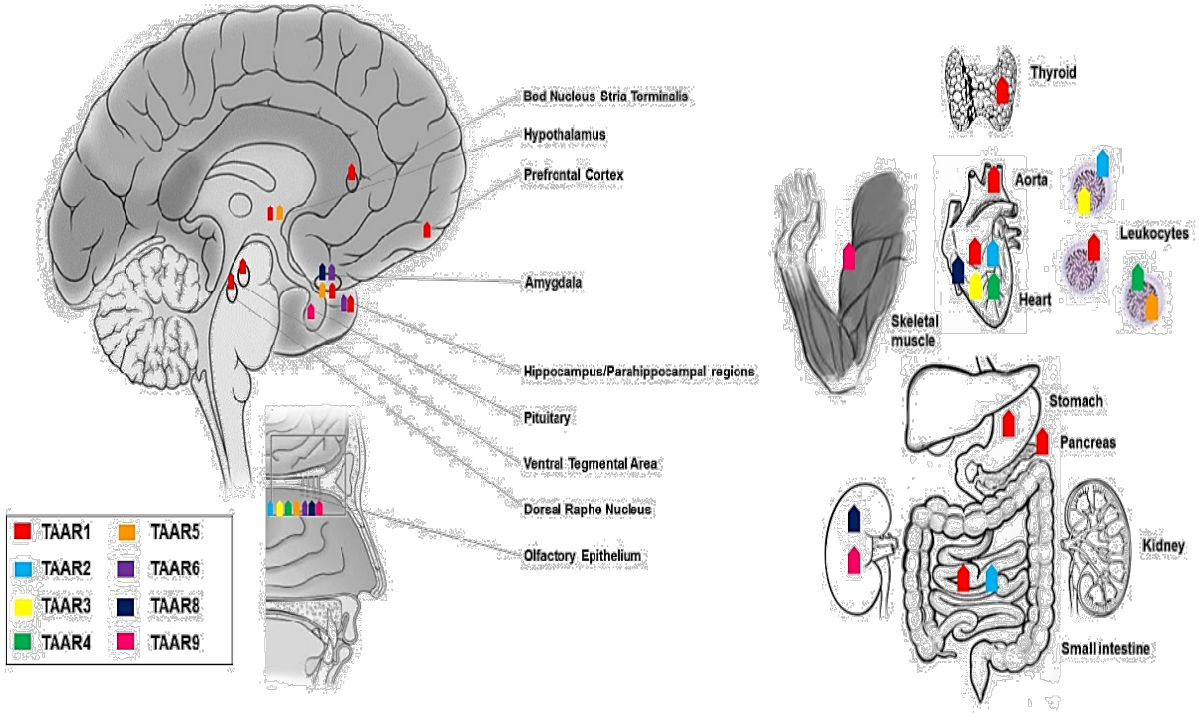


Figure 5: Anatomical distribution of trace amine-associated receptors (TAARs): expression of members of the TAAR family in brain, olfactory mucosa and across the body (Rutigliano, Accorroni et al. 2017).

Furthermore, there have been discussions about the relevance of different endogenous ligands of TAARs in relation to microbiota and their production.

Trace amines can be released and stored in nerve terminals alongside other classical neurotransmitters such as dopamine (DOP), norepinephrine, or serotonin (Premont, Gainetdinov et al. 2001); (Dewar, Dyck et al. 1988). Despite their lower abundance, trace amines play an essential role in modulating synaptic transmission in the vertebrate brain by influencing the activity of other classical neurotransmitters (Burchett and Hicks 2006); and (Gainetdinov, Hoener et al. 2018).

1.2.3 Trace amines and their synthesis

In the human body, biogenic amines, which are small organic compounds, play various biological roles as hormones, neuromodulators, or neurotransmitters (Zucchi, Chiellini et al. 2006). These compounds can be produced by microorganisms, plants, and mammals through processes such as decarboxylation of aromatic amino acids or amination and transamination of aldehydes and ketones (Halasz et al., 1994). Biogenic amines can be categorized into polyamines and monoamines, with monoamines further classified into classical neurotransmitters such as histamine, serotonin, catecholamines, and trace amines.

Trace amines, in particular, are produced from their precursor amino acids through the process of decarboxylation, facilitated by the enzyme aromatic L-amino acid decarboxylase (AADC). This process leads to the production of PEA, TRY, and TYM from L-phenylalanine, L-tryptophan, and L-tyrosine, respectively (Boulton and Wu 1972, Boulton and Wu 1973); (Saavedra 1974); (Snodgrass and Iversen 1974); (Silkaitis and Mosnaim 1976); (Dyck, Yang et al. 1983). Additionally, trace amines can also be produced as end products in the monoamine synthesis pathway when there is a lack of tyrosine hydroxylase or tryptophan hydroxylase activity. This is illustrated in Figure 5.

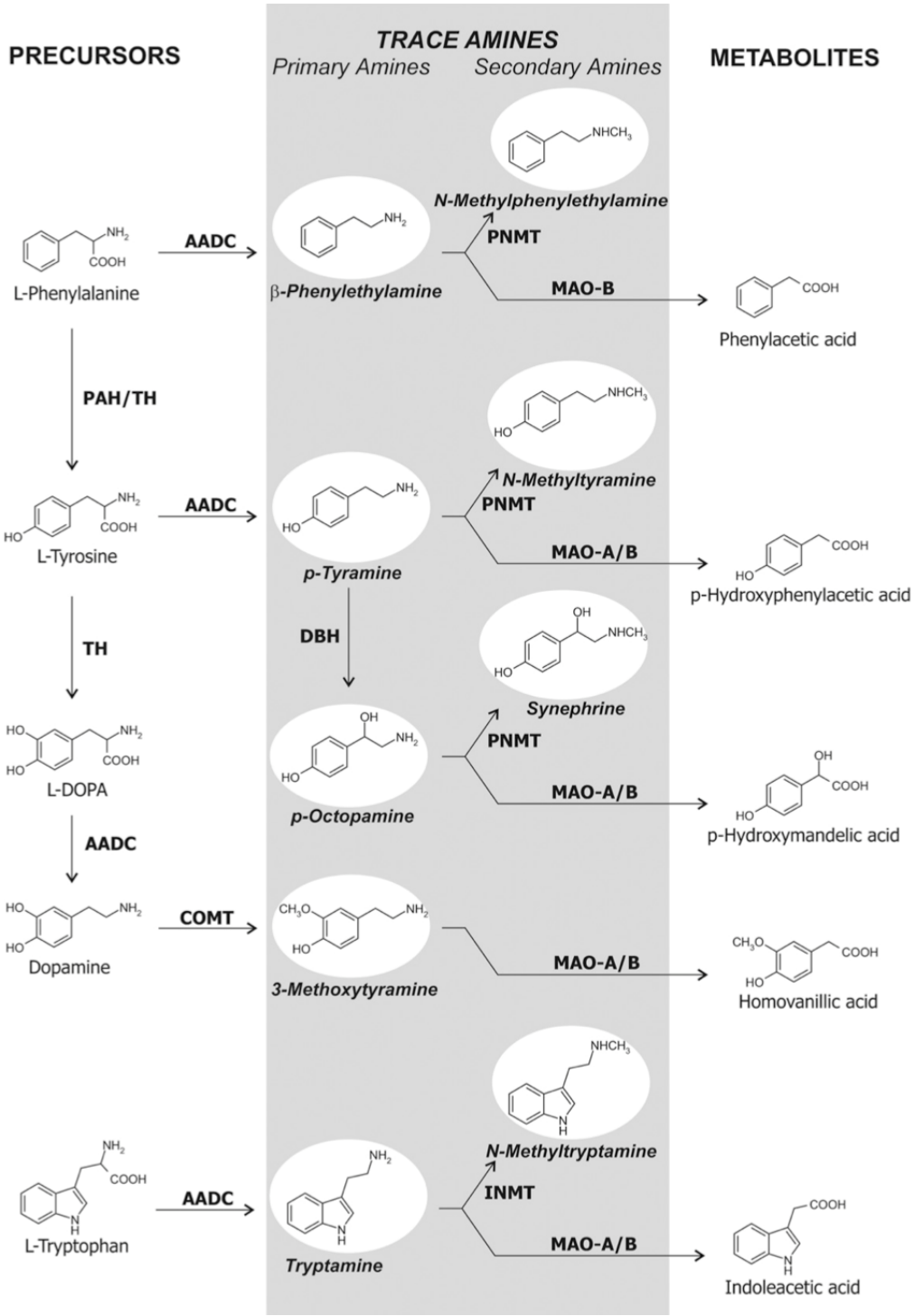


Figure 6. **Endogenous synthetic and metabolic routes for trace amines**. DBH, dopamine-b-hydroxylase; INMT, indolethylamine N-methyltransferase; PAH, phenylalanine hydroxylase; TH, tyrosine hydroxylase (Gainetdinov, Hoener et al. 2018).

The production of trace amines is not limited to neuronal cells but can also occur in various other cell types. The enzyme AADC, responsible for the decarboxylation of precursor amino acids to produce trace amines, is expressed in different cell types including glia (Juorio, Li et al. 1993); gastrointestinal tract cells (Lauweryns and Van Ranst 1988); (Vieira-Coelho and Soares-da-Silva 1993), kidney (Christenson, Dairman et al. 1970); (Lancaster and Sourkes 1972); (Aperia, Hokfelt et al. 1990), liver (Bouchard and Roberge 1979); (Ando-Yamamoto, Hayashi et al. 1987); (Dominici, Tancini et al. 1987), lungs (Lauweryns and Van Ranst 1988), pancreas (Lindstrom and Sehlin 1983); (Furuzawa, Ohmori et al. 1994); (Rorsman, Husebye et al. 1995), and stomach (Lichtenberger, Delansorne et al. 1982). This suggests that AADC present in these non-neuronal cells may convert precursor amino acids to their respective trace amines. However, the extent and significance of local trace amine production in these tissues, and its connection to TAAR ligands, have not been extensively studied.

While AADC is sufficient for the synthesis of PEA, TYR, and TRY, their precursor amino acids are not suitable substrates for AADC. Various studies have explored the regulation of AADC by different selective substrates (Bender and Coulson 1972); (Sims, Davis et al. 1973); (Sims and Bloom 1973); (Rahman, Nagatsu et al. 1981); (Siow and Dakshinamurti 1985). Additionally, several splice variants of the AADC enzyme involved in this regulation have been identified (O'Malley, Harmon et al. 1995); (Rorsman, Husebye et al. 1995); (Vassilacopoulou, Sideris et al. 2004). These findings highlight the complexity of AADC regulation and its role in trace amine synthesis.

1.2.4 Trace amines from different sources

In mammals, the predominant trace amines acting as neurotransmitters or neuromodulators include Tyramine (TYM), Phenylethylamine (PEA), Tryptamine (TRY), and Octopamine (OCT) (Khan and Nawaz 2016); (Miller 2011). However, these neurotransmitters are found in relatively low levels in brain tissue (Berry 2004). Studies have shown that these neurotransmitters can be produced by mammals, plants, and various other organisms (Branchek and Blackburn 2003). In bacteria, TYM and PEA production has been observed in *Escherichia coli* and certain food-borne bacteria

(Chander, Batish et al. 1988); (Marcobal, De las Rivas et al. 2012); (Spano, Russo et al. 2010). Consumption of trace amines in significant amounts may affect the gastrointestinal and nervous systems and can also have effects on blood pressure (Moracanin et al., 2015); (Sillia Santos, 1996). Some strains of Staphylococcus used in starter cultures are believed to be used as trace amine supplements, but it is important to note that they also pose some health risks (Sillia Santos, 1996).

Furthermore, *Staphylococcus piscifermentans* (Ansorena, Montel et al. 2002); (Stavropoulou, Borremans et al. 2015); (Straub, Kicherer et al. 1995) and *Staphylococcus xylosus* (Martuscelli, Crudele et al. 2000) have been found to produce cadaverine, putrescine, PEA, TYM, and TRY, although the specific genes responsible for this production have not been identified. Interestingly, in 2020, Luqman et al. (Luqman, Muttaqin et al. 2020) discovered that certain skin commensal bacteria, particularly Staphylococcal strains, can convert these trace amines from their corresponding amino acids by breaking down their carboxyl group using the decarboxylase enzyme SadA. The team also conducted shotgun metagenomics analysis of the skin microbiome and identified SadA homologs in seven bacterial phyla, including Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes, Acidobacteria, Chloroflexi, and Cyanobacteria, as well as in 23 genera within the phylum Firmicutes (Luqman, Zabel et al. 2020).

1.2.5 Trace amines production and role of different Decarboxylases

In vertebrates, the synthesis of trace amines is primarily mediated by the enzyme L-amino acid decarboxylase (AADC), which converts phenylalanine, tyrosine, and tryptophan into phenylethylamine (PEA), tyramine (TYM), and tryptamine (TRY), respectively (Boulton and Wu 1972); (Saavedra 1974); (Silkaitis and Mosnaim 1976); (Dyck, Yang et al. 1983).

Studies on trace amine production have mainly focused on food bacteria that produce biogenic amines, including Enterococci, Lactobacilli, Streptococci, Lactococci, Pediococci, and Oenococci (Marcobal, Martin-Alvarez et al. 2006); (Irsfeld, Spadafore et al. 2013); (Williams, Van Benschoten et al. 2014); (Barbieri, Montanari et al. 2019).

In 2018, Luqman et al. revealed the production of trace amines in different species of Staphylococcus and identified the SadA gene, which encodes the decarboxylation process, as being responsible for trace amine production in this genus (Luqman, Nega et al. 2018). Their study demonstrated that TRY, PEA, and TYM were produced by Staphylococcus through the pyridoxal phosphate (PLP)-dependent pathway. SadA, the Staphylococcal aromatic amino acid decarboxylase, showed high potential for converting precursor amino acids into trace amines, and SadA-producing Staphylococcus strains were found to be abundant in both the skin and the gut (Luqman, Muttaqin et al. 2020) (Luqman, Ebner et al. 2019) (Luqman, Nega et al. 2018).

Another PLP-dependent enzyme called tryptophan decarboxylase (TpDC) was characterized in two gut bacteria, *Clostridium sporogenes* and *Ruminococcus gnavus*, by (Williams, Van Benschoten et al. 2014). However, the study suggested that the activity of TpDC in bacteria is not widespread.

The expression of tyrosine decarboxylase (TDC) in gram-positive bacteria, particularly those involved in food fermentation, was investigated, revealing that TDC expression depends on various variables and conditions in bacteria (Torriani, Gatto et al. 2008). Tyramine-producing lactic acid bacteria were also studied in human feces (Ladero, Fernandez et al. 2009). However, the source of tyramine in the human body, whether from food, other polymers, or gut bacteria, remains uncertain. Although a gram-negative gut commensal called *Morganella morganii*, which can produce phenylethylamine from L-phenylalanine, has been identified, the gene responsible for this conversion is still unknown.

As food is considered a rich source of biogenic amines, investigations have been conducted on food bacteria from starter cultures to determine their production of cadaverine, putrescine, PEA, TRY, and TYM. Some bacteria found to be involved in trace amine production include *Staphylococcus piscifermentans* (Ansorena, Montel et al. 2002); (Stavropoulou, Borremans et al. 2015); (Straub, Kicherer et al. 1995) and *Staphylococcus xylosus* (Martuscelli, Crudele et al. 2000). However, the specific genes involved in this trace amine production process still need to be characterized.

While human AADC is involved in the internal synthesis of trace amines (Christenson, Dairman et al. 1970); (Juorio and Yu 1985); (Gainetdinov, Hoener et al. 2018), the question arises as to whether humans acquire trace amines through dietary intake or microbial contribution in trace amine conversion. Microbial conversion of precursor amino acids into trace amines plays a significant role in mammals and may also contribute to neurotransmission in humans.

1.2.6 Trace amines as advantageous for human and microbes

It has been reported that trace amines (TAs) can exhibit dual characteristics on different adrenergic receptors. On one hand, they can act as partial antagonists of the β2-adrenergic receptor (β2-AR), leading to increased cell mobility in the wounded area of the skin and accelerating the healing process (Luqman, Muttaqin et al. 2020); (Kleinau, Pratzka et al. 2011).

Studies have shown that TAs and dopamine produced by SadA-expressing *Staphylococcus epidermidis* can be beneficial for wound healing in humans (Luqman, Muttaqin et al. 2020). Epinephrine, which is produced by keratinocytes, normally decreases cell motility in wounded areas through its interaction with the β2-adrenergic receptor (β2-AR). However, the study revealed that in the presence of TAs, which act as antagonists to the β2-AR receptor, the activity of epinephrine is hindered. This leads to an increase in cell movement at the wound site, thereby promoting the wound healing process. The detailed process is illustrated in Figure 7. The beneficial mechanism of TAs was demonstrated in vitro through a gap-filling assay using tryptamine (TRY) and phenylethylamine (PEA) in a keratinocyte cell line (HaCaT cells), as well as in vivo using a mouse model.

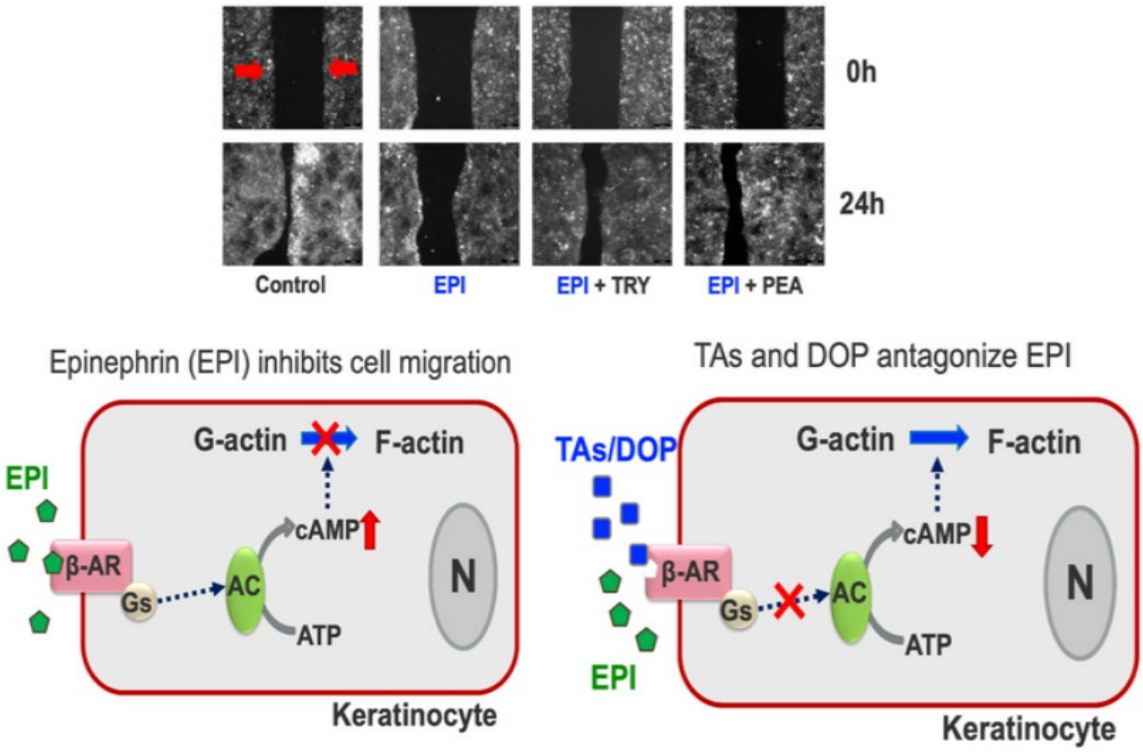


Figure 7: trace amines acting as partial antagonist on β2- adrenergic receptor, accelerate the healing process on skin (Luqman, Muttaqin et al. 2020).

Indeed, studies have revealed the agonistic activity of trace amines (TAs) on the α2-adrenergic receptor (α2-AR), which enhances the adherence and internalization process of gut commensals (Luqman, Nega et al. 2018); (Ma, Bavadekar et al. 2010); (Cornil and Ball 2008). Luqman et al. (2018) demonstrated the beneficial effects of TAs produced by gut Staphylococcus carrying the sadA gene in the HT-29 cell line. They observed increased internalization and adherence of bacterial cells (Figure 8). This highlights the potential role of TAs in modulating host-microbe interactions and the importance of trace amine production by skin commensals.

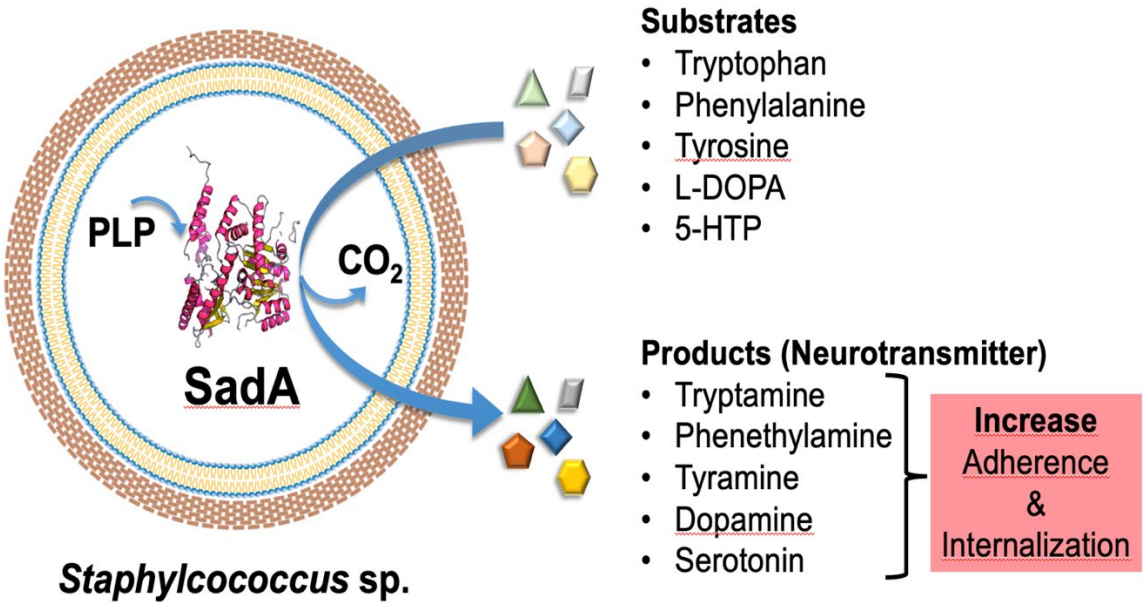
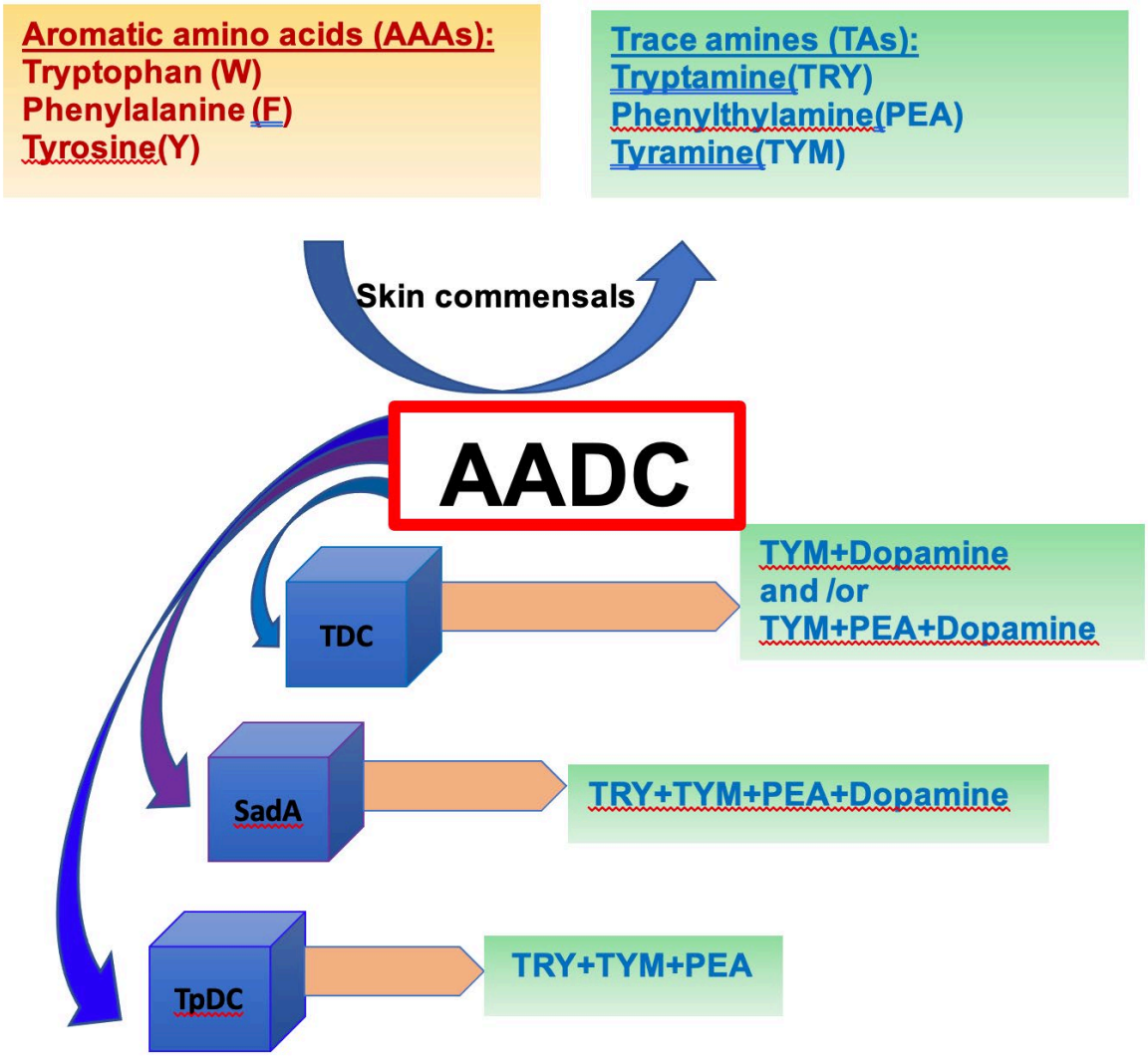


Figure 8: Trace amines act as agonist on α2-adrenergic receptor, boost the gut commensal's adherence and internalization process (Luqman *et al* 2018).

Considering the significance of trace amines and their production by skin commensal bacteria, our project goals involve the isolation of different skin bacteria, analysis of their trace amine production capability and patterns, and ultimately the identification of the responsible bacterial AADCs (aromatic L-amino acid decarboxylases) involved in the trace amine conversion process. This research will contribute to our

understanding of the role of skin microbiota in trace amine production and its potential impact on human health and well-being.

The objectives of this research work is schematically illustrated as follows.



2. Materials and Methods

2.1 Materials

Chemicals and media for general use were bought from the companies Roche (Mannheim), Carl Roth GmbH (Karlsruhe), Difco Laboratories (Augsburg), GibcoBRL Life Technologies GmbH (Eggenstein), Merck GmbH (Darmstadt), Serva Fein biochemika GmbH (Heidelberg), Peq Lab Biotechnologie GmbH (Erlangen), Sigma-Aldrich Chemie GmbH (Taufkirchen).

Special materials, chemicals, enzymes and purification kits for this study and the respective supplier are listed in the tables below.

2.1.1 Chemicals and Buffers

Table 1: Chemicals used in this study

Chemicals	**Supplier**
Agarose PEQ Fold universal	PeqLab, Erlangen
Peptone from casein tryptic digest Tryptic Soy Broth Tris (hydroxymethyl-aminomethane)	Sigma, Taufkirchen
D-(+)-glucose D-(+)-sucrose	Sigma
Glycerol	Sigma
Phosphoric acid	Sigma
Acetonitrile	Sigma
L-Tyrosine disodium salt	BioChemical
L-Phenylalanine	Sigma
Tryptophan	Sigma
L-DOPA	Sigma
Tyramine	Sigma
Phenylthylamine	Sigma
Tryptamine	Sigma
Dopamine	Sigma

Table 2: Buffers used in this study

Buffer	Processing
PBS	1 liter, pH 7.2
NaCl 8.0 g KCl 0.2 g Na2HPO4 1.77 g KH2PO4 0.24 g	
Tris-HCl	1 liter, pH 7.8
Tris	20 mM, adjust pH with HCl

Buffer	Concentration	Reference
PBS	10X	Sigma Aldrich

Table 3: Buffers used for Corynebacterium and LAB sample collection

Ingredients	amount	Reference
Disodium phosphate	12.49 g/L	Merck
Potassium dihydrogen phosphate	0.63 g/L	Merck
Triton X-100	1%	Sigma
pH=7.5		

2.1.2 Laboratory appliances

Table 4: Special laboratory appliances

Appliance	Supplier
Centrifuge 5804R	Eppendorf AG, Hamburg
Ice machine	Scotsman
Incubators	Hereaus Seperations technik GmbH, Osterode
Microcentrifuge Hermle Z233 M-2	Hermle-Labor technik, Wehingen
Milli-Q PF Plus water purification system	Millipore, Eschborn
pH-Meter	METTLER TOLEDO
Premium Freeze (-20 °C)	Liebherr
Refrigerator	Revco/Liebherr
Shaker	Innova44, NEW BRUNSWICK SCIENTIFIC
PCR machine	Applied biosystems by life technologies
Nanodrop	IMPLEN
Spectrophotometer	WPA biowave
HPLC	Agilent, Germany

2.1.3 Consumable materials and plastic articles

Table 5: Consumable material

Article	Supplier
1 mm electroporation cuvettes	Carl Roth GmbH, Karlsruhe
1.5 ml and 2 ml Eppendorf reaction tubes	Eppendorf, Hamburg
12 ml & 14 ml round bottom tubes	Eppendorf, Hamburg
15 ml and 50 ml conic Falcon™ tubes	Greiner Bio-One, Frickenhausen
200 µl reaction tubes	Molecular Bioproducts, San Diego
96 well plates, flat bottom	Greiner Bio-One, Frickenhausen
Lids for 96- well plates	Greiner Bio-One, Frickenhausen
Cotton swab collector	Eppendorf, Hamburg

2.1.4 Isolation of skin bacteria

A number of various media, dyes and antibiotics were used under this study for the purpose of isolation of skin bacteria from healthy volunteer's skin samples.

Table 6: Medias used in this study

Tryptic soy broth (TSB)

Ingredients	amount	Reference
Casein peptone	17 g	**Sigma Aldrich**
Dipotassium hydrogen phosphate	2.5 g	
Glucose	2.5 g	
Sodium chloride	5 g	
Soya peptone (Papein digest)	3 g	
Distilled H2O	1 liter	

Tryptic soy broth (TSB) agar

Ingredients	amount	Reference
Casein peptone	17 g	**Sigma Aldrich**
Dipotassium hydrogen phosphate	2.5 g	
Glucose	2.5 g	
Sodium chloride	5 g	
Soya peptone (Papein digest)	3 g	
Agar	15 g	
Distilled H2O	1 liter	
pH=7.3±0.2 (25 °C)		

SK salt agar (Schleifer-Krämer) for Staphylococcus

Ingredients	amount	Reference
Tryptone or peptone from casein	10.0 g	**(Schleifer and Krämer, 1980)**
Beef extract	5.0 g	
Yeast extract	3.0 g	
Glycerol	10.0 g	
Sodium pyruvate	10.0 g	
Glycine	0.5 g	
KSCN	2.25 g	
NaH2PO4 · H2O	0.6 g	
Na2HPO4 · 2H2O	0.9 g	
Agar	15.0 g	
Distilled H2O	1 liter	

Adjusted pH to 7.2. Autoclaved at 121°C for 15 min, cool down in water bath to 45°C and added 10 ml of a 0.45% sterile-filtered solution of sodium azide.

Cullum-Medium (MS agar) for Streptomyces

Ingredients	amount	Reference
Soya meal	20.0 g	**Hobbs et al.1989**
Mannitol	20.0 g	
Agar	15.0 g	
Distilled H2O	1 liter	

Lactic acid bacteria (LAB) Selective media

MRS broth (Man, Rogosa & Sharpe, 1960**)** for Rod-shaped lactic acid bacteria

Ingredients	amount	Reference
Proteose peptone	10.0 g	**Sigma Aldrich**
Beef extract	10.0 g	
Yeast extract	5.0 g	
Dextrose	20.0 g	
Polysorbate 80	1.0 g	
Ammonium citrate	2.0 g	
Sodium acetate	0.1 g	
Magnesium sulphate	2.5 g	
Manganese sulphate	0.05 g	
Dipotassium phosphate	2.0 g	
Distilled H2O	1 liter	
Decreasing pH to 5.4 by 100% Acetic acid acid		

M17 broth for Coccus-shaped lactic acid bacteria

Ingredients	amount	Reference
Ascorbic acid	0.5 g	**Sigma Aldrich**
Lactose	5.0 g	
Magnesium sulfate	0.25 g	
Meat extract	5.0 g	
Meat peptone (peptic)	2.5 g	
Sodium glycerophosphate	19.0 g	
Soya peptone (papainic)	5.0 g	
Yeast extract	2.5 g	
Tryptone	2.5 g	
Distilled H2O	1 liter	
pH 7.0±0.2 (25 °C)		

For Corynebacterium

Tellurite blood agar (TBA)

Ingredients	amount	Reference
Hoyle's tellurite agar base		
Peptic digest of animal tissue	10.0 g	**Sigma Aldrich**
Beef extract	10.0 g	
Sodium chloride	5.0 g	
Agar	15.0 g	
Distilled H2O	1 liter	
Final pH 7.8 +/- 0.2 at 25°C		

40 grams of agar base powder was suspended in 915 ml distilled water. Boiled to dissolve the medium completely. Sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cooled to 55°C and aseptically added 50 ml of laced blood and 35 ml of 1.0% Potassium Tellurite Solution. Mixed well and poured into sterile petri plates.

Corynebacterium Selective media (Jensen, M. G. et al 2023)

Ingredients	amount	Reference
Trypticase soy agar (TSA)	40 g	**Sigma Aldrich**
Yeast extract	5 g	**Sigma Aldrich**
Tween 80	10 ml	**Sigma Aldrich**
ultra-filtrated-water (UF-water)	1 L	

The mixture was autoclaved at 121 °C for 15 min. After that, 5 ml of furazolidone (6 µg/ml Furazolidone) from a stock solution (12 mg in 10 ml acetone) and 1 ml of Oil red O from 0.5 % stock solution (50 mg in 10 ml acetone) was added aseptically after cooling to approximately 50^0C.

Table 7: Dye used in this study

Dye	Source	Stock solution	Final concentration to be used	Dissolved in
Oil Red O	Sigma	50 mg/10 ml	0.5%	Acetone

The used dye was sterile filtered by passing through filters with 0.2 µm pore size (Filtropur S 0.2; Sarstedt AG & Co.) and dissolved in acetone. The addition of dye to the autoclaved liquid media was mediated after cooling to approx. 50°C because of their thermostability.

Table 8: Antibiotics used in this study

Antibiotics	Source	Stock solution	Final concentration to be used	Dissolved in
Furazolidone	Sigma	12 mg/10 ml	6 mg/ml	Acetone

The used antibiotics were sterile filtered by passing through filters with 0.2 µm pore size (Filtropur S 0.2; Sarstedt AG & Co.) and dissolved in acetone The addition of antibiotics to the autoclaved liquid media was mediated after cooling to approx. 50°C because of their thermostability.

2.1.5 Primers used in this study

For identification process of skin bacteria, 16s rRNA sequencing was done by using universal primers and also for AADC determination some primer sets were used.

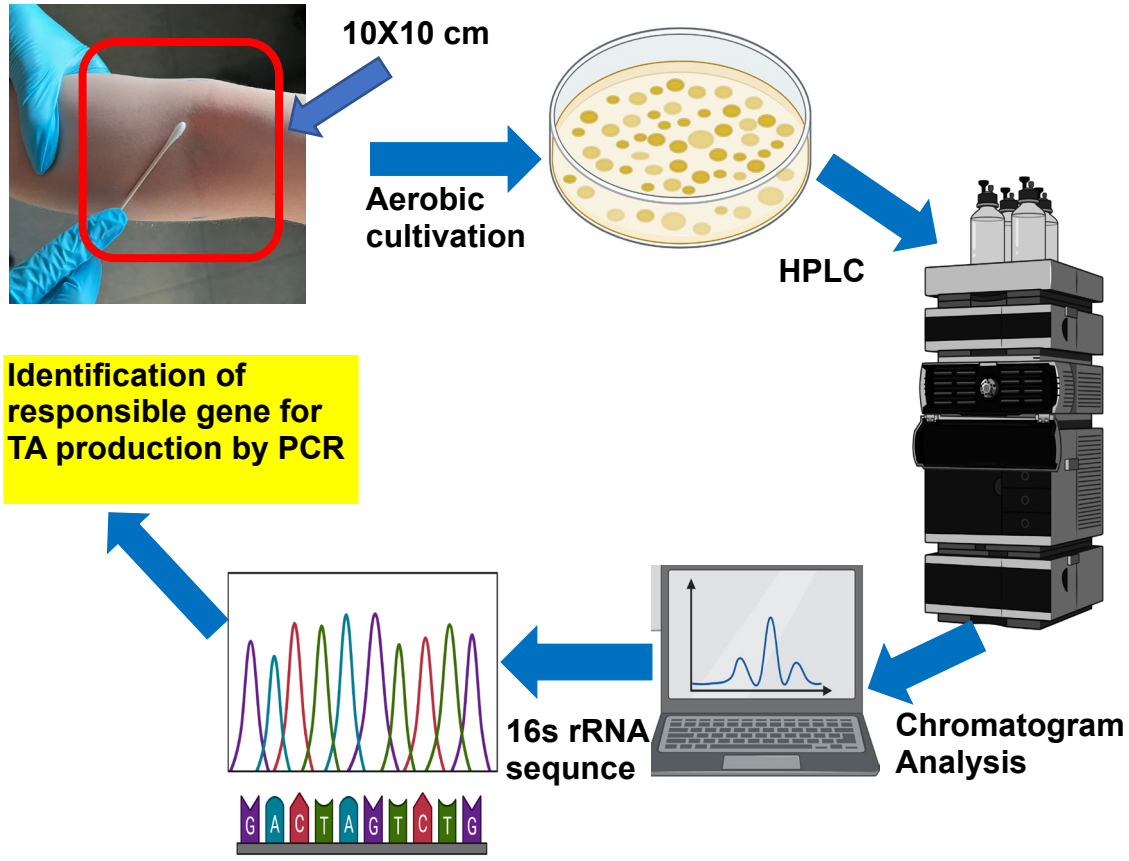
Table 9: Primers used in this study

Primers	Sequence 5‘-3‘
16R (Universal primer used for 16s sequencing)	AAG GAG GTG ATC CAA CCG CA
8F (Universal primer used for 16s sequencing)	AGA GTT TGA TCA TGG CTC AG
TDC Dec5f (Sebastiaan P, 2019)	CGTTGTTGGTGTTGTTGGCACNACNGARGARG
TDC Dec3r (Sebastiaan P, 2019)	CCGCCAGCAGAATATGGAAYRTANCCCAT
SadA F-G1 (*S.epidermidis*)	CKGATAAGATATGTGAWAGTCC
SadA R-G1 (*S.epidermidis*)	GTMTTTGTATCTGGYGGTTCWA
SadA 52-S (*S.coagulans*)	GCGTTACGTTGATACAGAGA
SadA 52-S (*S.coagulans*)	CCAGCACGATATGTATTTAGC

2.2 Methods

2.2.1 Experimental design

The following diagram shows shortly the experimental steps that we acted in accordance with under our research project



2.2.2 Skin swab sample collection

The initial step of our experiment involved the collection of skin samples from the antecubital fossa of 15 male and 15 female volunteers, representing a diverse demographic range in terms of age. Using sterile swab collectors, we meticulously obtained skin swab samples, ensuring the integrity and sterility of the collection process. These swab samples were then transferred into sterile Tryptic Soy Broth (TSB) and allowed to incubate at room temperature for 15 minutes to facilitate the release of microorganisms from the skin surface.

Subsequently, we performed serial dilutions of the swab samples, diluting them at ratios of 1:10 and 1:100 to obtain manageable concentrations suitable for analysis. These dilutions were then spread onto TSB agar plates using aseptic techniques, ensuring even distribution of the microbial suspension across the agar surface. The agar plates were carefully labeled and incubated under aerobic conditions at a controlled temperature of 30°C for a period of three days.

Following the incubation period, we meticulously examined each agar plate for the presence of microbial colonies. Depending on the colony-forming unit (CFU) count observed, representative colonies were selected from each plate for further analysis. These selected colonies were sub-cultured onto fresh TSB agar plates to obtain pure colonies, thus enabling the isolation and characterization of individual microbial strains present in the skin samples. Throughout this process, stringent measures were taken to maintain the environmental conditions consistent with the primary culture, ensuring the fidelity of the microbial growth and development.

2.2.3 Purification of skin isolates

The experimental process of purification of skin bacteria from the volunteers' samples is well-elaborated in the following descriptive steps to ensure the clarity and detail.

Isolation of phenotypically different colonies:

- After collecting skin samples from the volunteers, you isolated bacteria colonies exhibiting distinct phenotypes.

Two-steps purification process:

- In the first purification step, single bacterial colonies were streaked onto corresponding plates to maintain subculture.
- Subsequently, in the second purification step, single colonies from subcultured plates were transferred into 96-well plates and diluted with Tryptic Soy Broth (TSB) until a dilution of 10^{-5} was achieved.
- The 96-well plates were then incubated in a shaking incubator at 37°C for 6 hours to allow bacterial growth and proliferation.

Spreading on TSB agar plates:

- Following the incubation period, 80µl from the last two dilutions in the 96-well plates were spread onto TSB agar plates.
- These agar plates were then incubated overnight at 37°C to facilitate the growth of bacterial colonies.

Incubation in Erlenmeyer flasks:

- Single pure colonies from the overnight incubated agar plates were selected and inoculated into 100 ml Erlenmeyer flasks containing 10 ml of TSB.
- The flasks were aerobically incubated at 37°C for 24 hours in a shaker, allowing for optimal bacterial growth and metabolism.

Collection of culture supernatants:

- After the 24-hour incubation period, culture supernatants were collected from the Erlenmeyer flasks for further trace amine identification processes.
- The remaining bacterial cultures were preserved with 60% glycerol and stored at -80°C freezer for future use.

This systematic approach ensures the isolation of pure bacterial cultures and provides ample material for subsequent analyses. The chronological depiction of this process in the following figures will serve as a visual aid to enhance understanding and facilitate replication of the experiment.

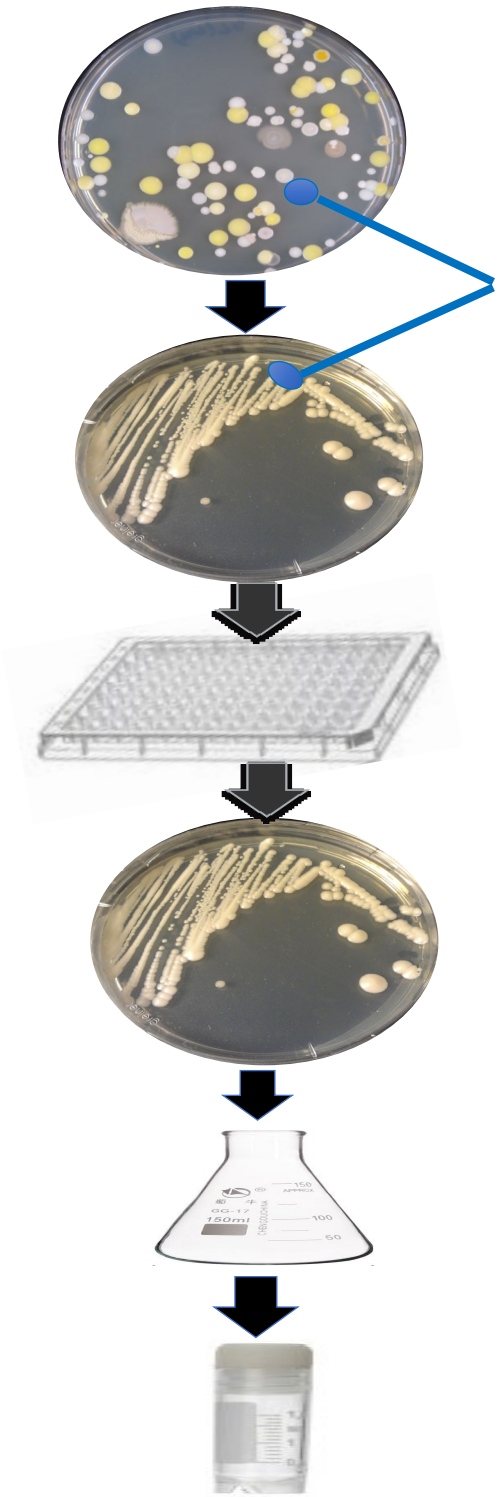


Figure 9: Steps of purification and storage of skin isolates.

2.2.4 Isolation of lactic acid bacteria and Corynebacterium

The specialized isolation procedures for lactic acid bacteria and Corynebacterium from healthy skin involved meticulous steps tailored to the unique growth requirements of these bacterial species.

For anaerobic isolation of lactic acid bacteria:

Preparation of swab samples:

- Cotton swabs were pre-moistened with aqueous sampling buffer to ensure efficient collection of skin samples.

Sample processing in Eppendorf tube:

- The swab samples were transferred into 2 ml Eppendorf tubes containing 1 ml of phosphate-buffered saline (PBS) to create a suitable environment for bacterial viability.

Vortexing and Centrifugation:

- The contents of the Eppendorf tubes were vortexed at the highest speed to ensure thorough mixing of the bacterial suspension.
- Subsequently, the tubes were centrifuged at 4°C and 1000 rpm for 2 minutes to facilitate the sedimentation of bacterial cells.

Supernatant removal and dilution:

- After centrifugation, the supernatant was carefully removed, leaving behind a bacterial pellet.
- The bacterial suspension was diluted with PBS to obtain dilutions of 10^{-1} and 10^{-2}.

Inoculation onto agar plates:

- Aliquots of 100 µl from the undiluted suspension, as well as from the 10^{-1} and 10^{-2} dilutions, were spread onto MRS (de Man, Rogosa, and Sharpe) and M17 agar plates.

Anaerobic incubation:

- The inoculated agar plates were placed in an anaerobic jar containing Anaerocult A and incubated at 37°C for 3 days to promote the growth of lactic acid bacteria under anaerobic conditions.

For Corynebacterium isolation:

The isolation procedure for Corynebacterium followed a similar protocol with slight modifications:

Sample processing:

- Swab samples were collected and processed as described above for lactic acid bacteria isolation.

Inoculation onto selective agar plates:

- Aliquots of the processed bacterial suspension were spread onto selective agar plates specifically designed for the isolation of Corynebacterium.

Aerobic incubation with CO2:

- The inoculated plates were incubated in an aerobic atmosphere supplemented with 5% CO2 at 37°C for 2-4 days to promote the growth of Corynebacterium species.

These specialized isolation procedures ensured the successful isolation and cultivation of lactic acid bacteria and Corynebacterium from healthy skin samples, providing valuable insights into the microbial composition of the skin microbiota

2.2.5 TAs identification from skin samples by HPLC

From each volunteer, distinct bacterial colonies exhibiting morphological variations were carefully selected for the identification of trace amines (TAs). Detailed criteria for colony selection are outlined in Table 1. Subsequently, a single pure colony from each selected morphotype was inoculated into 10 ml of Tryptic Soy Broth (TSB) and incubated aerobically at 37°C for 24 hours in a 100 ml Erlenmeyer flask with agitation at 120 rpm.

After incubation, the bacterial cultures were centrifuged, and 500 µl of the resulting supernatants were collected for analysis using reversed-phase high-performance liquid chromatography (RP-HPLC). The RP-HPLC analysis was conducted at room temperature with the following parameters: an Eclipse XDB-C18 column (4.6 x 150 nm; 5µm) (Agilent) equipped with an analytical guard column for Eclipse XDB-C18 column (4.6 x 12.5 mm; 5µm) (Agilent). A 15-minute linear gradient from 0.1% phosphoric acid to acetonitrile was applied, followed by a 5-minute wash with 0.1%

phosphoric acid. Each sample injection consisted of 10 µl, and the flow rate was maintained at 1.5 ml/min. Diode array detectors (DAD) were employed at wavelengths of 210 nm and 360 nm as reference points, with a total run time of 10 minutes per sample.

During the HPLC analysis, TSB media served as a negative control, where isolates were cultured to provide a baseline measurement of the three aromatic amino acids (AAAs). *Staphylococcus epidermidis* O47, known for its ability to convert AAAs to TAs, was utilized as a positive control to validate TA conversion. Additionally, the sadA deletion mutant strain of *Staphylococcus epidermidis* O47 was employed as a negative control to ensure the accuracy of our result analysis.

2.2.6 Identification of TA positive skin bacteria

For DNA extraction, we initiated the process by re-purifying the stored isolates through a 10-fold dilution into 96-well plates containing 100 µl of Tryptic Soy Broth (TSB). Following aerobic incubation, the cultures were streaked onto Tryptic Soy Agar (TSA) plates and incubated at 30°C for 24 hours. Single colonies were then selected and inoculated into 100 ml Erlenmeyer flasks containing 10 ml of TSB, followed by overnight incubation at 35°C with agitation at 120 rpm.

After overnight growth, 1.5 ml of the culture was transferred to Eppendorf tubes and centrifuged at 8000 rpm for 2 minutes. The resulting pellet was carefully resuspended in a solution containing lysostaphin (5 mg/ml concentration) and lysozyme (20 mg/ml concentration), followed by incubation at 37°C for 20 minutes. Subsequently, genomic DNA extraction was performed using the Quick DNA Microprep Kit (Zymo Research), following the manufacturer's protocol.

The purity and concentration of the extracted DNA were assessed using a Nanodrop spectrophotometer (IMPLEN NanophotometerR NP80). To amplify the 16S rRNA gene, universal primers (8F: AGAGTTTGATCATGGCTCAG and 16R: AAGGAGGTGATCCAACCGCA) and Q5 polymerase (New England Biolabs) were utilized in a polymerase chain reaction (PCR) assay. The PCR products were purified

using the Nucleospin Gel and PCR Clean-up kit (Macherey-Nagel) and then subjected to sequencing (GATC).

Bacterial species identification was accomplished by performing BLASTN analysis, comparing the obtained sequences of the amplified 16S rRNA gene from our skin isolates to the 16S rRNA database in EZBiocloud. This enabled accurate identification of the bacterial species present in our samples.

2.2.7 Authentication of TAs conversion using different AAAs substrates in minimal media

For this RP-HPLC analysis, we meticulously selected each of the trace amine (TA) positive bacterial species isolated from every volunteer. The HPLC was conducted with individual aromatic amino acid (AAA) substrates, including Tyrosine, Phenylalanine, Tryptophan, and also included L-Dopa, as well as with all these substrates combined.

Initially, the bacteria were cultured from freshly prepared plates, with a single colony picked and inoculated into 5 ml of Tryptic Soy Broth (TSB) in 15 ml Falcon tubes. The cultures were then incubated at 37°C with agitation at 140 rpm overnight in a slanted position. Following 17-18 hours of incubation, the optical density (OD) of the cultures was measured and adjusted to 0.1. Subsequently, the fresh cultures were transferred to 100 ml of TSB in 1 L Erlenmeyer flasks and incubated overnight under the same conditions as before.

After overnight incubation, 20 ml of culture was separately transferred to 15 ml Falcon tubes, and the OD of each culture was checked. The cultures were then centrifuged at 4°C at 4700 rpm for 15 minutes, and the supernatants were discarded. Subsequently, 4 ml of 1X phosphate-buffered saline (PBS) was added to each Falcon tube, and the contents were thoroughly mixed before centrifugation at 4°C at 4700 rpm for another 15 minutes. The resulting cell pellets were collected for further analysis.

In the subsequent step, the required volume of minimal media containing 5xPBS and 10% glucose with different substrates was added separately to each Falcon tube,

depending on the OD of each culture. The tubes were then incubated at 37°C with agitation at 140 rpm in a slanted position. Following 18 hours of incubation, all falcon tubes were centrifuged at 4°C at 4700 rpm for 15 minutes, and the supernatants were collected for HPLC analysis.

2.2.8 Identification of different aromatic amino acid decarboxylases (AADCs)

For the identification of genes responsible for decarboxylation in skin isolates exhibiting different trace amine (TA) conversion patterns, we employed PCR amplification with specific primer sets targeting Tyrosine decarboxylase (TDC) and Staphylococcus aromatic amino acid decarboxylase A (Particularly designed SadA for *S.epidermidis* and *S.coagulans*) genes. These genes were selected based on the most abundant TA patterns observed in the last RP-HPLC analysis.

For TDC identification, we utilized Q5 polymerase from New England Biolabs. The PCR reaction mixture consisted of template DNA isolated from representative positive skin isolates from each TA cluster, along with specific forward and reverse primers targeting the TDC gene.

For the identification of SadA genes, we employed ReproFast polymerase from Genaxxon Bioscience. Similar to TDC identification, the PCR reaction mixture contained template DNA from representative positive skin isolates, along with specific forward and reverse primers targeting the SadA gene.

The detail about these PCR is as follows:

Primers	Sequence 5‘-3‘	Thermal cycle
TDC Dec5f (Sebastiaan P, 2019)	CGTTGTTGGTGTTGTTGGCACNACNGARGARG	98^0c: 2.30 min 98^0c: 15 sec 50^0c: 30 sec 72^0c: 1.30 min 72^0c: 3 min
TDC Dec3r (Sebastiaan P, 2019)	CCGCCAGCAGAATATGGAAYRTANCCCAT	
SadA F-G1 (*S.epidermidis*)	CKGATAAGATATGTGAWAGTCC	98^0c: 5 min 94^0c: 30 sec 48^0c: 15 sec 72^0c: 24 sec 72^0c: 10 min
SadA R-G1 (*S.epidermidis*)	GTMTTTGTATCTGGYGGTTCWA	
SadA 52-S (*S.coagulans*)	GCGTTACGTTGATACAGAGA	98^0c: 5 min 94^0c: 30 sec 50^0c: 15 sec 72^0c: 22 sec 72^0c: 10 min
SadA 52-S (*S.coagulans*)	CCAGCACGATATGTATTTAGC	

Following PCR amplification, the resulting products were analyzed using agarose gel electrophoresis to confirm the presence of the expected amplicons corresponding to the TDC and SadA genes.

3.Results

3.1 Isolation of skin bacteria

3.1.1 Cultural characterization of skin bacteria

Skin bacteria were obtained from thirty healthy volunteers, specifically from the antecubital fossa, a region known to harbor a diverse array of microorganisms and is easily accessible for sample collection.

For the isolation of mesophilic aerobic skin bacteria, we utilized Tryptic Soy Broth (TSB) agar plates, a commonly used medium. Morphologically distinct colonies were observed on these plates, exhibiting variations in size, shape, and color, as illustrated in Figure 10.

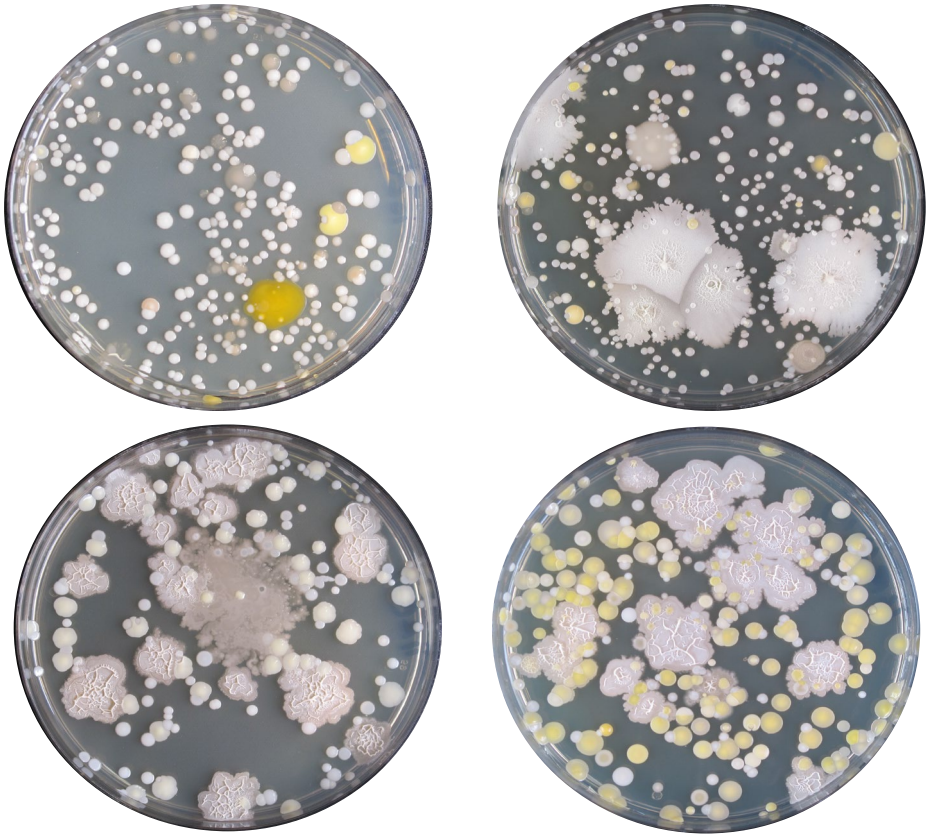


Figure 10: TSA plates with different colonies of mesophilic aerobic bacteria

Additionally, we employed selective media such as SK salt agar, Tellurite blood agar, Mannitol sucrose agar, MRS agar, and M17 agar to isolate specific bacterial species. Interestingly, no colonies were observed on Cetrimide agar, suggesting the absence of certain bacterial species targeted by this medium.

Although initial attempts to isolate Corynebacterium using Tellurite blood agar were unsuccessful, we successfully isolated different species of Corynebacterium using an alternative selective medium formulated specifically for this purpose. The colonies obtained on these selective media are depicted in Figures 11 and 12.

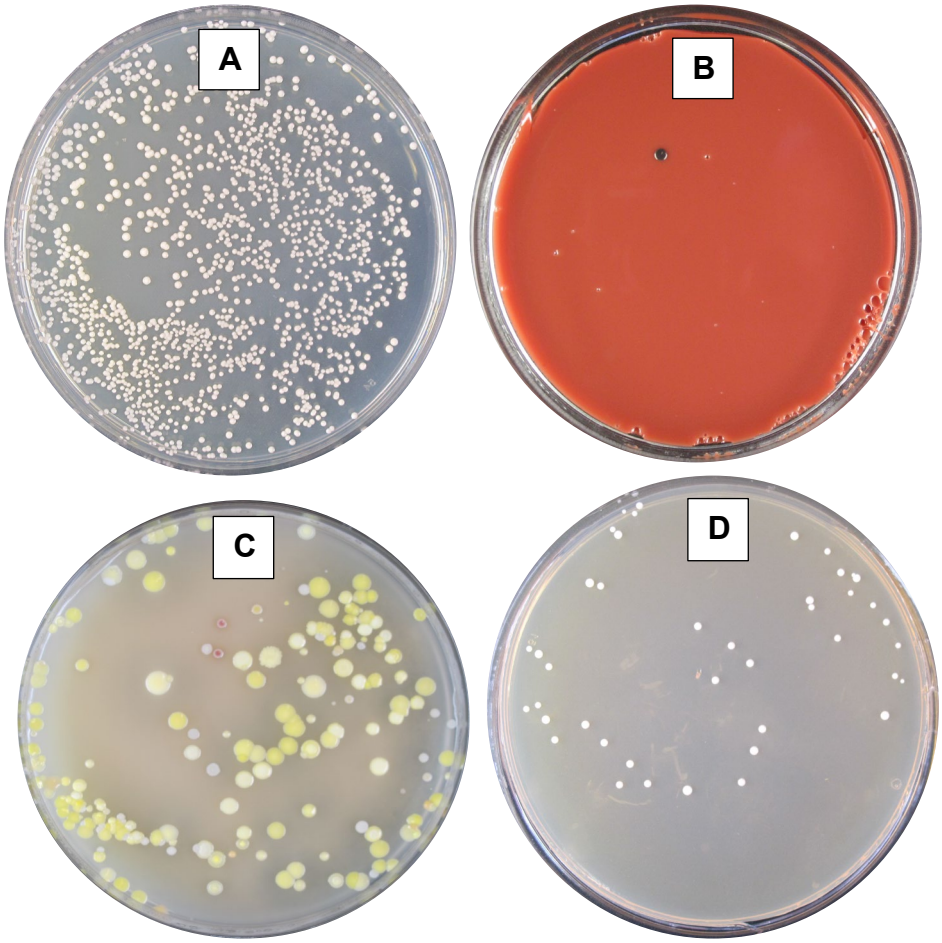


Figure 11: Some selective media with different colonies of mesophilic skin bacteria; (A) SK salt agar used for Staphylococcus, (B) Tellurite blood agar used for Corynebacterium, (C) MS agar used for Streptomyces, and (D) MRS agar used for LAB

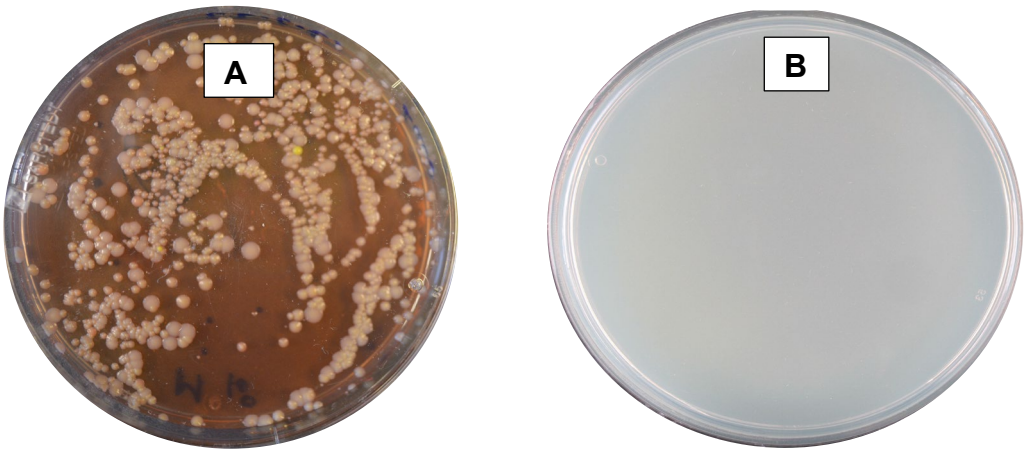


Figure 12: (A) Corynebacterium selective media exhibited pink color colony of Corynebacterium, (B) Cetrimide agar containing not any colony of Pseudomonas

However, our efforts to isolate lactic acid bacteria using MRSA and M17 agar were unsuccessful. This suggests that these media may not be conducive to the growth of lactic acid bacteria under the conditions tested.

Furthermore, we did not observe any targeted Streptomyces bacterial colonies on MS plates, although other non-selective bacterial species were identified on these plates. This highlights the importance of employing a variety of selective media to effectively isolate specific bacterial taxa from complex microbial communities such as those found on human skin.

3.1.2 Determination of abundance of skin bacteria

The colony forming unit (CFU) count per square centimeter (cm^2) of skin from each volunteer was determined by plating skin swab samples onto Tryptic Soy Broth (TSB) agar plates. The CFU count varied considerably among individuals, ranging from $19x10^{-1}$ to $1381x10^{-3}$ CFU/cm^2. This wide range of bacterial abundance on the skin of

different volunteers is depicted in Figure 13, which illustrates the CFU counts for each volunteer using a bar diagram.

Interestingly, our analysis did not reveal any statistically significant differences in CFU counts between male and female volunteers. Furthermore, we did not observe any substantial variations in bacterial counts across different age groups among the volunteers. These findings suggest that factors other than gender or age may play a more significant role in determining the bacterial abundance on the skin, highlighting the complexity of the skin microbiota composition and dynamics.

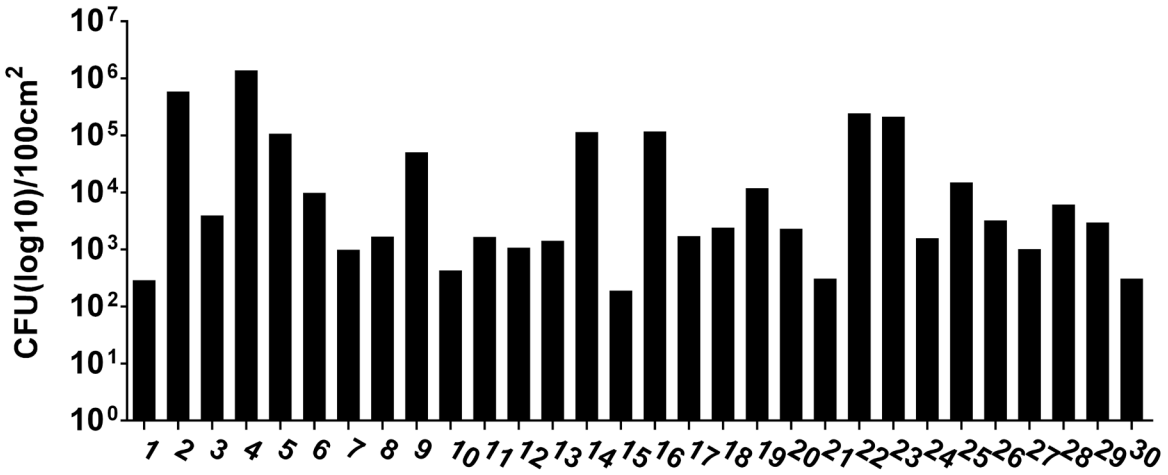


Figure 13: Bacterial count from each volunteer's skin sample

3.1.3 Isolation of skin bacteria

The isolation process yielded varying numbers of mesophilic skin bacteria from Tryptic Soy Broth (TSB) agar plates through aerobic cultivation. In total, we successfully isolated and purified 1909 morphologically distinct bacteria from these plates. The number of bacteria isolated from TSB agar plates varied depending on the individual skin sample, reflecting the impact of bacterial abundance on the skin on the number of isolates obtained per volunteer. A detailed summary of the isolation process, including the number of isolates obtained from each volunteer, is provided in Table 10.

The skin isolates from TSB agar plates, were prioritized for further analysis and recurrent experiments.

Additionally, we isolated and purified bacterial colonies from the specialized media mentioned earlier to investigate specific skin bacteria in our further experiments.

3.2 Determination of Trace amines (TAs) producing skin bacteria

3.2.1 Selection of colonies for TAs determination

In our research project, Tryptic Soy Broth (TSB) media served as the standard medium for isolating both aerobic and mesophilic skin bacteria. Colonies were selected based on the colony-forming unit (CFU) range observed on the agar plates for subsequent trace amine (TA) identification. A large number of colonies were observed on the TSB agar plates, spanning various CFU ranges, including >10,000, 1000-10,000, 500-1000, and 200-500. Depending on these CFU ranges, pure isolates were selected for the TA identification process using reverse-phase high-performance liquid chromatography (RP-HPLC). The sorting of colonies for TA identification is detailed in Table 10.

Table 10: Number of isolated colonies for TA determination from different volunteers

CFU range on plate	**TSB agar**	
	Colonies analyzed for HPLC	**Total**
>10,000	89 colonies X10 volunteer	890
1000-10,000	60 colonies X14 volunteer $^{+6*}$	846

500-1000	41 colonies X 2 volunteer +1*	83
200-500	22 colonies X 4 volunteer +2*	90
Total colonies collected for TA identification		**1909**

Legends:
+6*- six more colonies were selected due to different morphology
+2*- two more colonies were selected due to different morphology
+1*- one more colony was selected due to different morphology

3.2.2 HPLC analysis for TA determination

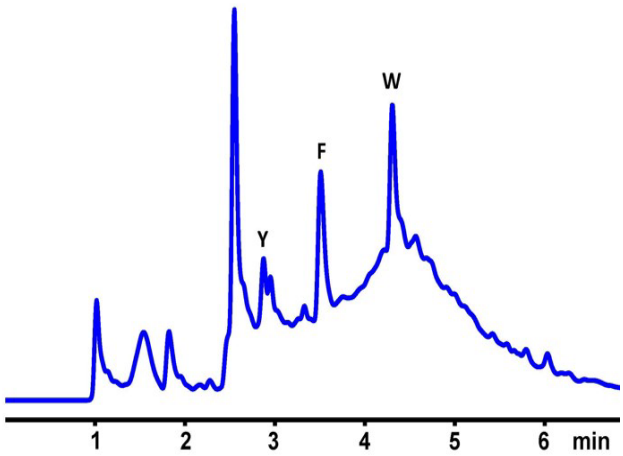
TSB media proved advantageous for isolating mesophilic aerobic skin bacteria, as all isolates were able to grow on it. Additionally, TSB contains relatively high amounts of aromatic amino acids (AAAs) such as Tryptophan (W), Phenylalanine (F), and Tyrosine (Y), as reported in a previous study (Luqman A. et al., 2018). We conducted reverse-phase high-performance liquid chromatography (RP-HPLC) to identify trace amine (TA) production by the skin isolates, screening TA producers from TA non-producers.

During RP-HPLC analysis, the column was calibrated with commercial AAA and TA standards, as well as with the supernatant of *S. epidermidis* O47, known to possess a very unspecific AAA-decarboxylase (SadA) that converts all three AAAs into the corresponding TAs, which are then excreted. The chromatograms of TSB and *S. epidermidis* O47 served as negative and positive controls, respectively, with an overlay of both controls displaying peaks of AAAs and TAs. The retention times of AAAs and TAs in the HPLC study were recorded.

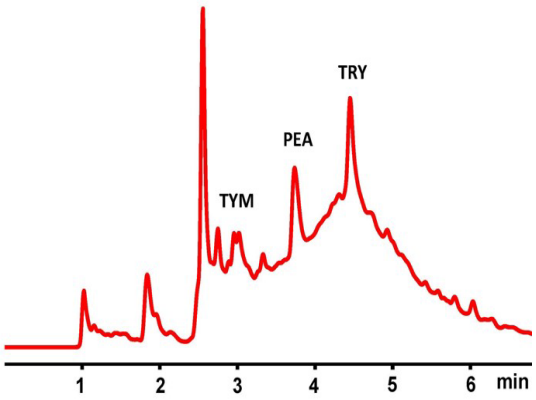
Table 11: Retention time of AAAs and TAs in HPLC

Standards		Retention time (Minute)
Aromatic amino acid (AAAs)	**Tyrosine (Y)**	**2.903**
	Phenyalanine (F)	**3.570**
	Tryptophan (W)	**4.321**
Trace amines (TAs)	**Tyramine (TYM)**	**2.791**
	Phenylthylamine (PEA)	**3.366**
	Tryptamine (TRY)	**4.536**

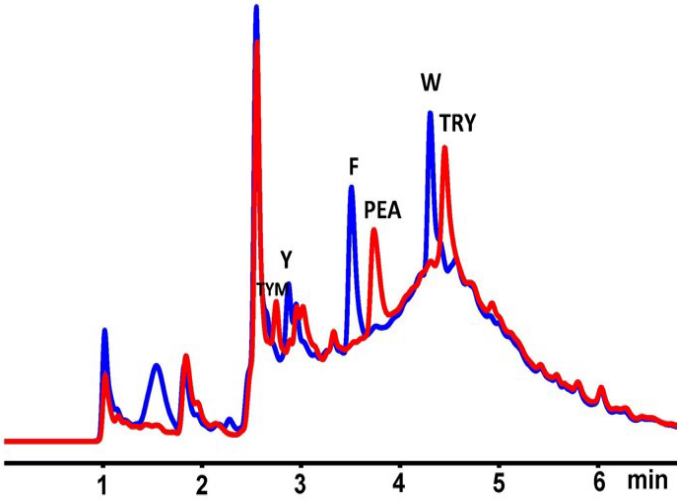
The HPLC chromatograms of skin isolates were analyzed by comparison with *S. epidermidis* O47 as a positive control and TSB media as a negative control. To authenticate our chromatogram analysis, we also cross-checked with a SadA mutant of *S. epidermidis* O47 in all our analyses.



TSB (Negative control)



Staphylococcus epidermidis O47 **(Positive control)**



Overlay of TSB and O47

Figure 14: HPLC chromatograms of TSB, *S.epidermidis* O47 and their overlay

Out of 1909 skin isolates, 269 isolates (14%) were able to convert at least one AAA into its corresponding TA.

Table 12: TA positive skin isolates from RP-HPLC analysis

No of colonies collected for TA identification	
Total no	**1909**
TA positive	**269**
%	**14**

During the HPLC investigation, varying numbers of TA-producing skin bacteria were discovered, with differences observed between individuals and their skin samples (Figure 15). The highest production rate (56%) was observed in volunteer number 12, while the lowest rate (3%) was observed in volunteer number 10. Interestingly, there

wasn't always a direct correlation between bacterial count and the rate of TA production within this range.

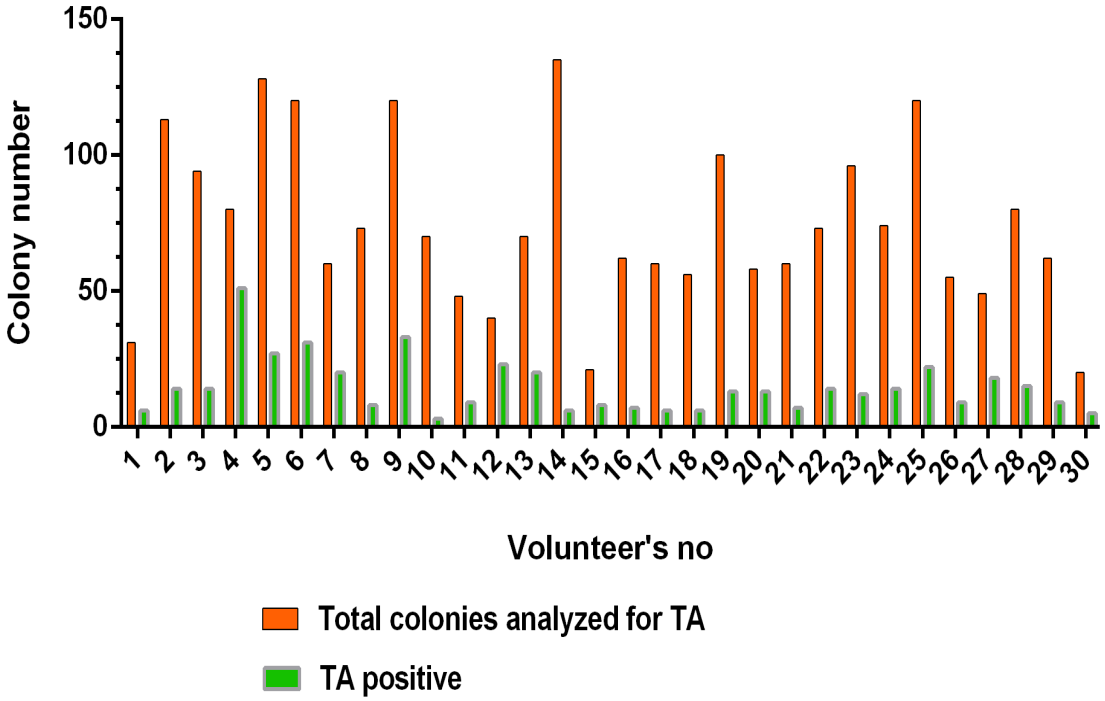
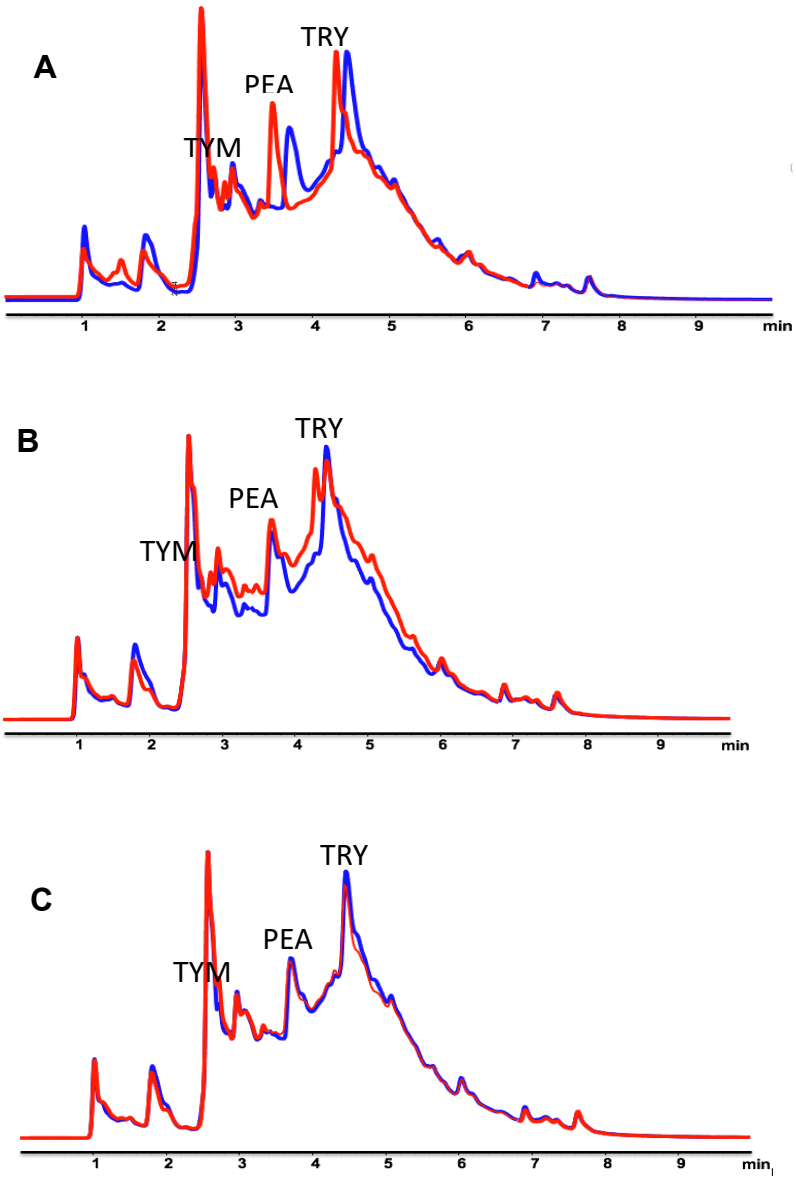


Figure 15: TA-positive Isolates in each volunteer

The TA identification process using HPLC provided insights into TA production diversity among skin bacteria. It was observed that bacterial isolates could metabolize single TAs as well as combinations of two or three TAs simultaneously. Among individual TAs, TYM producers (3.6%) were most abundant, followed by TRY producers (2.6%), and PEA producers (0.3%). The widest variety of TA production patterns was observed in TYM+PEA+TRY, accounting for 5.0% of the total TA producers. Table 13 provides the actual numbers and percentages of each TA variety produced by skin bacteria.

Table 13: TA distribution of isolated TA producing skin bacteria

	TYM	PEA	TRY	TYM+ PEA	PEA+ TRY	TYM+ TRY	TYM+PEA +TRY	Total Positive Isolates (out of 1909)
No. of TAs Positive	71	05	49	10	38	07	89	269
%	3.6	0.3	2.6	0.5	2.0	0.4	5.0	14



Retention time

Figure 16: Exemplary RP-HPLC chromatograms on different varieties of TAs conversion; (A) TYM positive peaks of skin sample that is red color overlayed with *S.epidermidis* O47 in blue color, (B) PEA+TRY positive peaks of skin sample that is red color overlayed with *S.epidermidis* O47 in blue color, (C) TYM+PEA+TRY positive peaks of skin sample that is red color overlayed with *S.epidermidis* O47 in blue color.

3.3 Bacterial species identification from the TA positive isolates

3.3.1 Distribution of TA positive skin bacteria

To identify the species of TA-producing skin isolates, we employed the 16S rRNA sequencing method. From the HPLC analysis, we identified 14% of bacterial isolates as TA-positive, with TA producer candidates predominantly belonging to the Staphylococcaceae family, followed by the Bacillaceae family.

Of the TA-producing bacterial isolates, 80% originated from the Staphylococcaceae family, while the remaining 20% were attributed to the Bacillaceae family. This distribution is illustrated in Figure 17, where a pie chart displays the percentage differentiation between TA non-producers and TA producers, highlighting the two main TA contender bacterial families.

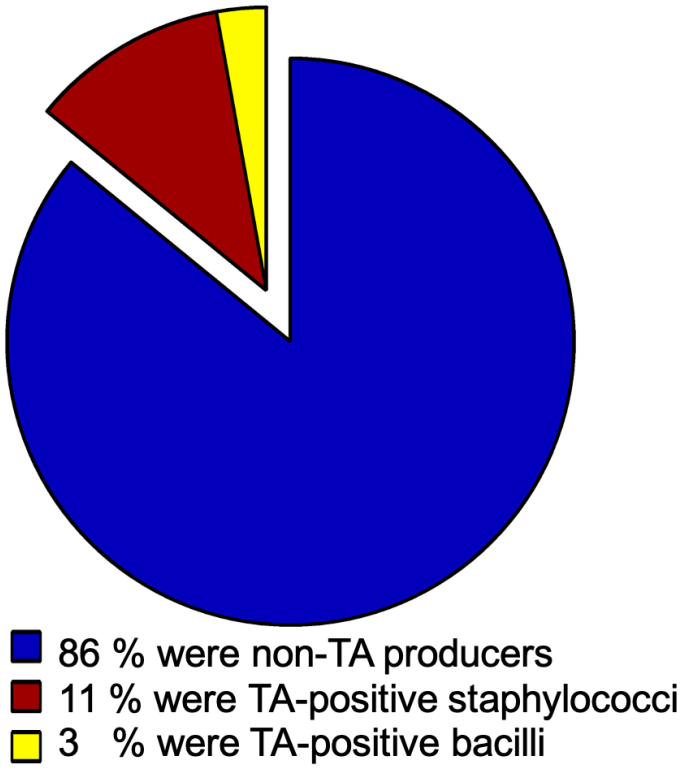


Figure 17: Distribution of TA producer families on skin

Table 14: Identified Bacterial species by 16s rRNA sequencing from TA positive skin isolates

Bacterial Family	Bacteria	No of Isolation	Total (Out of 1909)
Staphylococcacae	*Staphylococcus*		215 (80%)
Bacillacae	*Bacillus*	37	54 (20%)
	Peribacillus	07	
	Brevibacterium	06	
	Brevibacillus	03	
	Paenibacillus	01	
			Total: 269 (14%)

3.3.2 Distribution of TA positive bacterial species

In our investigation of bacterial species specialized in TA production within the skin microbial niche, we observed a predominant presence of Staphylococcus species, particularly *Staphylococcus epidermidis*, which accounted for 48% of the TA-producing species within the Staphylococcus genus. Additionally, other Staphylococcus species such as *Staphylococcus hominis* (31%), *Staphylococcus saccharolyticus* (5%), *Staphylococcus capitis* (7%), *Staphylococcus coagulans* (4%), and *Staphylococcus caprae* (5%) were identified as contributors to TA conversion. Some less prevalent Staphylococcus species involved in TA conversion included *Staphylococcus saprophyticus, Staphylococcus haemolyticus*, and *Staphylococcus pragensis*. This distribution of Staphylococcal species specialized in TA production is depicted in Figure 18.

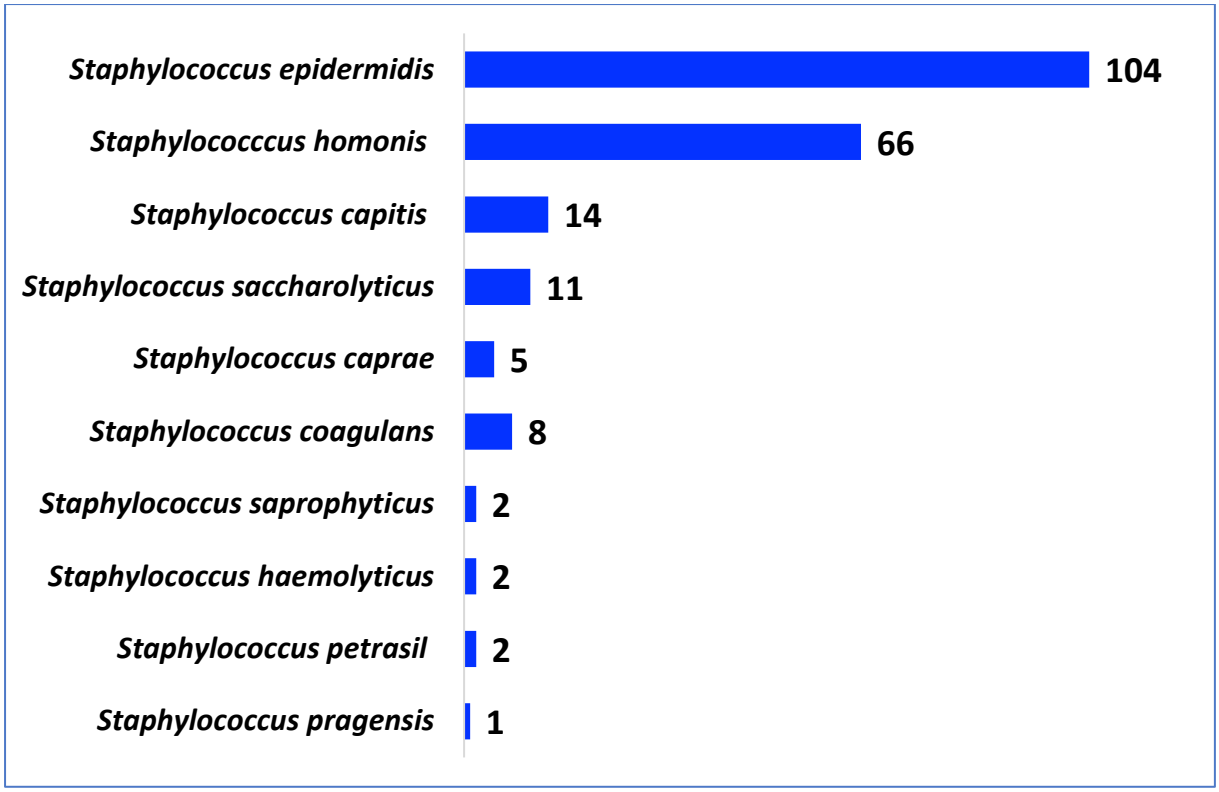


Figure 18: Number of TA-positive Staphylococci

Furthermore, Bacillus species also exhibited a notable percentage of TA production capability, with a diverse range of species identified as TA producers. Among these, *Bacillus licheniformis* stood out, representing approximately 22% of the total TA-producing Bacillus species. Other significant TA-producing Bacillus species included *Bacillus velezensis, Bacillus tequilensis, Peribacillus frigoritolerans, Peribacillus simplex, Bacillus siamensis,* and *Peribacillus butanolivorans,* along with *Brevibacillus borstelensis*. Figure 19 illustrates the distribution of these Bacillus species as a bar diagram.

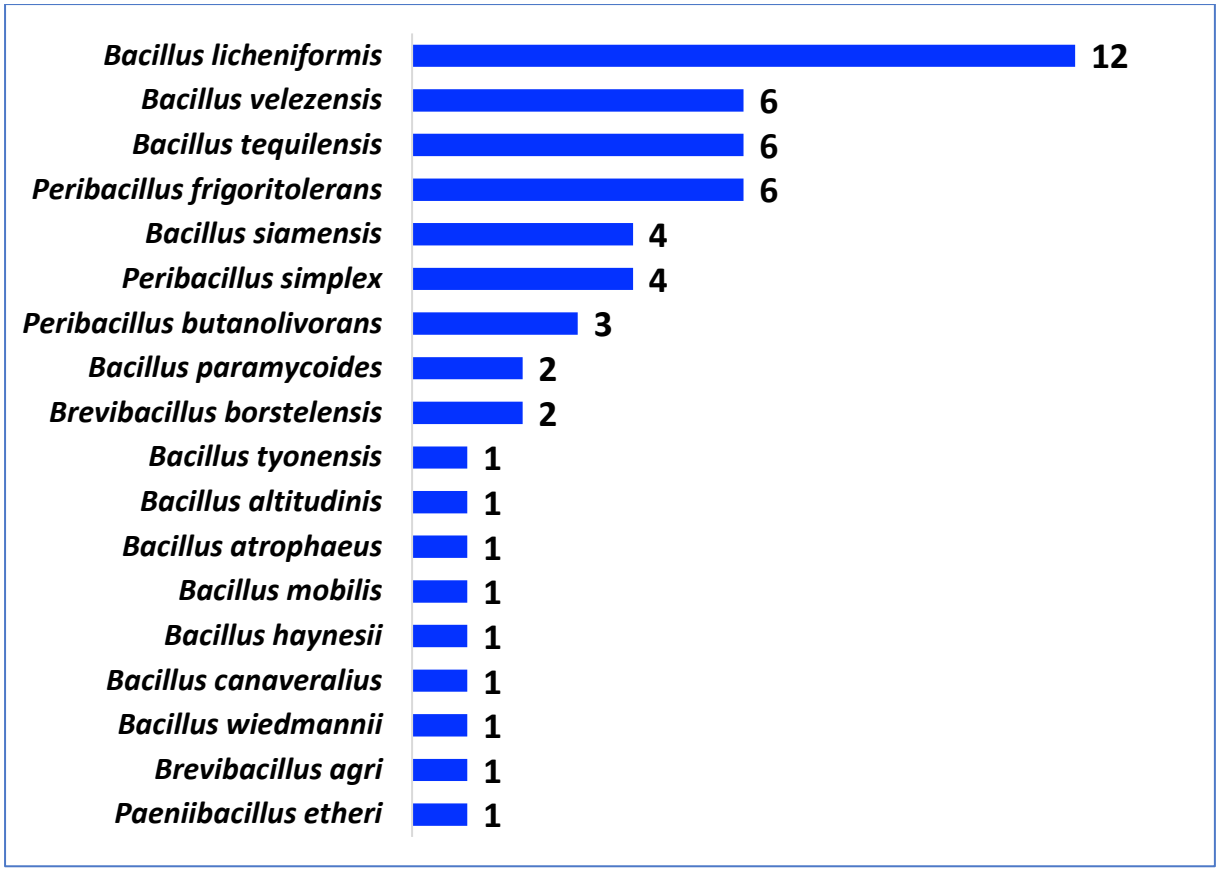


Figure 19: Number of TA-positive Bacilli

3.3.3 Identification of TA-non producer bacterial isolates

Following the HPLC analysis to categorize skin isolates into TA producers and non-producers, we conducted 16S rRNA sequencing on some of the non-producer isolates. Analysis of the sequencing results using EZ BioCloud revealed various bacterial species from three different bacterial families. This outcome indicated that certain Staphylococcal and Bacillus species were unable to convert AAAs into TAs. The list of TA non-producer bacteria is provided in Table 15.

Table 15: List of some mesophilic aerobic skin isolates not producing TAs

Bacterial species
Micrococcus endophyticus
Micrococcus luteus
Nialia circulans
Kocuria arsenatis
Bacillus albus
Bacillus cereus
Mixta intestinalis
Staphylococcus caledonicus

Additionally, despite efforts to isolate lactic acid bacteria (LAB) through anaerobic cultivation in special media, we were unsuccessful in isolating any lactobacillus or lactococcus species.

3.3.4 Special consideration for Corynebacterium and other selective bacteria

In our trace amine focusing project, we targeted additional skin bacteria by using special media in a CO2-enriched environment. Through 16S rRNA sequencing, we identified various species of Corynebacterium using a specialized media recipe, as well as several other bacterial species. However, during RP-HPLC analysis, all of these isolates exhibited negative results for TA production. The TA non-producer *Corynebacterium spp* and other bacterial species are listed in Table 16.

Table 16: Isolated bacteria from Corynebacteria special media

Isolated Bacterial species
Corynebacterium meitnerae
Corynebacterium kefirresidentii
Corynebacterium tuberculostericum
Corynebacterium bouchesdurhonense
Corynebacterium riegelii
Corynebacterium mucifaciens
Corynebacterium curieae
Corynebacterium fournieri
Corynebacterium gottingense
Corynebacterium parakroppenstedtii
Cutibacterium acne
Cutibacterium avidum
Streptococcus thermophillus

Streptococcus anginosus
Serratia nematodiphilia
Roseomonas mucosa

3.4 Divergence of TA in identified skin isolates

We further utilized the RP-HPLC method to verify the TA production patterns by treating bacterial cells individually with different substrates: Tyrosine, Phenylalanine, Tryptophan, and at this stage, we also added Levodopa (L-Dopa), as well as the combination of all these substrates. Over time, bacteria showed their decarboxylation capacity and converted AAAs into their corresponding TAs. Through this analytical approach, we gained valuable insight into TA production, discovering that bacterial isolates have the ability to convert not only a single TA but also multiple TAs at once, often in combination with the classical neurotransmitter Dopamine.

Among the TA conversion profiles, we identified two predominant patterns in different skin bacteria. The widest variety was observed in TYM+PEA+TRY+Dopamine, while TYM+PEA+Dopamine ranked second. Figure 20 showcases these HPLC chromatograms, featuring our calibrated *Staphylococcus epidermidis* O47 as a positive control, along with two exemplary chromatograms of skin isolates (Figure 21 and Figure 22).

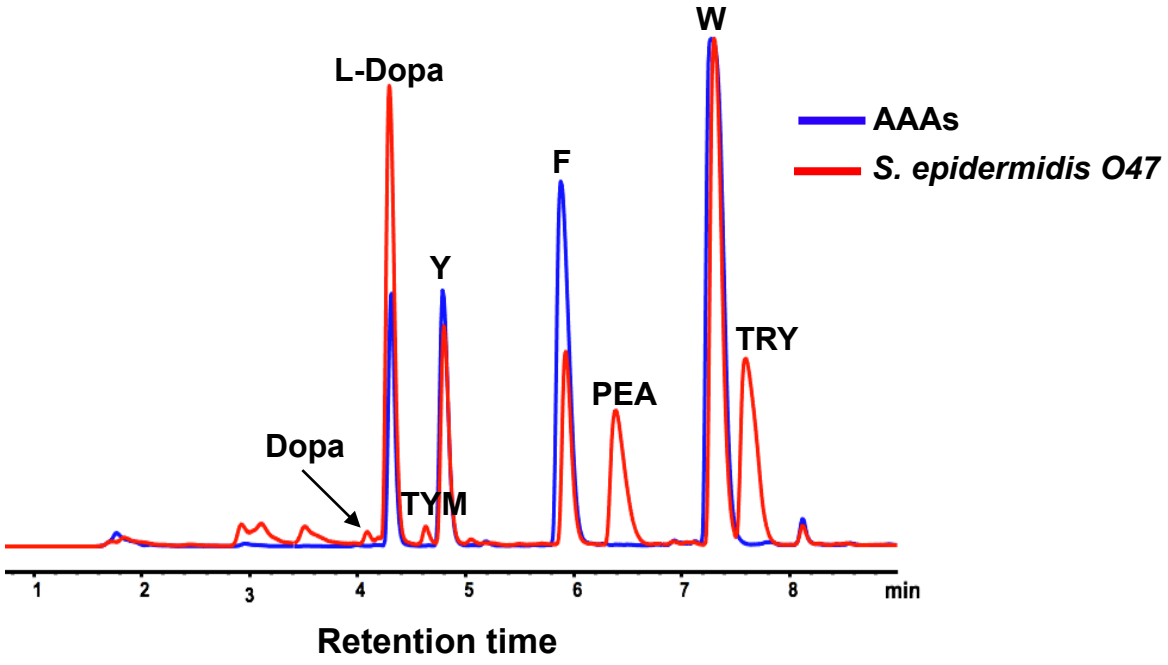


Figure 20: Chromatogram of *Staphylococcus epidermidis O47* (positive control) after 18 hrs incubation

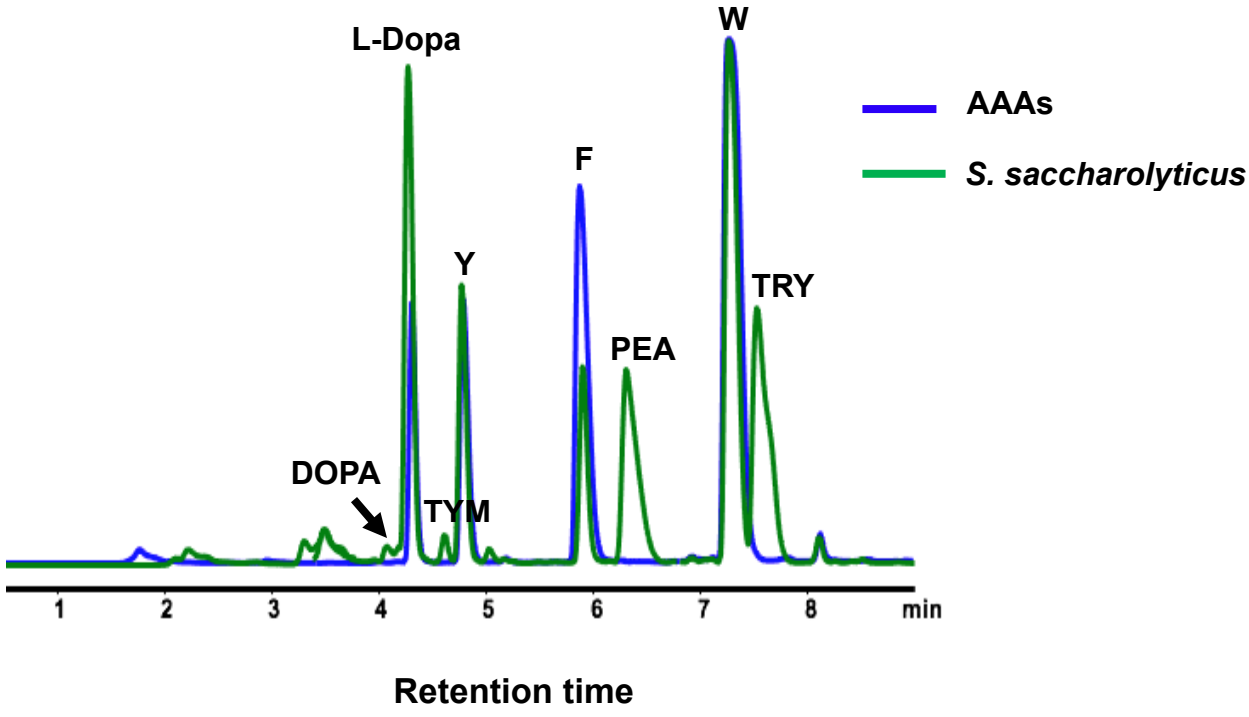


Figure 21: Exemplary TRY, PEA, TYM and DOPA producer Staphylococcal skin isolate chromatogram in RP-HPLC after 18 hrs incubation

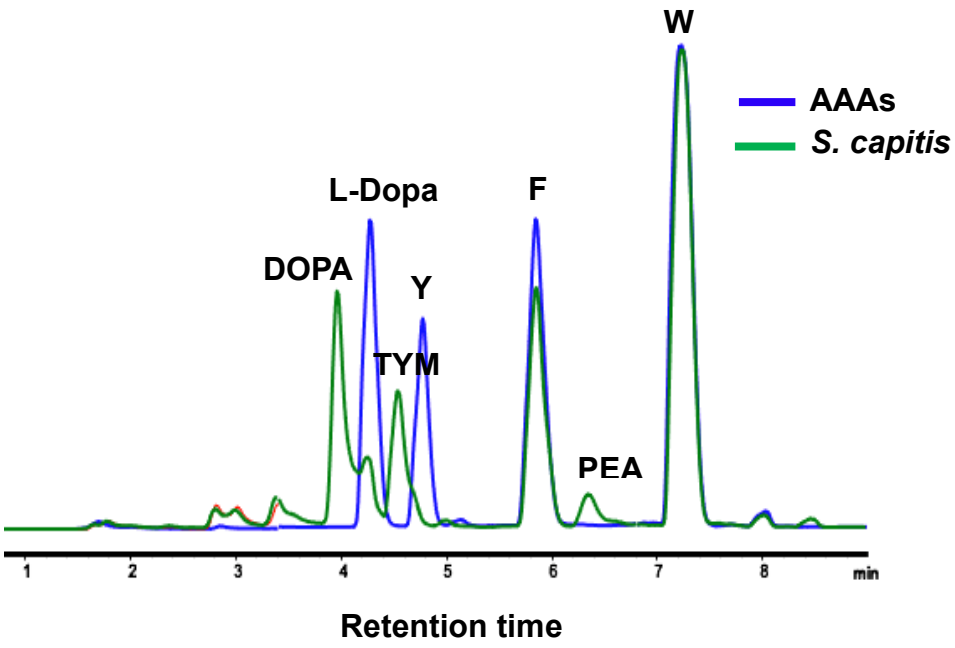


Figure 22: Exemplary PEA, TYM and DOPA producer Staphylococcal skin isolate chromatogram in RP-HPLC after 18 hrs incubation

We conducted a comparative study on the variability of TA production among bacterial isolates to determine the dispersion of TAs at the genus and species levels. Our analysis revealed that the Staphylococcal family had the highest number of TA producers, with its isolated members capable of converting TYM+PEA+TRY+Dopamine. Additionally, two species of Staphylococcus - *S. epidermidis* and *S. capitis* - were able to produce TYM+PEA+Dopamine. Conversely, two other Staphylococcus species - *S. haemolyticus* and *S. pragensis* - only exhibited TYM+PEA+Dopamine production.

The Bacillus group also played a significant role in various TA variants. From our skin isolates in the Bacillacae family, we identified sixteen species (refer to Fig 19), the majority of which (eight *Bacillus spp.)* demonstrated their ability to convert TYM+PEA+TRY+Dopamine. *B. tequilensis* and *B. paramycoides* were identified as producers of TYM+PEA+Dopamine, while *Peribacillus frigoritolerans* was found to

produce TYM+PEA+Dopamine and convert TYM+PEA+TRY+Dopamine, similar to Staphylococcus.

In addition to the comparative analysis results, we discovered additional clusters of TA conversion in various Bacillus species. Two distinct TA conversion patterns were identified, with four Bacillus species containing the TRY+Dopamine producer phenotype. On the other hand, three members of Bacilli were found to exhibit the TYM+Dopamine phenotype.

The highest disparity in TA conversion was displayed by *Bacillus licheniformis* and *Peribacillus frigoritolerans*. They were able to convert not only TYM+PEA+TRY+Dopamine but also produce TRY+Dopamine. *Peribacillus simplex* also exhibited three different TA production groups, including TYM+PEA+TRY+Dopamine, TYM+Dopamine, and TRY+Dopamine. All of these results are compiled below.

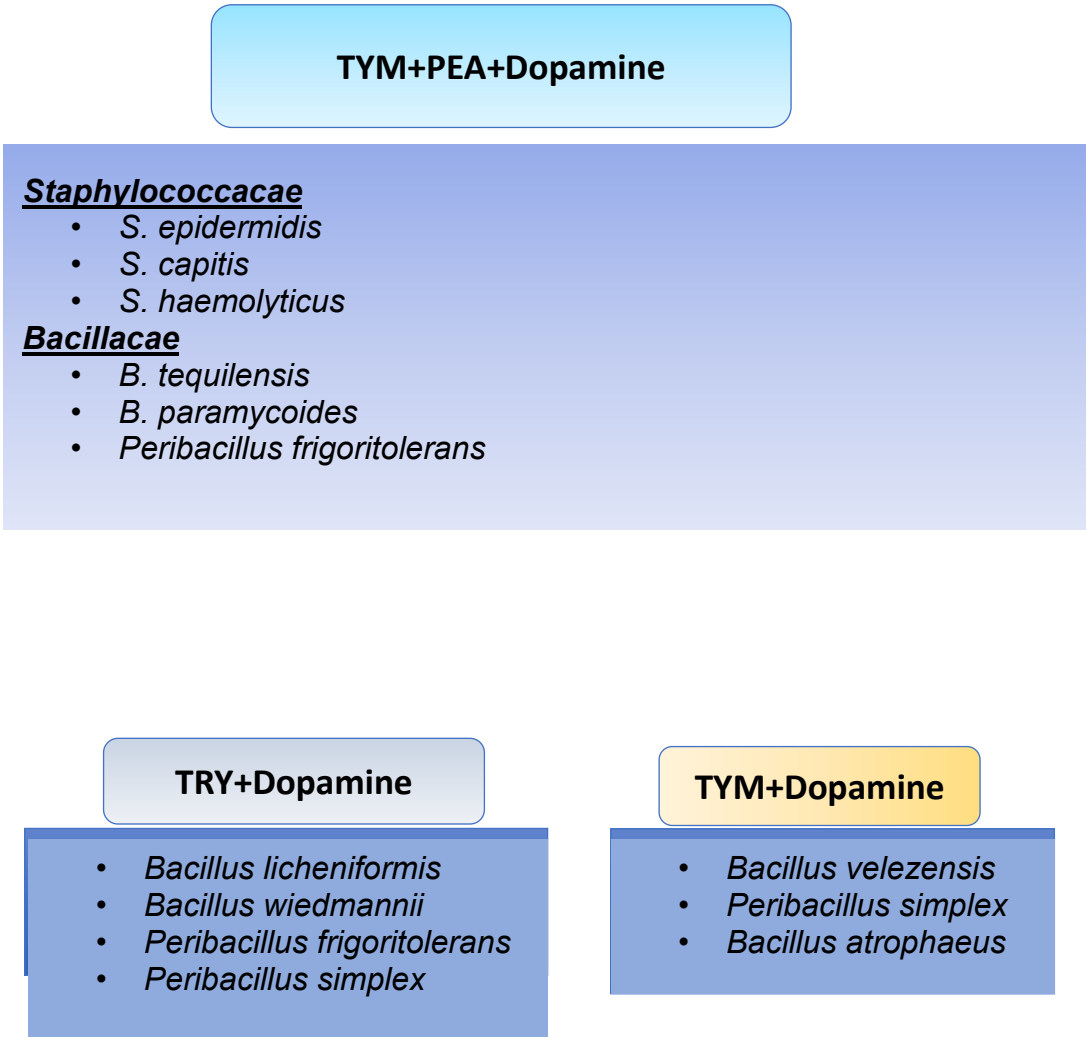
TRY+TYM+PEA+ Dopamine

Staphylococcaceae

- *S. epidermidis*
- *S. homonis*
- *S. saccharolyticus*
- *S. coagulans*
- *S. capitis*
- *S. caprae*
- *S. saprophyticus*
- *S. pragensis*

Bacillacae

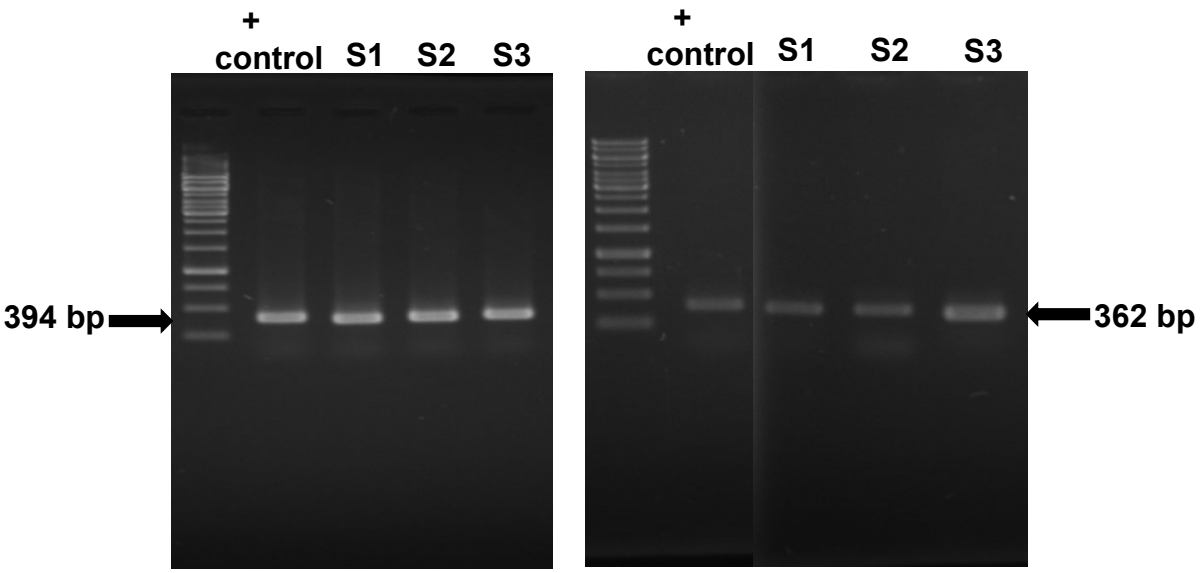
- *B. licheniformis*
- *B. canaveralius*
- *Peribacillus frigoritolerans*
- *Peribacillus simplex*
- *Peribacillus butanolivorans*
- *Brevibacillus borstelensis*
- *Brevibacillus agri*
- *Paeniibacillus etheri*



3.5 Identification of responsible gene of decarboxylation in skin isolates

One of the primary objectives of our research was to explore the different responsible genes for decarboxylase enzymes. These genes play a crucial role in aiding bacterial isolates to convert trace amines from their parent amino acids through the decarboxylation process. To achieve this goal, we focused on two pyridoxal 5' phosphate (PLP)-dependent aromatic amino acid decarboxylases (AAAD) known for their strong affinity for different aromatic amino acids.

Based on the abundance of trace amines in various skin isolates, we identified two key genes associated with specific conversion clusters. Among these genes, SadA is responsible for all three trace amine productions, along with the classical neurotransmitter Dopamine. To verify the presence of these genes, we selected isolated Staphylococcus species with known trace amine profiles. The following image of the gel displays the positive bands of skin isolates, along with the positive control, confirming the existence of the targeted genes.

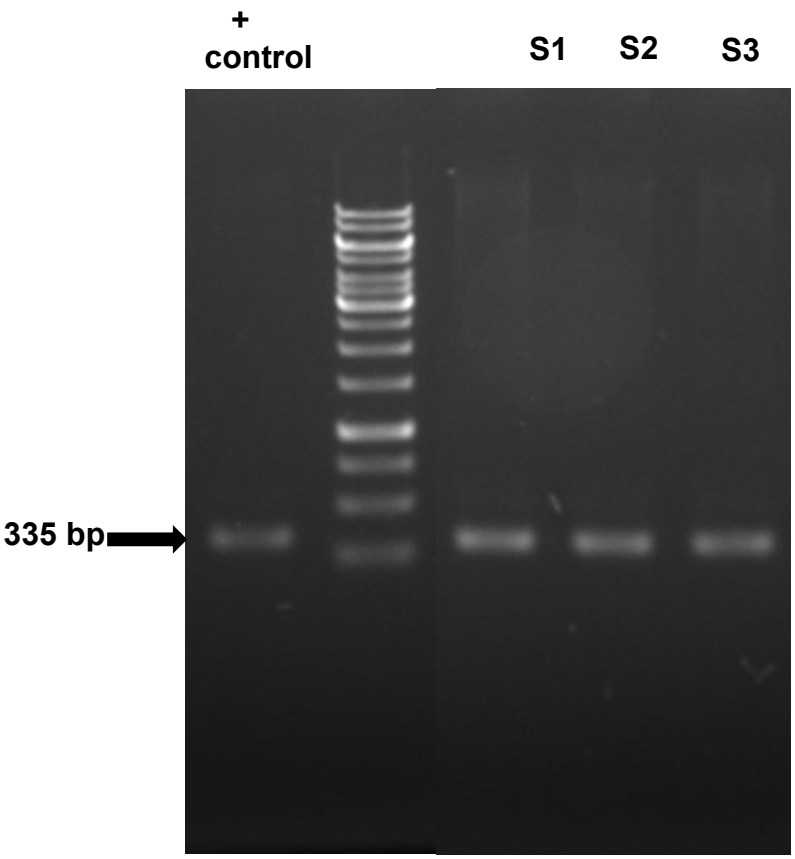


+ control: *S.epidermidis O47*
S1: Skin isolate 1,
S2: Skin isolate 2
S3: Skin isolate 3

+ control: *S.schliferi*
S1: Skin isolate 1,
S2: Skin isolate 2
S3: Skin isolate 3

Figure 23: Gel electrophoresis of SadA gene identification in skin isolates (1kb DNA ladder was used)

Given the enzyme's propensity to convert Tyrosine into Tyramine, it's plausible that the Tyrosine decarboxylase (TDC) enzyme may also exhibit affinity towards Phenylalanine and L-Dopa. In light of this, we sought to identify the TDC gene in certain skin isolates selected from the TYM+PEA+Dopamine group, which exhibited a high profile of trace amine conversion. The image below showcases the band corresponding to the TDC gene in the skin isolate, alongside the positive control, confirming the presence of the targeted gene.



+ control: *Enterococcus faecalis*
S1: Skin isolate 1,
S2: Skin isolate 2
S3: Skin isolate 3

Figure 24: Gel electrophoresis of TDC gene identification in skin isolates (1kb DNA ladder was used)

4. Discussion

4.1 Isolation of skin bacteria

The human skin serves as a potent barrier, inhibiting a wide variety of microbes. This is largely due to its structural composition, which attracts a diverse array of microorganisms. The diversity of these microbes is further facilitated by various extrinsic and intrinsic factors.

Our research project embarked on a study to isolate various skin bacteria from the antecubital fossa. We collected skin samples from the swabs of healthy male and female volunteers. During the isolation process, we determined the colony forming unit (CFU) count per 100 cm^2 area. Previous studies, such as Skowron, K. et al.,2021, have demonstrated variability in CFU counts across different skin sites. Typically, the CFU count on skin ranges from 10^3 to $10^4/cm^2$, but this can vary significantly depending on the site. In our study, we counted $19x10^1$ to $1381x10^3$ CFU/cm^2 from the forearms of thirty healthy volunteers.

In 2018, Luqman et al conducted a similar investigation on Staphylococcal colonization from the same anatomical site. They used SK salt agar media for colony counting, which ranged from 5 to 6000 CFU/cm^2.

To isolate diverse bacteria, particularly mesophilic aerobes from human skin, we utilized TSB agar media and other selective media. In our isolation procedure, *Staphylococcus spp.* was the most prevalent among the group of mesophilic aerobes, followed by *Bacillus spp*. and *Micrococcus spp*. However, we did not isolate any anaerobes using selective agar plates like MRSA and M17.

Culturing under CO_2 rich condition allowed us to isolate various *Corynebacterium spp*. using specially optimized media. According to the studies of Gallo, 2017; Byrd et al., 2018; McLoughlin et al., 2021, the majority of the skin microbiome is composed of four main groups of bacteria: Actinobacteria (36-51%), Firmicutes (24-34%), Proteobacteria (11-16%), and Bacteroides (6-9%).

When focusing specifically on skin bacteria isolated from different skin sites, *Staphylococcus*, *Cutibacterium*, and *Corynebacterium spp*. comprised the majority of the skin microbiome (Samaras, S. and Hoptroff, M., 2020).

4.2 Determination of trace amine producing skin bacteria

Trace amines, including Tyramine, Phenylalanine, and Tryptamine, are endogenous monogenic amines. Although they are structurally related to other classical neurotransmitters, their low tissue abundance distinguishes them from catecholamines and indolamines (Boulton, 1974, Berry, 2004).

The primary aim of this research project was to identify skin commensal bacteria that produce Trace amines (TAs). Our RP-HPLC analysis revealed that 14% of skin bacteria act as TA producers. Among these TA-positive skin isolates, *Staphylococcus spp.* were more prevalent than other skin bacteria, followed by Bacillus, which also contributes to TA production. Of these two TA-positive candidates, 80% of TA producers were from the Staphylococcaceae family, and the remaining 20% were from the Bacillaceae family (Table 13).

In contrast, Luqman et al. (2018) found that 20% of skin Staphylococcus was capable of TA production from 28 healthy volunteer skin samples in their trace amine research. Our investigation also revealed that the prevalence of TA-producing bacteria varied in individual skin samples, ranging from 56% to 3% (Fig. 15).

Evidence suggests that bacteria do not use AAA for their protein biosynthesis process. Instead, they utilize the imported AAAs and excrete them as TA via decarboxylation reaction (Luqman, A. et al 2018). Therefore, the availability of AAAs on the skin can accelerate TA production. Skin sweat, for instance, contains a high content of AAAs, approximately 60 µg/ml or 300 µM. Other studies have shown that the skin can carry around 10 mg/100cm^2 of AAA and 5 µg/100cm^2 of TAs (Luqman, A. et al 2020).

To address the question of the prevalence of TA-producing skin bacteria and their TA conversion pattern, we conducted RP-HPLC with skin isolates and compared them

with *Staphylococcus epidermidis* O47 as a positive standard for TA production. TSB media, a complete form of all AAAs for TA conversion, was used as a negative control in all our in vitro analyses. HPLC chromatograms of skin isolates showed significant variations in TA conversion patterns (Table 13). Among the TA producers, skin isolates did not always produce a single TA. They could also produce double and triple TAs. The highest number of TA production patterns was observed as TYM+PEA+TRY (5%). Considering single TA, TYM producers (3.6%) were more prevalent than other PEA and TRY producers. For the production of two TAs, PEA+TRY producers were more numerous than TYM+PEA and TYM+TRY producers.

4.3 Identification and distribution of TA positive bacterial species

Various research outcomes have noted that the predominant bacterial community on different anatomical sites of the skin primarily consists of staphylococci, coryneforms, micrococci, and bacilli. These are mainly isolated from the head, legs, and arms. While bacterial characterization and identification through different genomic approaches have revealed much more diversity than the conventional culture-based identification process, it's important to note that bacterial distribution is always dependent on the topographical position of skin sites (Grice, E. A. et al., 2009; Grice, E. A. & Segre, J. A., 2011).

Our project employed HPLC study to identify Trace Amine (TA) positive skin bacteria isolated from TSB agar media and some other special media. Out of 1909 purified mesophilic aerobic skin bacteria, 269 isolates showed positive peaks in the production of different TAs during HPLC analysis. To identify these TA producer bacterial species, we adopted the 16s rRNA sequencing method.

Out of the 14% TA positive isolates, 11% comprised 10 different species of Staphylococcus. Among the 215 TA conversion capable Staphylococcus, *Staphylococcus epidermidis* was the most prevalent, accounting for nearly half of all other *Staphylococcus spp*. Following *Staphylococcus epidermidis*, the second most prevalent was *Staphylococcus hominis* (66). Other TA producer *Staphylococcus spp*, such as *Staphylococcus capitis, Staphylococcus saccharolyticus, Staphylococcus*

coagulans, and *Staphylococcus caprae,* were identified in lesser numbers, 14, 11, 8 and 5 respectively (Fig. 18).

We also identified other Staphylococcal species as TA producers, with similar isolation numbers (Fig. 18). This smaller group of bacterial species includes *Staphylococcus saprophyticus, Staphylococcus haemolyticus, Staphylococcus petrasil* and *Staphylococcus pragensis*.

In line with our focus on TA-producing Staphylococcus from skin, Luqman, A. et al. (2020) conducted similar research. They identified four capable species from their TA-positive Staphylococcus isolates, which included 92.8% *Staphylococcus epidermidis*, 3.8% *Staphylococcus capitis*, 2.8% *Staphylococcus haemolyticus*, and 0.6% *Staphylococcus caprae*.

Our research project also yielded unique results by identifying different Bacillus species as TA converters. Of the 1909 TA-positive isolates, 54 were from 18 different species of Bacillus. Among this diverse group of Bacillus species, *Bacillus licheniformis* was the most common with 12 isolates. Other species, such as *Bacillus velezensis, Bacillus tequelensis,* and *Peribacillus frigoritolerans*, contributed 6 isolates each. *Bacillus siamensis* and *Bacillus simplex* each had 4 isolates, *Peribacillus butanolivorans* had 3, and *Bacillus paramycoides* and *Brevibacillus borstelensis* each had 2 isolates. In the 16s rRNA sequencing results, we also identified 9 other Bacillus species, each with only one isolate (Fig. 19).

This project, which focused on the skin microbiome's production of the neurotransmitter Trace amine, also validated the HPLC study of individual bacterial isolates from each volunteer's skin sample. We treated bacterial cells both separately and in combination with different substrates such as Tyrosine, Phenylalanine, Tryptophan, and the classical neurotransmitter L-Dopa. This was to test and evaluate the conversion pattern by different isolated bacterial species.

Most visible TA conversion patterns by various skin bacteria were TYM+PEA+TRY+Dopamine, and TYM+PEA+Dopamine. Almost all of the Staphylococcus species showed their ability to convert TYM+PEA+TRY+Dopamine, a result also mirrored in most of the Bacillus species. In the cluster of TYM+PEA+Dopamine producers, three species from Staphylococcus and Bacillus displayed this conversion pattern (Section 3.4). Despite these two main patterns of TA conversion, some Bacillus species could convert TYM+Dopamine and TRY+Dopamine from their precursor AAAs (Section 3.4).

Luqman et al.'s (2020) research on TA-producing Staphylococcus from skin revealed that their isolated Staphylococcus species were capable of producing three TAs, namely TYM, PEA, and TRY, all together. Interestingly, these isolates were also able to convert DOPA from L-DOPA along with other TAs.

In addition to Staphylococcus and Bacillus, other bacteria also demonstrated the ability to convert TAs, particularly TYM. These included various bacteria such as *Enterococcus faecalis* (Pessione et al., 2009), *Enterococcus faecium, Enterococcus durans* (Burdychova and Komprda, 2007), *Lactococcus lactis, Lactobacillus brevis* (Fernandez et al., 2004), and *Carnobacterium divergens* (Coton et al., 2004). This expands the scope of our understanding of TA production and conversion across a broader spectrum of bacterial species.

4.4 TA non producer bacteria isolated from skin

Under this trace amine-focused project, we also investigated some TA-negative skin isolates. Through the 16s rRNA sequencing approach, we identified different bacterial species from these TA-negative isolates. Selected from TSB agar plates, these included species from Staphylococcus, Bacillus, and Micrococcus (Table 15) that were incapable of converting TAs.

Additionally, the special media used in our research, aimed at targeting specific skin bacteria, revealed different TA non-converting *Corynebacterium spp*, and *Actinomycetes spp* (Table 16). However, it's worth noting that no other Streptomycetes

or Lactic acid bacteria were isolated in our project for trace amine study. This highlights the specificity and selectivity of TA conversion among different bacterial species.

4.5 Role of decarboxylases for the conversion of TAs

Trace amines (TAs) production is facilitated by aromatic amino acid decarboxylases (AADCs), which aid in the conversion of TAs from their parent amino acids (AAAs). Various AADCs are PLP-dependent enzymes that fall under four distinct subgroups. These subgroups are determined by their primarily aligned sequences and their conserved domains (Sandmeier, E., Hale, T. I. & Christen, P., 1994). Microbial AADCs enable some of the human commensals to convert TAs from their parent AAAs, which is beneficial for both humans and the bacteria itself (Luqman, A. et al., 2020 and 2018).

Based on our results regarding the trace amines production pattern created by different bacterial species, we further explored the AADCs responsible for two highly promising patterns from our TAs clusters. The NCBI protein blast with our reference genes guided us to identify the most probable genes in our TA-positive isolates. This in-depth analysis allows us to better understand the genetic mechanisms behind TA production and conversion.

Among the two highly repeated clusters by most of the bacteria, TYM+PEA+TRY+Dopamine was our primary focus for identifying the responsible gene for this conversion process. We conducted PCR with specific primer sets for this purpose. SadA (Luqman, A. et al., 2018) was our first target as a promising AADC for investigating our various isolated TA positive Staphylococcus species under this TAs conversion group, including *Staphylococcus epidermidis, Staphylococcus saccharolyticus, Staphylococcus coagulans, and Staphylococcus pragensis*.

From Luqman et al.'s research in 2020, we discovered that 20% of the 900 Staphylococcal isolates were producers of all three TAs, including DOPA (TYM+PEA+TRY+Dopamine). They also found that the majority of their isolates came

from *Staphylococcus epidermidis*, with more than 50% of strains carrying the SadA gene. The metagenomic analysis of healthy skin microbiota (n=27) also demonstrated the dispersion of SadA in 1100 species across seven bacterial phyla (Luqman, A. et al 2020b).

Our gene identification process then proceeded with TDC, due to its high affinity to Tyrosine, in some cases Phenylalanine, and also to the Tyrosine-related structure L-DOPA (Zhang, K. & Ni, Y., 2014; Moreno-Arribas, V. & Lonvaud-Funel, A., 1999). We selected some of our skin isolates under the TYM+PEA+Dopamine production group and identified TDC particularly in *Staphylococcus caprae* and *Staphylococcus haemolyticus*. The TDC gene was identified in different microbes such as *Lactobacillus brevis* (Zhang, K. & Ni, Y., 2014; Moreno-Arribas, V. & Lonvaud-Funel, 2011), *Enterococcus faecalis* (Borresen, T. et al., 1989), *Methanocaldococcus jannaschii* (Kezmarsky, N. D. et al.,2005), *Sporolactobacillus sp* (Coton, M. et al., 2011), and *Lactococcus lactis* (Thakur, M. & Azmi, W., 2009). This extensive research helps us better understand the genetic mechanisms behind TA production and conversion.

4.6 Outlook

For the future study, the following experiments would be suggested for better understanding of trace amine production by different microbes and also their underlying mechanisms.

- Cloning of the corresponding AADC gene in *E. coli* for in detail investigation
- Analyzing the substrate specificity

5. Summary and conclusion

The skin, our largest organ, forms a complex ecosystem teeming with microorganisms that collectively constitute the skin microbiota. This intricate microbial community plays crucial roles in maintaining skin health and overall well-being. Our research delved into the intricate dynamics of the skin microbiota, focusing on the production of trace amines by various commensal bacteria residing on human skin. Trace amines, including tyramine, phenylalanine, tryptamine, and dopamine, are biogenic amines known for their diverse physiological effects, including neurotransmission and modulation of immune responses.

The initial phase of our investigation involved isolating aerobic mesophilic bacteria from Tryptic Soy Broth (TSB) agar plates. To ensure a diverse representation, we collected skin samples from the antecubital fossa of thirty healthy volunteers, with an equal distribution of males and females. Employing a diligent two-step purification process, we successfully isolated 1909 bacterial strains from TSB media. Notably, our analysis revealed variations in colony-forming unit (CFU) counts among the volunteers ranging from $19x10^1$ to $1381x10^3$ CFU/cm^2, although no significant disparity was observed between male and female samples. This diversity underscored the richness of the skin microbiota and laid the foundation for further exploration.

Moreover, to target specific skin bacteria, we employed various specialized media, facilitating the isolation of bacterial colonies conducive to subsequent trace amine identification. The utilization of tailored media enabled us to capture a spectrum of bacterial species harboring diverse metabolic capabilities, thus enriching our understanding of the skin microbial community's functional diversity.

Subsequently, we employed Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) to identify trace amine producers among the isolated skin bacteria. This sophisticated analytical technique allowed us to detect and quantify trace amines produced by the bacterial strains. Through HPLC analysis, we identified that approximately 14% of the skin isolates exhibited the capacity to produce trace amines, highlighting the prevalence of this metabolic trait within the skin microbiota.

Notably, calibration of the HPLC column using *Staphylococcus epidermidis* O47 as a positive control and TSB as well as a sadA mutant of *Staphylococcus epidermidis* O47 as negative controls ensured the accuracy and reliability of our findings.

Furthermore, we elucidated the metabolic pathways underlying trace amine production by employing specialized media enriched with aromatic amino acids such as tyrosine, phenylalanine, and tryptophan. These media served as an optimal substrate for bacterial conversion of precursor amino acids into trace amines, facilitating their identification and quantification via HPLC chromatograms. The analysis revealed distinct patterns of trace amine production among different skin isolates, reflecting the metabolic versatility and diversity of the skin microbiota.

To validate the identity of trace amine-positive bacterial species, we employed 16S rRNA sequencing, a robust molecular technique capable of accurately identifying bacterial taxa. Our sequencing analysis revealed that 80% of the trace amine-positive isolates belonged to the Staphylococcaceae family, with *Staphylococcus epidermidis* emerging as the predominant species. Additionally, 20% of the isolates were attributed to the Bacillaceae family, with *Bacillus licheniformis* being the most prevalent species. This taxonomic characterization provided invaluable insights into the composition and diversity of trace amine-producing bacteria inhabiting the human skin microbiota.

Under the investigation procedure of trace amine capable bacteria on human skin, we also authenticated our isolated individual bacteria which showed positive in HPLC analysis with culture supernatant by treating the cell separately and cumulatively with Tyrosine, phenylalanine, Tryptamine along with L-DOPA. From this analytical approach, two clusters of TAs were making attention by most of the skin bacteria. This highly repeatable TAs pattern included TYM+PEA+TRY+Dopamine and TYM+PEA+Dopamine. Other clusters of TAs pattern we observed by isolated skin bacteria were Tyramine producer with Dopamine and Tryptamine producer along with Dopamine.

Our neurotransmitter investigation experiments also focused on identifying several non-TA producer skin isolates not only from TSB media but also from other special

media that we used under this studies. Those non-TA producer list from TSB media consisted with *Nialia circulans, Micrococcus endophyticus, Micrococcus luteus, Kocuria arsenatis, Bacillus albus, Bacillus cereus, Mixta intestinalis and Staphylococcus caledonicus.*

Additionally, from Corynebacterium special media we found various TA non producer *Corynebacterium spp* which includes *Corynebacterium meitnerae, Corynebacterium kefirresidentii, Corynebacterium tuberculostericum, Corynebacterium bouchesdurhonense, Corynebacterium riegelii, Corynebacterium mucifaciens, Corynebacterium curieae, Corynebacterium fournieri, Corynebacterium gottingense, Corynebacterium parakroppenstedtii* and some other bacteria such as *Cutibacterium acne, Cutibacterium avidum, Streptococcus thermophillus, Streptococcus anginosus, Serratia nematodiphilia, Roseomonas mucosa*.

Furthermore, we conducted experiments to elucidate the genetic determinants underlying trace amine production in skin bacteria. We tried to figure out the candidate aromatic amino acid decarboxylase (AADC) genes responsible for catalyzing the conversion of precursor amino acids into trace amines. PCR amplification of these genes allowed us to elucidate the genetic basis of trace amine biosynthesis in skin bacteria, shedding light on the molecular mechanisms driving this metabolic process.

In summary, our comprehensive investigation into the production of trace amines by skin commensal bacteria has unveiled the metabolic diversity and functional significance of the skin microbiota. Through a multidisciplinary approach encompassing microbiological, biochemical, and molecular techniques, we have elucidated the metabolic pathways, and genetic determinants of trace amine production in skin bacteria. These findings lay the groundwork for future research aimed at harnessing the therapeutic potential of skin microbiota-derived trace amines in promoting skin health and combating microbial infections.

References

1. Ando-Yamamoto, M., H. Hayashi, T. Sugiyama, H. Fukui, T. Watanabe and H. Wada (1987). "Purification of L-dopa decarboxylase from rat liver and production of polyclonal and monoclonal antibodies against it." J Biochem **101**(2): 405-414.

2. Ansorena, D., M. C. Montel, M. Rokka, R. Talon, S. Eerola, A. Rizzo, M. Raemaekers and D. Demeyer (2002). "Analysis of biogenic amines in northern and southern European sausages and role of flora in amine production." Meat Sci **61**(2): 141-147.

3. Aperia, A., T. Hokfelt, B. Meister, A. Bertorello, J. Fryckstedt, U. Holtback and I. Seri (1990). "The significance of L-amino acid decarboxylase and DARPP-32 in the kidney." Am J Hypertens **3**(6 Pt 2): 11S-13S.

4. Barbieri, F., C. Montanari, F. Gardini and G. Tabanelli (2019). "Biogenic Amine Production by Lactic Acid Bacteria: A Review." Foods **8**(1).

5. Bender, D. A. and W. F. Coulson (1972). "Variations in aromatic amino acid decarboxylase activity towards DOPA and 5-hydroxytryptophan caused by pH changes and denaturation." J Neurochem **19**(12): 2801-2810.

6. Berry, M. D. (2004). "Mammalian central nervous system trace amines. Pharmacologic amphetamines, physiologic neuromodulators." J Neurochem **90**(2): 257-271.

7. Borowsky, B., N. Adham, K. A. Jones, R. Raddatz, R. Artymyshyn, K. L. Ogozalek, M. M. Durkin, P. P. Lakhlani, J. A. Bonini, S. Pathirana, N. Boyle, X. Pu, E. Kouranova, H. Lichtblau, F. Y. Ochoa, T. A. Branchek and C. Gerald (2001). "Trace amines: identification of a family of mammalian G protein-coupled receptors." Proc Natl Acad Sci U S A **98**(16): 8966-8971.

8. Borresen, T., N. K. Klausen, L. M. Larsen and H. Sorensen (1989). "Purification and characterisation of tyrosine decarboxylase and aromatic-L-amino-acid decarboxylase." Biochim Biophys Acta **993**(1): 108-115.

9. Bouchard, S. and A. G. Roberge (1979). "Biochemical properties and kinetic parameters of dihydroxyphenylalanine--5-hydroxytryptophan decarboxylase in brain, liver, and adrenals of cat." Can J Biochem **57**(7): 1014-1018.

10. Boulton, A. A. (1974). "Letter: Amines and theories in psychiatry." Lancet **2**(7871): 52-53.

11. Boulton, A. A. and P. H. Wu (1972). "Biosynthesis of cerebral phenolic amines. I. In vivo formation of p-tyramine, octopamine, and synephrine." Can J Biochem **50**(3): 261-267.

12. Boulton, A. A. and P. H. Wu (1973). "Biosynthesis of cerebral phenolic amines. II. In vivo regional formation of p-tyramine and octopamine from tyrosine and dopamine." Can J Biochem **51**(4): 428-435.

13. Branchek, T. A. and T. P. Blackburn (2003). "Trace amine receptors as targets for novel therapeutics: legend, myth and fact." Curr Opin Pharmacol **3**(1): 90-97.

14. Brunkwall, L. and M. Orho-Melander (2017). "The gut microbiome as a target for prevention and treatment of hyperglycaemia in type 2 diabetes: from current human evidence to future possibilities." Diabetologia **60**(6): 943-951.

15. Bunzow, J. R., M. S. Sonders, S. Arttamangkul, L. M. Harrison, G. Zhang, D. I. Quigley, T. Darland, K. L. Suchland, S. Pasumamula, J. L. Kennedy, S. B. Olson, R. E. Magenis, S. G. Amara and D. K. Grandy (2001). "Amphetamine, 3,4-methylenedioxymethamphetamine, lysergic acid diethylamide, and metabolites of the catecholamine neurotransmitters are agonists of a rat trace amine receptor." Mol Pharmacol **60**(6): 1181-1188.

16. Burchett, S. A. and T. P. Hicks (2006). "The mysterious trace amines: protean neuromodulators of synaptic transmission in mammalian brain." Prog Neurobiol **79**(5-6): 223-246.

17. Burdychova, R. and T. Komprda (2007). "Biogenic amine-forming microbial communities in cheese." FEMS Microbiol Lett **276**(2): 149-155.

18. Byrd, A. L., Y. Belkaid and J. A. Segre (2018). "The human skin microbiome." Nat Rev Microbiol **16**(3): 143-155.

19. Carmona-Cruz, S., L. Orozco-Covarrubias and M. Saez-de-Ocariz (2022). "The Human Skin Microbiome in Selected Cutaneous Diseases." Front Cell Infect Microbiol **12**: 834135.

20. Chander, H., V. K. Batish, S. Babu and K. L. Bhatia (1988). "Amine production by Streptococcus lactis under different growth conditions." Acta Microbiol Pol **37**(1): 61-64.

21. Christenson, J. G., W. Dairman and S. Udenfriend (1970). "Preparation and properties of a homogeneous aromatic L-amino acid decarboxylase from hog kidney." Arch Biochem Biophys **141**(1): 356-367.

22. Cornil, C. A. and G. F. Ball (2008). "Interplay among catecholamine systems: dopamine binds to alpha2-adrenergic receptors in birds and mammals." J Comp Neurol **511**(5): 610-627.

23. Coton, M., M. Fernandez, H. Trip, V. Ladero, N. L. Mulder, J. S. Lolkema, M. A. Alvarez and E. Coton (2011). "Characterization of the tyramine-producing pathway in Sporolactobacillus sp. P3J." Microbiology (Reading) **157**(Pt 6): 1841-1849.

24. Coton, T., P. Kraemer, F. Simon, J. J. Morand, J. J. Depina, T. Lonjon and P. Hovette (2004). "[Alithiasic cholecystitis in association with visceral leishmaniasis in an immunodepressed patient]." Med Trop (Mars) **64**(4): 407-408.

25. Dewar, K. M., L. E. Dyck, D. A. Durden and A. A. Boulton (1988). "Involvement of brain trace amines in the behavioural effects of phenelzine." Neurochem Res **13**(2): 113-119.

26. Dinan, T. G. and J. F. Cryan (2015). "The impact of gut microbiota on brain and behaviour: implications for psychiatry." Curr Opin Clin Nutr Metab Care **18**(6): 552-558.

27. Dominici, P., B. Tancini, D. Barra and C. B. Voltattorni (1987). "Purification and characterization of rat-liver 3,4-dihydroxyphenylalanine decarboxylase." Eur J Biochem **169**(1): 209-213.

28. Dreno, B., E. Araviiskaia, E. Berardesca, G. Gontijo, M. Sanchez Viera, L. F. Xiang, R. Martin and T. Bieber (2016). "Microbiome in healthy skin, update for dermatologists." J Eur Acad Dermatol Venereol **30**(12): 2038-2047.

29. Dyck, L. E., C. R. Yang and A. A. Boulton (1983). "The biosynthesis of p-tyramine, m-tyramine, and beta-phenylethylamine by rat striatal slices." J Neurosci Res **10**(2): 211-220.

30. Fernandez, M., D. M. Linares and M. A. Alvarez (2004). "Sequencing of the tyrosine decarboxylase cluster of Lactococcus lactis IPLA 655 and the development of a PCR method for detecting tyrosine decarboxylating lactic acid bacteria." J Food Prot **67**(11): 2521-2529.

31. Findley, K., J. Oh, J. Yang, S. Conlan, C. Deming, J. A. Meyer, D. Schoenfeld, E. Nomicos, M. Park, N. I. H. I. S. C. C. S. Program, H. H. Kong and J. A. Segre (2013). "Topographic diversity of fungal and bacterial communities in human skin." Nature **498**(7454): 367-370.

32. Furuzawa, Y., Y. Ohmori and T. Watanabe (1994). "Immunohistochemical colocalization of serotonin, aromatic L-amino acid decarboxylase and polypeptide hormones in islet A- and PP-cells of the cat pancreas." J Vet Med Sci **56**(5): 911-916.

33. Gainetdinov, R. R., M. C. Hoener and M. D. Berry (2018). "Trace Amines and Their Receptors." Pharmacol Rev **70**(3): 549-620.

34. Gallo, R. L. (2017). "Human Skin Is the Largest Epithelial Surface for Interaction with Microbes." J Invest Dermatol **137**(6): 1213-1214.

35. Grandy, D. K. (2007). "Trace amine-associated receptor 1-Family archetype or iconoclast?" Pharmacol Ther **116**(3): 355-390.

36. Grice, E. A., H. H. Kong, S. Conlan, C. B. Deming, J. Davis, A. C. Young, N. C. S. Program, G. G. Bouffard, R. W. Blakesley, P. R. Murray, E. D. Green, M. L. Turner and J. A. Segre (2009). "Topographical and temporal diversity of the human skin microbiome." Science **324**(5931): 1190-1192.

37. Grice, E. A. and J. A. Segre (2011). "The skin microbiome." Nat Rev Microbiol **9**(4): 244-253.

38. Hannigan, G. D., J. S. Meisel, A. S. Tyldsley, Q. Zheng, B. P. Hodkinson, A. J. SanMiguel, S. Minot, F. D. Bushman and E. A. Grice (2015). "The human skin double-stranded DNA virome: topographical and temporal diversity, genetic enrichment, and dynamic associations with the host microbiome." mBio **6**(5): e01578-01515.

39. Honda, K. and D. R. Littman (2016). "The microbiota in adaptive immune homeostasis and disease." Nature **535**(7610): 75-84.

40. Irsfeld, M., M. Spadafore and B. M. Pruss (2013). "beta-phenylethylamine, a small molecule with a large impact." Webmedcentral **4**(9).

41. Juorio, A. V., X. M. Li, W. Walz and I. A. Paterson (1993). "Decarboxylation of L-dopa by cultured mouse astrocytes." Brain Res **626**(1-2): 306-309.
42. Juorio, A. V. and P. H. Yu (1985). "Effects of benzene and other organic solvents on the decarboxylation of some brain aromatic-L-amino acids." Biochem Pharmacol **34**(9): 1381-1387.

43. Kezmarsky, N. D., H. Xu, D. E. Graham and R. H. White (2005). "Identification and characterization of a L-tyrosine decarboxylase in Methanocaldococcus jannaschii." Biochim Biophys Acta **1722**(2): 175-182.

44. Khan, M. Z. and W. Nawaz (2016). "The emerging roles of human trace amines and human trace amine-associated receptors (hTAARs) in central nervous system." Biomed Pharmacother **83**: 439-449.

45. Kleinau, G., J. Pratzka, D. Nurnberg, A. Gruters, D. Fuhrer-Sakel, H. Krude, J. Kohrle, T. Schoneberg and H. Biebermann (2011). "Differential modulation of Beta-adrenergic receptor signaling by trace amine-associated receptor 1 agonists." PLoS One **6**(10): e27073.

46. Ladero, V., M. Fernandez and M. A. Alvarez (2009). "Isolation and identification of tyramine-producing enterococci from human fecal samples." Can J Microbiol **55**(2): 215-218.

47. Lam, V. M., S. Espinoza, A. S. Gerasimov, R. R. Gainetdinov and A. Salahpour (2015). "In-vivo pharmacology of Trace-Amine Associated Receptor 1." Eur J Pharmacol **763**(Pt B): 136-142.

48. Lancaster, G. A. and T. L. Sourkes (1972). "Purification and properties of hog-kidney 3,4-dihydroxyphenylalanine decarboxylase." Can J Biochem **50**(7): 791-797.

49. Lange, A. B. (2009). "Tyramine: from octopamine precursor to neuroactive chemical in insects." Gen Comp Endocrinol **162**(1): 18-26.
50. Lauweryns, J. M. and L. Van Ranst (1988). "Immunocytochemical localization of aromatic L-amino acid decarboxylase in human, rat, and mouse bronchopulmonary and gastrointestinal endocrine cells." J Histochem Cytochem **36**(9): 1181-1186.

51. Liberles, S. D. (2015). "Trace amine-associated receptors: ligands, neural circuits, and behaviors." Curr Opin Neurobiol **34**: 1-7.

52. Lichtenberger, L. M., R. Delansorne and L. A. Graziani (1982). "Importance of amino acid uptake and decarboxylation in gastrin release from isolated G cells." Nature **295**(5851): 698-700.

53. Lindstrom, P. and J. Sehlin (1983). "Mechanisms underlying the effects of 5-hydroxytryptamine and 5-hydroxytryptophan in pancreatic islets. A proposed role for L-aromatic amino acid decarboxylase." Endocrinology **112**(4): 1524-1529.

54. Lunjani, N., C. Hlela and L. O'Mahony (2019). "Microbiome and skin biology." Curr Opin Allergy Clin Immunol **19**(4): 328-333.

55. Luqman, A., P. Ebner, S. Reichert, P. Sass, C. Kabagema-Bilan, C. Heilmann, P. Ruth and F. Gotz (2019). "A new host cell internalisation pathway for SadA-expressing staphylococci triggered by excreted neurochemicals." Cell Microbiol **21**(9): e13044.

56. Luqman, A., M. Z. Muttaqin, S. Yulaipi, P. Ebner, M. Matsuo, S. Zabel, P. M. Tribelli, K. Nieselt, D. Hidayati and F. Gotz (2020). "Trace amines produced by skin bacteria accelerate wound healing in mice." Commun Biol **3**(1): 277.

57. Luqman, A., M. Nega, M. T. Nguyen, P. Ebner and F. Gotz (2018). "SadA-Expressing Staphylococci in the Human Gut Show Increased Cell Adherence and Internalization." Cell Rep **22**(2): 535-545.

58. Luqman, A., S. Zabel, S. Rahmdel, B. Merz, N. Gruenheit, J. Harter, K. Nieselt and F. Gotz (2020). "The Neuromodulator-Encoding sadA Gene Is Widely Distributed in the Human Skin Microbiome." Front Microbiol **11**: 573679.

59. Ma, G., S. A. Bavadekar, B. T. Schaneberg, I. A. Khan and D. R. Feller (2010). "Effects of synephrine and beta-phenethylamine on human alpha-adrenoceptor subtypes." Planta Med **76**(10): 981-986.

60. Marcobal, A., B. De las Rivas, J. M. Landete, L. Tabera and R. Munoz (2012). "Tyramine and phenylethylamine biosynthesis by food bacteria." Crit Rev Food Sci Nutr **52**(5): 448-467.

61. Marcobal, A., P. J. Martin-Alvarez, M. C. Polo, R. Munoz and M. V. Moreno-Arribas (2006). "Formation of biogenic amines throughout the industrial manufacture of red wine." J Food Prot **69**(2): 397-404.

62. Martuscelli, M., M. A. Crudele, F. Gardini and G. Suzzi (2000). "Biogenic amine formation and oxidation by Staphylococcus xylosus strains from artisanal fermented sausages." Lett Appl Microbiol **31**(3): 228-232.

63. Miller, G. M. (2011). "The emerging role of trace amine-associated receptor 1 in the functional regulation of monoamine transporters and dopaminergic activity." J Neurochem **116**(2): 164-176.

64. Moreno-Arribas, V. and A. Lonvaud-Funel (1999). "Tyrosine decarboxylase activity of Lactobacillus brevis IOEB 9809 isolated from wine and L. brevis ATCC 367." FEMS Microbiol Lett **180**(1): 55-60.

65. Murillo, N. and D. Raoult (2013). "Skin microbiota: overview and role in the skin diseases acne vulgaris and rosacea." Future Microbiol **8**(2): 209-222.

66. O'Malley, K. L., S. Harmon, M. Moffat, A. Uhland-Smith and S. Wong (1995). "The human aromatic L-amino acid decarboxylase gene can be alternatively spliced to generate unique protein isoforms." J Neurochem **65**(6): 2409-2416.

67. Oh, J., A. L. Byrd, M. Park, N. C. S. Program, H. H. Kong and J. A. Segre (2016). "Temporal Stability of the Human Skin Microbiome." Cell **165**(4): 854-866.

68. Pessione, E., A. Pessione, C. Lamberti, D. J. Coisson, K. Riedel, R. Mazzoli, S. Bonetta, L. Eberl and C. Giunta (2009). "First evidence of a membrane-bound, tyramine and beta-phenylethylamine producing, tyrosine decarboxylase in Enterococcus faecalis: a two-dimensional electrophoresis proteomic study." Proteomics **9**(10): 2695-2710.

69. Premont, R. T., R. R. Gainetdinov and M. G. Caron (2001). "Following the trace of elusive amines." Proc Natl Acad Sci U S A **98**(17): 9474-9475.

70. Rahman, M. K., T. Nagatsu and T. Kato (1981). "Aromatic L-amino acid decarboxylase activity in central and peripheral tissues and serum of rats with L-DOPA and L-5-hydroxytryptophan as substrates." Biochem Pharmacol **30**(6): 645-649.

71. Roeder, T. (2005). "Tyramine and octopamine: ruling behavior and metabolism." Annu Rev Entomol **50**: 447-477.

72. Rorsman, F., E. S. Husebye, O. Winqvist, E. Bjork, F. A. Karlsson and O. Kampe (1995). "Aromatic-L-amino-acid decarboxylase, a pyridoxal phosphate-dependent enzyme, is a beta-cell autoantigen." Proc Natl Acad Sci U S A **92**(19): 8626-8629.

73. Rozas, M., A. Hart de Ruijter, M. J. Fabrega, A. Zorgani, M. Guell, B. Paetzold and F. Brillet (2021). "From Dysbiosis to Healthy Skin: Major Contributions of Cutibacterium acnes to Skin Homeostasis." Microorganisms **9**(3).

74. Rutigliano, G., A. Accorroni and R. Zucchi (2017). "The Case for TAAR1 as a Modulator of Central Nervous System Function." Front Pharmacol **8**: 987.

75. Saavedra, J. M. (1974). "Enzymatic isotopic assay for and presence of beta-phenylethylamine in brain." J Neurochem **22**(2): 211-216.

76. Sandmeier, E., T. I. Hale and P. Christen (1994). "Multiple evolutionary origin of pyridoxal-5'-phosphate-dependent amino acid decarboxylases." Eur J Biochem **221**(3): 997-1002.

77. Sanford, J. A. and R. L. Gallo (2013). "Functions of the skin microbiota in health and disease." Semin Immunol **25**(5): 370-377.

78. Silkaitis, R. P. and A. D. Mosnaim (1976). "Pathways linking L-phenylalanine and 2-phenylethylamine with p-tyramine in rabbit brain." Brain Res **114**(1): 105-115.

79. Sims, K. L. and F. E. Bloom (1973). "Rat brain L-3,4-dihydroxyphenylalanine and L-5-hydroxytryptophan decarboxylase activities: differential effect of 6-hydroxydopamine." Brain Res **49**(1): 165-175.

80. Sims, K. L., G. A. Davis and F. E. Bloom (1973). "Activities of 3,4-dihydroxy-L-phenylalanine and 5-hydroxy-L-tryptophan decarboxylases in rat brain: assay characteristics and distribution." J Neurochem **20**(2): 449-464.

81. Siow, Y. L. and K. Dakshinamurti (1985). "Effect of pyridoxine deficiency on aromatic L-amino acid decarboxylase in adult rat brain." Exp Brain Res **59**(3): 575-581.

82. Skowron, K., J. Bauza-Kaszewska, Z. Kraszewska, N. Wiktorczyk-Kapischke, K. Grudlewska-Buda, J. Kwiecinska-Pirog, E. Walecka-Zacharska, L. Radtke and E. Gospodarek-Komkowska (2021). "Human Skin Microbiome: Impact of Intrinsic and Extrinsic Factors on Skin Microbiota." Microorganisms **9**(3).

83. Snodgrass, S. R. and L. L. Iversen (1974). "Formation and release of 3H-tryptamine from 3H-tryptophan in rat spinal cord slices." Adv Biochem Psychopharmacol **10**: 141-150.

84. Sonnenburg, J. L. and F. Backhed (2016). "Diet-microbiota interactions as moderators of human metabolism." Nature **535**(7610): 56-64.

85. Sotnikova, T. D., O. I. Zorina, V. Ghisi, M. G. Caron and R. R. Gainetdinov (2008). "Trace amine associated receptor 1 and movement control." Parkinsonism Relat Disord **14 Suppl 2**: S99-102.

86. Spano, G., P. Russo, A. Lonvaud-Funel, P. Lucas, H. Alexandre, C. Grandvalet, E. Coton, M. Coton, L. Barnavon, B. Bach, F. Rattray, A. Bunte, C. Magni, V. Ladero, M. Alvarez, M. Fernandez, P. Lopez, P. F. de Palencia, A. Corbi, H. Trip and J. S. Lolkema (2010). "Biogenic amines in fermented foods." Eur J Clin Nutr **64 Suppl 3**: S95-100.

87. Stavropoulou, D. A., W. Borremans, L. De Vuyst, S. De Smet and F. Leroy (2015). "Amino acid conversions by coagulase-negative staphylococci in a rich medium: Assessment of inter- and intraspecies heterogeneity." Int J Food Microbiol **212**: 34-40.

88. Straub, B. W., M. Kicherer, S. M. Schilcher and W. P. Hammes (1995). "The formation of biogenic amines by fermentation organisms." Z Lebensm Unters Forsch **201**(1): 79-82.

89. Thaiss, C. A., N. Zmora, M. Levy and E. Elinav (2016). "The microbiome and innate immunity." Nature **535**(7610): 65-74.

90. Thakur, M. and W. Azmi (2009). "Biotransformation of L-tyrosine to tyramine by the growing cells of Lactococcus lactis." Acta Microbiol Immunol Hung **56**(1): 101-114.

91. Torriani, S., V. Gatto, S. Sembeni, R. Tofalo, G. Suzzi, N. Belletti, F. Gardini and S. Bover-Cid (2008). "Rapid detection and quantification of tyrosine decarboxylase gene (tdc) and its expression in gram-positive bacteria associated with fermented foods using PCR-based methods." J Food Prot **71**(1): 93-101.

92. Ursell, L. K., J. L. Metcalf, L. W. Parfrey and R. Knight (2012). "Defining the human microbiome." Nutr Rev **70 Suppl 1**(Suppl 1): S38-44.

93. Vassilacopoulou, D., D. C. Sideris, A. G. Vassiliou and E. G. Fragoulis (2004). "Identification and characterization of a novel form of the human L-dopa decarboxylase mRNA." Neurochem Res **29**(10): 1817-1823.

94. Vieira-Coelho, M. A. and P. Soares-da-Silva (1993). "Dopamine formation, from its immediate precursor 3,4-dihydroxyphenylalanine, along the rat digestive tract." Fundam Clin Pharmacol **7**(5): 235-243.

95. Williams, B. B., A. H. Van Benschoten, P. Cimermancic, M. S. Donia, M. Zimmermann, M. Taketani, A. Ishihara, P. C. Kashyap, J. S. Fraser and M. A. Fischbach (2014). "Discovery and characterization of gut microbiota decarboxylases that can produce the neurotransmitter tryptamine." Cell Host Microbe **16**(4): 495-503.

96. Zhang, K. and Y. Ni (2014). "Tyrosine decarboxylase from Lactobacillus brevis: soluble expression and characterization." Protein Expr Purif **94**: 33-39.

97. Zucchi, R., G. Chiellini, T. S. Scanlan and D. K. Grandy (2006). "Trace amine-associated receptors and their ligands." Br J Pharmacol **149**(8): 967-978.

98. Andersen, B.M. *Prevention and Control of Infections in Hospitals: Practice and Theory*, 1st ed.; Springer Nature: Cham, Switzerland, 2018; pp. 337–437.

99. Boulton, A. A. (1974). "Letter: Amines and theories in psychiatry." Lancet **2**(7871): 52-53.

100. Buerger, S. The Skin and Oral Microbiome: An Examination of Overlap and Potential Interactions between Microbiome Communities. In *Skin Microbiome Handbook: From Basic Research to Product Development*, 1st ed.; Dayan, N., Ed.; Scrivener Publishing LLC: Beverly, MA, USA, 2020; pp. 45–58.

101. Chu, D. M. *et al.* Maturation of the infant microbiome community structure and function across multiple body sites and in relation to mode of delivery.*Nat. Med.* **23,** 314–326 (2017).

102. Cundell, A. M. (2018). "Microbial Ecology of the Human Skin." Microb Ecol **76**(1): 113-120.

103. Giacomoni, P. U., et al. (2009). "Gender-linked differences in human skin." J Dermatol Sci **55**(3): 144-149.

104. Halasz, A., Barath, A., Simon-Sarkadi, L., and Holzapfel, W. (1994). Biogenic- amines and their production by microorganisms in food. Trends Food Sci. Technol. *5*, 42–49.

105. McLaughlin, J., Watterson, S., Layton, A. M., Bjourson, A. J., Barnard, E., and McDowell, A. (2019). Propionibacterium Acnes and Acne Vulgaris: New Insights From the Integration of Population Genetic, Multi–Omic, Biochemical and Host–Microbe Studies. Microorganisms 7 (5), 128. doi: 10.3390/microorganisms7050128

106. Moracanin, S.V., Stefanovic, S., Radicevic, T., Borovic, B., and Djukic, D. (2015). Production of biogenic amines by lactic acid bacteria isolated from Uzicka sausages. Procedia Food Sci. *5*, 308–311.

107. Pinto, D., et al. (2021). "Effect of commonly used cosmetic preservatives on skin resident microflora dynamics." Sci Rep **11**(1): 8695.

108. Samaras, S.; Hoptroff, M. The Microbiome of Healthy Skin. In *Skin Microbiome Handbook: From Basic Research to Product Development*, 1st ed.; Dayan, N., Ed.; Scrivener Publishing LLC: Beverly, MA, USA, 2020; pp. 3–32.

109. Schroeder, B. O. (2019). "Fight them or feed them: how the intestinal mucus layer manages the gut microbiota." Gastroenterol Rep (Oxf) **7**(1): 3-12.

110. Silla Santos, M.H. (1996). Biogenic amines: their importance in foods. Int. J. Food Microbiol. *29*, 213–231.

Chapter two

Isolation and characterization of L-Forms in *Staphylococcus aureus* HG001 and JE2 (MRSA)

List of figures (Chapter two)

List of Tables (Chapter two)

Abstract

L-forms of *Staphylococcus aureus* play a role in recurrent and persistent infections. Stable L-forms can be used to study their growth behavior, metabolism and evolution. We plan to use such L-forms to better study the mode of action of antibiotics that target the bacterial membrane and the cell wall by fluorescence microscopy. In Gram (+) L-forms the thick cell wall is largely decreased, however, we don't know how much of the peptidoglycan biosynthesis is intact.

In this study, two strains of *Staphylococcus aureus* were selected, one was common laboratory strain HG001 and another one was methicillin resistant strain JE2 (MRSA). So, in this experimental process, we obtained good conversion to L-form by using Pen G 300 µg/ml for HG001 and 2000 µg/ml for JE2. The first task was to optimize a medium in which L-form conversion is easier, rapid and convenient. For the better growth and propagation of L-forms, two media were optimized in this study, DM3 agar and SMMP. Successful growth of cell wall defective state of *Staphylococcus aureus* was achieved with DM3 ager using the layer-by-layer method. The SMMP broth media could be improved by the addition of antioxidants which increased the stability of L-forms. In the DM3 agar medium, characteristic 'Fried egg' colonies were observed in primary culture and also in sub-cultured plates with Pen G treatment. *S. aureus* L-form cells are bigger in size than the parent cells, they are pleomorphic, e.g., vacuolated, budding or in proliferative forms. The cells in the optimized media were also characterized by Fluorescence microscopy. A slight disadvantage is that L-form growth was slower than that of the parent, the lag phase was largely extended, but they can grow for a long time in steady state. Interestingly, L-forms of HG001 and JE2 were lysostaphin resistant, in contrast to the parent they were apparently not lysed. In summary, L-forms might represent an interesting tool in studying the effect of CW- and membrane-active compounds.

1. Introduction

1.1 Staphylococci

The escalating prevalence of bacterial infections in both humans and animals is a global health concern, with a significant proportion of these infections attributed to Gram-positive bacteria. Among these, the Staphylococcus species, belonging to the Firmicutes phylum, Bacilli class, Bacillales order, and Staphylococcaceae family, have emerged as predominant pathogens over the last decade. These bacteria, characterized by their round shape with diameters ranging from 0.5 to 1.5 µm, are typically arranged in grape-like clusters, a characteristic that lends to their name derived from the Greek word 'staphyle', meaning 'bunch of grapes'. As non-motile, non-spore-forming, and facultative anaerobic Gram-positive organisms, they possess a robust outer layer of peptidoglycan and a cytoplasmic membrane beneath this layer, composed of a protein and lipid bilayer. The reproductive method of S. aureus, like most bacterial species, is binary fission, which contributes to its rapid proliferation and the widespread infections observed in recent years. Therefore, a comprehensive understanding of the structural characteristics and reproductive mechanisms of Staphylococcus species is crucial in devising effective strategies to combat the increasing incidence of these bacterial infections in humans and animals.

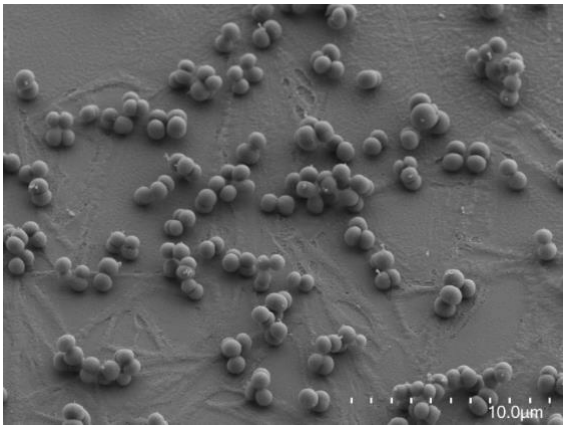


Fig.1: SEM-picture of *Staphylococcus aureus* (Photo curtesy of M. Nega).

The Staphylococcus species, bearing a genome size of 2000-3000 kbp, contain DNA of low G+C content (33-40 mol%) and an average genome size of 2-3 mbp (George and Kloos 1994);(Kloos et al. 1998). Generally, Staphylococci exhibit susceptibility to lysostaphin, furazolidone, and nitrofuran, while demonstrating resistance to erythromycin and bacitracin at lower levels.

To date, approximately 40 species and subspecies of the Staphylococcus genus have been identified. These bacteria are typically catalase and coagulase positive. However, it is important to note that not all *S. aureus* strains are coagulase-positive (Rayn and Ray, 2004).

S. aureus is a common commensal of humans and animals, predominantly found in the upper respiratory tract and on the skin. It is estimated that about 20-30% of the human population are long-term carriers of S. aureus, as part of the normal inhabitants on skin and in the nostril (Kluytmans, van Belkum, and Verbrugh 1997; Tong et al. 2015).

Despite its commensal nature, pathogenic strains of S. aureus can cause abscesses, respiratory infections, food poisoning, and occasionally life-threatening infections. Presently, this bacterium is considered one of the five most common causes of hospital-acquired infections and is also identified as a major cause of wound infections post-surgery.

In the field of veterinary medicine, *S. aureus* can cause several fatal diseases. This bacterium is a major cause of "Mastitis" in dairy cows and "Bumblefoot" in chickens (Poultry site, 2013). Infections in dogs, cats (Hanselman et al. 2009) and horses (Burton et al. 2008) by this pathogen are also not uncommon. Health care workers of dogs or pet owners may be considered as an important source of antibiotic-resistant *S. aureus*, especially during outbreaks (Boost, O'Donoghue and James, 2008).

A study conducted by Boost, O'Donoghue, and James in 2008 revealed that approximately 90% of S. aureus colonized within pet dogs were resistant to at least one

antibiotic. The nasal region is a significant site for transmission between dogs and humans (Boost, O'Donoghue, and James, 2008).

S. aureus HG001 is a derivative of NCTC 8325 modified by Herbert et al in 2010 where they repaired rsbU gene which is a positive regulator of the alternative sigma factor sigma(B).

The rapid spread of methicillin-resistant strains of *S. aureus* (DeLeo et al. 2010), (Graveland et al. 2011) has resulted in a challenging situation in medicine, complicating anti-Staphylococcal treatment in both humans and animals. The frequent isolation and severity of infection produced by Community-acquired (CA) MRSA strains of the USA300 type have become a major global health concern (Otto 2010), surpassing that of beta-lactam antibiotics like Penicillin.

1.2 L-form

Bacteria, as one of the most abundant forms of microorganisms, pervade every aspect of life. These organisms can be both beneficial, acting as commensals, and harmful, functioning as pathogens. The transformation from non-walled to walled form in a diverse group of bacteria is often similar and straightforward. The L-form mode of proliferation is considered a good model as it reflects the proliferation of primitive cells before the invention of the peptidoglycan cell wall.

The study of this L-form is crucial to unravel the mysteries surrounding the evolution of bacteria. The term "L-form," where "L" stands for "lacking," refers to a state of bacteria that is deficient or defective in its cell wall. This form was first discovered by Emmy Klieneberger in 1935 at the Lister Institute of London, which is the source of the "L" in its name. Over time, this L-form has been referred to by various names such as L-phase bacteria, L-variants, L-organisms, and CWD (cell wall deficient) bacteria (Allan, Hoischen, and Gumpert 2009).

Typically, a bacterium possesses a complete cell wall that not only protects it but also maintains its specific shape and size. When a bacterium is reformed without this protective covering, it is often misshaped and significantly less protected. This cell wall or some other parts of bacteria can sometimes be lost spontaneously by bacteria itself or can be induced forcefully (Dienes 1947); (Dienes and Weinberger 1951) and (Lederberg 1956).

The induction into the L-form can occur in both Gram-positive and Gram-negative bacteria (Burmeister and Hesseltine 1968); (Hubert et al. 1971); (Makemson and Darwish 1972) and (Williams 1963). From the aspect of pathogenicity in human and animal, *S aureus* is considered as the most significant pathogen. From a pathogenicity perspective, S. aureus is considered the most significant pathogen. This L-form can be formed from this organism in vitro (Fuller et al. 2005) ; (Banville 1964) and in vivo during infection (Michailova et al. 2007); (Tanimoto et al. 1995); (Owens 1987) or following the antibiotic treatment (Sears, Fettinger, and Marsh-Salin 1987). However, the mechanism leading to the development of the L-form in this bacterium remains unknown.

This thesis aims to explore the L-form of bacteria, its discovery, its formation, and its role in the pathogenicity of *S. aureus*, with a focus on understanding the unknown mechanisms that lead to the development of the L-form in this bacterium. Through this research, we hope to shed light on the evolution of bacteria and contribute to the broader understanding of these ubiquitous organisms.

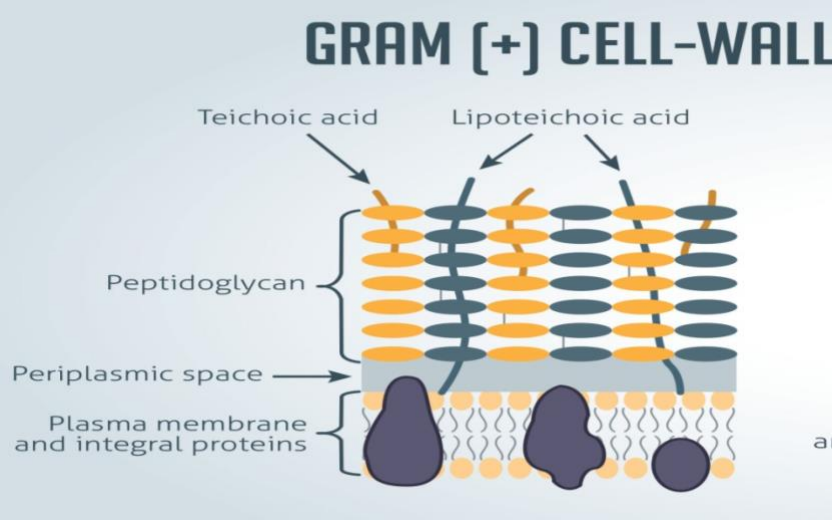


Fig. 2: Structural feature of Cell wall and Cell membrane of Gram-positive bacteria (https://www.technologynetworks.com/immunology/articles/gram-positive-vs-gram-negative-323007)

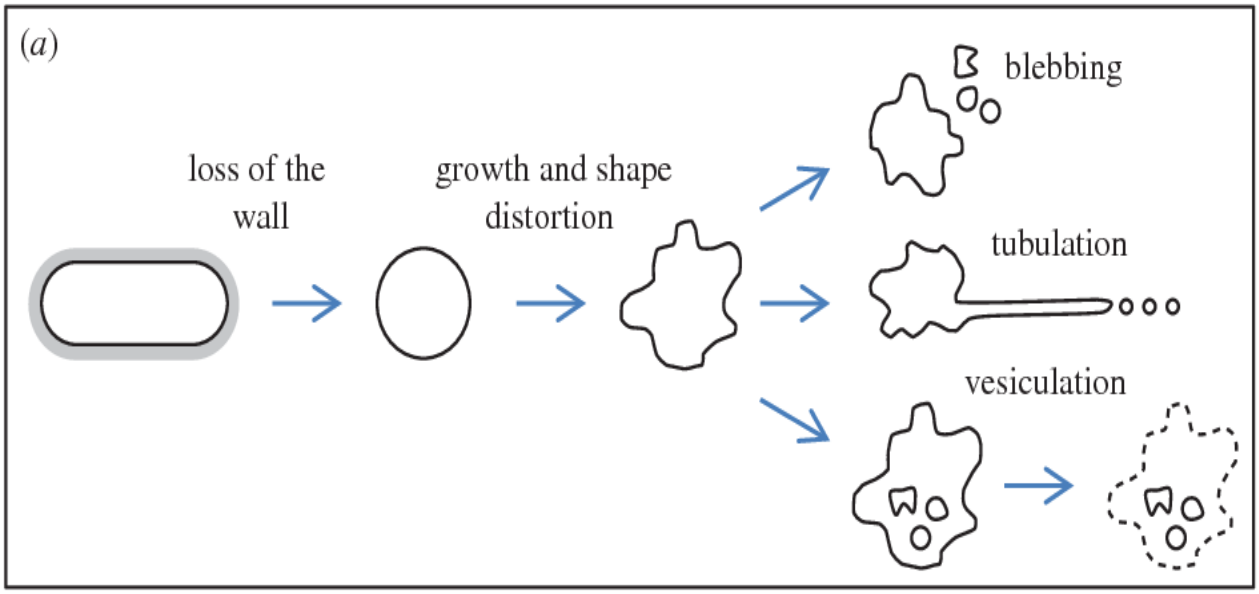


Fig. 3: L-form proliferation and its similarity to in vitro vesicle replication. (a) Schematic of L-form proliferation based on observations with defined primary L-forms of *B. subtilis* (Mercier, Dominguez-Cuevas, and Errington 2012) together with the vesiculation described by (Dell'Era et al. 2009) Comparison of L-form cells and replicating lipid vesicles.

Research into the L-form of bacteria has been ongoing and has included experiments on L-form organisms in mouse lungs after the inoculation of *Nocardia caviae* (Beaman 1980) and (Beaman and Scates 1981). Recent studies have suggested that this L-form may infect immunosuppressed patients following bone marrow transplantation (Woo et al. 2001). This unique form of bacteria has sparked a new era of research into antibiotic resistance (Fuller et al. 2005) and (Nature, 2019 https://www.nature.com/articles/s41467-019-12359-3).

Clinically, the L-form of bacteria holds potential significance. It can be used as a tool for determining high resistance to various essential antibiotics, particularly those that inhibit cell wall synthesis such as beta-lactams like Penicillin and Cephalosporins, or other cell wall inhibiting compounds. Despite the absence of an intact cell wall in the L-form state, fragments of the cell wall and related metabolites may still act as potent effectors of the immune system. These effectors include Toll-like receptors (TLRs), Peptidoglycan recognition proteins (PGRPs), and Nucleotide-binding oligomerization domain (NOD) receptor systems (Gottar et al. 2002); (Royet and Reichhart 2003) and (Takeda, Kaisho, and Akira 2003).

The exploration of the L-form of bacteria and its potential implications for antibiotic resistance and immune system interaction presents a promising avenue for future research. Understanding the mechanisms that underlie the formation and function of the L-form could lead to significant advancements in the treatment of bacterial infections, particularly in immunosuppressed patients. Furthermore, the potential of the L-form as a tool for determining antibiotic resistance could revolutionize the approach to developing new antibiotics and treating resistant bacterial strains. Therefore, continued research into the L-form of bacteria is not only necessary but also holds the potential for groundbreaking discoveries in the field of microbiology.

1.3 Varieties of L-form

Bacterial L-forms, depending on their sustainability and the presence of cell wall residues, can be classified into four categories: unstable spheroplast L-forms, stable spheroplast L-forms, unstable protoplast L-forms, and stable protoplast L-forms (Allan, Hoischen, and Gumpert 2009).

Stable L-forms are genetically distinct from the wild type of strain and are incapable of reverting to the classical bacteria. In contrast, unstable L-forms are genetically identical to their parent strain but retain the ability to revert to the classical bacteria (Allan, Hoischen, and Gumpert 2009) and (Glover, Yang, and Zhang 2009). A notable characteristic of stable L-forms is their ability to multiply indefinitely without a cell wall (Dell'Era et al. 2009).

Stable L-forms originate from the genetic variation of the wild type strain. Conversely, unstable L-forms derive from the wild type strain through changes in their phenotype or gene expression, which can be induced by cell wall synthesis inhibitors like beta-lactam antibiotics, or some cell wall-hydrolyzing enzymes under the appropriate osmotic conditions.

The growth conditions for unstable L-forms require certain elements that are absent in regular growth media, such as serum, osmoprotectants, and cell wall synthesis inhibitors (Allan, Hoischen, and Gumpert 2009), which are necessary throughout the growth of the L-form. However, the isolation of stable L-forms from the parent strain requires several steps including induction, selection, stabilization, and adaptation, with cell wall synthesis inhibitors only needed in the induction step (Allan, Hoischen, and Gumpert 2009).

The stable L-form model serves as a valuable tool in studying cell division, membrane organization, basic biological functions in L-forms, and their ability to cause diseases. Unstable L-forms are also important in examining the internal molecular processes of L-form formation. Consequently, there has been a surge of interest in new research exploring the molecular basis of L-form bacteria generation and their survival (Allan, Hoischen, and Gumpert 2009); (Devine 2012); (Domingue 2010); (Glover, Yang, and

Zhang 2009); (Hoischen et al. 1997); (Joseleau-Petit et al. 2007) and (Leaver et al. 2009). The study of these unique forms of bacteria could provide invaluable insights into understanding bacterial physiology, resistance, and pathogenicity.

1.4 Culture of L-form

The primitive L-form-like cells are generally considered fragile, unstable to high osmotic changes, and sensitive to desiccation. The cell wall of bacteria likely evolved during a critical moment in bacterial life at the cellular level, after which bacteria established their existence with a robust protective coat, enabling successful colonization on earth.

Bacterial L-forms have a significant impact in biology due to their potential role in latent and persistent infections (Dienes and Weinberger 1951); (Domingue and Woody 1997); (Allan, Hoischen, and Gumpert 2009) and (Domingue 2010). These L-form bacteria do not grow under normal cultural conditions; they require specific growth conditions such as a rich medium, serum, cell wall inhibitors like penicillin or other cell wall inhibiting antibiotics, osmotic protectants like sucrose, sodium chloride or glycine betaine, and sometimes antioxidants like Ascorbic acid, Glutathione, Uric acid, and soft agar.

L-forms can be developed from several cell-walled bacteria like Bacillus subtilis, *Staphylococcus aureus*, *Escherichia coli*, and *Listeria monocytogens*. Although many researchers have developed protocols for L-form generation from these bacteria (Dienes 1947), (Landman, Altenbern, and Ginoza 1958), (King and Gooder 1970) and (Joseleau-Petit et al. 2007), the variation in growth requirements can sometimes make this process tedious and limit the success rate in obtaining a pure culture of this form (Onoda et al. 1987); (Allan 1991) and (Dell'Era et al. 2009).

Typically, the L-form development process involves inhibiting peptidoglycan synthesis of the bacteria by antibiotics or by lysozyme, which can digest cell walls. An important consideration in culturing this variant is that the osmotic condition in the growth medium should resemble that of cytosol, which is an isotonic solution, to prevent bacterial cells from lysing due to osmotic shock (Joseleau-Petit et al. 2007). Moreover, L-forms may

have the tendency to revert to the normal complete form by regenerating their cell wall, which can be mitigated by culturing them in their growing media for an extended period.

While L-forms can be generated in liquid media, the best observable growth can be achieved on specialized solid media (Clasener 1972).

Despite the many ways to generate L-forms in vitro, the challenge of multiple types of L-forms and their difficult growing strategy should be considered when designing successful generation methods. The difficulty in establishing a standard protocol has resulted in a lack of clarity in this field, causing this research to be neglected(Allan, Hoischen, and Gumpert 2009).

However, it's never too late for recommenced researches on L-forms, and there have been emerging studies in recent years (Fuller et al. 2005), (Siddiqui et al. 2006), (Joseleau-Petit et al. 2007), (Leaver et al. 2009) and (Dell'Era et al. 2009). Additionally, advancements in nanotechnology and landscape ecology may aid in the induction of L-forms. Microfluidic devices can be made to inhibit the peptidoglycan synthesis of bacteria through excessive spatial confinement. Micro habitat patches might provide a new avenue for this research, where L-form-like cells can be derived (Mannik et al. 2009) using a microfluidics-based (synthetic) ecosystem implementing an adaptive landscape (Keymer et al. 2006) , selecting for shape-shifting phenotypes similar to L-forms. This approach could potentially revolutionize the understanding and manipulation of L-forms, opening new doors for research and application in the field of microbiology.

1.5 Characterization of L-form

Several published papers have delved into the morphological characterization of L-forms, their growth requirements, and the process of isolating them from humans and animals with chronic infections (Owens 1987); (Domingue and Woody 1997); (Schmidtke and Carson 1999) and (Mattman, 2001).

Typically, these L-form variants require a longer incubation period compared to other wild type bacteria. L-form colonies become visible to the naked eye after 3-5 days of incubation on L-form agar medium, while usual wild type colonies can easily be seen after just one day. On the agar medium, L-form colonies exhibit a unique "fried egg" appearance, where the center is dense with a bright vesicular periphery (Yabu 1986).

Early research and experiments have established that L-forms of bacteria exhibit pleomorphic shapes with variable sizes. In 1985, Yabu demonstrated that the use of lysostaphin and 6-aminopenicillanic acid (6-APA) in broth media could induce a stable L-form of Staphylococcus aureus from parent cells. Williams, in his work in 1963 on L-form preparation from Staphylococcus aureus, observed that L-form colonies of this bacteria are hard to touch on the surface of the agar. Microscopically, he found that the central part of the colony is formed deep within the agar. In a liquid medium, L-form growth appeared like microcolonies embedded in a slick material. Under a microscope, these microcolonies consisted of clusters of vesicles and granular debris. Typically, L-form colonies of *Staphylococcus aureus* measure up to about 2 mm in diameter, are smooth, domed, and have an entire edge (Marston 1961).

This detailed exploration of the morphological characteristics and growth requirements of L-forms provides a foundation for further research. Understanding these unique forms of bacteria could pave the way for new approaches to treating chronic infections. It also underscores the importance of continued research in this field, as the unique traits and behaviors of L-forms could hold the key to new discoveries in microbiology.

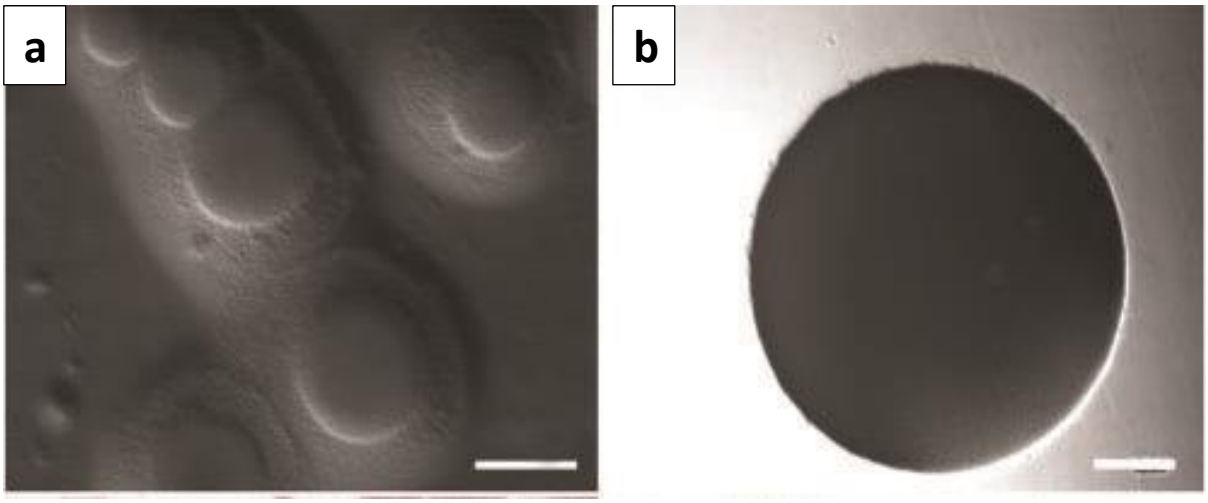


Fig. 4: Comparison of *S. aureus* L-form and Normal form morphologies (Scale bar 100 µm). (a) Typical 'fried-egg' morphology of *S. aureus* Newman L-form colonies on LIM. (b) Control classical *S. aureus* colony on BHI medium with sucrose control but without penicillin (Han et al. 2015).

Huber and Brinkley's groundbreaking work in 1977 utilized Brain Heart Infusion agar as an L-form induction medium to test the ability of *E. coli* K-12 strains (W3110 and BW25113) to convert to L-form variants (Huber and Brinkley 1977). This research laid the groundwork for subsequent studies on *E. coli* L-form isolation.

In 2009, Glover built on this earlier work by isolating L-forms from the same *E. coli* strains (Glover, Yang, and Zhang 2009). He found that the conversion rate to L-forms was most effective when the bacteria in the lag phase and stationary phase were directly inoculated into the L-form induction media. *E. coli* L-form colonies became visible after an incubation period of 48-72 hours, displaying the characteristic "fried egg" appearance. This consisted of peripheral growth on the surface of the agar with a dense center embedded into the agar. Examination of an agar block containing an L-form colony revealed numerous tightly packed coccoid cells.

When these coccoid cells were subcultured into the L-form induction media, they demonstrated aggregation and proliferation, resulting in the growth of new L-form colonies. This discovery marked a significant advancement in our understanding of the

growth and proliferation of L-forms, shedding light on their unique characteristics and behaviors.

The ability to reliably induce and isolate L-forms from *E. coli* strains has significant implications for further research into these unique bacterial L-forms. The insights gained from this work could potentially open new avenues for understanding bacterial physiology, antibiotic resistance, and chronic infections, thereby contributing to advancements in microbiology and medical science.

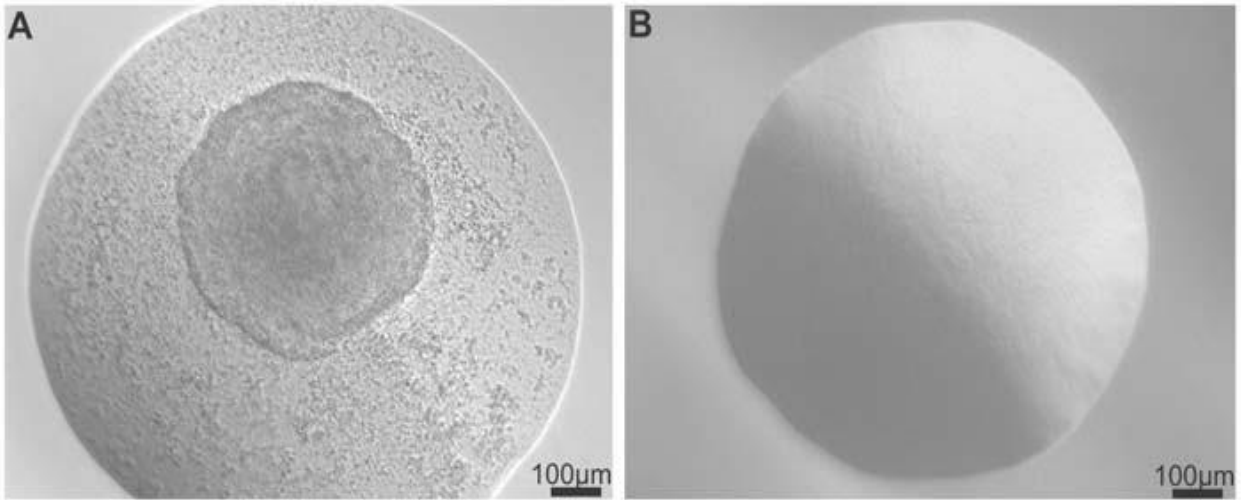


Fig. 5: Comparison of L-form *E. coli* and classical *E. coli* morphologies. (A) *E. coli* colony on L-form induction media (LIM) exhibiting typical ‘‘fried egg’’ morphology. (B) Classical *E. coli* colony on Brain Heart Infusion (BHI) agar (Glover, Yang, and Zhang 2009).

L-form from the rod shaped bacteria *Bacillus subtilis* turn into round shape under phase contrast microscope or by transmission electron microscope (Gilpin, Young, and Chatterjee 1973).

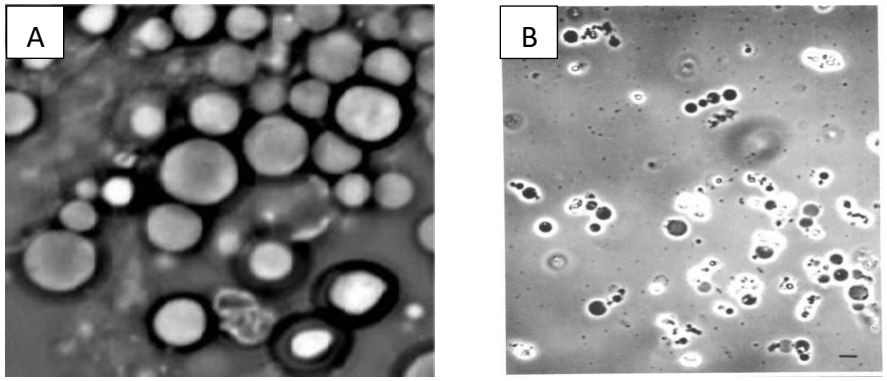


Fig. 6: (A)Phase contrast image of L-form cells from *Staphylococcus aureus* by PenG / Lysostaphin treatment (Kawai, Mickiewicz, and Errington 2018); (B) Morphological feature of L-form cells of *Bacillus subtilis* (Gilpin and Nagy 1976).

In case of *Streptococcus liquefaciens*, L-forms variants appear as large (up to 0.25 µm) vacuoles or foamy structures with uneven borders.

1.6 Objectives of the study

The successful conversion of L-forms from *S. aureus* can indeed serve as an effective tool for identifying the interactions of membrane inhibiting antibiotics. This can provide valuable insights for research into effective antibiotic therapies against Staphylococcal infections. This research also represents a significant step forward in understanding the unknown mechanisms of L-form preparation by *Staphylococcus aureus* and exploring the evolution of bacterial origin of life.

With these perspectives in mind, our research work aimed to achieve several objectives:

- **Optimization of DM3 medium for L-form from *Staphylococcus aureus* HG001 and JE2 growth**
- **Optimization of SMMP medium for L-form growth from those strains**
- **Characterization of L-form of HG001 and JE2**
- **Growth curve study of L-form from those strains**
- **Studying the resistance pattern of this L-form from HG001 and JE2 against different compounds or antibiotic candidates**

2. Materials and Methods

2.1 Materials

Chemicals and media for general use were bought from the companies Roche (Mannheim), Carl Roth GmbH (Karlsruhe), Difco Laboratories (Augsburg), GibcoBRL Life Technologies GmbH (Eggenstein), Merck GmbH (Darmstadt), Serva Fein biochemika GmbH (Heidelberg), Peq Lab Biotechnologie GmbH (Erlangen), Sigma-Aldrich Chemie GmbH (Taufkirchen).

Special materials, chemicals, enzymes and purification kits for this study and the respective supplier are listed in the tables below.

2.1.1 Chemicals and Buffers

Table 1: Chemicals used in this study

Chemicals	Supplier
Agarose PEQFold universal	PeqLab, Erlangen
Peptone from casein tryptic digest Tryptic Soy Broth Tris (hydroxymethyl-aminomethane)	Sigma, Taufkirchen
D-(+)-glucose D-(+)-sucrose	Sigma
Glycerol	Sigma

Table 2: Buffers used in this study

Buffer	Processing
PBS	1 liter, pH 7.2
NaCl	8.0 g
KCl	0.2 g
Na2HPO4	1.77 g
KH2PO4	0.24 g
Tris-HCl	1 liter, pH 7.8
Tris	20 mM, adjust pH with HCl

2.1.2 Laboratory appliances

Table 3: Special laboratory appliances

Appliance	Supplier
Centrifuge 5804R	Eppendorf AG, Hamburg
Ice machine	Scotsman
Incubators	Hereaus Seperations technik GmbH, Osterode
Microcentrifuge Hermle Z233 M-2	Hermle-Labor technik, Wehingen
Milli-Q PF Plus water purification system	Millipore, Eschborn
pH-Meter	Knick Elektronische Messgeräte, Berlin
Premium Freeze (-20 °C)	Liebherr
Refrigerator	Revco/Liebherr
Shaker	Heidolph-Elektro-GmbH, Kelheim
Tecan Reader Infinite M200	Tecan, Crailsheim

2.1.3 Consumable material and plastic articles

Table 4: Consumable material

Article	Supplier
1 mm electroporation cuvettes	Carl Roth GmbH, Karlsruhe
1.5 & 2 ml Eppendorf reaction tubes	Eppendorf, Hamburg
12 ml & 14 ml round bottom tubes	Eppendorf, Hamburg
15 ml and 50 ml conic Falcon™ tubes	Greiner Bio-One, Frickenhausen
200 µl reaction tubes	Molecular Bioproducts, San Diego
96 well plates, flat bottom	Greiner Bio-One, Frickenhausen
Lids for 96- well plates	Greiner Bio-One, Frickenhausen

2.1.4 Bacterial strains

Table 5: Bacterial strains used in this study

Species	Strain	Characteristics/ Genotype	References
Staphylococcus aureus HG001	wild type	Derivative of NCTC 8325 rsbU repaired	(Herbert et al. 2010)
Staphylococcus aureus USA300	JE2	methicillin resistant	(Kennedy et al. 2010)

2.1.5 Cultivation and storage of bacteria

2.1.5.1 Preculture of bacteria

For the preculture of *Staphylococcus aureus* HG001 and JE2 strains, we employed Tryptic Soy Broth (TSB) as the liquid medium of choice. TSB was meticulously prepared using deionized water to ensure the purity of the media components. Following preparation, the media underwent sterilization via autoclaving, a standard method employed to eliminate any potential contaminants. Once sterilized, the TSB was stored

at room temperature, maintaining its integrity and readiness for subsequent inoculation and cultivation of bacterial strains.

Table 6: Media used in this study

Medium	Composition	Concentration
Tryptic Soy Broth (TSB)	Casein peptone	17 g/l
	Dipotassium hydrogen phosphate	2.5 g/l
	Glucose	2.5 g/l
	Sodium chloride	5 g/l
	Soya peptone	3 g/l

2.1.5.2 Storage of bacteria

For the long-term preservation of L-form isolates, we employed a freeze medium to ensure the viability and stability of the bacterial cultures over extended periods. The preservation process involved the following steps:

- From a cultured plate containing L-form colonies, an agar block was carefully excised and transferred into a cryovial. The agar block was then inoculated onto DM3 agar within the cryovial by stab inoculation, ensuring the transfer of the L-form colony.

- Additionally, a portion of the L-form culture from a cultured broth in the logarithmic growth phase was transferred into the cryovial, with approximately 500 µl of the culture being added.

- Subsequently, the cryovial was filled with freeze medium up to a total volume of 1 ml. The freeze medium serves as a protective matrix, safeguarding the bacterial cells during the freezing process.

- For short-term storage, the cryovials were stored at 4°C, ensuring preservation at a refrigerated temperature. However, for long-term storage purposes, the cryovials containing the L-form bacteria were subjected to ultra-low temperatures. Specifically, they were stored at -20°C initially to facilitate the formation of ice crystals, followed by transfer to even lower temperatures of -80°C for prolonged preservation.

By employing this meticulous preservation protocol, we ensured the integrity and viability of the L-form isolates, facilitating their availability for future research endeavors and experimental analyses.

Medium	Composition	Concentration
Freeze Medium	Glycerol MgSO4 Tris/HCl pH 8.0	65 % 100 mM 25 mM

2.1.6 Antibiotic and Enzyme

The used antibiotics were sterile filtered by passing through filters with 0.2 µm pore size (Filtropur S 0.2; Sarstedt AG & Co.) and dissolved in distilled water. The addition of antibiotics to the autoclaved liquid media was mediated after cooling to approx. 50°C because of their thermostability.

Table 7: Antibiotics used in this study

Antibiotics	Source	Stock solution	Final concentration to be used	Dissolved in
Penicillin G Sodium salt	Sigma	10 mg/ml	300 µg/ml and 2000 µg/ml	H_2O

Table 8: Enzyme used in this study

Enzyme	Source	Concentration to be used	Dissolved in
Lysostaphin	Sigma	0.3 mg/ml	H_2O

Lysostaphin (0.3 mg/ml) was dissolved in H_2O_{bidest} and filter sterilized, pore size 0.45 µm. Aliquots of 1 ml were stored at -20°C.

2.1.7 Compounds used in solid media (DM3 agar)

To prepare L-forms from Staphylococcus aureus HG001 and JE2 strains, we utilized DM3 agar medium as the substrate for cultivation. DM3 agar medium provides an optimal environment for the induction and growth of L-form variants. This specialized medium is carefully formulated to support the unique requirements of L-form bacteria, facilitating their adaptation and proliferation.

Table 9: DM3 agar composition

Medium compounds	Optimized DM3 agar for L-form from *Staphylococcus aureus* (M and g/ Liter)
Agar	0.8% = (0.8 g/ 100 ml)
Glucose	50% = (500 g/L) (10 ml = 5 g)
Yeast extract	1.6 % = (16 g/L) (360 ml = 5.76 g)
BSA	5% = (50 g/ L) (10 ml = 0.5 g)
Casamino acid	1.2 % = (12 g/ L) (360 ml = 4.3 g)
MgCl2	1M = (95.211 g/ L) (20 ml = 1.9042 g)
Na succinate	1 M = (162.1 g/L) (500 ml = 81.5 g/L)
Dipotassium Phosphate (K2HPO4)	3.5 % = (35 g/L)
Monopotassium Phosphate (KH2PO4)	0.15 % = (15 g/L)

2.1.8 Compounds used in liquid media (SMMP broth)

To prepare L-forms from Staphylococcus aureus HG001 and JE2 strains, we utilized Synthetic Medium for Minimal Populations (SMMP) broth as the culture medium. This specialized broth medium provides an optimized environment for the induction and growth

of L-form variants. The process involved inoculating the bacterial strains into SMMP broth and incubating them under controlled conditions conducive to L-form development.

Table 10: SMMP media composition

Medium compounds	Optimized SMMP media for *Staphylococcus aureus* (with supplementation) (M and g/ Liter)
Sucrose	1M = (342.30 g/L)
Dextrose	4.0 g/ L
Beef extract	6.0 g/L
Yeast extract	6.0 g/L
BSA	5% = (50 g/ L)
Peptone	20 g/ L
Maleic acid	40 mM = (4.6 g/ L)
MgCl2	40 mM = (3.9 g/ L)
NaCl	14.0 g/ L
Dipotassium Phosphate (K2HPO4)	14.72 g/ L
Monopotassium Phosphate (KH2PO4)	5.28 g/ L
Supplementation:	
Antioxidants - L-Ascorbic acid	5%
- Glutathione (GSH)	3mM = (0.9 g/L)
Glycine Betaine	5 mM = (0.6 g/ L)

2.2 Methods

2.2.1 Preculture of bacteria

For the preculture of *Staphylococcus aureus* HG001 and JE2 strains, we employed Tryptic Soy Broth (TSB) medium. The preculture process involved the following steps:

Preparation of TSB Medium: TSB medium was prepared according to standard protocols and sterilized by autoclaving. Once cooled to room temperature, the medium was dispensed into 50 ml Erlenmeyer flasks, with each flask containing 10 ml of TSB medium.

Inoculation: Bacteria from both *Staphylococcus aureus* HG001 and JE2 strains were inoculated into the TSB medium in the Erlenmeyer flasks. A small volume of bacterial culture or a standardized inoculum was added to each flask to initiate the preculture.

Incubation: The inoculated TSB flasks were then placed in a shaking incubator set at 37°C. The flasks were subjected to continuous agitation at 140 rpm to ensure uniform mixing of the culture and optimal aeration. This incubation period allowed for the growth and proliferation of the bacteria, resulting in the development of an overnight culture.

Harvesting: After incubation overnight, the preculture was harvested from the TSB medium. The bacterial culture was then ready for further experimentation or downstream applications, such as subculture, inoculation into experimental media, or preparation of working cultures for subsequent assays.

By following this protocol, we were able to efficiently prepare precultures of *Staphylococcus aureus* HG001 and JE2 strains in TSB medium, providing a robust foundation for subsequent experimental procedures.

2.2.2 Induction of L-form on DM3 agar

For the preparation of L-forms from *Staphylococcus aureus* strains, a special process was followed using DM3 agar medium. Here's a detailed description of the procedure:

Preparation of DM3 Agar Plates: DM3 agar medium was prepared according to the specified formulation. For each plate, a total volume of 30 ml of DM3 agar was required.

Layering of Agar with Penicillin G (PenG): Initially, 15 ml of DM3 agar containing Penicillin G at a concentration of 300 µg/ml (for HG001 strain) or 2000 µg/ml (for JE2 strain) was poured onto the plate. This layer serves to inhibit the growth of the parent bacterial cells while allowing the L-form variants to develop.

Overlaying with Agar without PenG: After allowing the first layer of DM3 agar with PenG to solidify for a few minutes, the remaining 15 ml of DM3 agar without PenG was carefully overlayed onto the first layer. This step creates a dual-layered agar surface, with the top layer devoid of PenG, providing a conducive environment for the growth of L-form variants.

Inoculation with Overnight Culture: Following the preparation of the dual-layered agar plates, 100 µl of undiluted overnight culture of *Staphylococcus aureus* HG001 or JE2 strain was evenly spread over the surface of the plate. This inoculation step introduces the bacterial cells onto the agar surface, allowing them to proliferate and develop into L-form variants.

Incubation: The inoculated plates were then incubated at 37°C for a period of 3-7 days. During this incubation period, the bacterial cells undergo a transition to the L-form state, characterized by alterations in morphology and growth characteristics.

2.2.3 Subculturing of L-form

2.2.3.1 First subculturing

For the propagation of L-form bacteria, a subculturing process was employed using various culture media including DM3 agar, DM3 broth, and Synthetic Medium for Minimal Populations (SMMP) broth supplemented with Penicillin G (PenG). Here's a detailed description of the subculturing procedure:

Subculturing onto DM3 Agar: An agar block containing the L-form bacterial colony from the primary cultured plates was carefully excised. This agar block was then subcultured onto DM3 agar plates following the same process as primary culturing, as described earlier.

Subculturing into Liquid Media (DM3 and SMMP Broth): For broth culture, 10 ml of DM3 or SMMP broth supplemented with Penicillin G (PenG) at a concentration of 300 µg/ml for HG001 or 2000 µg/ml for JE2 was prepared in 50 ml Erlenmeyer flasks. The agar

block containing the L-form colony was inoculated into the liquid media in the Erlenmeyer flasks.

The inoculated flasks were then incubated aerobically at 37°C with constant shaking at 100 rpm. This shaking incubation ensures uniform distribution of nutrients and oxygen throughout the broth, promoting optimal growth of the L-form bacteria.

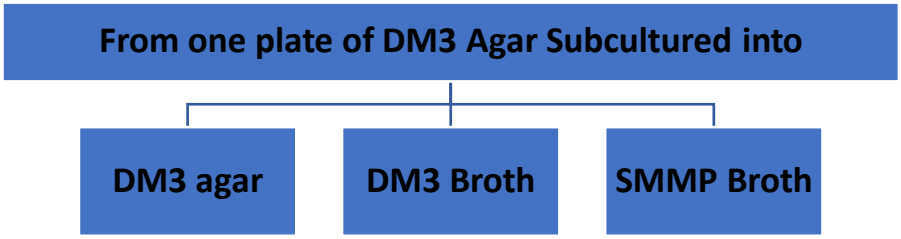
2.2.3.2 Second subculturing for getting pure culture

After the initial subculturing onto DM3 agar plates, the L-form-containing agar blocks were further inoculated onto plates treated with Penicillin G (PenG) as well as untreated plates of DM3 agar. Similarly, L-form cultures propagated in broth media were subcultured after 7 days of incubation.

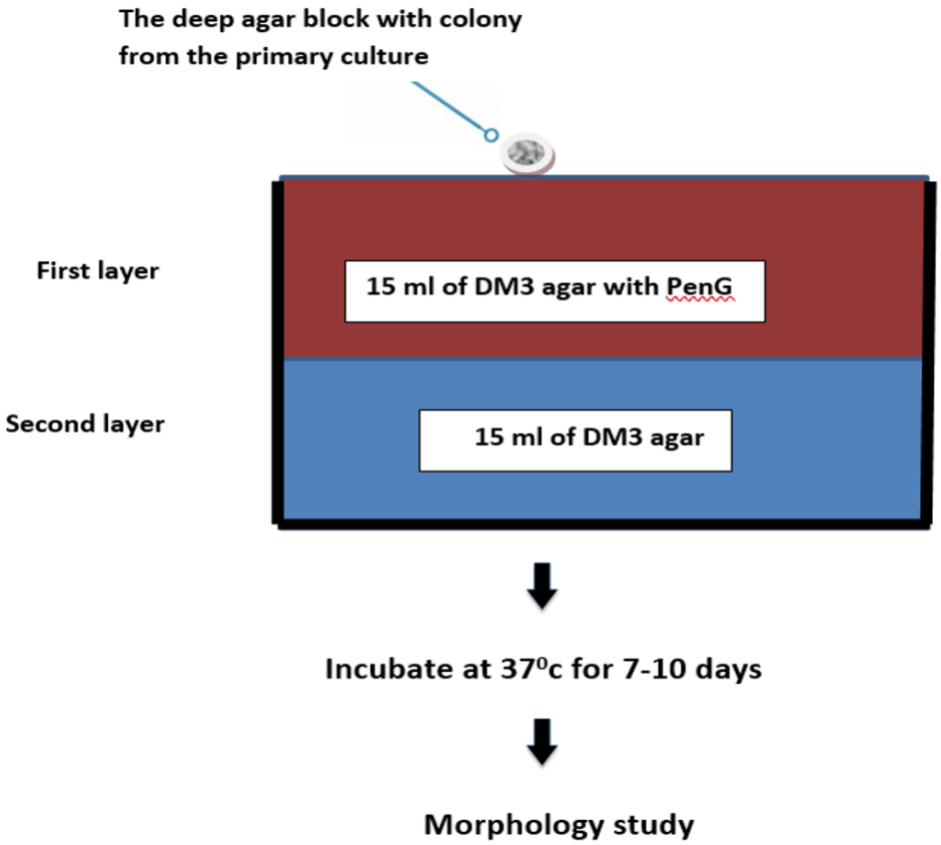
The subculturing process onto PenG-treated plates aimed to assess the susceptibility of the L-form variants to PenG, an antibiotic targeting cell wall synthesis. In contrast, inoculating onto untreated plates provided a control condition to compare the growth and morphology of L-form variants without antibiotic pressure. This approach allows for the observation of any alterations or differences in L-form growth and morphology in response to PenG treatment.

Following subculturing from broth media, the L-form cultures were similarly evaluated on PenG-treated and untreated DM3 agar plates to assess their response to the antibiotic under both solid and liquid culture conditions. This comprehensive approach enables a thorough investigation of the behavior and characteristics of L-form variants in the presence and absence of PenG, providing valuable insights into their antibiotic resistance and adaptation mechanisms.

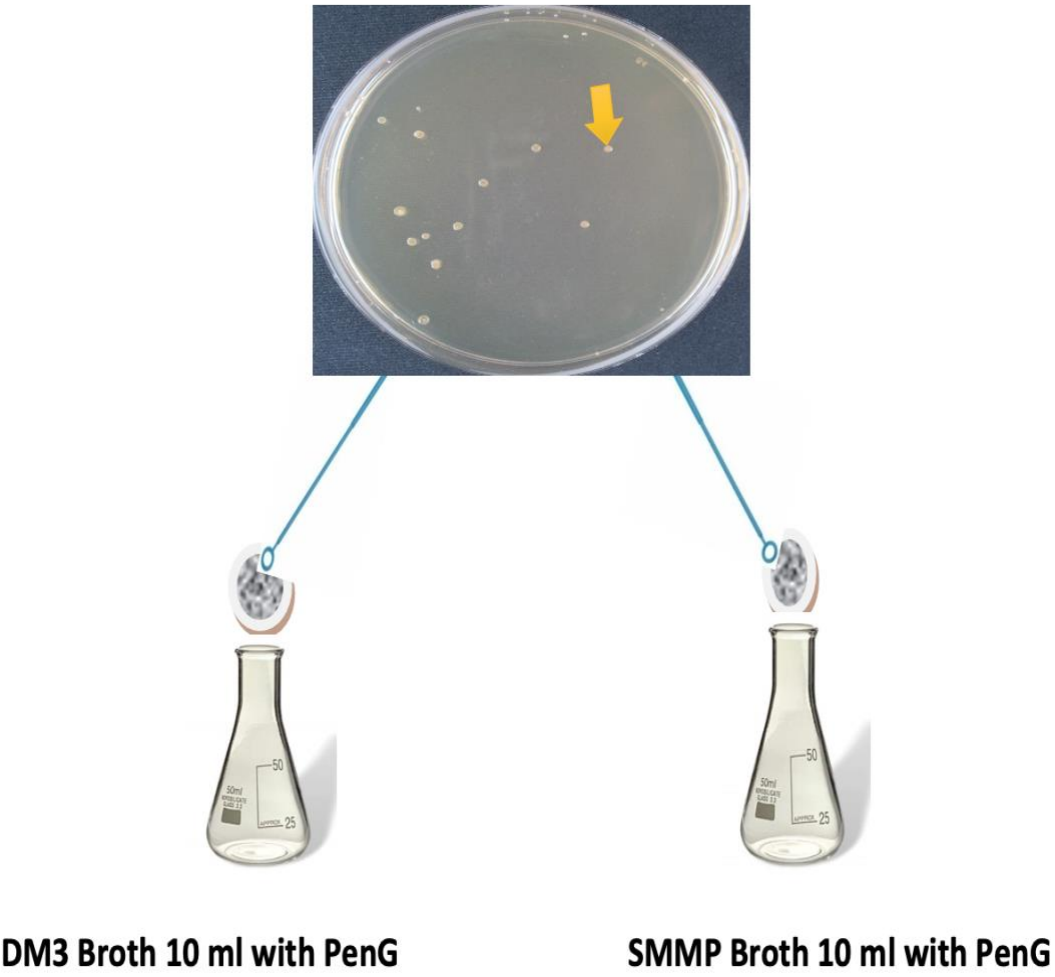
First Subculturing:



The schematic presentation of subculturing onto DM3 agar is as follows:



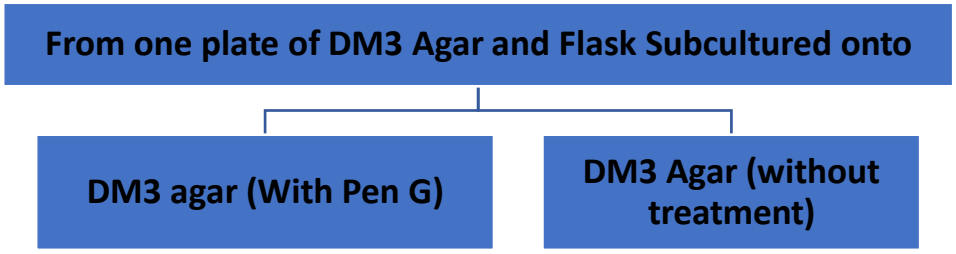
Subculturing into DM3 broth and SMMP broth:



DM3 Broth 10 ml with PenG

SMMP Broth 10 ml with PenG

Second Subculturing:



2.2.4 Formation of L-form in optimized SMMP Broth

Protocol of L-form preparation using SMMP Broth containing Vit C (with PenG)

For the preparation of overnight cultures of Staphylococcal strains, Tryptic Soy Broth (TSB) medium was utilized. Following this, L-form induction was carried out in 100 ml Erlenmeyer flasks. Here's a detailed description of the procedure:

- 100 ml Erlenmeyer flasks were employed for cultivation.
- In each flask, 20 ml of Synthetic Medium for Minimal Populations (SMMP) broth supplemented with Penicillin G (Pen G) was dispensed. For Staphylococcus aureus HG001, Pen G was added at a concentration of 300 µg/ml, while for JE2 strain, it was added at a concentration of 2000 µg/ml.
- Additionally, 250 µl of overnight culture of the respective Staphylococcal strains was inoculated into each flask to initiate the L-form induction process.
- Addition of L-Ascorbic Acid (Vitamin C) and Glutathione (GSH): L-Ascorbic acid (Vitamin C) and Glutathione (GSH) were filter-sterilized and added to the SMMP broth at certain concentrations. The purpose of incorporating these compounds was to evaluate their effects on L-form induction and compare their efficacy in promoting the formation of L-form cells.
- Incubation Conditions: Following the inoculation and addition of supplements, the flasks were tightly sealed and incubated at 37°C. The incubation was carried out in a shaking incubator set at 100 rpm to ensure uniform mixing and aeration of the broth culture.
- Monitoring L-form Formation: Flasks were examined daily under a microscope to observe the formation and development of L-form cells.
- The incubation period ranged from 3 to 10 days, with daily observations made to track the progression of L-form induction and growth.

Table 11: Radical scavengers used in this study

Radical scavengers	Source	Stock solution	Final concentration to be used	Dissolved in
L Ascorbic acid	Sigma	20% (Adjusted pH 7.0)	5%	H_2O
Glutathione	Sigma	5mM (Adjusted pH 7.0)	3mM	H_2O

2.2.5 Protocol of Fluorescence microscopy of L-form

At first 100 µl PBS was taken in the PCR tube.

↓

Then 50 µl of L-form culture was transferred into the tube and resuspended

↓

Centrifuged the tube for 2 minutes and discarded the supernatant

↓

Took 10 µl into a PCR tube and added DAPI, FM4-64 and Vancomycin tagged Bodipy FL in different concentration into the tube.

↓

The tubes were then left for 5 minutes in room temperature.

↓

1.5 µl of inoculum /cells was taken on the Glass slide with agarose and covered with coverslip

↓

Observed under fluorescence microscope

Table12: Fluorescent dyes used in this study

Flurescent dyes	Source	Final concentration to be used	Dissolved in
DAPI	Sigma	10 µg/ml	H_2O
FM4-64	Invitrogen	10 µg/ml	DMSO
Bodipy FL	Molecular probe	1 µg/ml	H_2O

2.2.6 Microscopy

The examination of S. aureus L-form bacteria involved the utilization of advanced microscopy techniques and image processing software. Here's a breakdown of the methodology:

Microscopy Equipment:

A Nikon Eclipse Ti-E microscope was used for observing "Fried egg" colonies grown on DM3 agar, as well as normal growth on control medium without penicillin.
Additionally, a Leica DML microscope was employed for observing L-form colonies on DM3 agar under 10X magnification.
For high-magnification examination, *S. aureus* L-form cells were observed using the Leica DML microscope at 100X magnification.

Image Processing:

Microscopic images were captured and processed using the Fiji software (Schindelin et al. 2012).This software offers powerful tools for image analysis and manipulation, allowing for the preparation of high-quality images suitable for publication.

Sample Preparation:

Samples for microscopy experiments, including cells and fluorescence microscopy, were mounted on microscopy slides coated with a thin layer of 1% agarose in water. This agarose layer served to immobilize the cells, enabling precise observation under the microscope.

Fluorescence Microscopy:

In fluorescence microscopy, specific cellular components such as the cell wall, DNA, and membranes were visualized using fluorescent stains.

Vancomycin Bodipy FL, 4′,6-diamidino-1-phenylindole (DAPI), and FM4-64 stains were employed to visualize the cell wall, DNA, and membranes, respectively.
Fluorescence microscopy experiments were conducted using the Zeiss LSM800 super-resolution microscope, known for its exceptional imaging capabilities and high-resolution output.

2.2.7 Growth curve study

To determine the growth curves of L-form variants of *S. aureus* HG001 and JE2 strains, as well as their wild-type counterparts, the following experimental procedures were undertaken:

Preparation of L-form Cultures:

- Overnight cultures of both strains were grown in Tryptic Soy Broth (TSB).
- 280 µl of the overnight culture was inoculated into 20 ml of Synthetic Medium for Minimal Populations (SMMPB) supplemented with 5% L-Ascorbic acid in 100 ml Erlenmeyer flasks.
- Penicillin G (PenG) was added to the media at concentrations of 300 µg/ml for HG001 and 2000 µg/ml for JE2 to induce L-form formation.
- The flasks were then incubated overnight at 37°C with agitation at 100 rpm.

Confirmation of L-form:

- L-form cultures were confirmed for each strain under a microscope following overnight incubation.

Adjustment of Starting OD:

- The optical density at 578 nm (OD_{578}) of the L-form cultures was adjusted to 0.1.
- A final volume of 20 ml of media was used in PenG-treated 100 ml flasks for each strain.

Incubation and Monitoring:

- Flasks were incubated at 37°C with agitation at 100 rpm.
- The OD_{578} was measured once a day for up to 7 days to track growth dynamics.

Additionally, to analyze the duration of the lag phase of the L-form variants, the following experiment was conducted:

Transfer of L-form Cultures:

- After 5 days of incubation in the previous growth curve determination experiment, L-form cultures were transferred to a new media.
- The starting OD_{578} was adjusted to 0.1, and 20 ml of media was prepared in PenG-treated 100 ml flasks.

Incubation and Monitoring:

- The flasks were incubated at 37°C with agitation at 100 rpm.
- OD_{578} was measured once a day for up to 7 days to assess the duration of the lag phase.

By systematically conducting these experiments and monitoring growth parameters, we aimed to elucidate the growth kinetics and lag phase characteristics of L-form variants of *S. aureus* HG001 and JE2 strains, providing valuable insights into their physiological behavior and adaptation mechanisms.

2.2.8 Lysostaphin assay

To confirm the presence of L-form bacteria from broth media, a Lysostaphin assay was performed. Here's a detailed protocol for the preparation of both wild-type (WT) and L-form cultures of Staphylococcus aureus HG001 and JE2 strains:

Preparation of WT of HG001 and JE2

- Took 2 ml of overnight culture of HG001 and JE2 wildtype in an Eppendorf tube.
- Centrifuged the tubes at 12,000 rpm for 2 minutes to pellet the bacterial cells. Discard the supernatant and carefully collect the precipitated cells.
- Added 250 µl of PBS to resuspend the bacterial cells thoroughly.
- Added 25 µl of Lysostaphin solution (0.3 mg/ml concentration) to the bacterial suspension.

- Incubated for 2 hrs at 37^0c to allow Lysostaphin to act on the bacterial cells.

Preparation of L-form of HG001 and JE2

- Transferred 2 ml culture of L-form of HG001 and JE2 wildtype in an Eppendorf tube.
- Centrifuged for 7500 rpm 2 mins minutes to pellet the L-form cells. If no significant precipitation occurs, gently remove some culture from the supernatant.
- Added 250 µl of PBS, mix well for resuspending the L-form cells thoroughly.
- Added 25 µl of Lysostaphin (0.3 mg/ml) to the L-form cell suspension.
- Incubated the tubes for 2 hrs at 37^0c

Following incubation, the samples can be further analyzed or processed to assess the effectiveness of Lysostaphin in lysing the bacterial cells. This assay helps confirm the presence of L-form variants by comparing their susceptibility to Lysostaphin with that of the wild-type bacterial cells.

3. Results

3.1 Induction and identification of L-form colonies on DM3 agar

Before using DM3 agar, this medium had to be optimized from the recommended DM3 agar by Chang and Cohen, 1979 **(Chang and Cohen 1979)**

Table 13: Comparison between the chemical constituents of recommended DM3 media with my optimized media

Medium compounds	DM3 agar (Chang and Cohen 1979) (M and g/ Liter)	Optimized DM3 agar for L-form from *Staphylococcus aureus* (M and g/ Liter)
Agar	1% = 1 g/ 100 ml	0.8% = 0.8 g/ 100 ml
Glucose	20% = 200 g/ L (25 ml = 5 g)	50% = 500 g/L (10 ml = 5 g)
Yeast extract	10% = 100 g/ L (100 ml = 10 g)	1.6 % = 16 g/L (360 ml = 5.76 g)
BSA	2% = 20 g/ L (5 ml = 0.1 g)	5% = 50 g/ L (10 ml = 0.5 g)
Casamino acid	5% = 50 g/ L (100 ml = 5 g)	1.2 % = 12 g/ L (360 ml = 4.3 g)
MgCl2	1M = 95.211 g/ L (20 ml = 1.9042g	1M = 95.211 g/ L (20 ml = 1.9042 g)
Na succinate	1 M = 162.1 g/L (500 ml = 81.5 g/L)	1 M = 162.1 g/L (500 ml = 81.5 g/L)
Dipotassium Phosphate (K2HPO4)	35 g/L	3.5 % = 35 g/L
Monopotassium Phosphate (KH2PO4)	15 g/L	0.15 % = 15 g/L

In the Gram-positive group, Bacillus is also an important member like as Staphylococcus. In the earlier days, some media ware used by many of researchers to get L-form from this *Bacillus subtilis*.

Here, the differential study of my optimized media with the recipe of media used for L-form preparation from *Bacillus subtilis* by two researchers is added as follows.

Table 14: Comparison among the chemical constituents in my optimized media with two other L-form induction media for *Bacillus subtilis*

Medium compounds	Optimized DM3 media *Staphylococcus aureus* (M and g/ Liter)	L-form induction media for *Bacillus subtilis* (g/ Liter) 1*	L-form induction media for *Bacillus subtilis* (g/ Liter) 2*
Glucose	50% = 500 g/L (10 ml = 5 g)	5 g/L	2 g/L
Yeast extract	1.6 % = 16 g/L (360 ml = 5.76 g)	-	-
BSA	5% = 50 g/ L (10 ml = 0.5 g)	-	-
Horse serum	-	-	20 ml
Casamino acid	1.2 % = 12 g/ L (360 ml = 4.3 g)	-	-
Casein hydrolysate	-	0.2 g/L	-
L-Tryptophane	-	0.05 g/L	0.05 g/L
L-lysine	-	0.05 g/L	-
L-methionine	-	0.05 g/L	0.05 g/L
MgCl2	1M = 95.211 g/ L (20 ml = 1.9042 g)	-	0.5 g/L
NaCl	-	70 g/L	-
Na succinate	1 M = 162.1 g/L (500 ml = 81.5 g/L)	-	90 g/L
Dipotassium Phosphate (K2HPO4)	3.5 % = 35 g/L	-	3.5 g/L

Monopotassium Phosphate (KH2PO4)	**0.15 % = 15 g/L**	**-**	**1.5 g/L**
MgSO4.7H2O	**-**	**0.005 g/L**	**-**
NH4NO3	**-**	**-**	**1 g/L**
(NH4)2SO4	**-**	**-**	**0.1 g/L**

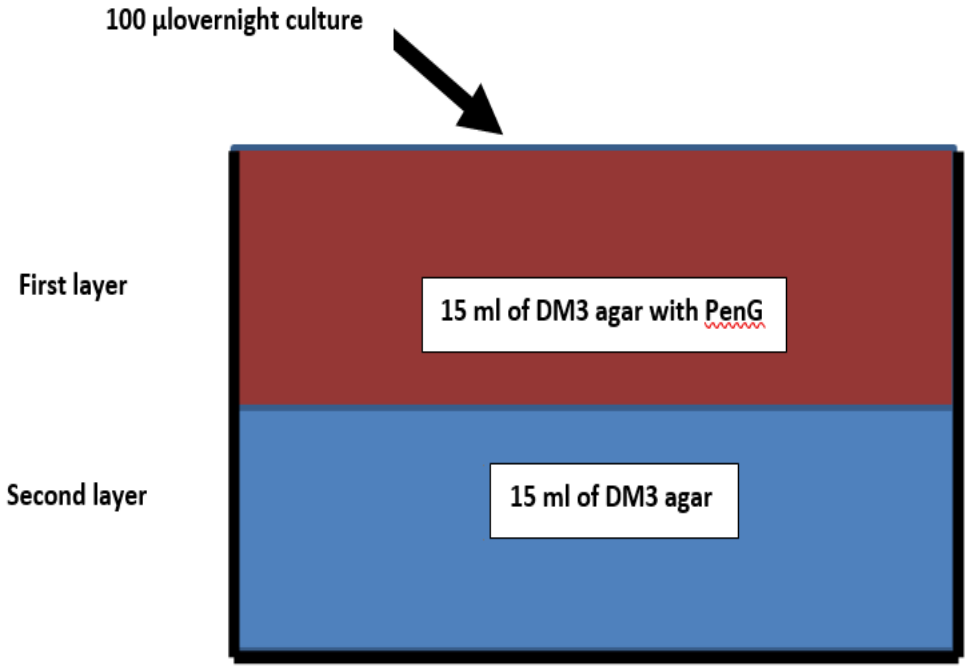
1* (Bettinger and Young 1975)

2* (Gilpin, Young, and Chatterjee 1973)

After analyzing all of their formulation for induction of L-form, I optimized some of the chemicals in my DM3 agar medium. Then that DM3 agar was tested for layer by layer pouring in the plates and got better growth of L-form of HG001 and JE2 strains.

The schematic presentation of layer-by-layer method is as follows:

Spreading of *S. aureus* HG001 / JE2 onto the DM3 Agar (30 ml) with Pen G by overlay method



The final volume of agar was used 30 ml in which first layer of 15 ml containing PenG but rest 15 ml of media was poured without PenG. Overnight culture of HG001 and JE2 in TSB were used. Both of the strains were spreaded on the top of the two layers of agar.

In case of HG001, 50, 150 and 300 µg/ml of PenG were used but 300 µg/ml showed better growth for L-form from HG001.

As JE2 strain is Methicillin resistant, that why 1000, 1500 and 2000 µg/ml PenG had to be tested for this strain and among these 2000 µg/ml showed better result for L-form preparation.

DM3 agar plates were incubated at 37 ^{0}c until 3-7 days for observing the morphological characters of L-form. Some of the characteristic colonies appeared after 3 days of incubation, majority growth of L-form colonies was observed after 5-7 days but the growth continues up to 10 days.

By the naked eyes, the colonies of L-form looked like so tiny, limited growth (Fig 8), on the other hand wild type colonies on DM3 agar were smooth, bigger and profuse growth like on TSA agar which is selective media of Staphylococcal growth (Fig 7). Another important point noticed that L-form colonies not well grown on the top of the agar, they embedded into the agar, and the colonies attached to the agar surface in such a way that it could not be scrapped off easily but wild types showed not this type of features. Morphologically the colonies look like ‘’Fried egg’’ under the inverted and light microscope (Fig 9,10,11,12).

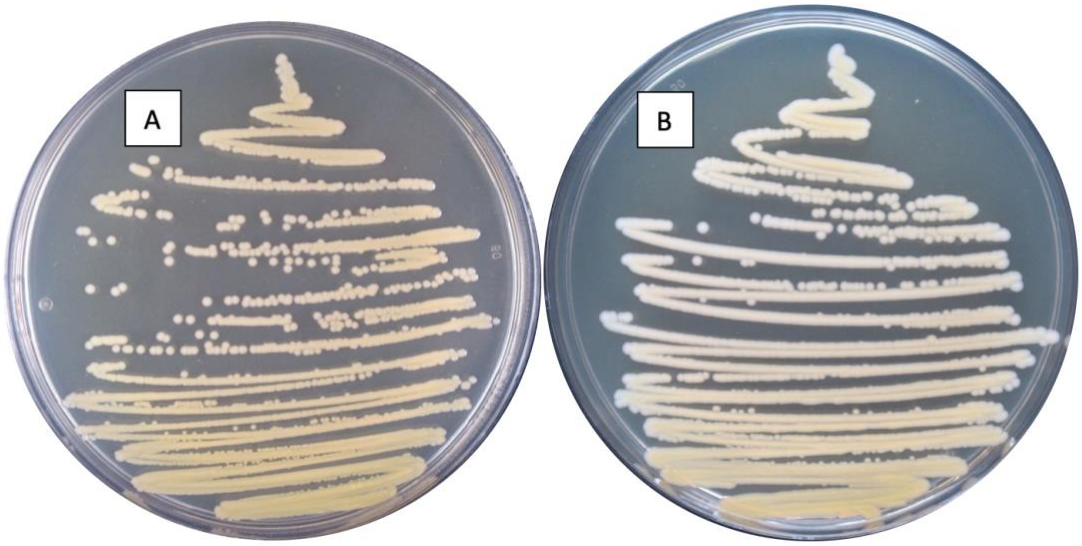


Fig. 7: Smooth Colonies on DM3 agar plates of WT of (A) HG001 and (B) JE2.

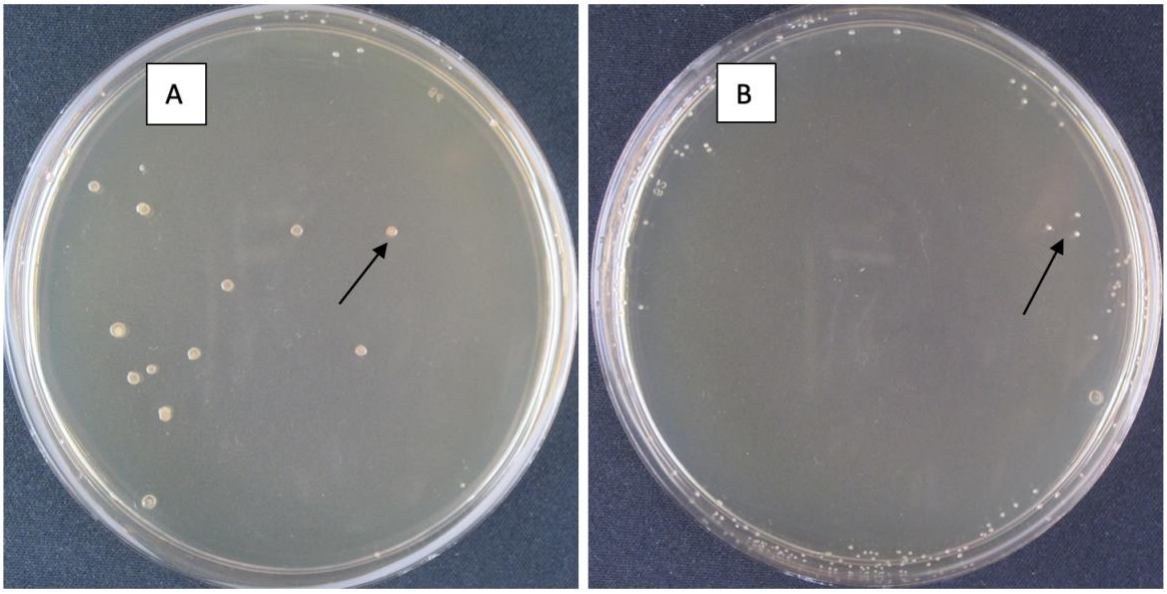


Fig. 8: Tiny Colonies on PenG treated DM3 agar plates of L-form from (A) HG001 and (B) JE2 seen after 5 days of incubation.

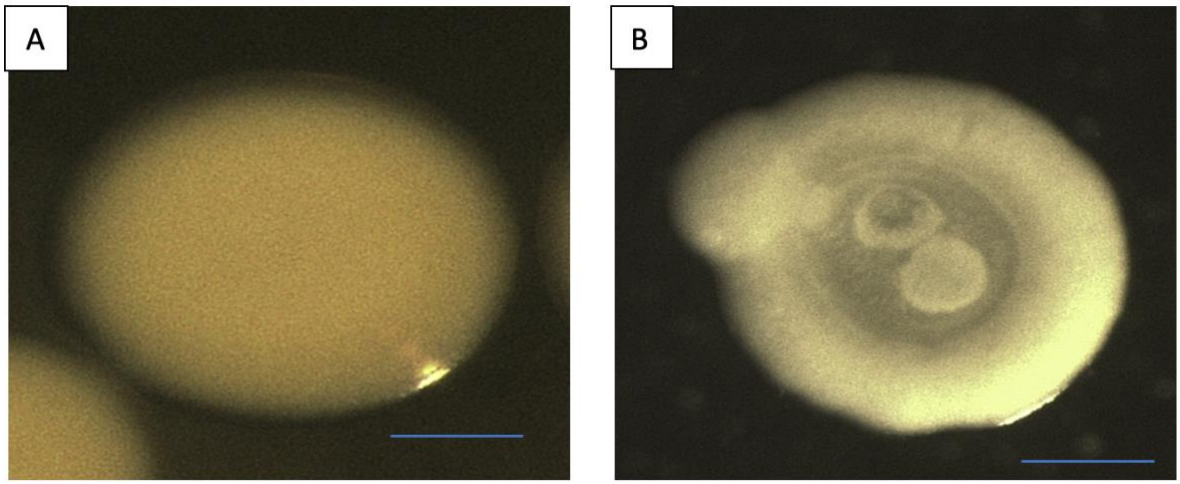


Fig. 9: Colonies of HG001 (A) WT and (B) L-form on Primary cultured plates of DM3 Agar under light Microscope (Leica DML 10X) after 7 days of incubation, here arbitrary scale bar was used.

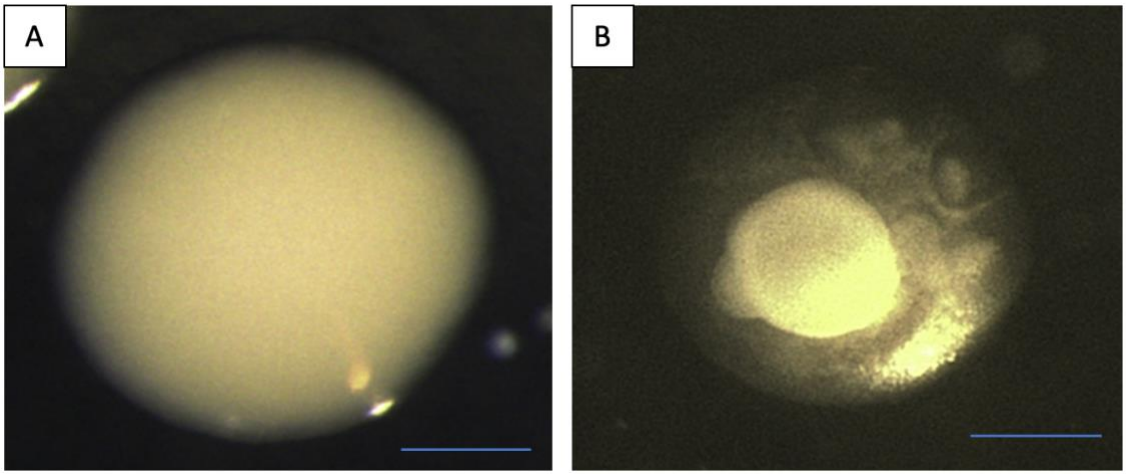


Fig. 10: Colonies of JE2 (A) WT and (B) L-form on Primary cultured plates of DM3 Agar under light Microscope (Leica DML 10X) after 7 days of incubation, here arbitrary scale bar was used.

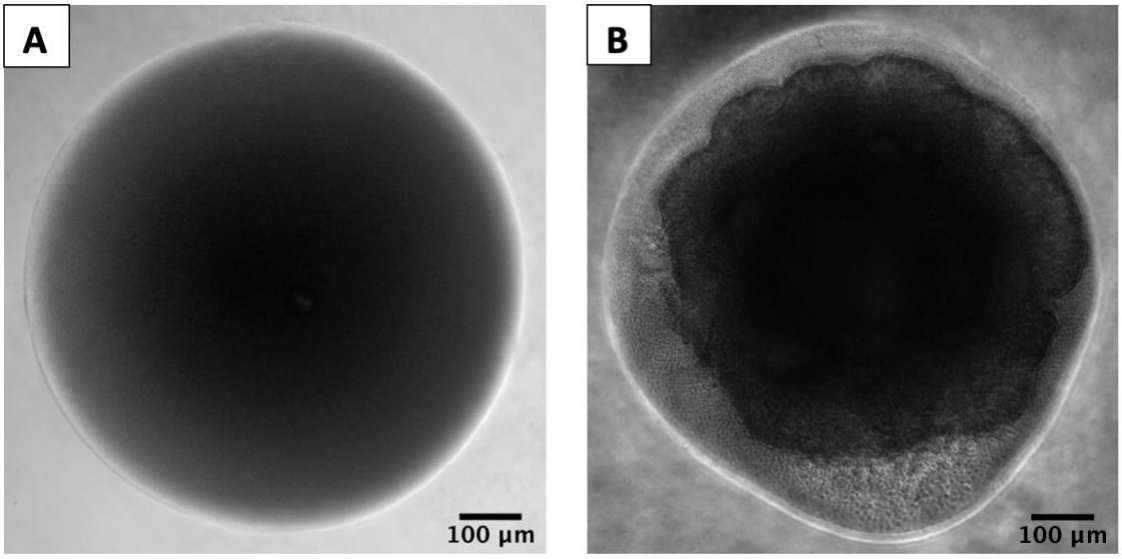


Fig. 11: (A) Wild type of HG001 in DM3 agar; (B) L-form colony of HG001 in PenG treated DM3 agar under Inverted Microscope (Nikon 10x) after 7 days of incubation.

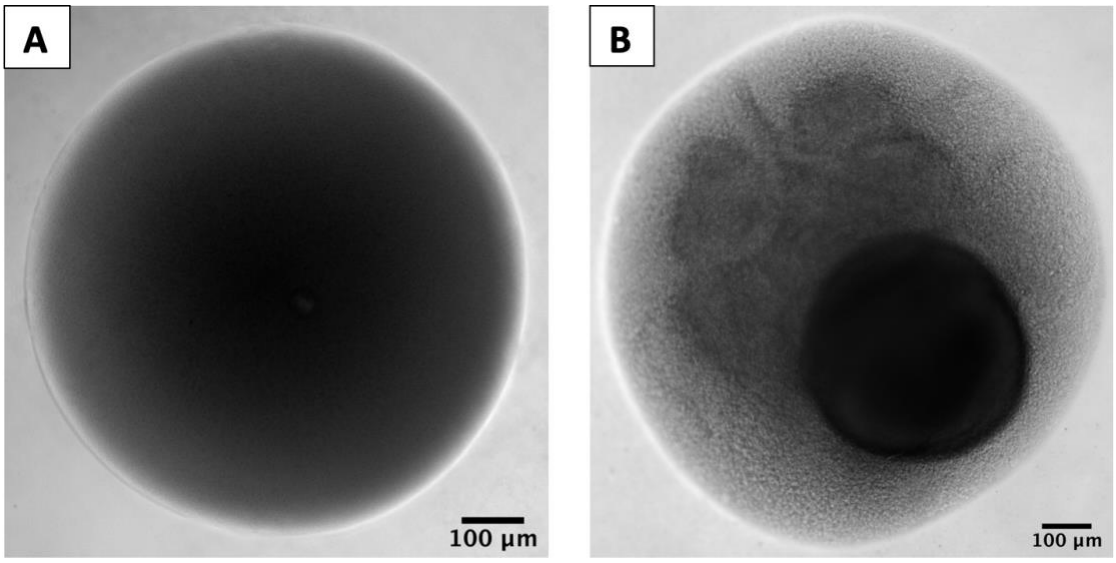


Fig. 12: (A) Wild type of JE2 in DM3 agar; (B) L-form colony of JE2 in PenG treated DM3 agar under Inverted Microscope (Nikon 10x) after 7 days of incubation.

3.2 Subculturing for Propagation and isolation of L-form

3.2.1 First subculturing

For propagation of L-form bacteria, from the primary cultured plates agar block containing bacterial colony was subcultured onto DM3 agar as well as DM3 broth and SMMP broth with PenG 300 µg/ml for HG001 and 2000 µg/ml for JE2. In DM3 agar, the process of subculturing was described in methods.

In case of broth culture, 10 ml of liquid media with PenG was used in 50 ml Erlenmeyer flask, then took agar block with colonies and incubated aerobically at 37°C by steadily shaking at 100 rpm.

Like as primary culture after 3 days of incubation characteristic fried egg like colonies were observed on PenG treated DM3 agar plates (Fig 13, 14) and as usual of L-form tiny colonies were seen by Naked eyes.

In broth cultures characteristic pleomorphic shaped, vacuolated cells were seen under microscope minimum after 10 days of incubation. But in the case of subculturing, the growth was very slower than primary culture in broth media.

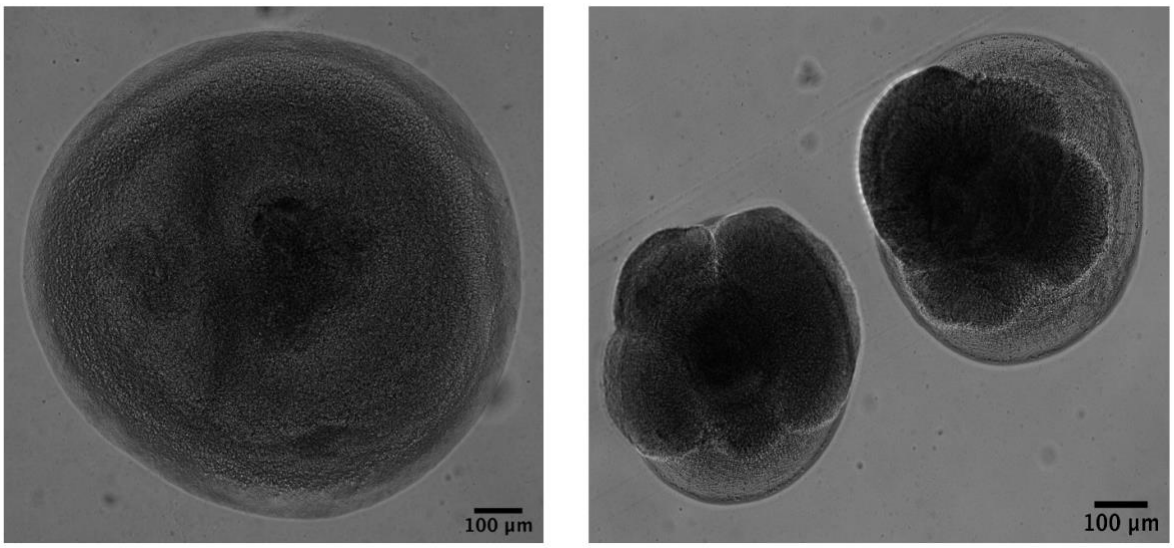


Fig. 13: Colonies of L-form of HG001 after first subculturing on PenG treated DM3 Agar under Inverted Microscope (Nikon 10x) after 7 days of incubation.

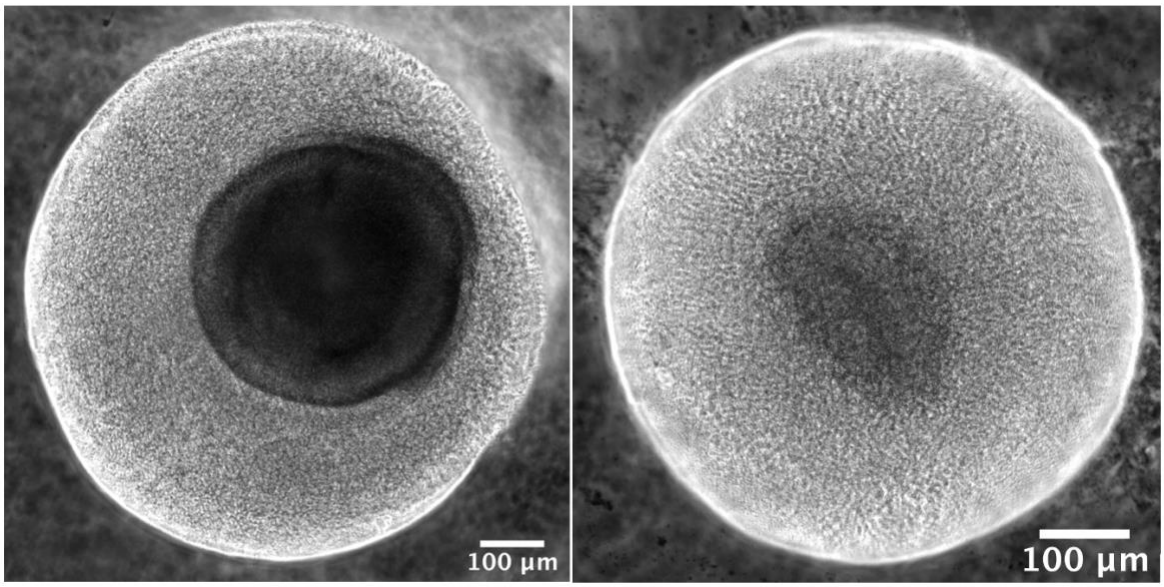


Fig 14: Colonies of L-form of JE2 after first subculturing on PenG treated DM3 Agar und.er Inverted Microscope (Nikon 10x) after 7 days of incubation.

3.2.2 Second Subculturing

For getting pure culture of L-form, subcultured again after 7 days of incubation from the first subcultured plate onto DM3 agar with PenG and without PenG. But in untreated plates of DM3 agar L-form reverted to wild type and in contrast, treated plates of DM3 agar exhibited L-form colonies (Fig 15). The process followed for subculturing onto the DM3 agar was described in methods. For getting more pure culture, subculturing was done two or three times by following the described process of primary culture.

In the case of subculturing, it was not easier to take one colony from the top of the agar. So, the agar block contained colony had to be transferred and macerated onto the new DM3 agar on the top. So that, the colony had to be opened from the agar block and then spreaded over the agar. After 3-7 days of incubation at 37 ^{0}c the typical fried egg colonies were get (Fig 16) and in naked eye, small colonies were found. On contrast, after 1 day reverted wild type colonies were found in untreated plates of DM3 agar (Fig 15).

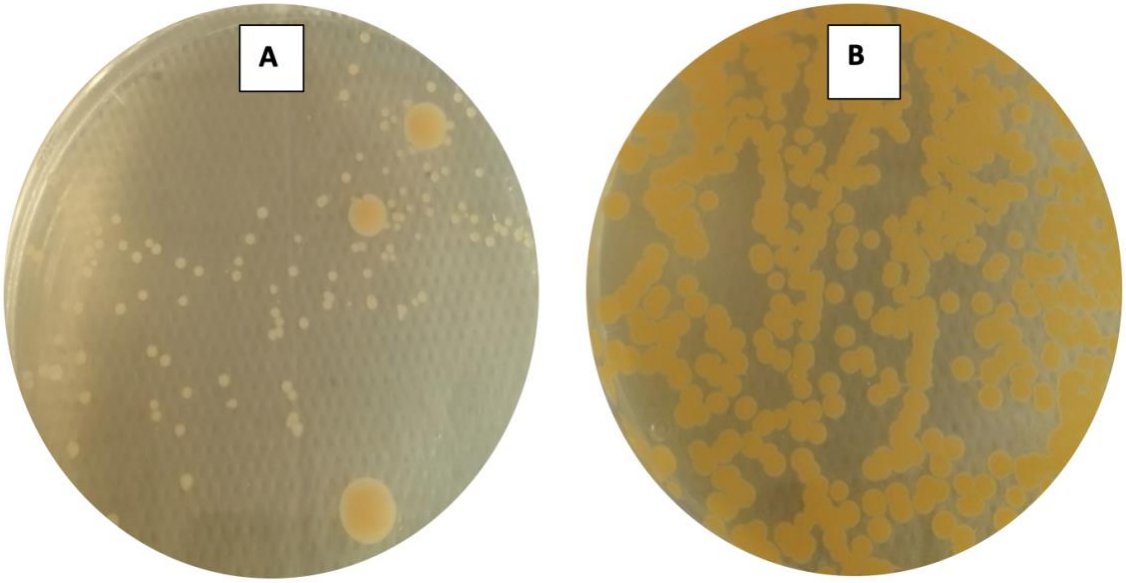


Fig. 15: (A) PenG treated plate of HG001 containing L-form colony; (B) Untreated DM3 agar plate of reverted wild type HG001 after first subculturing.

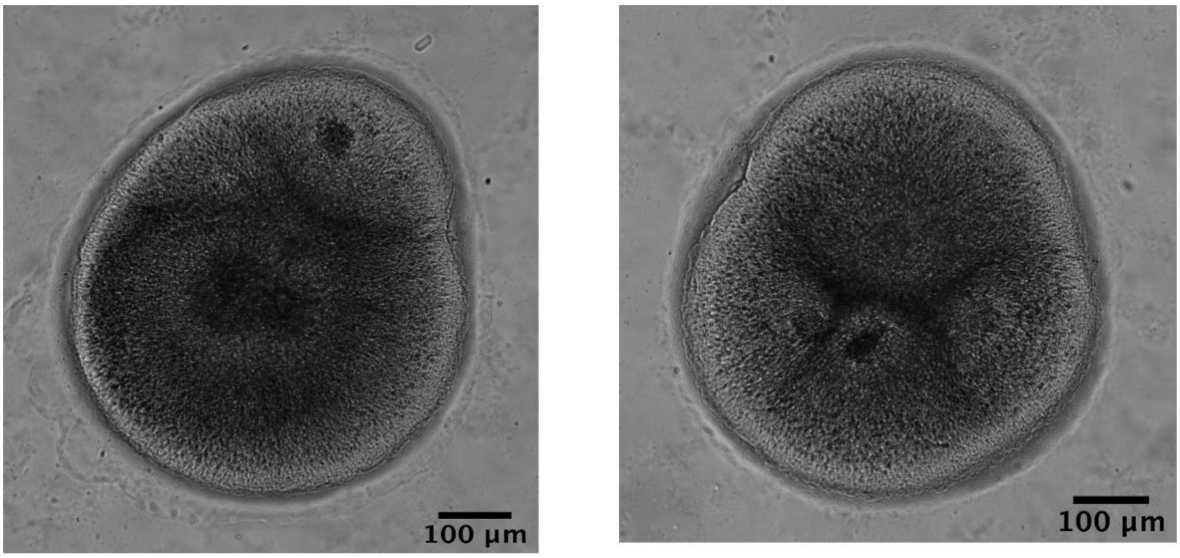


Fig. 16: L-form colony after Second subcultures on PenG treated DM3 Agar under Inverted Microscope (Nikon 10x) after 7 days of incubation.

3.2.3 Preserve Isolates of L-form

For restoration of L-form variants from the preserved vials, it should be cultured first on DM3 agar without Pen G treatment. Then first subcultured on DM3 Agar (layer by layer) with Pen G and again subcultured on the same media in the similar manner.

By repeated subculturing, we got the characteristic ‘’Fried egg’’ colonies in the PenG treated DM3 agar plates (Fig 17).

Another finding we got that the storage is better at -20^{0}c and -80 ^{0}c than 4^{0}c but in - 80 ^{0}c the cells were disturbed than -20^{0}c

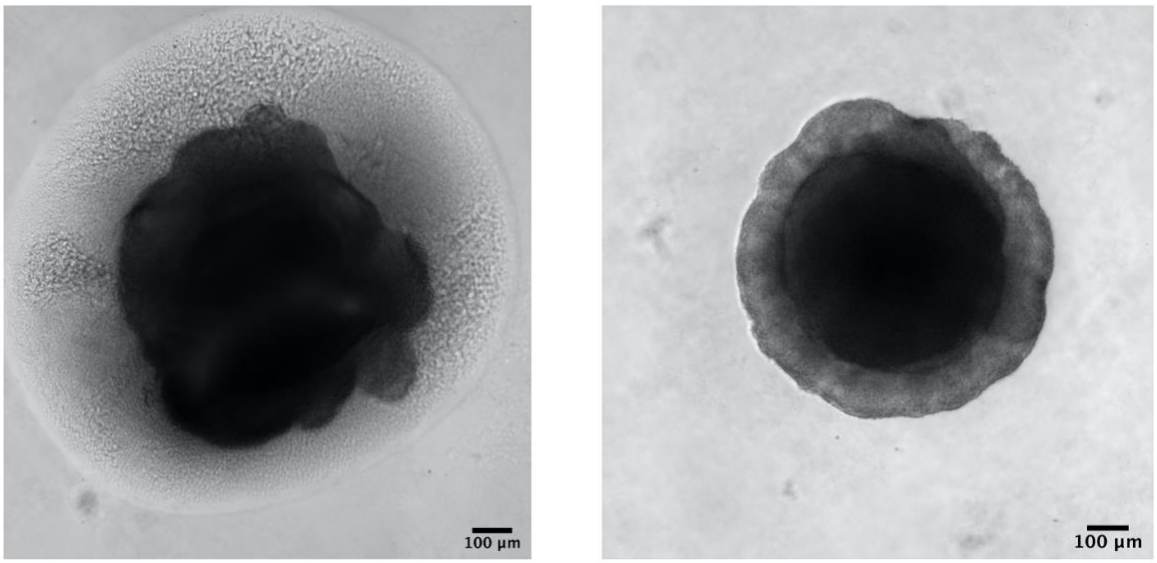


Fig. 17: L-form Colonies of preserve isolates from -20^{0}c in PenG treated DM3 agar after 7 days of incubation

3.3 Formation of L-form in SMMP Broth

3.3.1 Induction of L-form in SMMP broth

Before using SMMPB, this medium had to be optimized from the recommended SMMPB for protoplast transformation by Friedrich Goetz and B. Schumacher, 1987 and also by Jens et al, 1994 used for osmoregulation in *Bacillus subtilis.*

Table 15: Comparison among the chemical constituents used in my optimized media with recommended SMMPB and SMMPB used for *Bacillus subtilis*

Medium compounds	SMMP Broth 1* (M and g/Liter)	SMMP Broth for *Bacillus subtilis* (with supplementation) (g/ Liter) 2*	Optimized SMMP Broth for *Staphylococcus aureus* (with supplementation) (M and g/ Liter)
Sucrose	1 M = 342.30 g/L	-	1M = 342.30 g/L
Glucose	-	5 % = 50g/L	-
Dextrose	4.0 g/ L	-	4.0 g/ L
Maltose	-	Different conc.	-
Glycerol	-	Different conc.	-
Beef extract	6.0 g/L	-	6.0 g/L
Yeast extract	6.0 g/L	-	6.0 g/L
BSA	10 % = 100 g/L	-	5% = (50 g/ L)
Peptone	20 g/ L	-	20 g/ L
Tryptophane	-	0.02 g/ L	-
Phenylalanine	-	0.02 g/ L	-
Maleic acid	40 mM = 4.6 g/ L	-	40 mM = (4.6 g/ L)
MgCl2	40 mM = 3.9 g/ L	-	40 mM = (3.9 g/ L)
NaCl	14.0 g/ L	Different conc.	14.0 g/ L
KCl	-	Different conc.	-
Na3 citrate.2H2O	-	1 g/ L	-
Dipotassium Phosphate (K2HPO4)	14.72 g/ L	14 g/ L	14.72 g/ L
Monopotassium Phosphate (KH2PO4)	5.28 g/ L	6 g/ L	5.28 g/ L
Dipotassium Sulfate (K2SO4)	-	2 g/ L	-
MgSO4.7H2O	-	0.2 g/ L	-
(NH4)2SO4	-	2 g/L	-
Supplementation:			
Antioxidants			5%

- L-Ascorbic acid (Vit C)			
- Glutathione (GSH)			3mM = 0.9 g/L
Glycine Betaine		Different conc	5 mM = 0.6 g/ L
Choline		Different conc.	-

1* Improvements of protoplast transformation in *Staphylococcus carnosus*. By Friedrich Goetz and B. Schumacher, FEMS Microbiology Letters 40 (1987) 285-288.
2* (Boch, Kempf, and Bremer 1994)

For induction of L-form of *Staphylococcus aureus,* at first the reference media of protoplast transformation by Goetz et al,1987 SMMP broth was used. In this study, the final volume of SMMP broth was used 20 ml in 100 ml of Erlenmeyer flask. After overnight culture in TSB medium 250 µl was inoculated into the Pen G treated SMMP broth and incubated at 37^0c in 100 rpm shaking incubator. As like as agar media also in broth culture,300 µg/ml and 2000 µg/ml showed good result for HG001 and JE2 respectively. Diluted overnight culture into 10 times, 20 times and 50 times were also examined in this experiment but overnight culture of HG001 and JE2 showed better result.

The most observable result I found that L-form growth was not so abundant like wild type in TSB and SMMPB (Fig 18). But in this media, the L-form conversion was very slow and lots of cell debris were found due to cell damage.

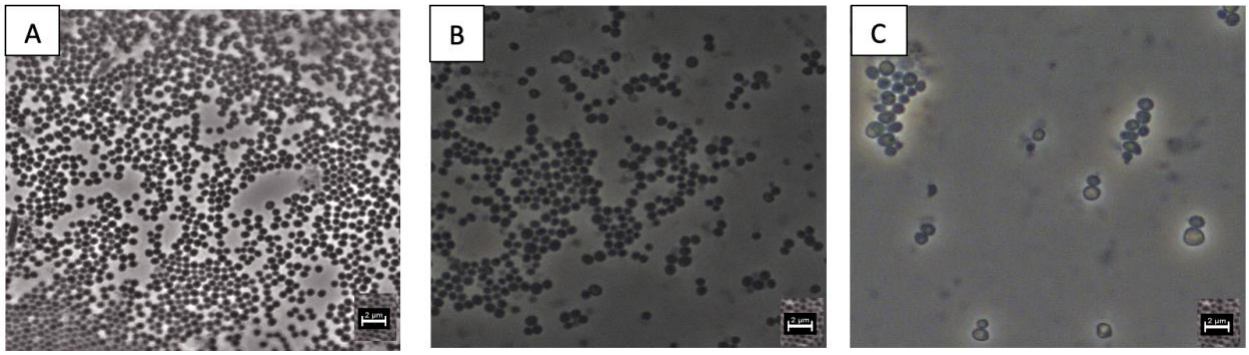


Fig. 18: Cells of HG001 in SMMP Broth; (A) Wild type in TSB, (B) Wild type in SMMPB and (C) HG001 L-form in SMMPB after 7 days (Scale bar 2µm) (Leica DML 100X).

3.3.2 Induction of L-form in SMMPB with Glycine betaine

With the view of minimization of cell lysis in SMMPB and acquisition of more L-form cells this media was optimized with some supplements. At first the SMMPB had to be enriched with osmoprotectant due to prevent the cell damage by osmotic stress in the media.

We tested the SMMPB with Glycine betaine as an osmoprotectant and the concentration was used 5 mM and 10 mM. Unfortunately, we got not significantly good result as expected (Fig 19).

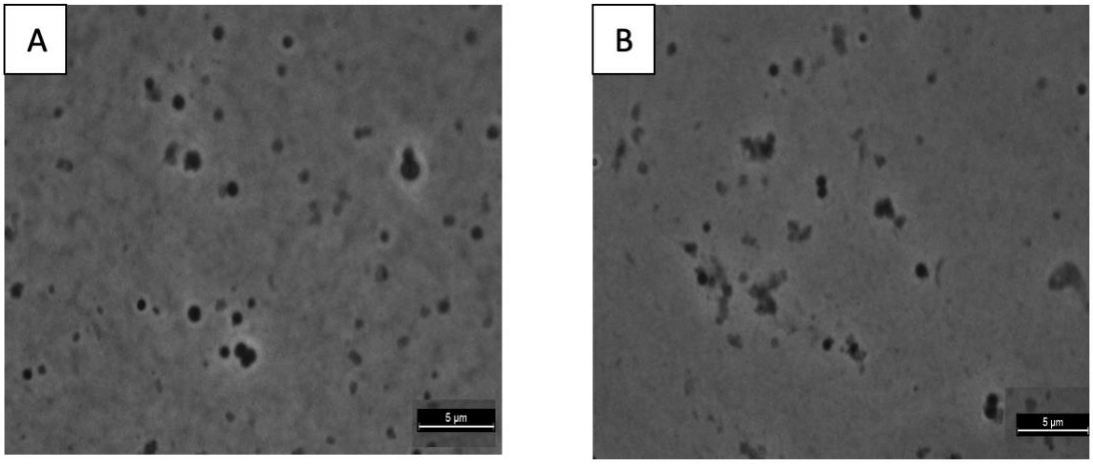


Fig. 19: Cells of HG001 in SMMP Broth with Betaine (A) L-form of HG001 in SMMPB with 10 mM Betaine (B) L-form of HG001 in SMMPB with 5 mM Betaine after 5 days of incubation (Scale bar 5 µm) (Leica DML 100X).

3.3.3 Induction of L-form in SMMPB with Glycine betaine and Ascorbic acid

In the next step, to robust this SMMPB in addition with Betaine some radical scavengers were experimented for minimizing the cell lysis by free radicals produced inside the cell along with osmotic balance.

For this study, we took L Ascorbic acid as an antioxidant in SMMPB with 5mM Glycine betaine. In this case, we tested 1%, 2% and 5% L Ascorbic acid in which 5% showed better result. Both supplementations showed desired result in L-form formation from HG001 and JE2 strains (Fig 20).

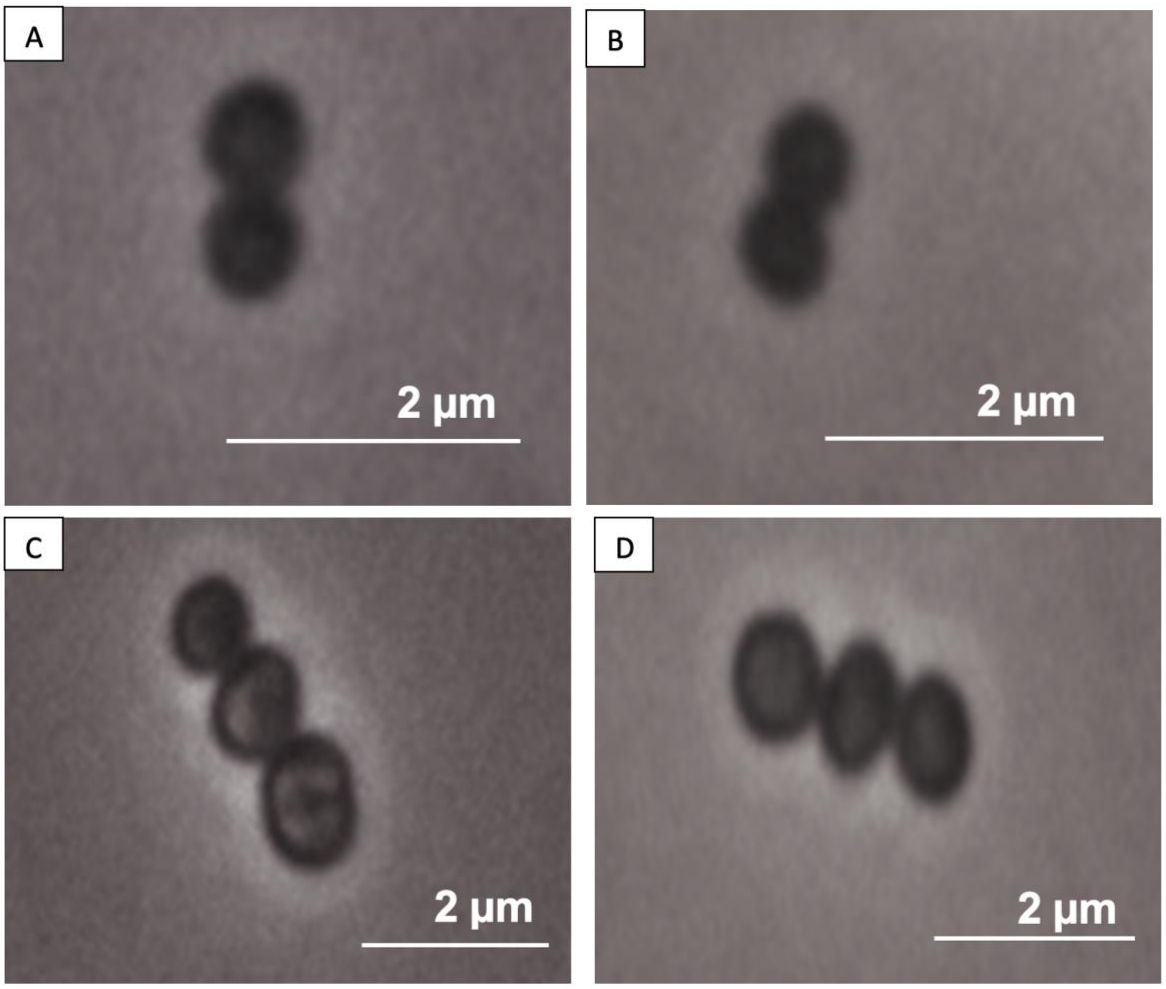


Fig. 20: Cells of HG001 and JE2 In SMMP Broth with 5 mM Betaine and 5% Ascorbic acid; (A) Wild type of HG001 Cell size about 0.75 µm, (B) Wild type of JE2 Cell size about 0.5 µm (C) L-form of HG001 Cell size about 1.00 µm and (D) L-form of JE2 Cell size about 0.75 µm (Leica DML 100X).

After getting good growth in SMMPB with these supplementations another experiment was carried out for evaluating that CO_2 either play a role as a growth promoter of L-form or not. For this evaluation, cultured flasks of L-form in this optimized media were put at 37 ^{0}c 100 rpm with 5% CO2 and also in normal shaking incubators for 5-7 days. After one day of incubation, characteristic L-form cells were observed under microscope from both incubators.

From this study the interesting results that I got, firstly L-form cells can be gained after one day of incubation but growth continues till 10 days or more. Second observation was that there was no significant difference in addition of CO2 in growth of L-form (Fig 21).

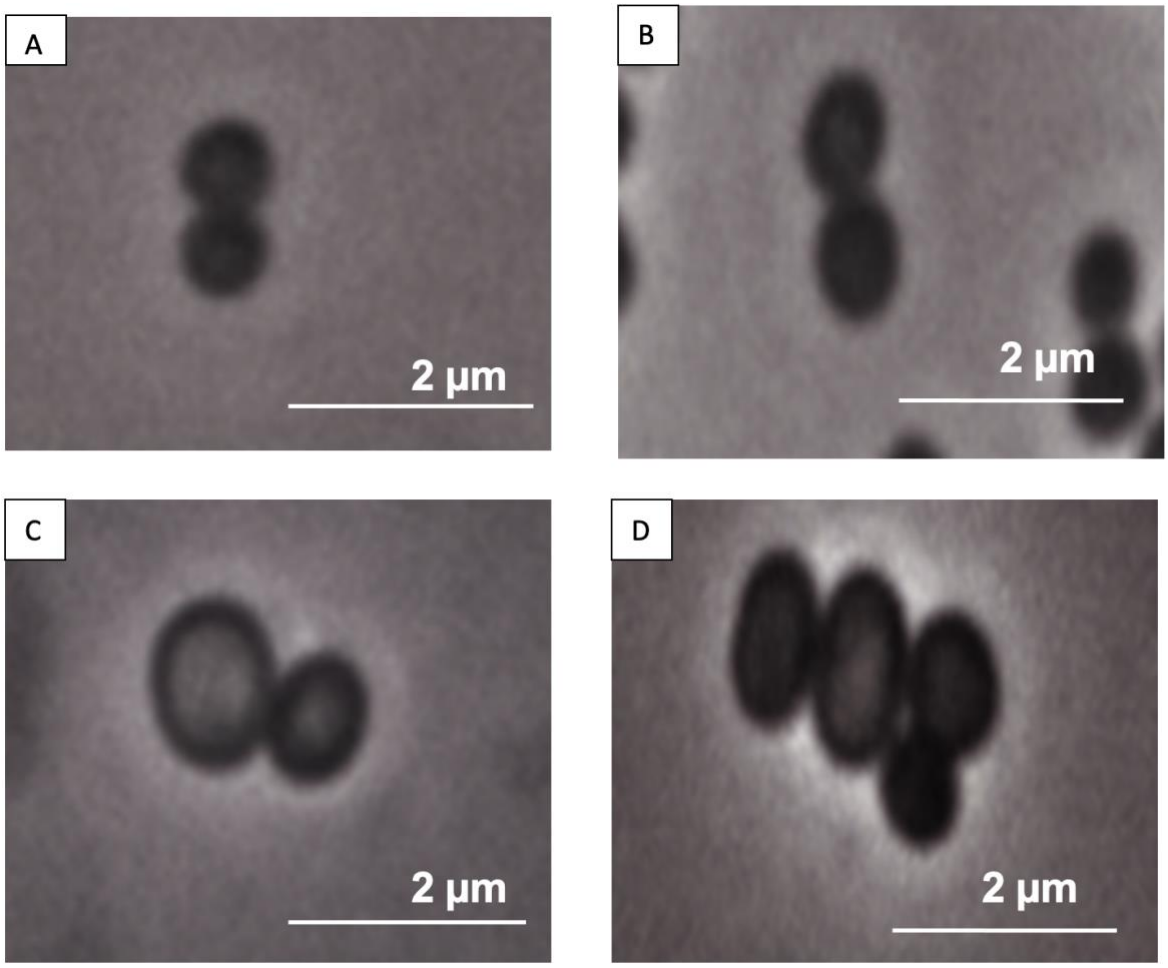


Fig. 21: Cells of HG001 in SMMP Broth with 5 mM Betaine and 5% Ascorbic acid in Normal shaking Incubator and in 5% CO_2 Incubator; (A) Wild type In SMMPB with Betaine and Ascorbic acid, (B) Wild type in TSB, (C) L-form in Normal Incubator and (D) L-form in CO2 Incubator (Leica DML 100X).

3.3.4 Induction of L-form in SMMPB with Ascorbic acid

In the optimization process, one factor I had to be considered when I faced some obstacles with Glycine betaine. Because it was suggested by the supplier that in dissolve condition Betaine cannot be stored above 2 days after preparation. That why, we optimized my SMMPB with only radical scavengers like L-Ascorbic acid as mentioned above and also with Glutathione.

Surprisingly, supplementation of SMMPB only with L-Ascorbic acid showed faster and better conversion of L-form from both strains (Fig 22, 23). Then it was possible to grow L-form within short time devoid of Betaine in SMMPB as we got the similar result with Betaine and Ascorbic acid in SMMPB and in only Ascorbic acid in SMMPB.

Another result was also found that use of Glutathione in SMMPB cannot facilitate more than Ascorbic acid.

In all these experiments, HG001 and JE2 wild types cells in both TSB and optimized SMMPB were tested as a control for the comparative analysis of wild type cell and L- form cell.

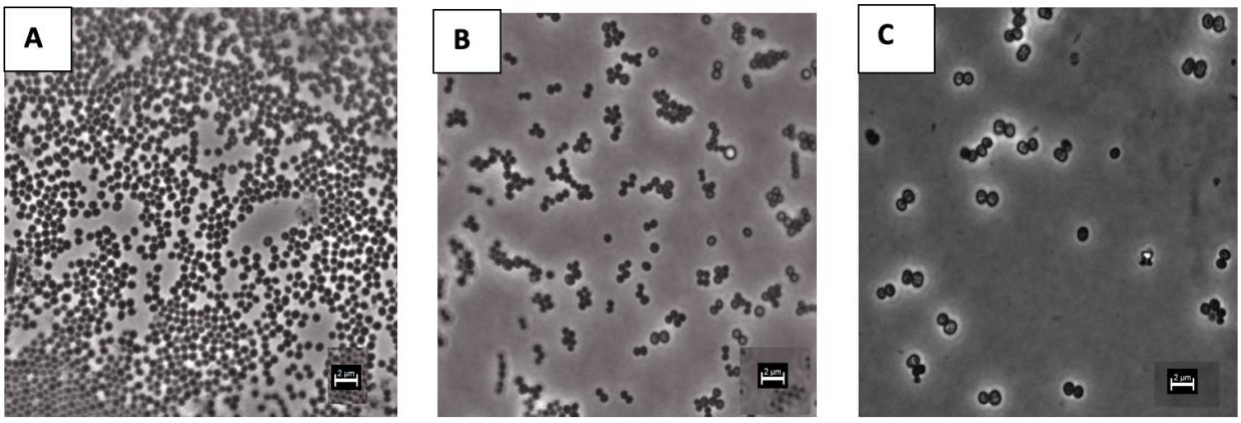


Fig. 22: Growth of (A) Wild type of HG001 in TSB; (B) Wild type of HG001 in SMMPB with Ascorbic acid and (C) L-form of HG001 in SMMPB with Ascorbic acid treated with PenG, scale bar 2µm (Leica DML 100X).

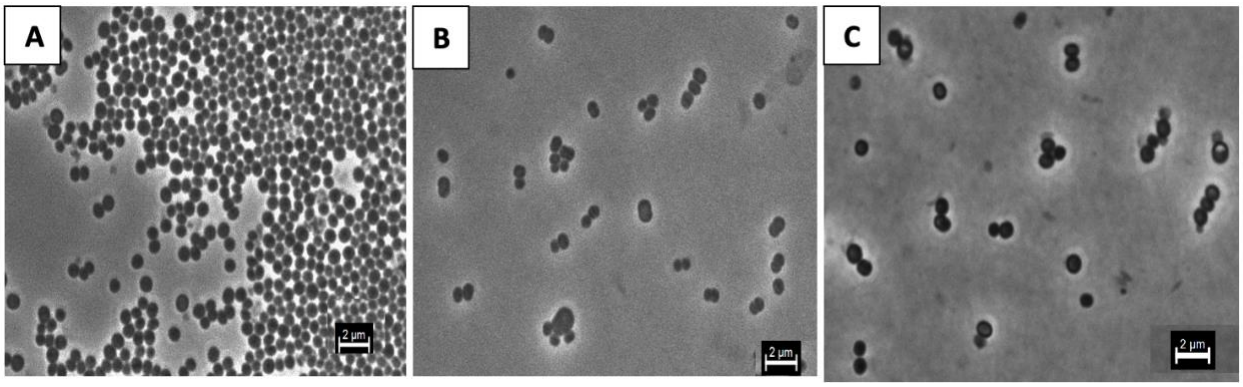


Fig. 23: Growth of (A) Wild type of JE2 in TSB; (B) Wild type of JE2 in SMMPB with Ascorbic acid and (C) L-form of JE2 in SMMPB with Ascorbic acid treated with PenG, scale bar 2µm (Leica DML 100X).

3.3.5 Microscopic characterization of L-form Cells in SMMPB

In the optimized SMMPB with Pen G, L-form cell looked like a vacuole surrounded by a thin covering, not so rounded, all cell's shape was not exactly similar, some were in budding form and also had clusters of proliferating cells (Fig 22,23). On contrast, wild type cells looked like evenly rounded, arranged as paired, clusters or single form which are their characteristic feature (Fig 22,23). The cell size of L-form of HG001 was observed 1µm (Fig 24) which was bigger than wild type because HG001 WT cell size was about 0.85 µm in SMMPB and TSB (Fig 24). The L-form of JE2 cell size was little bit smaller than HG001 L-form like 0.75 µm (Fig 25) and its wild type cell size was 0.5 µm (Fig 25). In this analysis, Phase contrast microscope was used (100X).

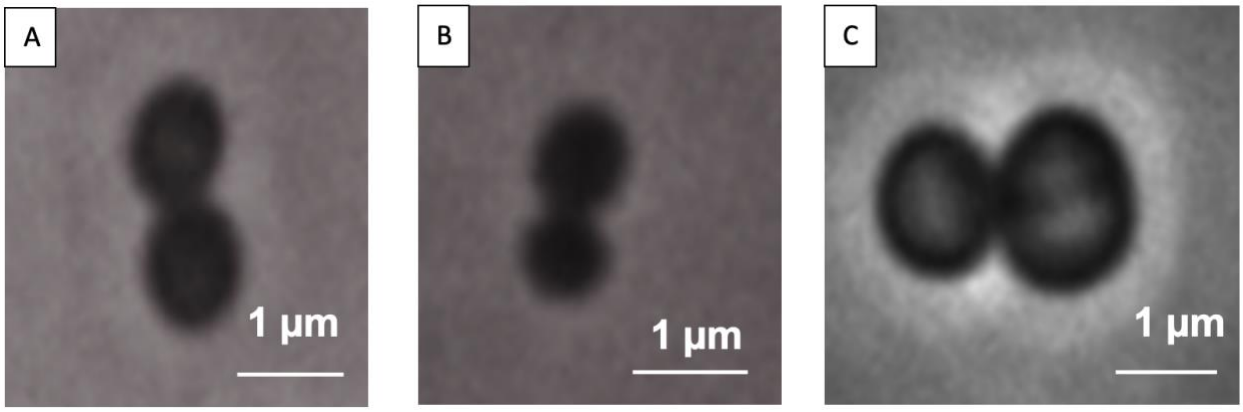


Fig. 24: Cells of HG001 In SMMP Broth with 5% Ascorbic acid; (A) Wild type in TSB Cell size about 0.85 µm, (B) Wild type in SMMPB with Ascorbic acid Cell size about 0.75 µm and (C) L-form of HG001 Cell size about 1.00 µm (Leica DML 100X).

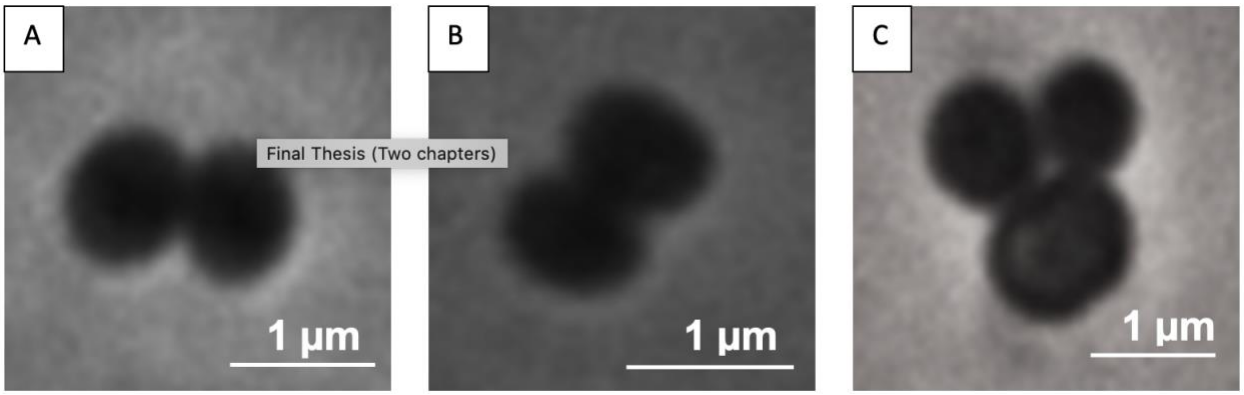


Fig. 25: Cells of JE2 In SMMP Broth with 5% Ascorbic acid; (A) Wild type in TSB Cell size about 0.50 µm, (B) Wild type in SMMPB with Ascorbic acid Cell size about 0.55 µm and (C) L-form of Cell size about 0.85 µm (Leica DML 100X).

3.4 Characterization through Fluorescence Microscope

For characterization *S. aureus*, Cell wall was stained with Vancomycin FL bodipy as green fluorescence, for cell membrane FM4 64 was used that fluorescence red color and in the case of DNA, DAPI was used that colored blue under fluorescence microscope. It was very closely observed that not in other two staining, only in FL Vancomycin L-form binding signal was lighter than WT in both strains.

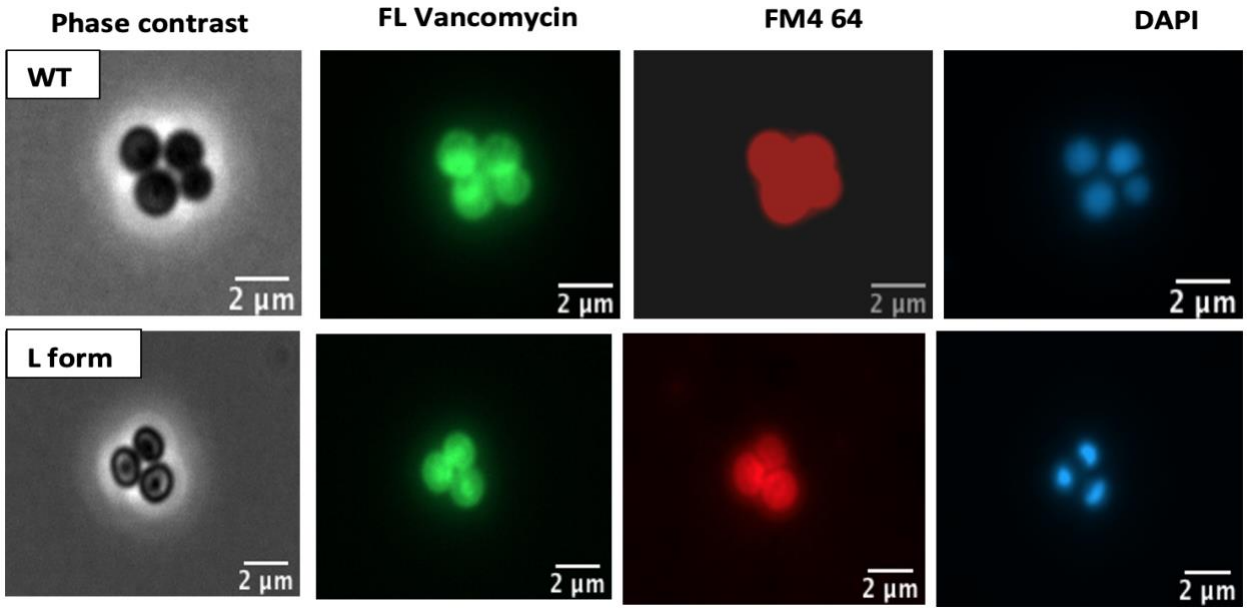


Fig. 26: HG001 WT and L-form cells in SMMPB with 5% ascorbic acid under Fluorescent microscope.

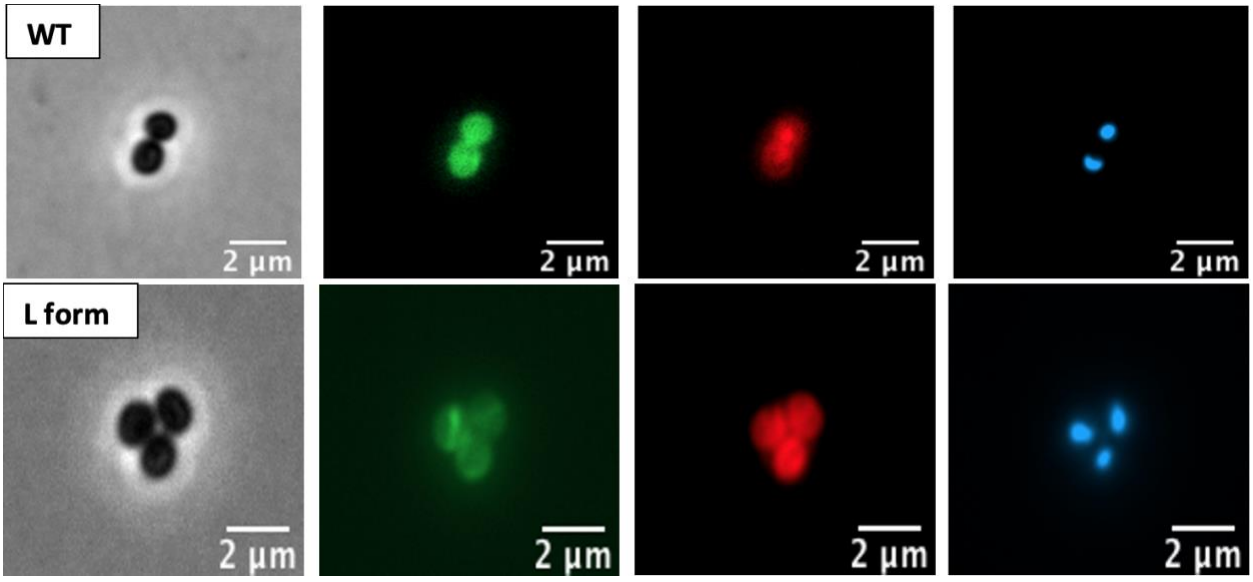


Fig. 27: JE2 WT and L-form cells in SMMPB with 5% ascorbic acid under Fluorescent microscope.

3.5 Growth curve study

To study the growth behaviour, OD was analyzed from the cultured flasks of the HG001, JE2 as well as wild types from both strains in optimized SMMPB as a control. L-form and WT cultures in optimized SMMPB were inoculated from an overnight culture to the starting OD_{578}= 0.1. The growth studies were performed as described in methods (section 2.2.7).

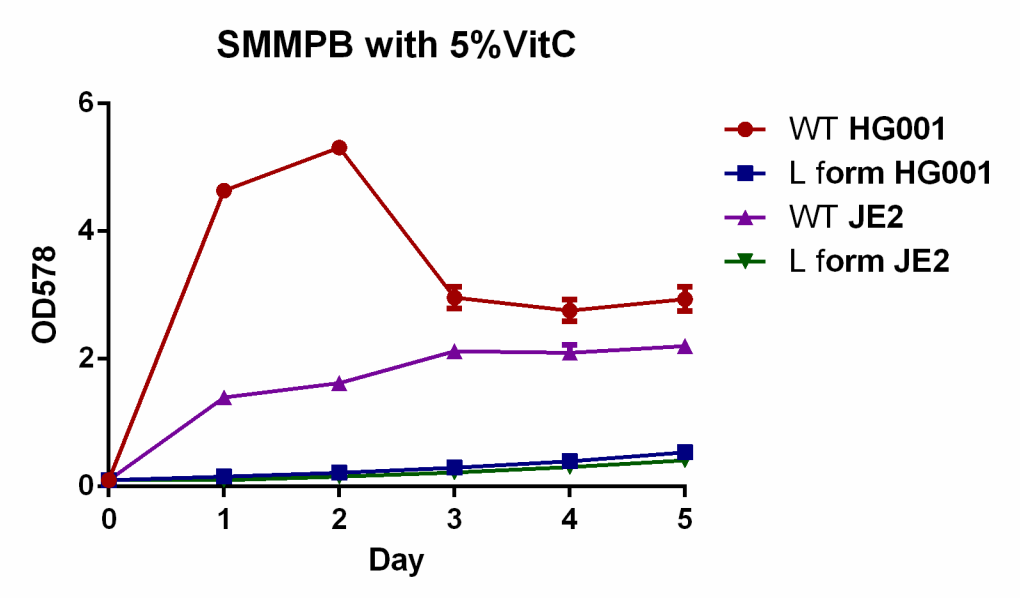


Fig. 28: Growth curve shows the growth pattern of L-form of HG001 and JE2 in comparison to their WT in the same optimized media.

Analysis of growth showed, L-form of both strain growth was very slow than wild types. Moreover, the lag phase of L-form variants from HG001 and JE2 was lengthier and it took around 1 day in slower way for getting into exponential stage. On the other hand, wild type strains quickly reached to log phase and grew faster (Fig 28).

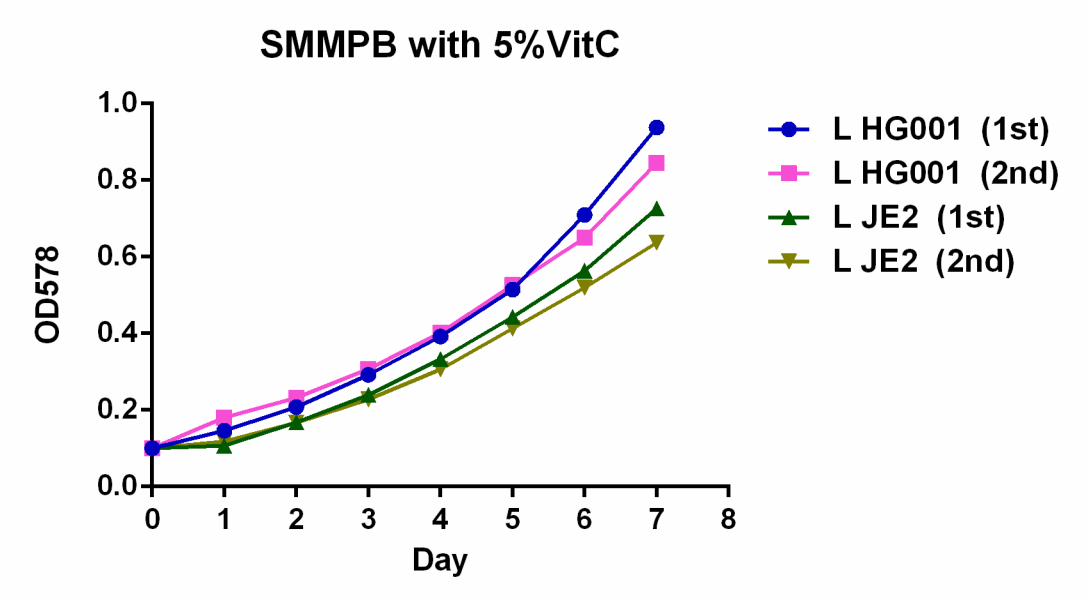


Fig. 29: Growth curve shows the comparative analysis in Lag phase as well as growth pattern of L-form of HG001 and JE2 in primary culture (1st) and subculture (2nd) in PenG treated optimized media.

For analysis of the time that required for adjustment stage of both strains, another growth curve was determined. The growth studies were performed as described in methods (section 2.2.7). Here, also the starting OD was 0.1. But unfortunately, no more variation was observed in the time consumed for lag phase of HG001 and JE2 (Fig 29).

It can be assumed from above growth curves that in the case of L-form in this optimized media the generation time was around 24 hrs for both of HG001 and JE2.

3.6 Lysostaphin assay

Cell wall defective state of HG001 and JE2 should be resistant to lysostaphin. For this analysis, lysostaphin assay was carried out with L-form of both strains as well as WT in both TSB and optimized SMMPB as a control. In this test, WT of HG001 and JE2 from both media showed clear culture due to lysis by lysostaphin whereas without lysostaphin treatment WT revealed turbidity for cellular growth after 2 hrs incubation. Interestingly, L-form of both strains with and without lysostaphin displayed slight turbidity as an indication of cellular growth. Moreover, this result was more understandable when observed under microscope. Because, WT of HG001 and JE2 with lysostaphin treatment exhibited cell lysis (Fig 30, 32). In contrast, WT of both strains without lysostaphin treatment shown huge cell growth (Fig 30,32). However, L-form of HG001 and JE2 from with and without lysostaphin treatment presented cell growth under microscope (Fig 31,33).

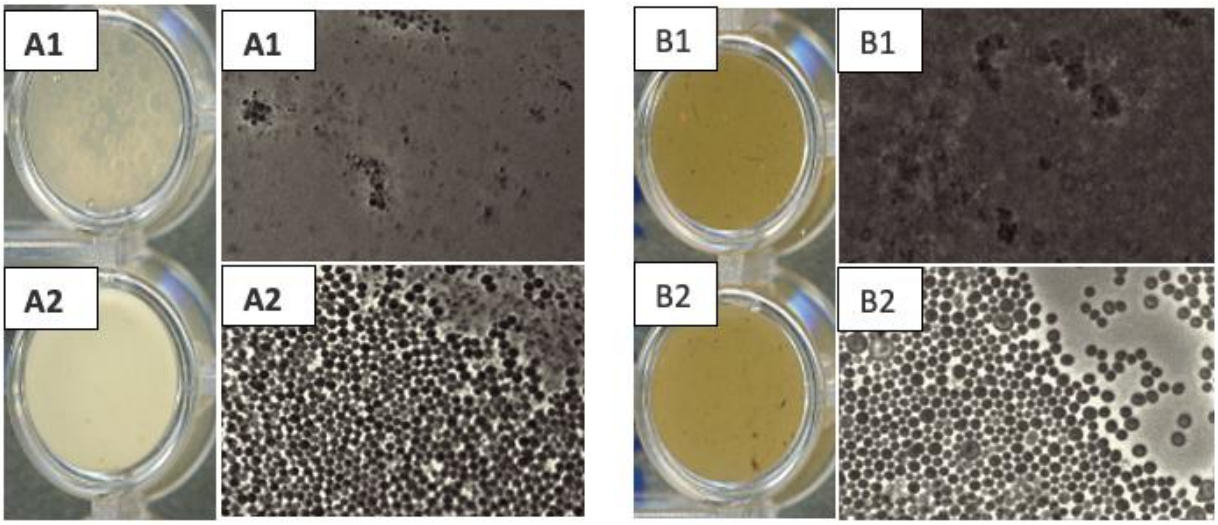


Fig. 30: (A) Lysostaphin assay with HG001 WT in T SB; A1 cells with Lysostaphin 0.3mg/ml, A2 cells without Lysostaphin. (B) Lysostaphin assay with HG001 WT in optimized SMMPB; B1 cells with Lysostaphin 0.3mg/ml, B2 cells without Lysostaphin.

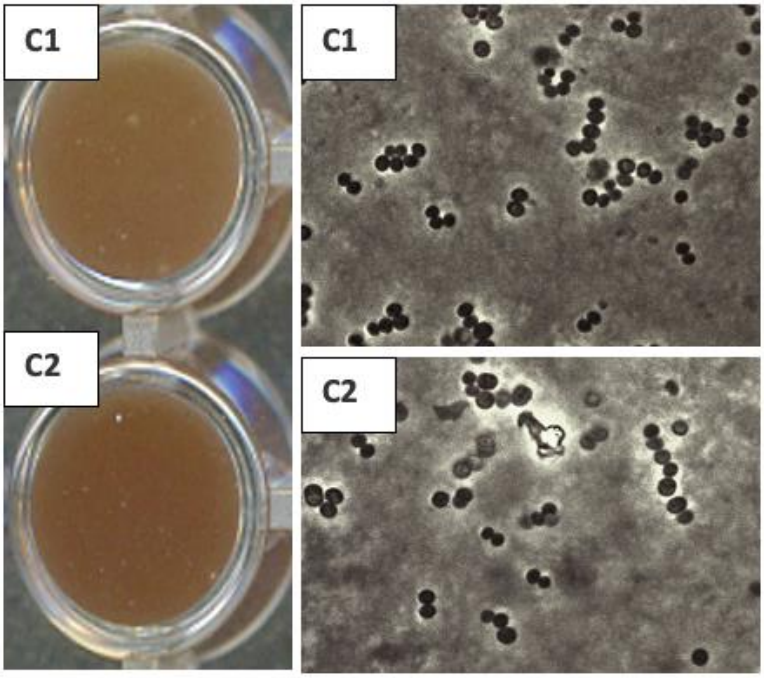


Fig. 31: (C) Lysostaphin assay with HG001 L-form; C1 cells with Lysostaphin 0.3mg/ml, C2 cells without Lysostaphin.

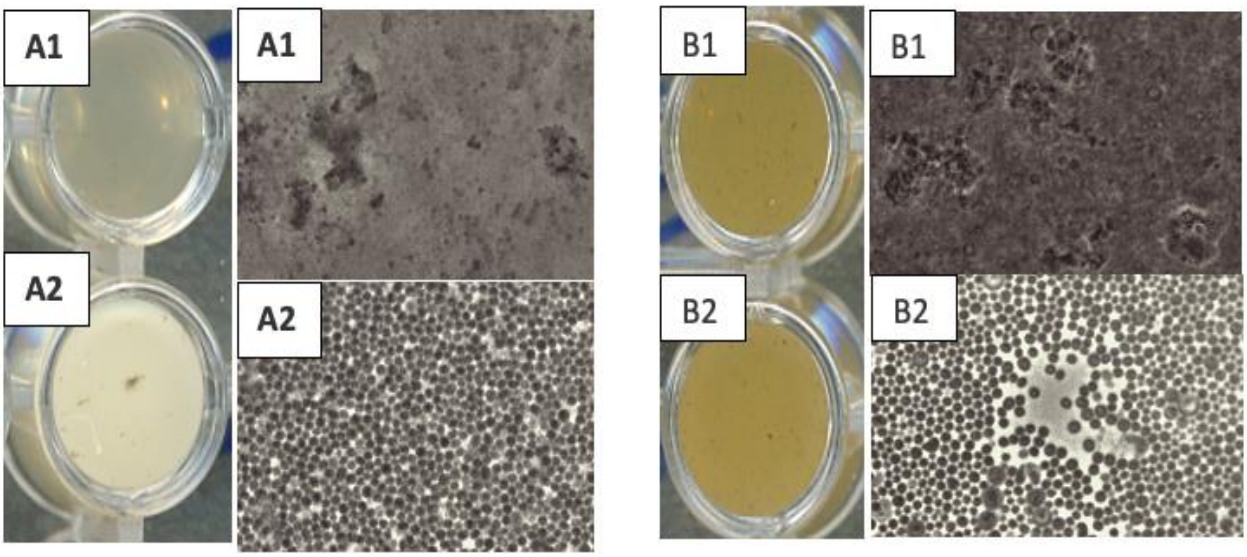


Fig. 32: (A) Lysostaphin assay with JE2 WT in TSB; A1 cells with Lysostaphin 0.3mg/ml, A2 cells without Lysostaphin. (B) Lysostaphin assay with HG001 WT in optimized SMMPB; B1 cells with Lysostaphin 0.3mg/ml, B2 cells without Lysostaphin.

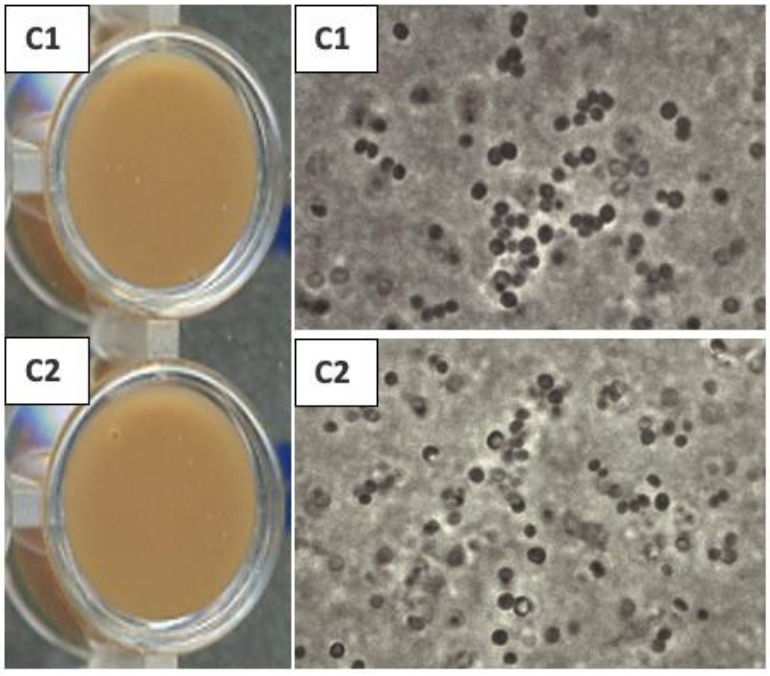


Fig. 33: (C) Lysostaphin assay with JE2 L-form; C1 cells with Lysostaphin 0.3mg/ml, C2 cells without Lysostaphin.

4. Discussion

4.1 Optimization of DM3 agar for propagation of L-form

The preparation of L-forms from *Staphylococcus aureus* involved the selection of two strains for this study: HG001, a wild-type strain, and JE2, a methicillin-resistant strain. This Gram-positive bacterium has a thick cell wall that plays a critical role in preventing the cell from mechanical damage, including cell lysis due to osmotic pressure and other stressors. The cell wall also maintains the shape of the cell and aids the cells in growth and multiplication.

For the in vitro propagation of cell wall-defective variants, the culturing conditions must be such that the defective form of bacteria can survive without any stress. In my optimization process, certain stress factors had to be considered for L-form bacteria to achieve a higher conversion of L-form in a shorter time and in an easier manner.

In this study, DM3 agar was used for the propagation of L-forms from these two strains. This medium was chosen because it is enriched and contains all the required nutrients for the stress control culture of defective cell-walled bacteria. For osmotic balance, this medium contains Na salts in the required amount, which is a crucial factor for propagating L-form in vitro.

DM3 agar, being a rich medium, is usually used for protoplast transformation. However, when I selected this medium for L-form preparation, I encountered some problems in propagating the L-form. In response, I optimized some of the chemicals in the proposed DM3 agar by Chang and Cohan (Chang and Cohen 1979) and I successfully prepared L-forms from both HG001 (Fig 11) and JE2 (Fig 12) within three days using the overlay method (Page 37). The comparative analysis of the optimized DM3 agar for L-form preparation with the Chang and Cohan proposed DM3 agar for protoplast transformation in *Bacillus subtilis* is shown in Table 13.

In this Gram-positive group, Bacillus is another important member, similar to Staphylococcus. In earlier days, some media were used by many researchers to obtain L-forms from Bacillus subtilis. So, in our study, we tried to follow their chemical setup and attempted to compare my recipe for L-form induction media of Staphylococcus aureus with their used media (Table 14).

4.2 Optimization of SMMP broth for propagation of L-form

In this research endeavor, the utilization of SMMP (Synthetic Minimal Medium with Phosphate) broth emerged as a pivotal component for inducing and cultivating L-forms from strains of *Staphylococcus aureus*. The expedited conversion of bacterial cells into L-forms within a single day of incubation underscored the efficacy of SMMP broth in this context. This medium's versatility has been extensively validated, notably in protoplast transformation studies conducted by Goetz and Schumacher in 1987, which solidified its reputation as a suitable medium for bacterial manipulation.

Despite its proven track record, the experimentation with SMMP broth revealed significant challenges. Notably, a notable portion of the bacterial cells experienced premature lysis before completing the transformation into L-forms. This phenomenon raised concerns regarding potential stressors within the medium, such as osmotic imbalances or oxidative stress, which could undermine the successful propagation of L-forms.

To address these challenges, a series of strategic adjustments were made to enhance the performance of SMMP broth. Initially, the incorporation of Glycine Betaine emerged as a logical intervention, drawing inspiration from previous studies by Jens et al. (1994) and James et al. (1992). (Boch, Kempf, and Bremer 1994) These studies underscored the efficacy of Glycine Betaine as an osmoprotectant in mitigating cellular stress, particularly in bacterial systems. However, despite the addition of Glycine Betaine, the

desired outcomes remained elusive, prompting further refinement of the experimental approach.

Subsequently, the introduction of L-ascorbic acid, a potent antioxidant with radical scavenging properties, was explored as a potential solution. The supplementation of SMMP broth with 5% L-ascorbic acid, in conjunction with 5 mM Glycine Betaine, yielded promising results (Fig. 20), indicative of improved cellular viability and resilience to oxidative stress. Nevertheless, it was noted that the stability of Glycine Betaine within the medium was time-sensitive, necessitating expedited utilization to prevent degradation.

In response to practical constraints associated with Glycine Betaine stability, experiments were conducted solely with L-ascorbic acid supplementation in SMMP broth. Remarkably, comparable outcomes to the combined supplementation regimen were achieved, reaffirming the pivotal role of L-ascorbic acid in enhancing the propagation of L-forms in *Staphylococcus aureus* (Fig 22,23).

To contextualize these optimizations, a comprehensive comparison was conducted between the reference SMMP broth and the refined formulations, alongside analogous preparations utilized in L-form induction studies involving *Bacillus subtilis* (Table 15). This comparative analysis provided valuable insights into the efficacy of the optimized SMMP broth in facilitating L-form propagation.

Furthermore, insights gleaned from studies by Kawai et al. in 2015 underscored the importance of radical scavengers in enhancing the growth of L-forms (Kawai et al. 2015), particularly in the context of oxidative stress-induced damage. Building upon these findings, attempts were made to integrate glutathione (GSH) into the SMMP broth, albeit with limited success compared to L-ascorbic acid supplementation.

Ultimately, the optimization process culminated in the development of a tailored SMMP broth formulation comprising solely of L-ascorbic acid supplemented with Penicillin G (PenG) as a cell wall inhibitor. Notably, Penicillin G consistently yielded successful outcomes in both DM3 agar and SMMP broth experiments, aligning with previous findings

by researchers such as (Han et al. 2015) on Staphylococcus, (Young and Armstrong 1969) on Streptococcus.

Moreover, the efficacy of Penicillin G, along with Cephalosporin, as potent cell wall-affecting antibiotics against which most Gram-positive and Gram-negative L-forms exhibit resistance, was reaffirmed by insights from Errington et al.'s review paper in 2016 (Errington et al. 2016). These collective findings underscored the robustness of the optimized SMMP broth formulation in facilitating the induction and propagation of L-forms from *Staphylococcus aureus* strains HG001 and JE2, thereby contributing to the advancement of bacterial research methodologies.

4.3 Morphological characterization of L-form on DM3 agar

In the morphological characterization of L-forms, tiny colonies resembling dew drops were observed on DM3 agar (Fig. 8). Visually, these colonies sometimes appeared as a central, deeper area surrounded by a thin covering. During our study, we noted that the L-form colonies adhered tightly to the agar surface, making it difficult to extract a colony from the agar without scraping. These observations parallel those of Williams in 1963 (Williams 1963), who reported that Staphylococcal L-forms exhibited a similar adherence to agar, with microscopic examination revealing deep center formation within the colony and surface spread beyond the central area. Similarly, Han et al. (2015) found comparable results in *S. aureus* L-forms (Han et al. 2015).

Microscopic examination revealed colonies resembling "Fried eggs" on DM3 agar under both light and inverted microscopy (Fig. 9, 10, 11, 12), a characteristic feature of L-forms as described by various researchers studying L-forms from different bacteria (Han et al. 2015), (Glover, Yang, and Zhang 2009) and (Xu et al. 2020). These researchers documented that the primary growth site appeared beneath the agar surface as a core area containing pleomorphic budding forms. In our observations, this phenomenon likely contributes to the "Fried egg" appearance of L-form colonies under the microscope.

4.4 Characterization of L-form cells in SMMP broth

Under microscopic scrutiny of the broth cultures, a remarkable phenomenon unfolded: the emergence of discernible large bodies merely a day after the initiation of incubation. These sizable vesicles, measuring approximately 1 µm in diameter, starkly contrasted with the usual dimensions observed in the wild type HG001 strain, which typically registers at around 0.85 µm in both Tryptic Soy Broth (TSB) and the specialized Synthetic Medium for Minimal Populations (SMMP) supplemented with Ascorbic acid (as illustrated in Fig. 24). Conversely, cells belonging to the JE2 strain exhibited a slightly reduced size compared to HG001, measuring at approximately 0.50 µm in both TSB and Ascorbic acid-enriched SMMPB. However, what truly intrigued was the observation that L-form cells boasted a size of about 0.75 µm (as depicted in Fig. 25).

These findings insinuate a potential correlation between the cell wall-defective state induced by Penicillin G activity and an amplification in cellular volume, evident through the expanded size of L-form cells. Furthermore, it's noteworthy to mention that the cellular morphology, including both size and shape, showcased a degree of variability, underscoring the dynamic nature of L-form populations.

Delving into historical experiments, the investigations conducted by Kunihiko Yabu during the mid-1980s provided valuable insights into the characteristics of unstable L-forms of S. aureus cultivated in liquid medium. Notably, large bodies emerged within a mere few days, distinctly discernible under phase-contrast microscopy.(Yabu 1985);(Yabu 1986).

Similarly, recent research endeavors by Patricia et al. in 2012 unveiled analogous phenomena with *Bacillus subtilis* L-forms (Dominguez-Cuevas et al. 2012). Additionally, pioneering studies by Weibull and Lundin in 1961 delved into the realm of stable L-forms of *Proteus mirabilis* (Weibull and Lundin 1961), unraveling the presence of akin large bodies in broth cultures, meticulously observed under phase-contrast microscopy. These collective findings not only accentuate the ubiquitous presence of large bodies across various L-form populations but also underscore their paramount importance in unraveling the intricacies of L-form biology and their potential implications in microbial physiology.

4.5 Characterization through Fluorescence microscope

In addition to morphological characterization, HG001 and JE2 cells cultured in the optimized media underwent further scrutiny through the application of various fluorescent dyes (as depicted in Fig. 26 and 27). Upon diligent analysis of the fluorescence microscopy results, a notable observation emerged: the signals emitted by FL Vancomycin were notably weaker in L-forms compared to their wild type counterparts. This intriguing phenomenon can be attributed to the altered structural composition of L-form cells, wherein the integrity of the peptidoglycan layer, crucial for the binding of Vancomycin, is compromised.

While these initial findings provide valuable insights into the differential response of L-form cells to fluorescent dyes, further experimentation is imperative to garner more comprehensive and statistically significant results. Future endeavors will focus on quantifying the signaling patterns of L-forms in comparison to wild types using fluorescence microscopy. By conducting parallel experiments under controlled conditions, a deeper understanding of the distinct fluorescence profiles exhibited by L-form cells can be achieved, shedding light on their unique biology and potential implications in microbial physiology and pathology.

4.6 Growth curve study

In the experiment focusing on growth curve determination, notable distinctions were observed between the L-forms of both HG001 and JE2 strains compared to their wild type counterparts. The growth rate of L-form variants was markedly slower, with a prolonged lag phase before reaching the exponential phase of growth. This sluggish progression continued until all available nutrients were consumed (as depicted in Fig. 28 and 29). Conversely, the wild type strains of HG001 and JE2 exhibited a more consistent growth pattern in the optimized media: after an initial rapid acceleration phase, growth proceeded at a slower, more sustained pace (Fig. 28).

Furthermore, when comparing the growth curves of primary cultures and subcultured flasks for both strains, no significant differences in the duration of the lag phase were observed (Fig. 29).

These findings are consistent with earlier research conducted by Kunihiko Yabu in 1985 and 1986, where S. aureus L-forms cultivated in liquid media treated with 6-aminopenicillanic acid (6-APA) and lysostaphin exhibited an extended lag phase (Yabu 1985); (Yabu 1986). Yabu's experiments, which involved measuring optical density (OD) daily and quantifying viable counts, also highlighted the prolonged lag phase characteristic of L-form bacteria.

Together, these observations underscore the distinctive growth dynamics of L-forms compared to their wild type counterparts, emphasizing the importance of understanding these differences in the context of bacterial physiology and adaptation.

4.7 Lysostaphin assay

In our study, we conducted experiments involving Lysostaphin treatment on both L-form variants of HG001 and JE2, as well as their wild type counterparts cultured in our optimized Synthetic Medium for Minimal Populations with Bacillus (SMMPB), with Tryptic Soy Broth (TSB) serving as a control. Our analysis revealed that L-form variants displayed resistance to Lysostaphin treatment (as illustrated in Fig. 31 and 33), whereas the wild type strains exhibited sensitivity to this compound (depicted in Fig. 30 and 32).

Previous research by Wadström and Vesterberg (1971) elucidated the mechanisms of action of Lysostaphin, highlighting its efficacy through three enzymes targeting the cell wall of Staphylococcus bacteria (Wadstrom and Vesterberg 1971). Additionally, Gre´gory Francius et al. (2008) provided further insights, stating that Lysostaphin specifically cleaves the peptidoglycan bridge of S. aureus, leading to bacterial lysis (Francius et al. 2008). In the case of L-forms, characterized by defective cell walls, this variant evaded

lysis by Lysostaphin. Conversely, wild type cells possess well-defined cell walls, rendering them susceptible to damage by Lysostaphin.

These findings underscore the importance of understanding the differential responses of L-form variants and wild type strains to antimicrobial agents like Lysostaphin, shedding light on potential avenues for targeted therapeutic interventions against Staphylococcus aureus infections.

4.8 Outlook

Looking ahead, future research endeavors will be directed towards the molecular characterization of *S. aureus* HG001 and JE2 strains, representing a significant challenge in unraveling the underlying molecular pathways governing L-form induction. Indeed, elucidating these pathways remains a formidable task, as the mechanisms underlying L-form formation continue to remain elusive. Furthermore, subsequent analyses will explore the response of these L-form isolates to a diverse array of antibiotics targeting cell wall and cell membrane components, thus providing valuable insights for combating Staphylococcal infections effectively. Ultimately, this research paves the way for a deeper understanding of L-form biology and its implications in antimicrobial resistance and therapeutic intervention strategies.

5. Summary and conclusion

Staphylococcus aureus exists in that group of bacteria which consists complete peptidoglycan covering as a protective shield for mechanical protection, biosynthesis, multiplication etc. Not only the complete form of this bacteria is infective but also its defective cell wall state can cause persistent and recurrent infections which is more detrimental for humans and animals. For the research purpose, by suppling suitable environment in vitro this defective state called L-form can be induced.

In this research endeavor, we achieved success in optimizing two distinct media formulations tailored for the cultivation of L-form variants of *Staphylococcus aureus*, providing a convenient and streamlined approach for their propagation. Notably, in our optimized DM3 agar medium, both experimental strains exhibited characteristic "Fried egg" colonies, a hallmark feature of L-form growth. Furthermore, the HG001 and JE2 strains readily transitioned into the L-form state in the optimized SMMP broth, demonstrating rapid conversion within a short timeframe. Utilizing this broth culture, we were able to observe large vacuolated L-form cells under phase contrast microscopy, further characterized through the application of various fluorescent dyes.

Moreover, our study revealed the inherently slow growth kinetics of L-form variants, particularly evident in the prolonged lag phase observed for each strain. Additionally, we investigated the activity of Lysostaphin against the L-form state of bacteria, shedding light on their altered susceptibility to antimicrobial agents.

In conclusion, our results shed on the characterization and mechanisms underlying this L-form formation in *S aureus*. This research findings can impact to develop a useful tool for identifying the different effective therapies against Staphylococcal infections. Thereby, a successful therapeutic strategy can advance to fight recurrent and latent infections by Staphylococcus.

5. References

1. Allan, E. J. 1991. 'Induction and cultivation of a stable L-form of Bacillus subtilis', *J Appl Bacteriol*, 70: 339-43.
2. Allan, E. J., C. Hoischen, and J. Gumpert. 2009. 'Bacterial L-forms', *Adv Appl Microbiol*, 68: 1-39.
3. Banville, R. R. 1964. 'Factors affecting growth of Staphylococcus aureus L-forms on semidefined medium', *J Bacteriol*, 87: 1192-7.
4. Beaman, B. L. 1980. 'Induction of L-phase variants of Nocardia caviae within intact murine lungs', *Infect Immun*, 29: 244-51.
5. Beaman, B. L., and S. M. Scates. 1981. 'Role of L-forms of Nocardia caviae in the development of chronic mycetomas in normal and immunodeficient murine models', *Infect Immun*, 33: 893-907.
6. Bettinger, G. E., and F. E. Young. 1975. 'Transformation of Bacillus subtilis: transforming ability of deoxyribonucleic acid in lysates of L-forms or protoplasts', *J Bacteriol*, 122: 987-93.
7. Boch, J., B. Kempf, and E. Bremer. 1994. 'Osmoregulation in Bacillus subtilis: synthesis of the osmoprotectant glycine betaine from exogenously provided choline', *J Bacteriol*, 176: 5364-71.
8. Boost, M. V., M. M. O'Donoghue, and A. James. 2008. 'Prevalence of Staphylococcus aureus carriage among dogs and their owners', *Epidemiol Infect*, 136: 953-64.
9. Burmeister, H. R., and C. W. Hesseltine. 1968. 'Induction and propagation of a Bacillus subtilis L-form in natural and synthetic media', *J Bacteriol*, 95: 1857-61.
10. Burton, S., R. Reid-Smith, J. T. McClure, and J. S. Weese. 2008. 'Staphylococcus aureus colonization in healthy horses in Atlantic Canada', *Can Vet J*, 49: 797-9.
11. Chang, S., and S. N. Cohen. 1979. 'High frequency transformation of Bacillus subtilis protoplasts by plasmid DNA', *Mol Gen Genet*, 168: 111-5.

12. Clasener, H. 1972. 'Pathogenicity of the L-phase of bacteria', *Annu Rev Microbiol*, 26: 55-84.
13. Cole, A. M., S. Tahk, A. Oren, D. Yoshioka, Y. H. Kim, A. Park, and T. Ganz. 2001. 'Determinants of Staphylococcus aureus nasal carriage', *Clin Diagn Lab Immunol*, 8: 1064-9.
14. DeLeo, F. R., M. Otto, B. N. Kreiswirth, and H. F. Chambers. 2010. 'Community-associated meticillin-resistant Staphylococcus aureus', *Lancet*, 375: 1557-68.
15. Dell'Era, S., C. Buchrieser, E. Couve, B. Schnell, Y. Briers, M. Schuppler, and M. J. Loessner. 2009. 'Listeria monocytogenes L-forms respond to cell wall deficiency by modifying gene expression and the mode of division', *Mol Microbiol*, 73: 306-22.
16. Devine, K. M. 2012. 'Bacterial L-forms on tap: an improved methodology to generate Bacillus subtilis L-forms heralds a new era of research', *Mol Microbiol*, 83: 10-3.
17. Dienes, L. 1947. 'The Morphology of the L(1) of Klieneberger and Its Relationship to Streptobacillus moniliformis', *J Bacteriol*, 54: 231-7.
18. Dienes, L., and H. J. Weinberger. 1951. 'The L-forms of bacteria', *Bacteriol Rev*, 15: 245-88.
19. Domingue, G. J. 2010. 'Demystifying pleomorphic forms in persistence and expression of disease: Are they bacteria, and is peptidoglycan the solution?', *Discov Med*, 10: 234-46.
20. Domingue, G. J., Sr., and H. B. Woody. 1997. 'Bacterial persistence and expression of disease', *Clin Microbiol Rev*, 10: 320-44.
21. Dominguez-Cuevas, P., R. Mercier, M. Leaver, Y. Kawai, and J. Errington. 2012. 'The rod to L-form transition of Bacillus subtilis is limited by a requirement for the protoplast to escape from the cell wall sacculus', *Mol Microbiol*, 83: 52-66.
22. Errington, J., K. Mickiewicz, Y. Kawai, and L. J. Wu. 2016. 'L-form bacteria, chronic diseases and the origins of life', *Philos Trans R Soc Lond B Biol Sci*, 371.
23. Francius, G., O. Domenech, M. P. Mingeot-Leclercq, and Y. F. Dufrene. 2008. 'Direct observation of Staphylococcus aureus cell wall digestion by lysostaphin', *J Bacteriol*, 190: 7904-9.

24. Fuller, E., C. Elmer, F. Nattress, R. Ellis, G. Horne, P. Cook, and T. Fawcett. 2005. 'Beta-lactam resistance in Staphylococcus aureus cells that do not require a cell wall for integrity', *Antimicrob Agents Chemother*, 49: 5075-80.
25. George, C. G., and W. E. Kloos. 1994. 'Comparison of the SmaI-digested chromosomes of Staphylococcus epidermidis and the closely related species Staphylococcus capitis and Staphylococcus caprae', *Int J Syst Bacteriol*, 44: 404-9.
26. Gilpin, R. W., and S. S. Nagy. 1976. 'Time-lapse photography of Bacillus subtilis L-forms replicating in liquid medium', *J Bacteriol*, 127: 1018-21.
27. Gilpin, R. W., F. E. Young, and A. N. Chatterjee. 1973. 'Characterization of a stable L-form of Bacillus subtilis 168', *J Bacteriol*, 113: 486-99.
28. Glover, W. A., Y. Yang, and Y. Zhang. 2009. 'Insights into the molecular basis of L-form formation and survival in Escherichia coli', *PLoS One*, 4: e7316.
29. Gottar, M., V. Gobert, T. Michel, M. Belvin, G. Duyk, J. A. Hoffmann, D. Ferrandon, and J. Royet. 2002. 'The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein', *Nature*, 416: 640-4.
30. Graham, J. E., and B. J. Wilkinson. 1992. 'Staphylococcus aureus osmoregulation: roles for choline, glycine betaine, proline, and taurine', *J Bacteriol*, 174: 2711-6.
31. Graveland, H., B. Duim, E. van Duijkeren, D. Heederik, and J. A. Wagenaar. 2011. 'Livestock-associated methicillin-resistant Staphylococcus aureus in animals and humans', *Int J Med Microbiol*, 301: 630-4.
32. Han, J., W. Shi, X. Xu, S. Wang, S. Zhang, L. He, X. Sun, and Y. Zhang. 2015. 'Conditions and mutations affecting Staphylococcus aureus L-form formation', *Microbiology (Reading)*, 161: 57-66.
33. Hanselman, B. A., S. A. Kruth, J. Rousseau, and J. S. Weese. 2009. 'Coagulase positive staphylococcal colonization of humans and their household pets', *Can Vet J*, 50: 954-8.
34. Herbert, S., A. K. Ziebandt, K. Ohlsen, T. Schafer, M. Hecker, D. Albrecht, R. Novick, and F. Gotz. 2010. 'Repair of global regulators in Staphylococcus aureus

8325 and comparative analysis with other clinical isolates', *Infect Immun*, 78: 2877-89.

35. Hoischen, C., K. Gura, C. Luge, and J. Gumpert. 1997. 'Lipid and fatty acid composition of cytoplasmic membranes from Streptomyces hygroscopicus and its stable protoplast-type L-form', *J Bacteriol*, 179: 3430-6.
36. Huber, T. W., and A. W. Brinkley. 1977. 'Growth of cell wall-defective variants of Escherichia coli: comparison of aerobic and anaerobic induction frequencies', *J Clin Microbiol*, 6: 166-71.
37. Hubert, E. G., C. S. Potter, T. J. Hensley, M. Cohen, G. M. Kalmanson, and L. B. Guze. 1971. 'L-forms of Pseudomonas aeruginosa', *Infect Immun*, 4: 60-72.
38. Joseleau-Petit, D., J. C. Liebart, J. A. Ayala, and R. D'Ari. 2007. 'Unstable Escherichia coli L-forms revisited: growth requires peptidoglycan synthesis', *J Bacteriol*, 189: 6512-20.
39. Kawai, Y., R. Mercier, L. J. Wu, P. Dominguez-Cuevas, T. Oshima, and J. Errington. 2015. 'Cell growth of wall-free L-form bacteria is limited by oxidative damage', *Curr Biol*, 25: 1613-8.
40. Kawai, Y., K. Mickiewicz, and J. Errington. 2018. 'Lysozyme Counteracts beta-Lactam Antibiotics by Promoting the Emergence of L-Form Bacteria', *Cell*, 172: 1038-49 e10.
41. Kennedy, A. D., S. F. Porcella, C. Martens, A. R. Whitney, K. R. Braughton, L. Chen, C. T. Craig, F. C. Tenover, B. N. Kreiswirth, J. M. Musser, and F. R. DeLeo. 2010. 'Complete nucleotide sequence analysis of plasmids in strains of Staphylococcus aureus clone USA300 reveals a high level of identity among isolates with closely related core genome sequences', *J Clin Microbiol*, 48: 4504-11.
42. Keymer, J. E., P. Galajda, C. Muldoon, S. Park, and R. H. Austin. 2006. 'Bacterial metapopulations in nanofabricated landscapes', *Proc Natl Acad Sci U S A*, 103: 17290-5.
43. King, J. R., and H. Gooder. 1970. 'Induction of enterococcal L-forms by the action of lysozyme', *J Bacteriol*, 103: 686-91.

44. Kloos, W. E., C. G. George, J. S. Olgiate, L. Van Pelt, M. L. McKinnon, B. L. Zimmer, E. Muller, M. P. Weinstein, and S. Mirrett. 1998. 'Staphylococcus hominis subsp. novobiosepticus subsp. nov., a novel trehalose- and N-acetyl-D-glucosamine-negative, novobiocin- and multiple-antibiotic-resistant subspecies isolated from human blood cultures', *Int J Syst Bacteriol*, 48 Pt 3: 799-812.
45. Kluytmans, J., A. van Belkum, and H. Verbrugh. 1997. 'Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks', *Clin Microbiol Rev*, 10: 505-20.
46. Landman, O. E., R. A. Altenbern, and H. S. Ginoza. 1958. 'Quantitative conversion of cells and protoplasts of Proteus mirabilis and Escherichia coli to the L-form', *J Bacteriol*, 75: 567-76.
47. Leaver, M., P. Dominguez-Cuevas, J. M. Coxhead, R. A. Daniel, and J. Errington. 2009. 'Life without a wall or division machine in Bacillus subtilis', *Nature*, 457: 849-53.
48. Lederberg, J. 1956. 'Bacterial Protoplasts Induced by Penicillin', *Proc Natl Acad Sci U S A*, 42: 574-7.
49. Makemson, J. C., and R. Z. Darwish. 1972. 'Calcium requirement and magnesium stimulation of Escherichia coli L-form induction', *Infect Immun*, 6: 880-2.
50. Mannik, J., R. Driessen, P. Galajda, J. E. Keymer, and C. Dekker. 2009. 'Bacterial growth and motility in sub-micron constrictions', *Proc Natl Acad Sci U S A*, 106: 14861-6.
51. Marston, J. 1961. 'Observations on L-forms of staphylococci', *J Infect Dis*, 108: 75-84.
52. Mercier, R., P. Dominguez-Cuevas, and J. Errington. 2012. 'Crucial role for membrane fluidity in proliferation of primitive cells', *Cell Rep*, 1: 417-23.
53. Michailova, L., V. Kussovsky, T. Radoucheva, M. Jordanova, and N. Markova. 2007. 'Persistence of Staphylococcus aureus L-form during experimental lung infection in rats', *FEMS Microbiol Lett*, 268: 88-97.

54. Onoda, T., A. Oshima, S. Nakano, and A. Matsuno. 1987. 'Morphology, growth and reversion in a stable L-form of Escherichia coli K12', *J Gen Microbiol*, 133: 527-34.
55. Otto, M. 2010. 'Basis of virulence in community-associated methicillin-resistant Staphylococcus aureus', *Annu Rev Microbiol*, 64: 143-62.
56. Owens, W. E. 1987. 'Isolation of Staphylococcus aureus L-forms from experimentally induced bovine mastitis', *J Clin Microbiol*, 25: 1956-61.
57. Royet, J., and J. M. Reichhart. 2003. 'Detection of peptidoglycans by NOD proteins', *Trends Cell Biol*, 13: 610-4.
58. Schenck, L. P., M. G. Surette, and D. M. Bowdish. 2016. 'Composition and immunological significance of the upper respiratory tract microbiota', *FEBS Lett*, 590: 3705-20.
59. Schindelin, J., I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J. Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, and A. Cardona. 2012. 'Fiji: an open-source platform for biological-image analysis', *Nat Methods*, 9: 676-82.
60. Schmidtke, L. M., and J. Carson. 1999. 'Induction, characterisation and pathogenicity in rainbow trout Oncorhynchus mykiss (Walbaum) of Lactococcus garvieae L-forms', *Vet Microbiol*, 69: 287-300.
61. Sears, P. M., M. Fettinger, and J. Marsh-Salin. 1987. 'Isolation of L-form variants after antibiotic treatment in Staphylococcus aureus bovine mastitis', *J Am Vet Med Assoc*, 191: 681-4.
62. Siddiqui, R. A., C. Hoischen, O. Holst, I. Heinze, B. Schlott, J. Gumpert, S. Diekmann, F. Grosse, and M. Platzer. 2006. 'The analysis of cell division and cell wall synthesis genes reveals mutationally inactivated ftsQ and mraY in a protoplast-type L-form of Escherichia coli', *FEMS Microbiol Lett*, 258: 305-11.
63. Takeda, K., T. Kaisho, and S. Akira. 2003. 'Toll-like receptors', *Annu Rev Immunol*, 21: 335-76.
64. Tanimoto, A., Y. Kitagaki, M. Hiura, H. Fujiwara, K. Iijima, and S. Ikawa. 1995. '[Methicillin-resistant Staphylococcus aureus forming the fried egg appearance colonies isolated from a patient with septicemia]', *Rinsho Byori*, 43: 1061-5.

65. Tong, S. Y., J. S. Davis, E. Eichenberger, T. L. Holland, and V. G. Fowler, Jr. 2015. 'Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management', *Clin Microbiol Rev*, 28: 603-61.
66. Wadstrom, T., and O. Vesterberg. 1971. 'Studies on endo-beta-acetylglucosaminidase, staphylolytic peptidase, and N-acetylmuramyl-L-alanine amidase in lysostaphin and from Staphylococcus aureus', *Acta Pathol Microbiol Scand B Microbiol Immunol*, 79: 248-64.
67. Weibull, C., and B. M. Lundin. 1961. 'Factors influencing the growth of a stable L-form of Proteus mirabilis', *J Bacteriol*, 81: 812-8.
68. Williams, R. E. 1963. 'L-forms of Staphylococcus Aureus', *J Gen Microbiol*, 33: 325-34.
69. Wollina, U. 2017. 'Microbiome in atopic dermatitis', *Clin Cosmet Investig Dermatol*, 10: 51-56.
70. Woo, P. C., S. S. Wong, P. N. Lum, W. T. Hui, and K. Y. Yuen. 2001. 'Cell-wall-deficient bacteria and culture-negative febrile episodes in bone-marrow-transplant recipients', *Lancet*, 357: 675-9.
71. Xu, Y., B. Zhang, L. Wang, T. Jing, J. Chen, X. Xu, W. Zhang, Y. Zhang, and J. Han. 2020. 'Unusual features and molecular pathways of Staphylococcus aureus L-form bacteria', *Microb Pathog*, 140: 103970.
72. Yabu, K. 1985. 'Conversion of Staphylococcus aureus cells to stable L-forms in a liquid growth medium', *Microbiol Immunol*, 29: 157-61.
73. Young, L. S., and D. Armstrong. 1969. 'Induction, colonial morphology, and growth characteristics of the L-form of Streptococcus liquefaciens', *J Infect Dis*, 120: 281-91.

74. "Staphylococcosis, Staphylococcal Arthritis, Bumble Foot". The Poultry Site. Retrieved 22 October 2013.

75. Friedrich Goetz and B. Schumacher. 1987. Improvements of protoplast transformation in *Staphylococcus carnosus*. FEMS Microbiology Letters 40: 285-288.

76. Klieneberger, E. 1935. The natural occurrence of pleuropneumonia-like organisms in apparent symbiosis with Streptobacillus moniliformis and other bacteria. *J Pathol Bacteriol,* 40: 93–105.
77. Mattman, L.H. 2001. Cell wall deficient forms: stealth pathogens. New York: CRC Press. 416 p.
78. Ryan, K.J., C.G. Ray. 2004. *Sherris Medical Microbiology* (4th ed.). McGraw Hill. ISBN 978-0-8385-8529-0.

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Abbreviations (Chapter one)

S.	**Staphylococcus**
B.	**Bacillus**
S.epidermidis	***Staphylococcus epidermidis***
AAA	**Aromatic amino acid**
TA	**Trace amine**
TRY	**Tryptamine**
PEA	**Phenylthylamine**
TYM	**Tyramine**
OCT	**Octapamine**
DOPA / Dopa	**Dopamine**
W	**Tryptophan**
F	**Phenylalanine**
Y	**Tyrosin**
L-Dopa / L-DOPA	**Levodoapa**
CFU	**Colony forming unit**
RP-HPLC	**Reverse phase high performance liquid chromatography**
AADC	**Aromatic amino acid decarboxylase**
SadA	**Staphylococcal aromatic amino acid decarboxylase**
TDC	**Tyrosine decarboxylase**
TpDC	**Tryptophan decarboxylase**
β2-AR	**β2-adrenergic receptor**
α2-AR	**α2-adrenergic receptor**
DNA	**Deoxyribonuecleic acid**
RNA	**Ribonucleic acid**
GPCRs	**G protein-coupled receptors**
TAAR	**Trace amine associated receptor**
OD	**Optical density**
PCR	**Polymerase chain reaction**

Symbols:

%	**percent**
<	**Less than**
>	**Greater than**
μ	**Micro**
^{0}C	**Degree Celsius**

Abbreviations (Chapter two)

S. aureus	***Staphylococcus aureus***
MRSA	**Methicillin Resistant *Staphylococcus aureus***
SMM	**Spizizen's Minimal Media**
approx.	**Approximately**
bp	**Base pairs**
BSA	**Bovine serum albumin**
Da	**Dalton**
dest.	**Distilled**
Fig.	**Figure**
g	**Gram**
h	**Hour**
kD	**Kilo Dalton**
kb	**Kilo base**
l	**Liter**
M	**Molar**
m	**Milli**
mol	**Mol**
MW	**Molecular weight**
OD	**Optical density**
rpm	**Rotations per minute**
sp.	**Species**
Tris	**Tris (hydroxymethyl- aminomethane)**
UV	**Ultraviolet**
Vit	**Vitamin**
GSH	**Glutathione**
CW	**Cell wall**

Symbols

°C	**Degree Celsius**
µ	**Micro**
%	**Percentage**