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Mini Review

Probiotic Lactobacillus Strains and Their Antimicrobial Peptides to Counteract Biofilm- Associated Infections- A Promising Biological Approach

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Abstract

Biofilms keep the intimate relationship between human body and resident microbes. According to National Institutes of Health (NIH), the development of extracellular microbial communities, called biofilms contribute approximately 75% of pathogenic infections to human. The formation of biofilm confers several advantages during pathogen colonization and tolerates extreme conditions like exogenous stress caused by anti-infective agents. The interpretation and exploitation of anti-biofilm properties would help in future challenges, particularly in the control of human infections. The proven scientific evidence with regard to cellular association and exopolysaccharide production by probiotic bacteria could play an important role as anti-biofilm tools. These extracellular components may directly interact with the biofilms as they are actively transported to the bacterial environments via cytoplasmic membrane. The interactive ability of these extracellular metabolites to treat pathogenic biofilms is gaining significant research interest and their possibility to use as anti-biofilm agents. In this review, the extracellular probiotic bacterial markers and molecular approaches to control pathogenic biofilms have been reviewed and future perspectives and research interests are discussed as well.

Introduction

Gastrointestinal tract and biofilms - an overview

The term 'biofilm' represents a structured group of bacteria enclosed in a pre-formed polymeric matrix that adhered to living or inert surface [1] that will have the effect of microbiome functionality. Biofilm forming flora of the gastrointestinal tract comprising lactobacilli may have some protective mechanism. In contrast, adherent structured microbial cells in the respiratory tract and oral cavity are well-characterized and are associated with respiratory infections, periodontitis and dental caries [2,3]. Bacteria employ on a particular phenotype during formation of biofilm [4,5]. Biofilm- forming bacterial strains have the ability to populate to enhanced thermo tolerance and resistance to freeze-drying, and to compete resident biofilm-forming pathogens with a non-pathogenic property [6]. Several studies revealed the divergent gene expressions between biofilms and plank tonic cells [7]. Moreover, biofilm will attain higher resistance to destruction by the use of bactericidal antibiotics [8]. The significant factors for the optimum functionality and survival are the colonization and an expression of the health-promoting properties of probiotics in digestive tract [9]. Bacteria must be of acid pH tolerant and bile toxicity resistant that is prevalent in the digestive tract to survive in the gut [10]. This extends and stabilizes gastrointestinal tract and helps to control pathogenic bacteria by competitive inhibition or steric barrier, in spite of a variety of defensive host cell immune responses [11]. Bacteria colonizing the digestive tract grow well in the form of biofilms [12] and the majority of the research work on probiotics is conducted on plank tonic cells. Several researchers have investigated on the effect of diverse environmental factors on biofilm forming *Lactobacillus* strains isolated from diverse niches. Slama et al. [13] reported that the LAB strains isolated from Tunisian traditional fermented food showed a significant reduction of the biofilm formation by Listeria species. Similarly, Das et al. [14] Lakhtin et al. [15] Tahmourespou and Kermanshah [16] also reported the efficacy of the different LAB strains isolated from different fermented food products showed in their potential ability to reduce the biofilm formation by human pathogens. In the post-genomic era, rapid screening techniques such as Metagenomics, transcriptomics, proteomics and metabolomics, have very much helped to categorize probiotic strains [17] and to know the mechanisms by which several lactic acid-producing bacteria assist to maintain human health and the many functions associated to these species in the gut [18]. They provide nutrition, aid the host to digest foods, struggle for space and nutrients with potential pathogens and persuade the production of antimicrobial peptides through an interaction with intestinal epithelial cells.

Progress on anti-biofilm approaches

An intensive study on microbial biofilms began only a couple of decades back with the rediscovery of natural biotic systems, predominant bacteria attached to surfaces [11]. Earlier Henrici [19] who reported "it is demonstrated that for the most bacteria in water are not only planktonic organisms, but also grow upon submerged surfaces". Biofilms are comprised of either single or several microbial species and express on a variety of biotic and abiotic surfaces. Mixed-species of biofilms exist in most environments, but single-species biofilms last in a variety of pathogenic infections and on the surface of therapeutic embeds [20]. Biofilms of single-species are vitally important in the current area of research. Biofilm forming Pseudomonas aeruginosa is the most premeditated single-species, Gram-negative bacterium. Escherichia coli, Pseudomonas fluorescents and Vibrio cholera have also been studied for biofilm producing potential. The Gram-positive biofilm forming bacteria include *Enterococcus species*, *Lactobacillus species*, and *Staphylococcus species* have been investigated. Studies report that biofilms are on stable point in a life cycle that consists of instigation, maturation, maintenance, and dissolution [21]. Bacteria commence biofilm formation in response to temperature, growth conditions, nutrient availability, etc. Even though these conditions vary widely, the Gram-negative organisms, with the exception of *Myxococcus Xanthus* and *E. coli* O517:H7, endure a shift-over from free-living plank tonic to sessile, surface adhered cells in response to a nutrient-rich medium. These biofilms persist as long as the availability of fresh nutrients, but when they are deprived of nutrients, they detach from the surface and reform to a plank tonic mode. Therefore O'Toole et al. [21] proposed that the starvation response pathway is a part of the total biofilm developmental cycle. It is noteworthy that most microorganisms were able to prepare the transition in life on a biotic or a biotic surface, irrespective of their physiological parameters. Even though these factors are essential in the initial cell to cell interactions and also cell surface, they are not complete by themselves.

Recent investigations on the ability of some of the probiotic strains (*Lactobacillus* acidophilus DSM 20079, *Lactobacillus* plantarum 299v, *Lactobacillus* paracasei DSMZ 16671, *Lactobacillus* reuteri strains PTA 5289, *Lactobacillus* rhamnosus GG, and *L. reuteri* SD2112, etc.) to hinder the growth of S. mutants and the *in vitro* biofilm formation has been evaluated, and these results support that the antibacterial activity of *Lactobacilli* seems to be pH-dependent and strain-specific [22]. *Lactobacilli* have also been shown to reduce Streptococcal adhesion [23] not much on glass surfaces, but in particular on saliva-coated hydroxyapatite [24].

The anti-biofilm potential of some probiotics against biofilm forming enteropathogens has also been reported, despite the fact that the results obtained so far are very few and conflicting. On the other hand, there are studies evaluating that probiotics are able to inhibit biofilms of intestinal pathogens, but further different experimental results seem to support the improvement of biomass of the enteropathogens biofilm in the presence of probiotics [23]. Bacterial surfaces are heterogeneous, and can change greatly in response to their environmental factors. Therefore, a bacterium cannot be precisely modeled as a sphere with a homogeneous surface. A comprehensive understanding of the essential bacterial components, meticulous for biofilm development and the mechanisms that control their production and its activity are necessary.

Probiotic *Lactobacillus* for the biofilm eradication

The use of probiotics in the treatment of diarrheal diseases has been proposed for many years [24]. The presence of *Lactobacillus species* in the gastrointestinal tract has gained significance due to health-promoting effects [25]. The probiotic mechanism involves the diversity in function of the intestinal microbiota for nutrients, competitive inhibition of attachment of pathogens to the surface, production of antagonistic substances and modulation of intestinal immunity Preidis et al. [26] proved a transitory increase in phylogenetic diversity and constancy taxa of fecal microbiome 24 h following single probiotic *L. reuteri* gavages in mice. The diversity in microbial communities is associated with increased ecological stability [27]. One of the methods to screen potential probiotic strains is adhesion to the mucous and epithelial cells, an essential part of the immune modulation, pathogen elimination and enhanced contact with the gut mucosa [28]. To study the quantitative adhesion potential a range of methods such as radioactive labeling, quantitative culturing, fluorescence in situ hybridization-FISH [29], or in vitro model systems viz., immobilized mucus and cell-culture models [30,31] have been developed. However, studies report that probiotic lactobacilli do not colonize GIT permanently but are beneficial to the hosts only for a short period later they stopped being administered [32].

The genus *Lactobacillus* in rats, mice, chickens and pigs are autochthonous to the proximal gut region [33]. The epithelial adhesion formed by lactobacilli in parts of the stomach, esophagus and crop illustrates distinctive characteristics of the bacterial biofilm formation [34]. The probiotic bacteria get strongly adhered to the intestinal epithelium surface and became well-established in a matrix of extracellular polymeric material [35,36]. Living in a biofilm is a selective advantage for microbes from the ecological point of view that provide a protected niche permitting to interact directly with the host, and to longer survival in the GIT with greater metabolic and beneficial efficiency [37]. There are a number of genetic and environmental factors that affect the formation of these microbial structures within the GIT [38]. The hierarchically ordered genetic factors can control the chronological development of biofilm formation and these genetic switches generally turn on in response to the changes in external stimuli such as microbe-microbe interactions, shear stress, host-microbe interactions and the presence of oxygen [39]. Most of the adherent bacteria form in the natural environment in the form of surface-attached biofilms, where they are bound within a self-produced extracellular matrix that protects them against unfavorable environmental conditions [17].

Genes that are transferred horizontally between bacteria are contributing significantly to bacterial evolution. While gene transfer within a mono-species result in the formation of specific traits, interspecific transfer of a gene may cause entirely new genetic combinations, which rarely impose some serious health issues to human [40]. Biofilm formation is a result of interbacterial interactions. Biofilms can be both single and multispecies, but the development of a stable and mature biofilm is always the product of abundant social interactions that have evolved through adaptation [40]. Diverse probiotic LAB species have been used as therapy for different biofilm-forming pathogens (Table 1).

Table 1: Effects of different probiotic Lactic acid bacteria against pathogenic biofilms.

Sl No.	Biofilm Forming Pathogen	LAB used to Control	Possible mode of action	Reference
1.	*Listeria monocytogenes*, *Salmonella Typhimurium* and *Escherichia Coli*	*Lactococcus lactis* VB69, *Lactobacillus lactis* VB94, *Lactobacillus sakei* MBSa1, and *Lactobacillus curvatus* MBSa3	Pathogen Exclusion mechanism	[71]
2.	*Candida albicans*	*Lactobacillus rhamnosus*, *Lactobacillus casei*, and *Lactobacillus acidophilus*	Production of exometabolites	[72]
3.	*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa, Bacillus cereus* and *Candida albicans*	*Lactobacillus helveticus*	Production of Biosurfactants with antiadhesive potential	[73]
4.	*Bacillus cereus*RSKK-863, *Listeria monocytogenes* ATCC 7644, *Enterococcus fecalis* ATCC 25175,*Pseudomonas aeruginosa* ATCC 72853	*L. delbrueckii* ssp.*bulgaricus* B-3, *L.delbrueckii* ssp. *bulgaricus* A-12, *L. fermentum* LB-69, *L. paracasei* LB-8, *L. plantarum* GD-2, and *L. rhamnosus* GD-11	Exopolysaccharide production	[74]
5.	*Bacillus cereus* and *pseudomonas aeruginosa*	*Lactobacillus plantarum* and *Lactobacillus pentosus*	Production of antibiofilm metabolites from the Cell free supernatant	[75]
6.	*Klebsiella pneumonia and Pseudomonas aeruginosa*	*Lactobacillus plantarum* and *Lactobacillus pentosus*	Production of bactiriocin like inhibitory substances	[76]
7.	*Candida albicans*	*Enterobacter faecalis*	Disruption of biofilm by exopolysaccharide production	[77]
8.	*Acinetobacter baumannii*, *Escherichia coli*, and*Staphylococcus aureus* (MRSA)	*Lactobacillus jensenii* and *Lactobacillus rhamnosus*	Production of Biosurfactants	[78]
9.	*Listeria monocytogenes*	*Lactobacillus plantarum*	Production of inhibitory compounds from the extracts	[79]

LAB Markers Responsible for Anti-Biofilm Property

Lactobacillus is a large component of GIT biofilm and has been very commonly used to identify bacterial feature that allow lactobacilli to survive in the GIT. Several genes encoding large cell surface proteins putatively involved in adhesion to the intestinal epithelium and biofilm formation are harbored in its genome [41]. Cell surface peptides of probiotic LAB were established to be effective as anti-biofilm agents (Table 2). The cell surface proteins MucBP and Lar 0958 are responsible in adhering *Lactobacillus* to the mucus [42]. A Large Surface Protein (Lsp) adhering to the epithelium of the fore stomach has been functionally characterized [43]. The Exopolysaccharide (EPS) -producing enzymes, GtfA and Inu of *L. reuteri* TMW1 induce cell aggregation, in vitro biofilm formation and colonization in the mouse gastrointestinal tract [44]. *L. reuteri* also recorded with high-frequency genes encoding pathways, improving oxidative stress (glutathione synthesis) and acid tolerance (urea degradation, γ-amino butyrate, arginine pathway) [45]. In addition, expression of pathways altering the structure of the bacterial cell wall (Cyclopropane-fatty-acyl-phospholipid synthase, DltA) was related with acid resistance [46]. Walter et al. [37] proved the inactivation of dltA gene from *L. reuteri* and reported a reduction in strain competitiveness *in vivo*; however the adherence was not altered. The LysM/YG proteins demonstrate the characteristics of proteins that persuade aggregation in lactobacilli, probably by the N-terminal LysM domain binding the peptidoglycan and C-terminal YG-motif to carbohydrate moieties [47].

Probiotic therapy through molecular approach

Comparative genomics explored that the evolution of *Lactobacillus* resulted in host restricted phylogenetic lineages concentrating particular hosts [48]. The ability to form epithelial biofilms by *L. reuteri* 100-23 in the mouse fore stomach is solely dependent on the host origin of the strain. Analyses performed showed a fundamentally diverse genomic evolution in a species of

Table 2: Antibiofilm cell surface adhesion peptides implicated in probiotic-pathogen interaction.

Sl No	LAB	Inhibitory peptides	Function	Reference
1	*L. johnsonii*	LTA, elongation factor Tu (EF-Tu), and heat shock protein (GroEL)	Adherence	[80]
2	*L. brevis*	S-layer proteins (SlpA)	Adherence, protection against stressors (low pH, bile, etc.), and enhancement of barrier function	[81]
3	*L. rhamnosus* GG	Peptides NPSRQERR and PDENK	Antimicrobial activity	[82]
4	*L. rhamnosus*	Fimbriae, mucus binding factor (MBF)	Adherence, protection against pathogen, and antiapoptotic effects on intestinal epithelial cells	[83]
5	*L. casei*	EPS, sortase-dependent proteins (SrtA)	Maintenance of barrier function, increased mucus production, and immunomodulation	[84]
6	*Lysinibacillus fusiformis* S9	Glycolipid	Inhibit biofilm formation of *E. coli and Streptococcus mutants*	[85]
7	*Lactobacillus rhamnosus*	Unspecified protein	Inhibit biofilm formation of *A. baumannii, E. coli, and S. aureus*	[86]
8	*Lb. plantarum* PA21	pMG36e-GFP	Antibiofilm activity	[87]
9	*Lactobacillus rhamnosus*GG	surface antigen NLP/P60 (gi l199598074)	Human mucus binding protein	[88]
10	*L. acidophilus* NCFM	acmB (lba0176) N –acetylglucosaminidase, a surface protein	Intestinal adhesion and modulation of the mucosal immune system	[89]

L. reuteri 100-23 and human isolate *L. reuteri* F275 [49]. The host specificity of the strain is mediated by a serine-rich surface adhesin Lr70902 (Fap1-like protein) [50]. Genome hybridization proved that a sourdough isolate *L. reuteri* LTH2584 model had genome content in the similar line of model as rodent isolate 100-23. The proteins with proven competitiveness of *Lactobacillus species* in cereal fermentation were also highly effective in biofilm formation, what substantiated with the projected model of collective intestinal origin for the rodent and sourdough isolates [51]. These remarks regarding the change in microbial niches need to be elucidated when choosing lactobacilli for any therapeutic applications and for appropriate use of probiotics.

Quorum Sensing (QS) mediated mechanisms such as the lucks gene and the pheromone peptide plantaricin A (Plna), could play an essential role in the regulation of the microbial interactions in intestinal human systems [52]. Calasso et al. [53] demonstrated the exoproteome of *L. plantarum* DC400 when grown in the presence of Plna or the co-culture with other *Lactobacillus species*, reports that *L. sanfranciscensis* DPPMA174 has significantly increased the capacity of *L. plantarum* DC400 to bind to Caco-2 cells and to form biofilms. In addition, the relation between these two strains proved *L. plantarum* DC400 to elevate the levels of proteins responsible for stress resistance, promote a immune modulation (via GroEL and/or DnaK) [53]. De Angelis et al. [54] investigated the exoproteome of *L. plantarum* DB200, choosing this strain for its capability to form biofilms and to bind to Caco-2 cells. The protein analysis by two-dimensional difference gel electrophoresis (2D-DIGE) revealed a varied exoproteome between biofilm-forming cells and plank tonic cultures. Consequently, the high levels of stress proteins (Dnak, GroEL, ClpP, GroES and catalase) expression in cells forming a biofilm showed their improved survival under environmental stress conditions (heat, acid and ethanol) [54].

Even though the availability of genome sequences will undoubtedly advance the field of probiotics, they need to be proved with the functional studies. Different methodologies have been developed for significant comparisons of varied gene expression, for example, by comparing expression profiles of a strain grown in vitro under standard laboratory conditions with those of strains grown in vivo or in GIT-related simulations. Amongst the genes differently expressed in the GIT ecosystems, potential genes contributing to the alteration and the survival of the microbes in the host environment are likely to be present. Some of the methods that are yet to be used for practical applications for the analysis of differential gene expression of lactobacilli under appropriate conditions are wide genome comparisons of RNA profiles using microarrays [55], evaluation of protein profiles using Two-Dimensional (2D) difference gel electrophoresis [56], *In Vivo* Expression Technology (IVET) with a promoter probe library [17], and Differential Display PCR (DD-PCR) [57] Therefore, these molecular techniques can be considered as complementary for the identification of bacterial pathogens and their interaction with the host GIT system, further the efficacy of probiotic approach can be determined.

Current Biofilm Control Strategies and Limitations

Clinical trials carried out with beneficial bacteria and predominantly LAB makes use of the inhibition of growth of pathogens and to protect the intestinal mucosa from the colonization of adverse bacteria [58]. This probiotic approach is anti-biofilm in nature as these aggregates formed on mucosal cells and a biotic surface has comparable molecular properties [59]. However, in general molecular data of the biofilm mode of life obtained from the pre-formed aggregates on biotic surfaces, and biofilm host immune response remains to be discovered. The potential strategy used to productively control biofilm formation as by the use of essential components of probiotics [60].

Conventional anti-biofilm therapies aim to target bacterial species without taking into consideration that most biofilm-related infections are due to mixed microbial biofilms [56]. As, there is no ideal system to totally eradicate biofilm, the solution would be the concurrent application of agents implementing synergic potential to both control the biofilms and kill bacteria [61]. A multidisciplinary approach is essential to elucidate the genetic networks, exploring complex community interactions and to replace them in their evolutionary and ecological context. Biofilm and mixed biofilm forming species modeling tools are to be made available, including heterotrophy parameters [62]. Three-dimensional system models of biofilm dynamics have been proposed as tools for studying mechanisms of protection against microbial inhibitors in biofilms [63]. They could be useful to investigate the effects of anti-biofilm compounds, in particular to assess their efficacy and to explore their impact on the emergence of new groups of resistant microbes [64].

Future directions and Concluding remarks

Several reports from medical device submissions have been received by the Food and Drug Administration that hold potential anti-biofilm agents [64]. However, existing in vitro and in vivo assays are not effective to predict biofilm effect in humans and it is necessary to introduce reliable and potential alternatives to clinical studies for the evaluation of anti-biofilm agents with standardized anti-biofilm methodologies and evaluation methods that can establish association with clinical outcomes. Potential targets with the elucidation of the mechanisms of action of several anti-biofilm and bactericidal agents have been depicted so far. It is important, considering bacterial anti-biofilm agents that will be an essential tool in the future to establish a frame to help industrial and academic institutions to explore their potential ability in agreement with health and nutrition policy [65]. Because of the administrative complications of these strategies, other potential applications such as vaccine therapy must be considered. FomA an outer membrane protein involved in bacterial co aggregation is preferentially potential target for developing an oral vaccine against the bacterium Fuso bacterium nucleatum, and this can be considered as a potent anti-biofilm vaccine [66]. Not only novel and specific vaccines are required, but it is necessary first to more fully explore the interactions between biofilm and the immune system of the host, a domain as yet unexplored [67]. The mechanism of probiotics comprises the diversity and function of the intestinal micro biota for nutrients, competitive inhibition of pathogen attachment, and production of antagonistic substances and modulation of intestinal immunity. On the other hand, consumption of traditional fermented foods, the rich source of Lactic Acid Bacteria (LAB) has the probiotic effect for healthy gut (Figure 1).

On the whole, the effect of probiotic counterparts in gene expression modification of pathogens within biofilm could represent an essential anti-biofilm target with a dual-purpose- to control bacterial colonization and to inhibit the expression of virulence

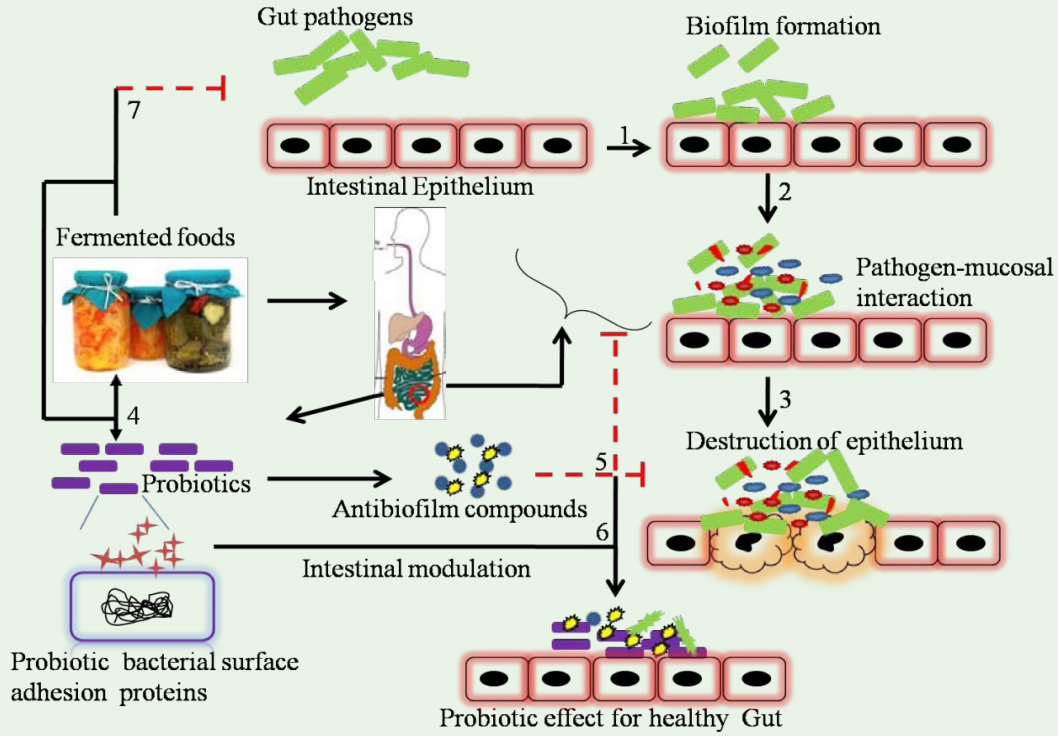

Figure 1: Proposed mechanism of probiotic approach for the suppression of biofilm forming pathogens in the human gut intestinal epithelium at different check points. Probiotic-pathogen interaction favours the blocking of pathogenic biofilm adhesion (1), mucosal interaction (2) and maturation steps (3) by competitive inhibition of pathogen attachment or direct intestinal modulation by the action of probiotic surface adhesion proteins through indirect immune response or by the action of produced antibiofilm compounds (4-6). On the other hand, consumption of traditional fermented foods which are the rich sources of probiotics favours the probiotic therapy for a healthy gut (7).

factors. Some Lactobacilli can down-regulate the expression of the virulence genes of gut pathogens [68,69,70]. The literature suggests, that the virulence capability of bacteria is generally opposed to the biofilm formation potential [71], suggesting that biofilm forming bacteria stay within a specific ecological niche and induces adverse effects. Further experiments are needed to assess the in vivo potential of anti-biofilm agents that can assure to possess therapeutic benefits that are target specific, highly effective and safe alternatives [64]. However, in particular the potential side effects on beneficial bacteria of the host gut and the development of antimicrobial resistant substances should also be given consideration along with the risks and benefits for a healthy gut.

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