Breaking Bacteria’s Biofilm

with

Helen Padarin ND

Presented LIVE
Wednesday 10th of JULY 2013

Partners webinar offer: use HML2013 coupon code to receive 20% off McGraw Hill publications
Breaking Biofilm
The Key to Resolving Resistant Dysbiosis

Helen Padarin
www.nourish-ed.com

What Is Biofilm?

Invisibility cloak for bugs.
Mycorrhiza in a highly evolved community structure.
1000x more resistant to antimicrobial therapy than planktonic microbes.

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3528225/

Biofilm Bacteria

Bacteria preferentially exist in complex, surface-attached organisations known as biofilms.

Bacteria in biofilms express a different set of genes than their planktonic counterparts and have markedly different phenotypes.


There is a perception that single-celled organisms are asocial, but that is misguided.
When bacteria are under stress - which is the story of their lives - they team up and form this collective called a biofilm.
If you look at naturally occurring biofilms they have very complicated architecture. They are like cities with channels for nutrients to go in and waste to go out.

--Andre Levchenko, PhD, Johns Hopkins University
Biofilm Bacteria

Biofilm bacteria communicate with each other, and have mechanisms to diffuse nutrients and dispose of waste. Biofilms provide bacteria with distinct advantages, including antimicrobial resistance and protection from host defenses. Thus, bacteria exist in a far more complex fashion than previously thought and can best be thought of as "self-assembling multicellular communities."

What's the Significance?

60-80% of all infections are caused by bacteria growing in biofilm, as opposed to planktonic bacteria. Part of what is known as the Th1 bacterial pathogens, which according to many researchers, collectively cause chronic disease. IBD, autoimmune illnesses, CFS, fibromyalgia, cancer, recurrent infections...

Biofilm Implications

Although a focus on the planktonic form of bacteria has been useful in understanding acute infections, chronic infections are much better understood as biofilm illnesses. Biofilms have been shown to be involved in chronic otitis media, chronic tonsillitis, cholesteatomas, and device-associated infections.

A New Understanding

Now that basic research has demonstrated that the vast majority of bacteria exist in biofilms, the biofilm concept of disease is beginning to spread throughout the clinical world. Understanding that many infections are actually biofilm related is fundamental to developing rational strategies for treatment and prevention.

Some Background on Biofilm......

Where is Biofilm?

- Slimy rocks.
- Dental plaque.
- Soil.
- Waterways.
- Gut lumen / wall.
- Space stations
- Surgical instruments

Candida Biofilm
Fig. 3. Pseudomonas biofilm formation around and inside polyethylene terephthalate (PET) suture material after 72 hours incubation.

Fig. 5. After conclusion of laser shockwave impact, total disruption of biofilm occurred. Note planctonic (green) fluorescing in fluid medium that was once protected inside biofilm structure.

How Did We First Learn About Biofilm?
Discovery of Biofilm

1950's booming oil exploration in the US.
Organised microbial colonies deep below ocean surface could make or break successful oil acquisition.

Discovery of Biofilm

In this industrial environment, study of biofilms taught us:
1. how adherent communities, as opposed to planktonic bacteria behaved
2. how biofilm protects pathogens

The Story Continues.....

Ties with Marine Biology

Concurrent study into the Angler fish (light bulb fish) taught us:
Light is emitted by saccules of Vibrio bacteria (ubiquitous in the ocean) as they replicate and secrete auto-inducers once the population reaches a certain size = Quorum Sensing.
Quorum Sensing

When microbial cells reach a certain density, they begin to produce communicator molecules called auto-inducers.

When a cell receives an appropriate number of signals it knows there are enough other cells to produce a certain response - generally gene activation or deactivation.

Quorum Sensing

Group behaviours initiated by quorum sensing include:
1. Biofilm production
2. Virulence
3. Bioluminescence
4. Sporulation

Role of Biofilm

Protection
1. Physical protection from environment
2. Antibiotic resistance
3. Stealth and invisibility from host immune system
25

Environmental Protection

Believed to provide protection against environmental stresses like heat-shock and desiccation as well as against the host immune system and antimicrobial agents.

26

Antibiotic Resistance

- Renders microbes 1000x more resistant to antibiotics.
- Biofilm infections respond only transiently to antibiotics.
- Facilitates exchange of antibiotic resistant genetic material between organisms.
- May contain antibiotic hydrolyzing enzymes, e.g. B-lactamase - neutralising B-lactam a/b's

27

Antibiotic Resistance - Metabolism

- Different regions of the biofilms contain subpopulations in different metabolic stages depending on the oxygen and nutrients available.
- Most antibiotics require rapidly growing cells in order to kill or inhibit pathogens.
- Slow metabolic rate = antibiotic resistance.

28

Persister Resister

FIG. 2 Model of biofilm antibiotic resistance based on persister survival. An initial treatment with antibiotics kills planktonic cells and the majority of biofilm cells. The immune system kills planktonic persister, but the biofilm persister cells are protected from host defenses by the extracellular polysaccharide matrix. After the antibiotic concentration drops, persisters resuscitate the biofilm and the infection relapses (35).

Certain bacterial pathogens are able to evade the host immune system and persist within the human host. The consequences of persistent bacterial infections potentially include increased morbidity and mortality from the infection itself as well as an increased risk of dissemination of disease. Eradication of persistent infections is difficult, often requiring prolonged or repeated courses of antibiotics. During persistent infections, a population or subpopulation of bacteria exists that is refractory to traditional antibiotics, possibly in a non-replicating or metabolically altered state. This review highlights the clinical significance of persistent infections and discusses different in vitro models used to investigate the altered physiology of bacteria during persistent infections. We specifically focus on recent work establishing increased protection against oxidative stress as a key element of the altered physiologic state across different in vitro models and pathogens.

Introduction

For many bacterial pathogens, the host immune system successfully eliminates the invading bacteria and the infection resolves. In certain infections, however, bacteria evade the host immune system and persist within the host. In some cases these persistent infections are asymptomatic for long periods of time, but can undergo future reactivation into clinically significant disease, or can be associated with malignancy or subsequent disease dissemination. Alternatively, some persistent infections result in clinically apparent, chronic symptoms. In these cases, even standard treatment with antibiotics often fails to effectively sterilize persistent infections, and prolonged or repeated courses of antibiotics are required for successful eradication. At an extreme, lifelong chronic suppression with antibiotics can be required in the absence of eradication.

Many factors contribute to the ability of pathogens to establish persistent infections, including both host and bacterial factors. Certain pathogens appear uniquely adapted to evade the host immune system and persist in infected individuals for decades in the absence of symptoms, for example *Mycobacterium tuberculosis* or *Salmonella Typhi*. Other pathogens like *Pseudomonas aeruginosa* or *Escherichia coli* can cause both symptomatic acute and chronic infections, with specific changes in the host facilitating the establishment of a persistent infection. The first section of this review highlights the clinical significance of persistent infections and the wide range of strategies employed by bacteria to survive the host immune system response (see Table 1 for examples of bacteria associated with persistent infections). In the second section, we discuss different in vitro models used to investigate the physiology of bacteria involved in persistent infections. Despite differences, many models share a common theme: bacteria adapt to environmental stresses imposed by the host by entering a different physiologic state. A key element of this different physiologic state is a non-replicating or slowly replicating growth rate, which may have the additional benefit of contributing to a pathogen’s defense against antibiotic exposure. Walsh McDermott first suggested in the 1950s that the relative metabolic state of bacteria affects antibiotic efficacy, causing cells to become “indifferent” to antibiotics, thereby relating the physiologic state of bacteria to antibiotic efficacy.

One of the most significant environmental stresses encountered by bacteria is the host oxidative immune response. In addition, studies have suggested that treatment with bactericidal antibiotics may result in increased oxidative stress via the Fenton reaction, though this finding remains controversial with more recent studies questioning this mechanism of cell death. Increased antioxidant capabilities may therefore protect a bacterium from both the host immune response as well as antibiotic therapy. In this review we specifically focus on recent work demonstrating the role of increased defenses against oxidative stress in various in vitro models for persistent infections. Increased antioxidant capabilities may protect a bacterium from the host immune response as well as facilitate survival during antibiotic exposure, thereby enabling the establishment of a persistent infection.

Clinical Significance of Persistent Infections

Asymptomatic persistent infections. Several persistent infections are clinically asymptomatic yet still have significant consequences for their human host. In some cases these consequences represent an increased risk of developing clinically significant disease at a later time, exemplified by *M. tuberculosis* and *Treponema pallidum*.
High Dose Antibiotics Promote Biofilm Growth

High dose antibiotics promote the growth of biofilm by leaving behind persister cells, which have become wiser.

Better results with either low dose, pulsed antimicrobials or with non-antimicrobial treatment approaches.

Persister Cells in Immunocompromised Patients

Persister cells form with particular ease in immunocompromised patients because the immune system is unable to help the antibiotic "mop up" all the biofilm cells.

Paradoxically, an antibiotic in a constant, high-dose manner (in which the antibiotic is always present) helps persisters persevere.

Stealth Function

Biofilm protects host immune cells from detecting microbial overgrowth.

Due to multiple factors, including decreased efficacy of antibodies and antimicrobial peptides against bacteria in biofilms, decreased phagocytic uptake, and decreased sensitivity to polymorphonuclear leukocyte-mediated killing.

Therefore, test results for antibodies may come back negative, even when there is biofilm infection.
How Is Biofilm Made?

Creation of Biofilm

Biofilm formation is induced by a variety of stresses, including nutrient limitation, iron limitation and cell envelope stress.

Quorum Sensing and the production of autoinducers is the signalling process which initiates the production of biofilm.

Biofilm Production

Biofilm formation. Planktonic cells adhere to the surface and proliferate. During biofilm maturation, extracellular matrix and quorum sensing molecules are produced. Mature biofilm is characterized by a large number of matrix, slow-growing microbial cells at the center, and biofilm detachment and spread of infection.

Biofilm Composition
Biofilm Composition

1. The microbes that live in it
2. A glycocalyx (sugar coat) / EPS made from polysaccharides and positively charged ions: Ca, Mg, Fe
3. Dismutases and oxidases for protection
4. Proteins
5. 99% water

Biofilm Composition

Due to water content, anything that does penetrate it diffuses quickly.
Food channels containing siderophores (Fe chelators). Fe is essential for bacterial growth, virulence and replication.

How will a patient with biofilm infection present to you in clinic?

Biofilm in Disease

Because biofilm is novel to most practitioners, it is important to gain an understanding to recognise that strategies developed to treat planktonic bacteria are ineffective against bacteria in a biofilm.
Biofilm Infections

Infection by planktonic bacteria is nearly impossible.

Biofilm formation causes persistent tissue and foreign body infections resistant to treatment with antimicrobial agents.

Up to 80% of human bacterial infections are biofilm associated.

Most frequently caused by Staph epidermidis, Pseudomonas aeruginosa, Staph aureus and enterobacteria such as E. coli.

http://www.mcbood.org/pic/oral23022725

Biofilm in Disease

Oral biofilms have been studied and treated for years.

Strep mutans in plaque accelerates tooth decay.

More recently we are learning of the role of biofilm in disease throughout the body.

Biofilm in Disease

1. Protects microbes from the host immune system
2. Enables communication and adaptation of microbes (e.g. MRSA)
3. Enables microbes to persist, multiply and overwhelm the host

Biofilm and Disease

Recent evidence of biofilm colonies has accumulated for its role in:

1. Otitis media
2. Sinusitis

Paucity of studies of biofilm in GIT (accessibility), though known to adhere to gut wall and particulates in gut lumen.
Biofilm in Disease

Eradicating ear, nose and throat biofilm is difficult when treating single-organism or mixed flora biofilms.

Antibiotic therapy is often ineffective against biofilms and treatment may need to focus on nonantibiotic therapies that reduce, disrupt, or eradicate biofilms.


Biofilm Implicated In...

- Barrett's oesophagitis
- Ulcerative colitis
- Bronchiectasis
- Recurrent UTIs
- Pneumonia
- Vaginosis
- Blepharitis
- Periodontal dx

- Deep breast pain in lactating women
- Diabetic wounds
- Chronic cough
- Protracted bacterial bronchitis
- Candidiasis / thrush
- Acne vulgaris
- CF pulmonary infection

Table 5. Pathogenic bacteria associated with periodontal bacterial infections

<table>
<thead>
<tr>
<th>Pathogenic bacteria</th>
<th>Persistent disease</th>
<th>Storage mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroides gingivalis</td>
<td>Lactobacillus</td>
<td>Intracellular growth</td>
</tr>
<tr>
<td>Porphyromonas gingivalis</td>
<td>Streptococcus</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>Prevotella intermedia</td>
<td>Actinomyces</td>
<td>Intracellular colonization</td>
</tr>
<tr>
<td>Fusobacterium nucleatum</td>
<td>Campylobacter</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>Veillonella</td>
<td>Intracellular colonization</td>
</tr>
</tbody>
</table>

Direct Evidence of Bacterial Biofilms in Otitis Media

J. Christopher Post, MD, PhD

Objectives/Hypothesis: Bacteriologic studies of otitis media with effusion (OME) using highly sensitive techniques of molecular biology such as the polymerase chain reaction have demonstrated that traditional culturing methods are inadequate to detect many viable bacteria present in OME. The presence of pathogens attached to the middle-ear mucosa as a bacterial biofilm, rather than as free-floating organisms in a middle-ear effusion, has previously been suggested to explain these observations. The suggestion has been speculative, however, because no visual evidence of such biofilms on middle-ear mucosa has heretofore been collected. The hypotheses motivating the current study were: 1) biofilms of nontypable Hemophilus influenzae will form on the middle-ear mucosa of chinchillas in an experimental model of OME, 2) these biofilms will exhibit changes in density or structure over time, and 3) biofilms are also present on tympanostomy tubes in children with refractory post-tympanostomy otorrhea. The objective of this study was to collect visual evidence of the formation of bacterial biofilms in these situations.

Study Design: Laboratory study of bacteriology in an animal model and on medical devices removed from pediatric patients.

Methods: Experimental otitis media was induced in chinchillas by transbullar injection of nontypable H. influenzae. Animal study of bacteriology in an animal model and on medical devices removed from pediatric patients. Methods: Experimental otitis media was induced in chinchillas by transbullar injection of nontypable H. influenzae. Animals were killed in a time series and the surface of the middle-ear mucosa was examined by scanning electron microscopy (SEM) for the presence of bacterial biofilms. Adult and fetal chinchilla uninfected controls were similarly examined for comparison. In addition, tympanostomy tubes that had been placed in children's ears to treat OME and removed after onset of refractory otorrhea or other problems were examined by SEM and by confocal scanning laser microscopy for bacterial biofilms, and compared with unused control tubes. Results: Bacterial biofilms were visually detected by SEM on the middle-ear mucosa of multiple chinchillas in which H. influenzae otitis media had been induced. Qualitative evaluation indicated that the density and thickness of the biofilm might increase until at least 96 hours after injection. The appearance of the middle-ear mucosa of experimental animals contrasted with that of uninfected control animals. Robust bacterial biofilms were also visually detected on tympanostomy tubes removed from children's ears for clinical reasons, in contrast with unused control tubes. Conclusions: Bacterial biofilms form on the middle-ear mucosa of chinchillas in experimentally induced H. influenzae otitis media and can form on tympanostomy tubes placed in children's ears. Such biofilms can be directly observed by microscopy. These results reinforce the hypothesis that the bacterial aggregates called biofilms, resistant to treatment by antibiotics and to detection by standard culture techniques, may play a major etiologic role in OME and in one of its frequent complications, post-tympanostomy otorrhea. Key Words: Otitis media, otitis media with effusion, bacterial biofilms, chinchilla, tympanostomy tubes.

Laryngoscope, 111:2083–2094, 2001

AIMS AND OBJECTIVES

The aims and objectives of this thesis and the study it is based on are to demonstrate that bacterial biofilms are associated with, and can explain some of the clinical features of, otitis media with effusion (OME). A brief background will be helpful in explaining the importance and motivation of the study, and the prior experimentation that led to this novel inquiry into the etiology of a common illness.

Clinical Importance

Otitis media (OM) is a ubiquitous disease, with the costs of treatment estimated to be over $5 billion per year in the United States.1 Otitis media is the most common reason for an ill child to visit a pediatric health care provider, and chronic OME is the most common indication for surgery.2 It is also the most common reason for anti-
**Wound Biofilm**

![Diagram of Wound Biofilm]

**Gut-Brain-Skin Axis in Acne**

1. 
2. 
3. 
4. 
5. 
6. 
7.

**Biofilm in MINDD's**

MIND Disorders:

1. PANDAS (Paediatric Neuropsychiatric Disorder Associated w Strep)
2. PITAND (Paediatric Infection-triggered Autoimmune Neuropsychiatric Disorders)
3. Gut and immune disorders in autism
4. Fibromyalgia
5. Lyme disease
6. Depression, anxiety

**Biofilms and Inflammation**

In 1999 Costerton discovered that **biofilms damage tissues primarily by triggering inflammation.**

He suggested an innovative approach to treating chronic diseases by **using immune modulators instead of antibiotics.**

He says that the 'antibiotics that have been designed to kill free-floating bacterial cells work poorly against cells growing in slime-enclosed biofilms.'
The Biofilm Protocol

Pioneered by Dr Anju Usman.
In use since about 2008.
An approach to address systemic biofilm populations.

Premise of Biofilm Protocol

An advanced “weed, seed and feed” protocol.
First breakdown or weaken the biofilm so immune modulators and antimicrobials will be more effective against microbes within the biofilm.
Less chance of leaving behind persister cells.

The Biofilm Protocol

- 4 step process
- Each step done daily or a weekly cycle
- Minimum of 3 month duration
- Up to 2 years duration
The 4 Steps

1. Lysis
2. Antimicrobial
3. Mop up
4. Nourish and repopulate

I: Lysis

Take on empty stomach.
Enzymes may be dependent on type of biofilm.
Fibrinolytics:
- Serratiopeptidases (esp for staph)
- Nattokinase (esp. for Strep)

A new anti-infective strategy to reduce the spreading of antibiotic resistance by the action on adhesion-mediated virulence factors in Staphylococcus aureus.

Serratiopeptidase (SPEP)

Results suggest that SPEP could be developed as a potential "anti-infective agent" capable of hindering the entry of S. aureus into human tissues, and also impairs the ability of this pathogen to adhere to prostheses, catheters and medical devices.

Serratiopeptidase

"Proteases, in particular the metalloprotease serratiopeptidase, can interfere with adhesion and invasion of eukaryotic cells and biofilm formation in staphylococci and their use could represent a viable treatment for the development of novel combination therapies."

Data suggest that treatment with this natural enzyme may provide a useful tool in the prevention of the initial adhesion of Listeria monocytogenes to the human gut.

Prevents prosthesis biofilm infection.
A new anti-infective strategy to reduce the spreading of antibiotic resistance by the action on adhesion-mediated virulence factors in *Staphylococcus aureus*\textsuperscript{a}*	extcopyright*

Rosanna Papa\textsuperscript{a,1}, Marco Artini\textsuperscript{a,1}, Andrea Cellini\textsuperscript{a}, Marco Tilotta\textsuperscript{a}, Eugenio Galano\textsuperscript{b}, Pietro Pucci\textsuperscript{b,c}, Angela Amoresano\textsuperscript{b}, Laura Selan\textsuperscript{a,*}

\textsuperscript{a} Department of Public Health and Infectious Diseases, Sapienza University, Piazzale Aldo Moro 5, 00185 Rome, Italy
\textsuperscript{b} Department of Chemical Sciences, Federico II University, Complesso Universitario Monte Sant’Angelo, Via Cinthia 4, 80126 Naples, Italy
\textsuperscript{c} CEINGE Advanced Biotechnology Scarl, Via Gaetano Salvatore 486, 80145 Naples, Italy

**Abstract**

*Staphylococcus aureus* is a flexible microbial pathogen frequently isolated from community-acquired and nosocomial infections. *S. aureus* expresses a wide array of secreted and cell surface-associated virulence factors, including proteins that promote adhesion to damaged tissue and to the surface of host cells, and that bind proteins in blood to help evade immune responses. Furthermore, surface proteins have a fundamental role in virulence related properties of *S. aureus*, including biofilm formation. The present study evaluates the anti-infective capabilities of a secreted protein of *Serratia marcescens* (serratiopeptidase, SPEP), in impairing some staphylococcal virulence-related properties, such as attachment to inert surfaces and adhesion/invasion on eukaryotic cells. SPEP seems to exert its action by modulating specific proteins. It is not assessed if this action is due to the proteolytic activity of SPEP or to a specific mechanism which triggers an out/inside signal. Proteomic studies performed on surface proteins extracted from SPEP treated *S. aureus* cultures revealed that a number of proteins are affected by the treatment. Among these we found the adhesin/autolysin Atl, SdrD, Sbi, EF-Tu and EF-G. EF-Tu and EF-G are known to perform a variety of function, depending on their cytoplasmic or surface localization. All these factors can facilitate bacterial colonization, persistence and invasion of host tissues. Our results suggest that SPEP could be developed as a potential “anti-infective agent” capable to hinder the entry of *S. aureus* into human tissues, and also impairs the ability of this pathogen to adhere to prostheses, catheters and medical devices.

© 2013 The Authors. Published by Elsevier Ltd. All rights reserved.

1. Introduction

*Staphylococcus aureus* (*S. aureus*) is a flexible microbial pathogen frequently isolated from community-acquired and nosocomial infections [1,2]. This microorganism can also be found as a part of the normal human resident flora and up to two-thirds of the healthy population are permanently or transiently colonized by *S. aureus* [3]. The rapid emergence of hospital associated, antibiotic resistant *S. aureus* strains is a major epidemiological problem worldwide [4,5]. Moreover, the increased use of medical devices is associated with a significant risk of intravascular and systemic infections by staphylococci, which frequently causes persistent infections on catheters, shunts, vascular and orthopedic prostheses, and other implanted devices [6–9]. The ability of *S. aureus* to adhere on both eukaryotic cells and abiotic surfaces via cell wall proteins and to form biofilm are important virulence factors in chronic infections associated with implanted biomaterials, which are particularly difficult to eradicate [10–13].

Hence, not surprisingly, the interest in the development of alternative anti-infective approaches for the prevention and treatment of staphylococcal infections has increased in recent years [14–17]. A successful strategy should not affect processes essential for bacterial survival in order to avoid the rapid appearance of escape mutants. An innovative approach should target *S. aureus* major virulence factors without affecting bacterial viability.
I: Lysis

DPP-IV (proteolytic)
Polysaccharide specific:
- Cellulase, hemicellulase, pectinase
- Chitinase (esp for fungal biofilm)
- B-Glucanase

Chelators
- Oral di-Na EDTA
- Lactoferrin
Other:
- Xylitol (attachment and development)
- ACV
- Honey (for wounds)
- Cinnamon oil (for Candida biofilms)

2: Antimicrobial

- Given 15-60 minutes after step 1 (Lysis)
- Herbs - dependent on stool test results and symptoms
- Pharmaceutical antibiotics last resort
- Rotation recommended

2: Antimicrobials

- Berberine cont.
- Oregano
- Artemisia
- Echinacea
- Gentian
- Neem
- Tea tree
- Honey
- Undecylenic
- Lauric acid
- CSE
- Black Walnut
3: Clean Up

- Roughly 2 hr post antimicrobials
- Bind and eliminate endotoxins released by killed microbes
- To prevent / lessen die-off / detox effect
- Die-off and detox are often significantly greater when addressing biofilm

3: Clean Up

Signs of die-off / detox:
- Exacerbation of existing symptoms
- Cold / flu like symptoms
- Muscle aches, headaches, fever
- Behavioural symptoms

3: Clean Up

- Slippery elm
- Psyllium
- Citrus pectin
- Chitosan
- Chlorella
- Charcoal
Candida species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options


Department of Clinical Analysis, Laboratory of Clinical Mycology, Faculty of Pharmaceutical Sciences, UNESP, Araraquara, Brazil

The incidence of fungal infections has increased significantly, so contributing to morbidity and mortality. This is caused by an increase in antimicrobial resistance and the restricted number of antifungal drugs, which retain many side effects. Candida species are major human fungal pathogens that cause both mucosal and deep tissue infections. Recent evidence suggests that the majority of infections produced by this pathogen are associated with biofilm growth. Biofilms are biological communities with a high degree of organization, in which micro-organisms form structured, coordinated and functional communities. These biological communities are embedded in a self-created extracellular matrix. Biofilm production is also associated with a high level of antimicrobial resistance of the associated organisms. The ability of Candida species to form drug-resistant biofilms is an important factor in their contribution to human disease. The study of plants as an alternative to other forms of drug discovery has attracted great attention because, according to the World Health Organization, these would be the best sources for obtaining a wide variety of drugs and could benefit a large population. Furthermore, silver nanoparticles, antibodies and photodynamic inactivation have also been used with good results. This article presents a brief review of the literature regarding the epidemiology of Candida species, as well as their pathogenicity and ability to form biofilms, the antifungal activity of natural products and other therapeutic options.

Introduction

The incidence and prevalence of invasive fungal infections have increased since the 1980s, especially in the large population of immunocompromised patients and/or those hospitalized with serious underlying diseases (Arendrup et al., 2005; Espinel-Ingroff et al., 2009). Candida species belong to the normal microbiota of an individual’s mucosal oral cavity, gastrointestinal tract and vagina (Shao et al., 2007), and are responsible for various clinical manifestations from mucocutaneous overgrowth to bloodstream infections (Eggimann et al., 2003). These yeasts are commensal in healthy humans and may cause systemic infection in immunocompromised situations due to their great adaptability to different host niches. The genus is composed of a heterogeneous group of organisms, and more than 17 different Candida species are known to be aetiological agents of human infection; however, more than 90 % of invasive infections are caused by Candida albicans, Candida glabrata, Candida parapsilosis, Candida tropicalis and Candida krusei (Pfaller et al., 2007). The expanding population of immunocompromised patients that use intravenous catheters, total parenteral nutrition, invasive procedures and the increasing use of broad-spectrum antibiotics, cytotoxic chemotherapies and transplantation are factors that contribute to the increase of these infections (Ortega et al., 2011). The pathogenicity of Candida species is attributed to certain virulence factors, such as the ability to evade host defences, adherence, biofilm formation (on host tissue and on medical devices) and the production of tissue-damaging hydrolytic enzymes such as proteases, phospholipases and haemolysin (Silva et al., 2011b).

Currently, an increase in the number of yeasts that are resistant to antifungal drugs is recognized worldwide; therefore, the use of in vitro laboratory tests may aid the doctor in choosing an appropriate therapy (Ingham et al., 2012). The ability of Candida species to form drug-resistant biofilms is an important factor in their contribution to human disease. As in the vast majority of microbial biofilms (Rajendran et al., 2010), sessile cells within C. albicans biofilms are less susceptible to antimicrobial agents than are planktonic cells (Kuhn & Ghannoum, 2004). The progression of drug resistance within Candida biofilms has been associated with a parallel increase in the maturation process.
3: Clean Up

Die off creates an acidic environment

Buffering agents:
- Buffered C
- Bicarbonate

4: Nourish

Diet is essential - the first step to begin on the protocol.

Without addressing diet, impossible battle.

4: Diet for Dysbiosis

- Low starch
- Low sugar, no added sugar
- Fermented foods most commonly required
- GFCF
- GAPS diet / BED

4: Diet for Dysbiosis

Base meals around:
- Good fats (ghee, coconut / olive / flax / hemp / fish)
- Animal proteins
- Loads of non-starchy vege, incl. fermented vege (raw and/or cooked, depending on person)
4: Diet for Dysbiosis

Special mention:
- Bone broths
- Fermented veggies
- Marshmallow root tea
- Xylitol

4: Repopulate

- Taken in the evening (away from step 2)
- Probiotics determined by stool testing
- Special mention:
  - E. coli
  - Saccharomyces boulardii

S. boulardii

Increases the activities of **intestinal brush border enzymes** such as disaccharidases, a-glucosidases, alkaline phosphatases, and aminopeptidases.

Secretes a leucine aminopeptidase that appears to support against **allergies** to dietary proteins following acute gastroenteritis.

S. boulardii

Augments the intestinal absorption of D-glucose coupled with sodium that may enhance uptake of **water and electrolytes** during diarrhoea.

Increases stool concentrations of **SCFAs** that nourish colon mucosal cells.
77

S. boulardii

Modulates immunity by boosting secretion of IgA and increasing crypt cell Ig receptors.

Inhibits inflammatory cytokine pathways and secretes a factor that blocks C. difficile toxin A activities.

Useful for antibiotic-associated diarrhoea, C. diff disease, IBD, IBS, traveller’s diarrhea, Blastocystis hominis, and giardia.


78

E. coli Nissle 1917

The probiotic E. coli strain Nissle 1917 is, in addition to some Lactobacilli sp, one of the best-studied probiotic strains.

Initial therapeutic success was noted in the management of GIT infectious disorders and infections affecting the urinary tract; the focus shifted later to chronic inflammatory conditions.

The unique combination of fitness and survival factors to support intestinal survival, the lack of virulence, and obvious probiotic properties make this microorganism a safe and effective candidate in the treatment of chronic inflammatory bowel diseases.


79

E. coli Nissle 1917

Shows effects in IBS especially in patients with altered enteric microflora, e.g. after gastroenterocolitis or antibiotics.

Enhances the wound-healing migration of human enterocytes.

Maintenance therapy for ulcerative colitis.

Protects gastric mucosa against stress-induced erosions due to anti-inflammatory and vasodilatory actions.

80

E. coli Nissle 1917

Studies show positive results when strain is used in Crohn’s disease, pouchitis, irritable bowel syndrome or necrotizing enterocolitis, but especially in the prevention of relapse in patients with ulcerative colitis.

Double blind, randomised controlled trials comparing the efficacy of E. coli Nissle 1917 to that of mesalazine, which is part of the first-line treatment in ulcerative colitis have shown that E. coli Nissle 1917 is as efficient as mesalazine to prevent relapse.

Genotoxicity of *Escherichia coli* Nissle 1917 strain cannot be dissociated from its probiotic activity

Maïwenn Olier,1,5,6,7,8 Ingrid Marcq,2,5,6,7 Christel Salvador-Cartier,7 Thomas Secher,2,3,4,5 Ulrich Dobrindt,6 Michèle Boury,2,3,4,5 Valerie Bacquè,1 Marie Penary,4,5,6 Eric Gautier,4,5 Jean-Philippe Nougyardè,2,4,5 Jean Fioramonti7 and Eric Oswald2,3,4,5,7,8

Neuro-gastroenterologie et Nutrition, UMR INRA/ENVT 1331; Toulouse, France; 2INRA; USC 1360; Toulouse, France; 3Inserm, UMR1244; Toulouse, France; 4CNRS, UMR5282; Toulouse, France; 5Université de Toulouse, UPS; Centre de Physiopathologie de Toulouse Purpan (CPTP); Toulouse, France; 6Universität Münster; Institut für Hygiene; Münster, Germany; 7CHU Toulouse; Hôpital Purpan; Service de bactériologie-Hygiène; Toulouse, France

**Keywords:** Colibactin, colitis, *Escherichia coli*, probiotic, genotoxin, inflammation, DSS, CD4+ CD45RBbright T-cell transfer model

**Abbreviations:** ANOVA, analysis of variance; cfu, colony forming unit; DMEM, Dulbecco’s modified Eagle’s medium; DSS, dextran sodium sulfate; FACS, fluorescence activated cell sorting; FBS, fetal bovine serum; IBD, inflammatory bowel diseases; IEC, intestinal epithelial cells; IL, interleukin; MPO, myeloperoxidase; UC, ulcerative colitis; WT, wild-type

*Correspondence to: Maïwenn Olier and Eric Oswald; Email: maiwenn.olierr@toulouse.inra.fr and eric.oswald@inserm.fr

Oral administration of the probiotic bacterium *Escherichia coli* Nissle 1917 improves chronic inflammatory bowel diseases, but the molecular basis for this therapeutic efficacy is unknown. *E. coli* Nissle 1917 harbors a cluster of genes coding for the biosynthesis of hybrid nonribosomal peptide-polypeptide(s). This biosynthetic pathway confers the ability for bacteria to induce DNA double strand breaks in eukaryotic cells. Here we reveal that inactivation of the clbA gene within this genomic island abrogated the ability for the strain to induce DNA damage and chromosomal abnormalities in non-transformed cultured rat intestinal epithelial cells but is required for the probiotic activity of *E. coli* Nissle 1917. Thus, evaluation of colitis severity induced in rodent fed with *E. coli* Nissle 1917 or an isogenic non-genotoxic mutant demonstrated the need for a functional biosynthetic pathway both in the amelioration of the disease and in the modulation of cytokine expression. Feeding rodents with a complemented strain for which genotoxicity was restored confirmed that this biosynthetic pathway contributes to the health benefits of the probiotic by modulating its immunomodulatory properties. Our data provide additional evidence for the benefit of this currently used probiotic in colitis but remind us that an efficient probiotic may also have side effects as any other medication.

**Introduction**

Probiotics, when administered alive and in adequate amounts, are supposed to be safe and confer health benefits to the host.1 As they are generally marketed as “natural” cures, probiotics benefit also from a positive public image among patients. Nonetheless, physicians need scientific guidance and additional investigations to definitively state on efficacy of probiotics before they can be routinely recommended in clinical practice.2 Several probiotic bacteria have been identified as promising in the management of inflammatory bowel disease (IBD),3-5 but only few studies identified the mode of action of these bacteria.6-9 A better understanding of the mechanisms by which probiotics promote health remains critical to fully optimize their safety assessment for human use.10

*Escherichia coli* Nissle 1917 (Mutaflor®) has been commercially available for almost one century and successfully used in humans for almost one century and successfully used in humans as an oral treatment for a number of intestinal disorders.11 A number of studies have shown positive results when this strain is used in conditions such as Crohn disease, pouchitis, irritable bowel syndrome or necrotizing enterocolitis12 but is especially used in the prevention of relapse in patients with ulcerative colitis (UC). Double blind randomized controlled trials comparing the efficacy of *E. coli* Nissle 1917 to that of mesalazine, which is part of the first-line treatment in patients with UC, have shown that *E. coli* Nissle 1917 is as efficient as mesalazine to prevent relapse of UC.13-15 In addition, the probiotic treatment is associated with a prolonged remission without any reported adverse effects. However, although *E. coli* Nissle 1917 is one of the best studied probiotic bacterial strains, the genetic determinants governing the probiotic nature of *E. coli* Nissle 1917 are yet to be elucidated.16-17

We have previously identified in the genome of *E. coli* Nissle 1917 a cluster of genes named “phs island” that allow production of a hybrid peptide polypeptide genotoxin, called Colibactin.18 Up to date, purification of Colibactin has failed and its structure remains unsolved but we previously reported that *E. coli* strains harboring this phs island are able to induce DNA damage in vivo and trigger genomic instability and gene mutations in mammalian cells.19 Given a possible contribution of Colibactin to the development of sporadic colorectal cancer, we wished to explore whether inactivation of this biosynthetic pathway contributes to the health benefits of the probiotic by modulating its immunomodulatory properties. Our data provide additional evidence for the benefit of this currently used probiotic in colitis but remind us that an efficient probiotic may also have side effects as any other medication.
ORIGINAL ARTICLE

Bacteria, biofilm and honey: A study of the effects of honey on ‘planktonic’ and biofilm-embedded chronic wound bacteria

PATRICIA MERCKOLL, TOM Øystein Jonasen, Marie Elisabeth Vad, Stig L. Jeansson & Kjetil K. Melby

From the Department of Microbiology, Oslo University Hospital, Ullevål and the Faculty of Medicine, University of Oslo, Oslo, Norway

Abstract
Chronically infected wounds are a costly source of suffering. An important factor in the failure of a sore to heal is the presence of multiple species of bacteria, living cooperatively in highly organized biofilms. The biofilm protects the bacteria from antibiotic therapy and the patient’s immune response. Honey has been used as a wound treatment for millennia. The components responsible for its antibacterial properties are now being elucidated. The study aimed to determine the effects of different concentrations of ‘Medihoney™ therapeutic honey and Norwegian Forest Honey 1) on the real-time growth of typical chronic wound bacteria; 2) on biofilm formation; and 3) on the same bacteria already embedded in biofilm. Reference strains of MRSE, MRSA, ESBL Klebsiella pneumoniae and Pseudomonas aeruginosa were incubated with dilution series of the honeys in microtitre plates for 20 h. Growth of the bacteria was assessed by measuring optical density every 10 min. Growth curves, biofilm formation and minimum bactericidal concentrations are presented. Both honeys were bactericidal against all the strains of bacteria. Biofilm was penetrated by biocidal substances in honey. Reintroduction of honey as a conventional wound treatment may help improve individual wound care, prevent invasive infections, eliminate colonization, interrupt outbreaks and thereby preserve current antibiotic stocks.

Introduction
Chronic wound care is costly and difficult. It is estimated that 1% of the UK and US populations have a chronic sore, with wound care costs in Britain alone estimated at £1 billion per y [1]. The chronic sore is often a painful, exuding, malodorous portal for invasive infection and a reservoir for antibiotic resistant bacteria, capable of causing outbreaks.
Honey has been used as a wound treatment for millennia [2]. It is once again being recognized that honeys of differing botanical provenance have varying levels of antibacterial and wound healing activity [2]. In 1991 a survey of the antibacterial activity of local honeys was carried out in New Zealand [3]. This has lead to the licensing of standardized, sterilized Leptospermum plant species honey as a wound treatment. The granting of a licence was a pivotal moment, as it allows for reproducible clinical trials and in vitro experiments. Several studies have now confirmed the bactericidal nature of therapeutic honeys against many species of bacteria, including strains resistant to antibiotics [4,5]. However, most in vitro honey studies have used methods traditionally used for assessing the likely antibiotic sensitivity of bacteria to serum levels of antibiotics – the reporting of minimum inhibitory concentration on agar plates. Honey, meant for topical application, is not itself an antibiotic, but a complex mixture of substances with biocidal effects [2]. Therefore, biokinetic studies of bacterial growth related to concentration of biocide through time, may be more appropriate [6]. A further difficulty of generalization of results from in vitro studies conducted on agar plates, is that bacteria are tested in their most vulnerable ‘planktonic’ form. Bacteria associated with chronic infections most often live in a biofilm – a highly organized, slimy, bacteria-secreted polysaccharide layer attached to a surface, be it the patient’s tissue or a prosthetic part [7,8]. The biofilm protects the bacteria from the
Biofilms Prevent Healing

An important factor in the failure of sores to heal is the presence of multiple species of bacteria living cooperatively in highly organised biofilms.

Biofilm protects the bacteria from antibiotic therapy and the patient's immune response.

Honey has been used as a wound treatment for millennia.

Honey Penetrates Biofilms

Strains of MRSE, MRSA, ESBL Klebsiella pneumoniae and Pseudomonas aeruginosa were incubated with dilution series of the honeys for 20 hours.

Honey was bactericidal against all the strains of bacteria.

Biofilm was penetrated by biocidal substances in the honey.

Honey for Wounds

Reintroduction of honey as a conventional wound treatment may help improve individual wound care, prevent invasive infections, eliminate colonisation, interrupt outbreaks and thereby preserve current antibiotic stocks.
Additional Considerations
Things to keep in mind when you are considering biofilm treatment

Avoid supplementing with Ca, Mg, Fe unless absolutely necessary.
- Timing.
Symbiotics create biofilm too. Hence suitable probiotic use is essential.

Cautions
Set the foundation -
- Inflammation
- Basic gut work
- Begin heal and seal protocol
- Liver support
Die-off cycles.

Biofilm in a Nutshell
A factor in up to 80% of chronic / recurrent infections.
Implication in chronic inflammatory diseases - autoimmune, fibromyalgia etc.
Implicated in MIND disorders.
Requires different treatment than planktonic infections.
Treatment takes 3-24 months, must be done gradually and monitored regularly.
Beneficial microbes build biofilm too - important to repopulate the human microbiome with these.
Best to complete stool testing or OAT to determine treatment products and duration.