Microbial Biosurfactants: Current trends and applications in Agricultural and Biomedical industries

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Running title: Bioactivity of Microbial biosurfactants

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Summary

Synthetic surfactants are becoming increasing unpopular in many applications due to previously disregarded effects on biological systems and this has led to a new focus on replacing such products with biosurfactants that are biodegradable and produced from renewal resources. Microbially derived biosurfactants have been investigated in numerous studies in areas including: increasing feed digestibility in an agricultural context, improving seed protection and fertility, plant pathogen control, anti-microbial activity, anti-biofilm activity, wound healing and dermatological care, improved oral cavity care, drug-delivery systems and anti-cancer treatments. The development of the potential of biosurfactants has been hindered somewhat by the myriad of approaches taken in their investigations, the focus on pathogens as source species and the costs associated with large- scale production. Here we focus on various microbial sources of biosurfactants and the current trends in terms of agricultural and biomedical applications.

Key words: rhamnolipids, sophorolipids, lipopeptides, wound healing, anticancer.

Introduction

It is now accepted that widespread use of synthetic surfactants negatively affects the environment. An area of particular concern relates to the use of synthetic surfactants that are utilised in abundance by various industries, including pharmaceutical and medical manufacturing, the food and feed industry, agriculture, environmental remediation and the petroleum industry. Environmental concerns in developed countries and increasingly worldwide have resulted in increasing legal and societal pressure for these substances to be biodegradable and produced sustainably using renewable substrates. These requirements have led to intensification of research and more recently the development of new technologies involving biogenic surface-active substances of microbial origin i.e. biosurfactants, (Marchant and Banat 2012a; Santos *et al.* 2016).

Biosurfactants, have many advantages over chemically produced surfactants, such as high biodegradability and low ecotoxicity, and can be easily produced from renewal energy resources (Makkar and Cameotra 2002). These microbially derived surface-active substances are widely used in the pharmaceutical, food, cosmetic, textile, oil and agricultural industries (Figure 1). They can be used as anti-fungal as well as antibiofilm agents (Gudiña *et al.* 2010; Banat *et al.* 2014a; Diaz de Rienzo *et al.* 2015; Haque *et al.* 2016). In a microbiological context, there is a particular interest in those biosurfactants produced by bacteria and their anti-bacterial, antifungal and anti-viral properties. In addition, these compounds also have a range of possible therapeutic and biomedical benefits. Despite the potential of biosurfactants the fact that the significant producers namely *Pseudomonas* and *Bacillus* are potentially pathogenic has proved a drawback hence the interest in yeasts and yeast-like fungi including *Starmerella bombicola* and non-pathogenic, bacteria which are generally seen as not posing a risk in terms of toxicity or pathogenicity. There is increasing evidence that biosurfactants as well as displaying the industrially valuable properties of detergency, emulsification, and foaming may also have significant bioactivities applicable to human and animal health (Fu *et al.* 2008; Shao *et al.* 2012; Fracchia *et al.* 2015).

The focus of many reviews in the area have been on the biosurfactants themselves and indeed recent reviews include those, which have focussed specifically on applications in agriculture or industry (Minif and Ghribi 2016; Santos *et al.* 2016; Singh *et al.* 2019). This review focuses on microbial biosurfactants and current trends in agricultural and health related applications.

Classification and structure of microbial biosurfactants of interest

Biosurfactants are classified according to their molecular weight and categorised, by their microbial origin and composition. The high molecular weight biosurfactants include the lipopolysaccharides but those of main interest are the low molecular weight glycolipids and lipopeptides (LP's) and phospholipids. Of the glycolipids (Minif and Ghribi 2016), which include trehalolipids, cellobiose lipids, mannosylerythritol lipids (MELs), rhamnolipids, (derived from mainly *Pseudomonas*) and

76 sophorolipids (SL's), (derived from *Candida* and related species) are of the most interest. The
77 glycolipids (Marchant and Banat 2012b) and the LP's (derived mainly from *Bacillus* spp) are the
78 biosurfactants of most interest in terms of their therapeutic potential of those investigated thus far.

79 Rhamnolipids are amphipathic in nature comprising hydrophobic and hydrophilic moieties which
80 enable them to reduce surface and interfacial tensions. The antimicrobial property of rhamnolipids is
81 attributed to their permeablising effect which leads to disruption of the bacterial cell plasma
82 membrane (Sotirova *et al.* 2008; Fracchia *et al.* 2015; Diaz de Rienzo *et al.* 2016a; Diaz de Rienzo *et*
83 *al.* 2016b; Diaz de Rienzo *et al.* 2016c), their ability to compromise cell surface charge (Kaczorek
84 2012) and ability to change bacterial cell hydrophobicity (Sotirova *et al.* 2009). They also have the
85 ability to prevent and obstruct biofilm formation making the constituent bacteria more susceptible to
86 antimicrobial agents (for a comprehensive review of the potential applications of rhamnolipids see
87 Chen *et al.* 2017).

88 Sophorolipids (SL's), are produced by yeasts. They have a dimeric carbohydrate sophorose linked to a
89 long-chain hydroxyl fatty acid through a glycosidic bond (for a recent detailed review of
90 Sophorolipids see de Oliveira *et al.* 2015). It is rapidly becoming apparent that the range of
91 biosurfactant congeners produced by a microorganism may have very different types and extents of
92 bioactivity and therefore it is important to use highly purified individual congeners to assign
93 unequivocally an activity to a specific congener. In the case of SL's the acidic and lactonic forms
94 show very different properties (Van Bogaert *et al.* 2007). In addition to the properties of detergency
95 and bioactivity the effectiveness of acidic SL's as a capping agent has been studied in the synthesis of
96 various metal-based nano-particles (Kasture *et al.* 2007; Dhar *et al.* 2011). Singh *et al.* (2013)
97 reported the mesoscale molecular assembly of SL using pulse UV laser processing technique. The
98 available reports suggest that SL could be utilised as a carrier system for drug delivery by exploring
99 its structure-forming attributes. Lactonic (LT) forms are more hydrophobic (Joshi-Navare *et al.* 2013)
100 and have been, reported to have better biocide activities (Ito *et al.* 1980) spermicide, cytotoxic and
101 proinflammatory activities. Work by Shao and co-workers suggest that the LT form possessed
102 anticancer activity (Shao *et al.* 2012) however more recent work (Callaghan *et al.* 2016) suggest this

is not the case albeit in another model system when using highly purified congeners. The acidic forms are better foaming agents, have higher water solubility (Hirata *et al.* 2009) and have shown potential in the food, bioremediation and cosmetics industries (Ma *et al.* 2011). SL's bear two different polar heads on the two ends of the lipophilic core this referred to as 'asymmetric bolas'. Being, amphiphilic, in nature, they tend to form self-assemblies or 'liposomes' (Rodrigues, 2015) with unique structural and physiochemical properties as well as functionality (Dubey *et al.* 2013) and biofilm disruption activity (Diaz De Rienzo *et al.* 2015), (for a review of the applications of SL's see de Oliveira *et al.* 2015).

Lipopeptides (LP's) are, composed of lipid moieties attached to a peptide chain and have biological activities including antimicrobial and anti-cancer. The most characterised LP's are Daptomycin and polymixin B, which are microbial-derived LP antibiotics. Surfactin (SUR), iturin and fengycin are among the best, known LP's and have a myriad of potential applications (Fracchia *et al.* 2015) (for a comprehensive review of lipopeptides see Mnif and Ghribi 2015)

Antimicrobial and antifungal properties of biosurfactants

Given the rise in antibiotic resistance, the need to identify new anti-microbials and find a means of rehabilitating current antibiotics used in medicine has become clear. There has been a global call to arms (WHO, 2017) in terms of efforts both nationally (DoH and DEFRA 2013) and internationally (CDC, 2015) to meet the challenge of antibiotic resistance. Biosurfactants are, ideally placed to answer the call in terms of their applications including; bactericidal, bacteriostatic, biofilm formation inhibition, biofilm disruption, synergistic and adjuvant effects with antibiotics.

Properties of biosurfactants include inhibition of bacterial and fungal growth (Kim *et al.* 1998, Lotfabad *et al.* 2010; Diaz de Rienzo *et al.* 2016a,). Biosurfactants produced by *S. saprophyticus* SBPS 15 showed antibacterial activity against *K. pneumonia. E. coli. V. cholera, B. subtilis* and *S. aureus* (Mani *et al.* 2016). Rhamnolipid has been, reported to have biofilm disruptive capability *against B. pumilus* (Dusane *et al.* 2010). The biosurfactant SUR can control the growth of *Listeria*

monocytogenes in food (Sabate and Audisio 2013) and some Gram-positive bacteria like *B. pumulis, M. flavus* (Das *et al.* 2007). LP's can damage and penetrate lipid containing negatively charged cell membranes. It has been suggested that a charge imbalance develops at the cell surface interface as a results of the polar element attempting to preserve solubility. This results in a loss of cell morphology leading to pore formation in the lipid containing cell membrane of Gram-negative bacteria causing cell damage/death.

In the case of rhamnolipids there, is clear evidence that they reduce bacterial growth in the exponential phase, which suggests that these compounds may have an influence on normal cell division. Diaz de Rienzo *et al.* (2016a) suggest that rhamnolipids and SP's may have different mechanisms of action against different microorganisms. They postulate that rhamnolipids inhibit the growth in the exponential phase but that the antimicrobial effects of SP's occurs between the exponential and stationary phases and, as evidenced by the enhanced effect produced by the inclusion of caprylic acid in this study, may be more comparable with conventional antibiotics than rhamnolipids. The differing results found when identical microorganisms are, challenged with biosurfactants in antimicrobial assays versus biofilm assays is a case in point. Often these assays give contradictory results for the same organisms in the presence of the same biosurfactant because of the different mechanism/mode of action at work.

The scientific literature also suggests that rhamnolipids may be more effective against Gram positive bacteria than Gram negative bacteria due to the presence of an outer membrane in Gram negative bacteria which can work to exclude biosurfactant molecules (Sotirova *et al.* 2008; Bharali and Konwar 2011) Another suggestion, is that rhamnolipids cause cell membrane damage by insertion of acyl tails causing cell leakage of cytoplasmic components (Yalçin and Ergene 2009). Sana *et al.* (2018) showed that both *E. coli* and *S. aureus* were sensitive to rhamnolipid and that because of its' hydrophilic and hydrophobic parts it interacts with the non-polar part of the cell membrane. The membrane disintegrates leading to penetration of the cell wall and plasma membrane by pore formation and subsequent leakage of inner cytoplasmic materials leading to cell death (Meincken *et al.* 2005, Ortiz *et al.* 2006). Another possibility is that rhamnolipid inserts its' shorter acyl tails into

156 the cell membrane and attacks the configuration of the cell wall and plasma membrane (Sanchez *et al.*
157 2006; Yalçin and Ergenen 2009,) alternatively, the membrane permeability produced by rhamnolipid
158 may be, enhanced by its interaction with the phospholipid component of the plasma membrane (Ortiz
159 *et al.* 2006). In terms of SL's, the vigorous membrane distorting potentiality of SUR is dependent on
160 the size of the peptide ring with the peptide moiety penetrating into the cell membrane and generating
161 a variance of charge at the site of action on the membrane surface (Heerklotz and Seelig 2001). These
162 mechanisms might help explain how the lipopeptide produced by *B. stratosphericus* (Sana *et al.* 2018)
163 has an antibacterial effect against both *S. aureus* and *E. coli.*

164

165 The anti-adhesive activity of biosurfactants is also an important property particularly if you are
166 seeking to prevent biofilm formation (Galié *et al.* 2018). Biofilm formation plays a key role in the
167 survival of both pathogenic (Kumar *et al.* 2017) and non-pathogenic microorganisms. The process of
168 surface attachment and the growth of heterogeneous cells within a matrix can be considered generic
169 i.e. common to both pathogenic and non-pathogenic microorganisms. In pathogens, the mechanisms
170 of attachment to and colonisation of surfaces are key and there are numerous examples of clinically
171 relevant biofilm formers e.g. *Pseudomonas* in the lungs (Lopes 2015); *Pseudomonas* on contact
172 lenses (El-Ganiny *et al.* 2017) and *Staphylococci* in orthopaedic implants and breast implants (Arciola
173 *et al.* 2015; Seng *et al.* 2015). While biofilms can be composed of multiple species or a single species
174 it is the case that many diseases including nosocomial infections are essentially biofilm associated
175 diseases associated with individual species e.g. *Mycoplasma pneumonia, Candida albicans,*
176 *Pseudomonas aeruginosa, Staphylococcus epidermidis, Mycobacterium tuberculosis* and
177 *Mycobacterium abscessus*. Key to the success of these biofilms are the advantages they afford to their
178 pathogenic inhabitants principally: drug tolerance, avoidance of the host immune responses and
179 recalcitrance of infection. The literature suggests, that biosurfactants can play an important role in
180 preventing biofilm formation on surfaces e.g. silicon (Rodrigues *et al.* 2006, Ceresa *et al.* 2015),
181 titanium (Ciandrini *et al.* 2016) and polystyrene plates (Gomez *et al.* 2016). Gudiña *et al.* (2015)
182 showed that glycoprotein biosurfactant from *L.agilis* inhibited the adhesion of *S. aureus* and Madhu &

Prapulla (2014) in their evaluation of a glycoprotein from *L. plantarum* CFR2194, also showed inhibition of *S. aureus* adhesion. Importantly, workers (Gudiña *et al.* 2015) have also shown the anti-adhesive properties can also be affected by the carbon source in the medium in which the producer strain is grown. Hence, changes in the proportion of carbohydrate, lipid and protein present in polymeric fractions of microbial biosurfactants can play a role in their biological effectiveness.

Quinn *et al.* (2013) have shown that Rhamnolipid is effective in inhibiting *S. aureus, B. subtilis* and *M. luteus* single species biofilms and that they were in fact more effective than broad-spectrum antibiotics used in the study. Rivardo *et al.* (2009) demonstrated the anti-adhesion activity of two biosurfactants produced by *Bacillus* spp therefore preventing human bacterial pathogens from producing bacterial biofilms. Rivardo and co-workers (2011) have also shown the synergistic effect of lipopeptide biosurfactant with antibiotics against *E. coli* CFT073 biofilm. It has, been previously demonstrated that the use of biosurfactants preventively i.e. prophylactically can prevent the formation of fungal biofilms (Dusane *et al.* 2012).

Immunocompromised and transplant patients and those with medical implants are highly susceptible to fungal infections such as those caused by *Candida albicans* and other *Candida* species and *Candida auris* in particular (Schwartz and Patterson 2018). Haque *et al.* 2016 found the SL derived from *Starmerella bombicola* MTCC1910 inhibited *C. albicans* hyphal growth and biofilm formation as well as reducing the viability of preformed biofilms. Additionally, when used with amphotericicn B (AmB) or fluconazole (FLZ) two potent anti-fungal agents the SL combination was, found to act synergistically against biofilm formation and preformed biofilm. Sarwar and co-workers (2018a, 2018b) in their investigations of microbial biosurfactants from *Bacillus* species found that LP extracts displayed antifungal activity against *Fusarium moniliforme, Fusarium oxysporum, Fusarium solani* and *Tricoderma atroviride*. Additionally, the LP extracts showed haemolytic activity and ~~their~~ potential as biocontrol agents against various *Fusarium* and *Trichoderma* species.

Fengycin is a cyclic lipodecapeptide produced by *Bacillus subtilis* strains, and appears to act by increasing the plasma membrane permeability of the target cell (Vanittanakom *et al.* 1986). Fengycin has been shown to exhibit strong fungitoxic activity specifically against filamentous fungi, inhibiting some enzymes (Loeffler *et al.* 1986; Steller and Vater 2000). The antifungal mechanism of fengycin may be as a result of its physicochemical properties due to its amphiphilic characteristics and affinity for lipid bilayers. Roy *et al.* (2013) in studies with fengycin did not show any antibacterial effects but did show anti-fungal activity of a fengycin-like peptide from *Bacillus thuringiensis* strain SM1 against *Candida albicans* and showed that treated cells displayed membrane blebs suggesting loss of contact between the cell membrane and the cell wall.

As previously mentioned the focus of research has now moved from the potential antimicrobial effects of biosurfactants themselves to how, they might act in unison with current antibiotics to maintain or even improve their efficacy. In the face of antibiotic resistance, these may include inhibitory or antibacterial adjuvant activities against various microorganisms (Fracchia *et al.* 2012; Joshi-Navare and Prabhune 2013)

The presence of a trans-envelope multidrug resistance (MDR) pump in some Gram-negative bacteria suggests that they may be resistant to a number of antibiotics (Girish and Smith 2008). This could be, overcome since both rhamnolipids and LP act on cell surfaces only. LP biosurfactant antimicrobial properties are associated with their lytic membrane properties. Basit *et al.* (2018) revealed that cationic lipopeptides exhibited significant antibacterial and antifungal activity against *S. aureus, E. coli, P. aeruginosa, K. pneumonia, A. niger* and *C albicans*. In addition, they showed antiviral activity against Newcastle disease virus (NVD). In susceptibility testing the largest zones of inhibition were, found against *S. aureus* and the smallest against *Aspergillus flavus*. These results were in accordance with previously reported antibacterial, antifungal and antiviral activity of biosurfactants (Gomaa 2013; Jemil *et al.* 2017; Borsanyiova *et al.* 2016).

Diaz de Rienzo and co-workers (2016a) showed that pre-formed biofilms of *P aeruginosa* PA01, *E coli* NCTC 10418, *B. subtilis* NCTC 10400 and *S. aureus* ATCC 9144 on glass coverslips were disrupted with SL's (5%) in the absence of an adjuvant i.e. caprylic acid. Domalson *et al.* (2018) in their investigation of short proline rich LP's revealed an amphiphilic non-haemolytic non-cytotoxic L-lipopeptide that significantly potentiated the activity of minocycline and rifampicin against multi-drug resistant MDR and XDR clinical isolates of *Pseudomonas aeruginosa*. Ghribi and Ellouze-Chaabouni (2011) isolated a biosurfactant producing strain *B. subtilis* SPB1 (HQ392822) and identified antimicrobial activity against microorganisms with multi-drug resistant profiles (Ghribi *et al.* 2012). Rossi *et al.* (2016) showed that some strains of biosurfactant producing *Staphylococcus haemolyticus* had antimicrobial activity against a range of Gram positive and Gram-negative bacteria and sub-inhibitory concentrations of the biosurfactant were able to decrease biofilm formation and showed synergistic effects with tetracycline.

The antimicrobial effects of SL's are dependent on the SL structure and class of bacteria examined. SL's have been shown to have virucidal and antibiotic adjuvant characteristics (Shah *et al.* 2005; Joshi-Navare and Prabhune 2013). A study using natural SL mixtures with a variety of sugar head groups reported antimicrobial activity against a range of predominately Gram-positive bacteria (Shah and Prabhune 2007). Equally important given the renewed focus on maternal sepsis both in the developed and developing world are biosurfactant studies carried out in rat models of peritonitis. Bluth *et al.* (2006) demonstrated that SL's block the lethal effects of septic shock in rats in a caecal ligation and puncture model of experimental sepsis and Hardin and co-workers (2007) showed that SL's derived from C. bombicola (now *Starmerella bombicola*) can improve sepsis survival. Di-rhamnolipid preparations have also been found to be successful in treating chronic decubitis ulcers (Piljac *et al.* 2008) and in the enhanced healing of full-thickness burn wounds (Stipcevic *et al.* 2006).

Inhibition of Biofilm formation

Some of the most promising candidates for the inhibition of biofilms have come from biosurfactants since they have strong anti-adhesive, anti-microbial and biofilm disruption properties (Banat *et al.* 2014a; Sharma *et al.* 2014). It has been proposed that biosurfactants play an important role in

organisms that produce them by partially disrupting the developing biofilm and maintaining channels for gas and nutrient diffusion and it is thus not surprising that they are effective in disrupting biofilms at appropriate concentrations. Researchers in this area point to the dispersal of a biofilm of pathogenic bacteria by decreasing bacterial cell viability and the reduction of bacterial adhesion properties as evidence of the effectiveness of biosurfactants. The suggested mechanism of action may be related to the binding of the biosurfactant molecules to cell wall components or the cell surface resulting in severe changes in outer membrane hydrophobicity. The insertion of biosurfactants into the bilayer structure of cell membrane may result in disruption of its integrity. The effects on both Gram-negative and Gram-positive bacteria may be due to the release of LPS molecules from the outer membrane or due to the formation of transmembrane pores resulting in increased permeability of the cell wall (Sotirova *et al.* 2008; Rivardo *et al.* 2009), (for further discussion of the various roles of biosurfactants see Satpute *et al.* 2016).

Previously, numerous studies have shown that biosurfactants inhibit biofilm formation by preventing adhesion of microorganisms to solid surfaces (Kuiper *et al.* 2004; Rodrigues *et al.* 2004; Rivardo *et al.* 2009; Janek *et al.* 2012). Mukherji and Phrunane (2014) reported anti-biofilm activity of SL against *Vibrio cholerae*, indicating that ~~the biofilm inhibitory activity of SL~~ it is likely to be broad-spectrum. The morphological changes to microbial cells as, a result of SL treatment (Haque *et al.* 2016) may go some way towards explaining the broad-spectrum nature of SL's and other biosurfactants (Haque *et al.* 2016). These changes could be associated with loss of cell membrane integrity resulting in cell death as reported previously for tetracycline-SL or cefaclor-SL combination treatment against *S. aureus* and *E. coli* respectively (Joshi-Navare and Prabhune, 2013). Furthermore, deformation of cells and loss of cell membrane integrity have been reported as the mechanisms of antimicrobial activity of many biosurfactants (Gudñia *et al.* 2013).

Importantly, Rhamnolipids have been, shown to be active against pre-existing bacterial biofilms of *S. typhimurium* (Leis *et al.* 2005). *Salmonella* remains an important cause of food-poisoning infections and has recently seen a resurgence in the EU primarily as, a result of zoonotic infections (EFSA and ECDC 2017). *Salmonella* causes gastroenteritis and in some cases septicaemia (Wang *et al.* 2013a).

Salmonella enterica is able to grow on stainless steel surfaces, resulting, in a 3D structure with several layers of cells, which may present different morphologies depending on the available nutrients (Wang *et al.* 2013b). Untreated steel is more easily colonised by *Salmonella* than polished or finished steel (Schlisselberg and Yaron 2013). In dry conditions, *S. enterica* has, been shown to survive in a biofilm on stainless steel for over a year (Morita *et al.* 2011). However, in contrast to other pathogens glass surfaces are not as easily colonised by *Salmonella* (De Oliveira *et al.* 2014). Given the continued disease burden caused by *Salmonella* a number of workers have investigated the potential of various biosurfactants against *Salmonella* including SUR's produced by *B. subtilis*. SUR's have been, reported to inhibit the growth of biofilms of *Salmonella* spp cultivated on PVC microtiter plates and urethral catheters (Mireles *et al.* 2001).

Nano-particles

Nanoparticle- based therapeutics have been considered as some of the most promising platforms in drug delivery applications due to their ability to increase drug accumulation in solid tumours by enhanced permeability and retention (EPR) and MDR reversal through bypassing or inhibiting P-gp activity (Bao *et al.* 2016). Furthermore, Basak *et al.* (2014) reported that SL capped ZnO nanoparticles mediated *C. albicans* cell death occurs via membrane bursting followed by oozing out of proteins and intracellular materials. In addition to functioning as a cyclic lipopetide the biosurfactant, SUR has, been found to exhibit versatile bioactive features including adjuvant for immunisation and anti-tumour properties. Based on its unique amphipathic properties SUR has the potential for self-assemble (under certain conditions) into nanoparticles to function as a drug carrier for loading hydrophobic drugs. Combining the anticancer activity of SUR and the characteristics of nanoparticles such as EPR effects and MDR reversal, might improve cancer chemotherapy by designing SUR as a carrier to load anticancer drugs. In an investigation by Huang and co-workers (2018), SUR was assembled by a solvent-emulsion method to load the anticancer drug doxorubicin (DOX). The DOX@SUR assembly was shown to induce stronger cytotoxicity against DOX-resistant human breast cancer MCF-7/ADR cells compared to free DOX. The DOX@SUR nanoparticles

exhibited enhanced cellular uptake and decreased cellular efflux. Moreover, *in vivo* DOX@SUR nanoparticles accumulated more efficiently in tumours than free DOX. The DOX@SUR showed stronger, tumour inhibition activity and fewer side effects in MCF-7/ADR-bearing nude mice suggesting that SUR-based nanoparticles might be used as potential anticancer drug carriers to reverse MDR in cancer chemotherapy.

Current trends and applications

Applications in agriculture

Biosurfactants are integral components of many commercial products in a variety of agricultural applications, for both plant and farm animal production systems. Furthermore, biosurfactants, due to their low organismal and environmental impact, (low toxicity, low irritation response/hypo-allergenicity) while exhibiting high digestibility as well as high biodegradability appear to offer excellent advantages over their synthetic and other natural counterparts.

In farm animal production, nutritional/dietary manipulation is one of the main directions of biosurfactant applications. Natural biosurfactants, such as plant derived alkyl polyglucosides (APG) have been, shown to be effective in ruminant nutrition, due to their positive effects on physiological and production parameters in e.g. ruminants. Both ruminal and intestinal digestibility of organic matter are, increased together with ruminal microbial protein synthesis resulting in increased duodenal microbial flow of nitrogen (Yuan *et al.* 2010). Additionally, APG may have positive indirect effects in terms of its ability to modify the rumen microbial community as it increases total volatile fatty acid production in the rumen *in vivo*. APG has the ability to increase the activities of ruminal carboxymethyl cellulase and xylanase (Yuan *et al.* 2010), together with its ability to modify ruminal fatty acids composition and decrease the population of *Ruminococcus albus in vivo* (Zeng *et al.* 2012) hence providing a favorable ruminal environment. Available research would indicate that microbial biosurfactants may have similar effects to those ascribed to APG in ruminant nutrition, e.g. rhamnolipid (produced by *Pseudomonas aeruginosa*) has shown increased activity of xylanase, and

overall increased degradation rates of organic matter *in vitro* (Liu *et al.* 2011). Past research has also acknowledged that incorporation of yeast cultures with emulsified glyco-protein into ruminant diets can improve the digestibility of organic matter, including digestibility of cellulose and hemicellulose (Wiedmeier *et al.* 1987) and more recent work (Feye *et al.* 2016) suggests that *Saccharomyces cerevisiae* fermentation products may mitigate faecal shedding of antibiotic resistant *Salmonella* in poultry (fed Original XPC™). Any development that can reduce the potential for the spread of antibiotic resistance in the agrarian environment (Conwell *et al.* 2017) is to be welcomed. Aside from improving the activity of fibrolytic enzymes in ruminant nutrition, microbial biosurfactants with their emulsifying properties have been suggested for improved digestibility of fats/oils in animal diets. Fats/oils are normally, added to animal diets as an inexpensive source of energy however, their use is limited by the animal's physiological ability to digest high levels of dietary fats/oils. Thus, more recent livestock and poultry feed additives consisting of lysophospholipids, of undisclosed origin have appeared on the market claiming enhanced effects on emulsification of nutritional fats/oils and hence improved digestion of fats/oils and improved absorption of other nutrients (for more information see: Lysoforte®, Kemin Industries, Inc., USA). It is possible that specific microbial biosurfactants could be, introduced to emulsify fats/oils in animal feed for specific age groups of animals or to decrease the cost of feed by increasing the oil/fat content above the level of animal/physiological ability to effectively digest without the negative effects on animal health. Hence, the inclusion of biosurfactants may prove to be financially effective in animal production. Other avenues for further exploration, may involve designer microbial biosurfactants that would aim to modify the ruminal microbiome and favour a bacterial "ruminotype" associated with low methane production over those with high methane outputs e.g. species belonging to *Ruminococcus* (Kittelmann *et al.* 2014).

More recently the potential of biosurfactants in seed protection and growth stimulation have been investigated, showing the effectiveness of LP's (Toral *et al.* 2018) against phytopathogens including *Botrytis cinerea* and that of rhamnolipids (Borha *et al.* 2016) against *Fusarium verticillioides* a major pathogen of maize. In addition, rhamnolipids have shown potential as biopesticides (Soltani Dashtbozorg *et al.* 2016), fungicides (Sha *et al.* 2015) and as anti-zoospore agents (Miao *et al.* 2015).

Sha *et al.* (2012) attributed the antifungal effect of cell-free culture broth of rhamnolipids to surface activity and rupture of plasma membranes.

Health related applications

Applications in Wound healing

A wide variety, of bioactive metabolites, including biosurfactants are, viewed as having potential for dermatological applications including wound healing. Zouari *et al.* (2016b) evaluated the *in vitro* antioxidant activities and the wound healing potential of *Bacillus subtilis* SPB1 LP on excision wounds induced in experimental rats. They found a significant increase in the percentage of wound closure compared with untreated and CICAFLORA™ treated groups. Biopsies treated with SPB1 LP's showed entirely re-epithelised wounds with perfect epidermal regeneration. It has been, suggested that the free-radical scavenging properties of the LP's help to prevent inflammation and improve tissue formation, re-epithelisation and differentiation of epidermis (Jemil *et al.* 2017). In addition, SPB1 has been shown previously to inhibit multidrug resistant bacteria (Ghribi *et al.* 2012) and show activity against phytopathogenic fungi (Minif *et al.* 2016). Gupta *et al.* (2017) investigated accelerated wound healing in rat tissue *in vivo* using a glycolipid produced by *B. licheniformis* SV1 containing ointment and found re-epithelisation and fibroblast cell proliferation in the early stage of wound healing with more rapid collagen deposition in the later stages. It has been suggested that the wound healing properties exhibited by those LP's investigated may be as a result of their ability to reduce oxidative stress through the prevention of reactive oxygen species (ROS) production. Ohadi *et al.* 2017 in their study of wound healing in rats showed that the LP produced by *Acinetobacter junii* B6 increased free-radical scavenging activities and improved histopathological remission. Lydon and co-workers (2017) tested a highly purified preparation of micelle-forming non-acetylated acidic SL that contained 90% C18 congener suggesting that acidic sophorolipids can be used as a component of antimicrobial creams to reduce the risk of wound infection during healing.

Dermatological applications

393 The anti-bacterial preservatives used in the majority of personal care products are synthetic and can
394 cause skin irritation and allergic reactions by interaction with keratin or collagen and elastin and
395 encourage the removal of lipids from the skin surface and affect the skin cells themselves (Bujak,
396 2015). On the other hand, biosurfactants are composed of lipid and proteins and are compatible with
397 the skin cell membrane (Stipcevic *et al.* 2013). While the majority of biosurfactant related work is
398 focussed on biosurfactants that are produced extracellularly by microorganisms much less work has
399 been carried out on cell-bound biosurfactants many of which are produced by e.g. probiotic
400 Lactobacilli strains which have the added advantage of being non-toxic, biodegradable and
401 environmentally friendly (Satpute *et al.* 2016). Vecino *et al.* (2018) investigated the anti-microbial
402 and anti-adhesive properties of cell-bound biosurfactants, produced by *Lactobacillus pentosus* (PEB),
403 which are characterised as glycolipid molecules, against several microorganisms found amongst
404 human skin flora. The performance of PEB was compared against the glycolipids produced by
405 *Lactobacillus paracasei* (PAB). The PEB showed anti-microbial activity against *P. aeruginosa,*
406 *Streptococcus agalactiae, S. aureus, E. coli, Streptococcus pyogenes* and *C. albicans*, which was
407 comparable with the results from PAB. Importantly, extracts prepared with phosphate buffered saline
408 (PBS) were more effective than phosphate buffer (PB) in the case of *P. aeruginosa, S. aureus* and *E.*
409 *coli*. Those extracted in PBS had a higher lipid content while those extracted in PB had a higher
410 carbohydrate content. Both PEB and PAB showed anti-adhesive properties against all the
411 microorganisms tested except for *E. coli* and *C. albicans*. PAB produced biosurfactants with a lower
412 content of lipids than those produced by PEB. However, Sharma and Saharan (2016) investigated the
413 antimicrobial of glycolipid from *Lactobacillus helveticus* and found higher anti-microbial activity
414 against *E. coli* and *S. epidermidis*. On the other hand, Gudina and co-workers (2015) working with
415 *Lactobacillus agilis* found no anti-microbial activity against *E. coli* or *C. albicans*. Ashby and co-
416 workers (2011) investigated the potential of biopolymer embedded SL's to improve the antimicrobial
417 potential of SL's against *Propionibacterium acnes* and found the efficacy varied depending on the
418 biopolymer matrix. Interestingly, when different carbon sources and different fermenting conditions
419 are applied then the same strain can produce different biosurfactants with different anti-microbial
420 properties (Singh *et al.* 2014).

In nature *P. aeruginosa* releases rhamnolipids to form vesicles or micelles and sheds flagellin. Meyer-Hoffert and co-workers (2011) demonstrated that rhamnolipid secretion facilitates the expression of antimicrobial protein psoriasis in human healthy skin via flagellin. Flagellin will activate keratinocytes to induce the expression of the antimicrobial protein psoriasin, which can kill *P. aeruginosa*. Therefore, healthy skin can prevent colonisation of pathogens before pathogens can develop strategies to disrupt the immune defence response. Antimicrobial hydrogels incorporating biosurfactants (Paniagua-Michel *et al.* 2014) have been studied as an auto-defense mechanism for combating drug resistant infections associated with the skin, because polymeric gels exhibit many properties avoiding the freely dissolved condition, which enable them to remain in place, on the skin, while maintaining antimicrobial activity (Li *et al.* 2013). These characteristics suggest potential for wound healing, implant/catheter coatings and skin infections.

Oral care

In the natural environment, biosurfactants have, been found to contribute to innate oral care. Biosurfactant producers such as *Steptococcus mitis* in the oral cavity can discourage the adhesion of *S. mutans*. In their study of the effectiveness of rhamnolipids derived from non-pathogenic *Burholderia thailandensis* E264, Elshikh and co-workers (2017) identified a 3-4 log decrease in bacterial viability amongst oral pathogens (The potential of biosurfactants in oral cavity care has been reviewed in detail by Elshikh *et al.* 2016). Bouassida and co-workers (2017) examined the potential of *Bacillus subtilis* SPB1 lipopeptide in toothpaste formulation and showed that lipopeptide-based product exhibited an important antimicrobial activity against *Enterobacter* sp and *Salmonella typhimurium*. Previous reports on the effectiveness of *Bacillus subtilis* SPB1 strain (HQ392822) revealed a wide spectrum of actions including antimicrobial activity towards microorganisms with multidrug resistant profiles (Ghribi *et al.* 2012) antifungal activity against phytopathogenic fungi (Mnif and Ghribi 2016) and antidiabetic and anti-lipidemic properties in alloxan-induced diabetic rats (Zouari *et al.* 2016a).

Drug delivery systems, including vaccines

446 The use of biosurfactants as drug delivery agents offers attractive applications such as passive
447 immunisation particularly where drug treatment options are limited. For instance, the treatment of
448 candidiasis is difficult due to the limited availability of antifungal drugs and their toxicities and severe
449 side effects in humans (Laniado-Laborin and Cabrales-Vargas 2009; Nett, 2014). These issues can be,
450 overcome by incorporating anti-fungal drugs into various drug delivery systems (Schinabeck *et al.*
451 2004; Ramage *et al.* 2013). Vesicular drug delivery systems including liposomes and noisomes are
452 thought to be particularly important for targeted delivery of drugs and to minimise undesirable side
453 effects (Jain *et al.* 2014).

454 Liposomes stand as promising candidates with wide applicability based on a drug delivery approach
455 including vaccination (Loew *et al.* 2011, Davitt and Lavelle 2015). Mannosylerythritol lipid-A, a type
456 of glycolipid biosurfactant that contains cationic liposomes has been shown to promote gene
457 transfection efficiency by five to seven times with mammalian cultured cells (Inoh *et al.* 2001).
458 Liposomes are made up of two hydrophobic tails and may or may not contain cholesterol in the
459 structure whereas noisomes are non-ionic surfactant based vesicles made up of single hydrophobic
460 chain, which makes them eminently suitable as carrier molecules in drug delivery applications (Kazi
461 *et al.* 2010; Khan and Irchhaiya 2016). Noisomes are constructed by hydration with or without the
462 amalgamation of cholesterol or other lipids (Kazi *et al.* 2010). The hydrophilic core of the noisome
463 provides an ideal environment for hydrophilic drugs since hydrophobic drugs are mainly localised to
464 the hydrophobic regions i.e. the lipid layer. Haque *et al.* 2017 compared the efficiency of SL-
465 Amphotericin B (AmB) niosome with a commercially available formulation of AmB and found fewer
466 fungal hyphae in biofilm treated with the SL-Amb noisome whereas more budding cells were found in
467 biofilm treated with Phosome (Amphotericin B) alone. Fungal pseudohyphae/true hyphae are thought
468 to be one of the most important virulence factors in *C. albicans* (Mayer *et al.* 2013). It is suggested
469 that SL-AmB niosomes may interfere with gene expression, downregulating expression of hyphal
470 genes. This is, supported by other work indicating that antifungal drugs inhibit such genes (Cheng *et*
471 *al.* 2009; Vediyappan *et al.* 2010).

Lipopeptide biosurfactants have also been shown to enhance the humoral immune response additionally they are non-toxic and non-pyrogenic making them prospective adjuvants in vaccines. The WHI fungin has, been shown to produce the SUR lipopetide, which has been suggested as a potential adjuvant for immunization through the oral route (Gao *et al.* 2013). Additionally Mittenbuhler and co-workers (2003) have suggested that LP's increased the humoral immunity to the tetanus toxoid, without a decrease in serum IgG levels in a mouse model. Work by Basit *et al.* (2018) in an investigation of LP's as adjuvant in inactivated low pathogenicity avian influenza H9N2 vaccine suggest that biosurfactant based vaccine increased the titre of antibodies in both broiler and layer chickens and showed comparable immunogenicity to oil based vaccine.

Anticancer potential of biosurfactants

The LP's, glycolipids and other types of biosurfactants owing to their structural novelty and diverse biophysical properties have emerged as possible broad-spectrum agents for cancer chemotherapy/biotherapy and as safe vehicles or ingredients in drug delivery formulations. However, while it is possible to show cancer cell killing activity *in vitro* the *in vivo* evidence is limited, and in many cases contradictory suggesting that in the short-term biosurfactants have limited clinical use except for topical or gut application. However, some studies have shown that lipopeptides and glycolipids can selectively inhibit the proliferation of cancer cells and disrupt cell membranes causing their lysis through apoptosis pathways (Gudina *et al.* 2013). Furthermore, the evidence from the literature suggests that the anti-cancer effects are based mostly on mixtures of congeners. There is a need to separate out these congeners in order to fully elucidate their individual anticancer effects.

The LP's and SL's are the biosurfactants most studied in terms of anti-cancer potential. The LP's are composed of a peptide and a fatty acid chain and have been shown to exhibit anti-tumour activity *in vitro* (Zhao *et al.* 2018). Reports on the *Bacillus* LP's namely, SUR, Iturin and Fengycin suggest that they possess anti-tumour activities. Iturin has been shown to inhibit the proliferation of MDa-MB-231 cancer cells (Dey et al 2015). Fengycin can block non-small cell lung cancer cell 95D and inhibit the growth of xenografted 95D cells in nude mice (Yin et al 2013). Recently, Zhao *et al.* (2018) showed the *B. subtilis* LP's consisting of a majority of iturin exhibited promising potential in inhibiting

chronic myelogenous leukaemia *in vitro* via simultaneously causing paraptosis, apoptosis, and inhibition of autophagy. The anticancer mechanisms of Bacillus LP's have been extensively studied and SUR has been found to display an anti-proliferative effect via apoptosis induction, cell cycle arrest and survival signalling suppression.

Amongst the suggested uses of SL's are their potential in human cervical cancer treatment. Li *et al.* (2017) showed induction of apoptosis of HeLa cells and inhibition of cancer cells in tumour bearing mice but the vast majority of studies have been conducted *in vitro* (Table 1). However, the more recent studies have included xerograph and *in vivo* studies. In therapeutic and preventative xerograph models of B16-EGRFRvIII melanoma cells the self-adjuvant LP vaccine micelles effectively prevented tumour growth as well as tumorigenesis (Chen *et al.* 2018). Different anticancer mechanism for SL's have, been proposed including a role in differentiation and apoptosis. While it is well accepted that SLs have anticancer activity *in vitro,* Li *et al.* (2017) is one of the few studies to suggest anti-tumour activity *in vivo*. Moreover, there are conflicting reports in the literature including Callaghan *et al.* (2016) suggesting that lactonic SL's may increase tumour burden in Apc min+/- mice.

Future trends and conclusions

The two main obstacles to the further development of biosurfactant applications and unlocking their potential remain the large numbers of assays and approaches to this type of work. Microbial biosurfactants are produced as mixtures of congeners and the proportions of congeners will vary based on producer strain, growth conditions and growth medium (Singh *et al.* 2014, Diaz de Rienzo *et al.* 2016a). Since different congeners have different properties and activities the use of 'mixtures' in experiments leads to confusing results. There is also the problem of endotoxin contamination of biosurfactants produced by Gram negative bacteria and very few investigators have taken steps to ensure that their experimental material is free of such highly bioactive molecules. Although expensive and time consuming bioactivity needs to be determined with pure single congeners. The different

assays currently employed may be providing different kinds of information on the mode of action of biosurfactants and the mechanism of action of biosurfactant either singly or in combination with other therapies against pathogenic microorganisms. There is a need for the standardisation of approaches and methodologies associated with biosurfactant research (recently reviewed in detail by Irorere *et al.* 2017).

The evidence of the efficacy of different biosurfactants from different microorganism in differing contexts remains a challenge. There is good evidence of the effectiveness of biosurfactants in terms of antimicrobial activity and there is increasing evidence of the benefits of biosurfactants in terms of wound healing, dermatological applications and oral care (Elshikh *et al.* 2017). There is promising work in the area of drug delivery but in the area of cancer treatment where biosurfactants might prove most efficacious there remains much conflicting data. It has to be pointed out, however, that their anticancer applications are likely to be limited to situations where topical application is possible e.g. skin or oral or for gastrointestinal administration.

The target market is of fundamental importance to any scale of biosurfactant production. To date developments have been limited for industrial applications such as bioremediation due to the deficit in the investment required and the feasibility of viable industrial production (Banat *et al.* 2014b). Therefore, the potential applications discussed here in terms of healthcare therapeutics are much more promising given the value added nature of such products and their likely benefit to human health. The cost benefits would appear to be more favourable (Marchant and Banat 2012a) in terms of the biomedical applications because, production is viable on a small-scale. Of the range of potential applications discussed here, it is likely that the innate antimicrobial nature of many biosurfactants and the ability of some of these to act in synergy and/or as adjuncts to current therapeutics in the context of the ever increasing threat of antibiotic resistance that may prove the most beneficial.

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Conflict of Interest

The authors declare no conflict of interest

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Table 1. Effects of Biosurfactants on various cancer cell lines

Biosurfactants class	Biosurfactant name	Source	Reference	Effect on Cell Line
Lipopeptide	Surfacin	*Bacillus subtilis*	Kim *et al.* 2007	Suppression of LoVo (colon carcinoma) cell line
Lipopeptide	Surfacin	*Bacillus natto TK-1*	Cao *et al.* 2010	Killing of MCF-7 (human breast cancer) cell line
Lipopeptide	Iturin	*Bacillus subtilis*	Zhao *et al.* 2018	Inhibition of K562 leukemia cells
Glycolipid	Mannosylerythritol lipid -A Mannosylerythritol lipid -B	*Candida Antarctica T-34*	Isoda *et al.* 1997	Induced HL60 (leukemia cell line) differentiation
Sophorolipid	Sophorolipid	*Candida bombicola* ATCC 22214	Joshi-Navaere *et al.* 2011	Increased in LN-229 differentiation
Sophorolipid	di-acetylated lactonic C18:1	*Wickerhamiella domercqiae*	Chen *et al*. 2006	Apoptosis in H7402 (liver cancer) cells
Sophorolipid	cetyl alcohol sophorolipid	*Candida bombicola* ATCC 22214	Nawale *et al.* 2017	Anti-proliferation of HeLa cells
Sophorolipid	Various derivatives	*Candida bombicola* ATCC 22214	Fu *et al.* 2008	Killing of human pancreatic cancer cells
Sophorolipid	Various derivatives	*Wickerhamiella domercqiae*	Shao *et al.* 2012	Inhibition of oesophageal cancer cells
Sophorolipid	Various derivatives	*Starmerella bombicola*	Ribeiro *et al.* 2015	Killing of MDA-MB-231 breast cancer cells