UNIVERSIDADE FEDERAL DE SANTA CATARINA
CENTRO TECNOLÓGICO
PROGRAMA DE PÓS-GRADUAÇÃO EM ENGENHARIA DE ALIMENTOS

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Antimicrobial properties of the biosurfactant mannosylerythritol lipids-B against *Cutibacterium acnes* ATCC 6919 and *Listeria monocytogenes* CLIST 4165

Florianópolis-SC
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Tese submetida ao Programa de Pós Graduação em Engenharia de Alimentos da Universidade Federal de Santa Catarina como requisite parcial para obtenção do título de doutor em Engenharia de Alimentos.

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Florianópolis-SC

2023

Ficha de identificação da obra elaborada pelo autor,
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Coelho, Ana Letícia Silva
Antimicrobial properties of the biosurfactant mannosylerythritol lipids-B against Cutibacterium acnes ATCC 6919 and Listeria monocytogenes CLIST 4165 / Ana Letícia Silva Coelho ; orientador, Débora de Oliveira, coorientador, Bruno Augusto Mattar Carciofi, coorientador, Cristiano José de Andrade, 2023.
103 p.

Tese (doutorado) - Universidade Federal de Santa Catarina, Centro Tecnológico, Programa de Pós-Graduação em Engenharia de Alimentos, Florianópolis, 2023.

Inclui referências.

1. Engenharia de Alimentos. 2. acne vulgaris. 3. microbiologia preditiva. 4. antimicrobiano. 5. segurança de alimentos. I. de Oliveira, Débora. II. Carciofi, Bruno Augusto Mattar. III. de Andrade, Cristiano José IV. Universidade Federal de Santa Catarina. Programa de Pós Graduação em Engenharia de Alimentos. V. Título.

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O presente trabalho em nível de Doutorado foi avaliado e aprovado em 21 de agosto de 2023, pela banca examinadora composta pelos seguintes membros:

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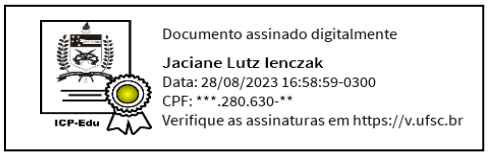

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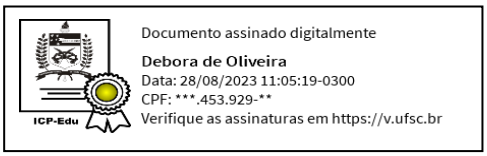

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Florianópolis, 2023

I dedicate this work to my parents and siblings.

ACKNOWLEDGMENTS

I express my most sincere thanks to my advisors, Prof. Dr. Débora de Oliveira, Prof. Dr. Bruno AM Carciofi, and Prof. Dr. Cristiano J. de Andrade for the opportunity and guidance.

Thanks to Dr. Denise A. Laroque for her teachings, enormous patience, motivation, and dedication.

I thank Ana G. Massia for her help with the mathematical modeling of data.

I am very grateful to Prof. Dr. Patrícia Poletto for her guidance during the teaching internship and her dedication and precious input for my training.

I am very grateful to my family, who always give me full support and help during my Ph.D. study.

I thank Solange C. Carreiro and Onélia A. Andreo for their encouragement and support and for believing in me when I didn't believe in myself. Thank you very much!

I thank Prof. Dr. Gislaine Fongaro for making the Applied Virology Laboratory available.

Thanks to Doris Sobral M. Souza and Raphael D. Cadamuro for accompanying the analysis with Nanovue.

Thanks to my colleagues in the pathogens' laboratory, Fernando, Dani, Sandra, Ana, Denise, and Abadio for the company and technical support.

Thanks to the PPGEAL secretariat, especially Fernando Curbani, for his availability.

Finally, I would like to thank everyone who directly or indirectly contributed to this work.

I thank CAPES for the financial support.

RESUMO

Manosileritritol lipídio-B (MEL-B) é um glicolipidio/biosurfactante natural produzido principalmente pela levedura *Pseudozyma tsukubaensis*. Esse composto apresenta várias propriedades interessantes, pode-se salientar: emulsionante, antimicrobiana, ação hidratante e anti-idade. Assim, em vista das propriedades deste composto para cuidados com a pele, investigou-se o efeito do MEL-B sobre o crescimento da *Cutibacterium acnes* ATCC 6919, o agente etiológico da acne vulgaris. A susceptibilidade da cepa ao glicolipidio foi avaliada pela técnica de macrodiluição, nas concentrações 128, 192, 256 e 512 µg/mL, incubação por 8 e 16 horas a 37 °C. Mensurou-se o dano de membrana do MEL-B sobre a cepa pela quantificação de material genético extravasado do meio intracelular, empregando-se espectrofotometria (260 nm). Os resultados indicaram que o MEL-B estimulou o crescimento da *C. acnes* em 1 a 3 log UFC/mL comparativamente às amostras sem MEL-B. Ademais, o biosurfactante não ocasionou dano de membrana sobre a linhagem. Assim, os dados sugerem a inadequação do MEL-B em produtos destinados ao cuidado da pele acneica. Outra abordagem desse estudo foi investigar o efeito do MEL-B sobre *Listeria monocytogenes* CLIST 4165 cultivada em salsicha hot dog. Os ensaios de atividade antimicrobiana foram primeiramente realizados em caldo de soja trypticase (TSB), pelo método de macrodiluição, sob agitação (210 rpm), por 3 e 6 horas, a 37 °C, e com diferentes concentrações de MEL-B (4-10000 µg/mL). Em seguida, ensaios similares foram conduzidos com salsicha hot dog, a 30 °C, com incubação por 20, 34 e 48 horas, e concentração do glicolipidio de 0,01, 0,1, 1, 10, 100, 500 e 1000 µg/mL. Os parâmetros cinéticos da *L. monocytogenes* em salsicha hot-dog foram obtidos pelo ajuste do modelo de Baranyi e Roberts aos dados de crescimento da cepa na presença ou não de MEL-B (0,01 e 500 µg/mL) sob 15 e 30 °C. Os ensaios em TSB evidenciaram uma alta susceptibilidade da cepa ao MEL-B, com concentração mínima inibitória (CMI) de 4 de µg/mL e concentração bactericida mínima (CBM) de 1000 µg/mL. Os ensaios de atividade antimicrobiana com salsicha hot dog mostraram que após 20 horas de incubação houve diminuição na contagem bacteriana (0,5 a 1,5 UFC/mL) para amostras tratadas com MEL-B em relação àquelas sem o glicolipidio. Após 48 horas, a redução na contagem bacteriana foi de aproximadamente 2 log UFC/mL para as amostras com MEL-B comparativamente aquelas sem o composto. Observou-se que para as condições 0,01 µg/mL/15°C, 500 µg/mL/15 °C e 0,01 µg/mL/30 °C, houve uma diminuição nos parâmetros cinéticos, fase lag e velocidade específica máxima de crescimento. Já a condição de 500 µg/mL/30 °C, estimulou o desenvolvimento da cepa reduzindo a fase lag e aumentando a velocidade específica máxima de crescimento. Conclui-se assim, que o MEL-B é estimulante e antimicrobiano para a *C. acnes* e *L. monocytogenes,* respectivamente. A atividade antimicrobiana do MEL-B sobre a *L. monocytogenes* cultivada em salsicha hot dog abre possibilidades para estudos futuros sobre o potencial conservante e emulsificante do glicolipidio na indústria de alimentos, seja isolado ou em conjunto com outros compostos com propriedades similares.

Palavras-chave: acne vulgaris; antimicrobiano; microbiologia preditiva; microbioma da pele, segurança de alimentos.

RESUMO EXPANDIDO

Introdução

Manosileritritol lipídios (MEL) são glicolípidos de natureza microbiana, que têm atraído particular interesse nas áreas cosmética, médica e farmacêutica. A literatura reporta quatro homólogos tradicionais de MEL, os quais apresentam uma estrutura básica semelhante, diferindo em relação à presença/ausência de grupos acetil nos carbonos C4 e C6: MEL-A é diacetilado nas posições C4 e C6, MEL-B e MEL-C são monoacetilados em C6 ou C4, respectivamente, enquanto MEL-D não tem grupo acetil. A atividade antimicrobiana desses compostos foi comprovada para *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* e *Enterococcus* resistente à vancomicina. Dentre as propriedades biológicas do MEL-B, destacam-se seu potencial como antimicrobiano, hidratante, anti-idade e antitumoral. No campo de *skincare* já foi demonstrado que o MEL-B possui propriedade hidratante similar à da ceramida (um composto de referência na área). Ademais, esse glicolipídio é capaz de reverter o processo de foto-envelhecimento em fibroblastos danificados por radiação UV-A, além de promover o clareamento da pele. Dados da literatura relatam que mudanças adversas na microbiota da pele podem desencadear processos inflamatórios, podendo culminar em doenças como a acne vulgaris, ocasionada pela *Cutibacterium acnes*. Nesse sentido, as propriedades mencionadas acima levantam questões sobre uma possível aplicação do MEL-B em produtos de cuidados com a pele destinado à pele acneica, condição na qual prevalece o desenvolvimento da bactéria patogênica *C. acnes*. No que concerne a cepas de matrizes alimentares, a atividade antimicrobiana do MEL-A (um homológo do MEL-B) já foi evidenciada em células vegetativas e esporos de *Bacillus cereus* e *L. monocytogenes.* Contudo, a atividade antimicrobiana do MEL-B em cepas bacterianas no setor alimentício ainda é pouco explorada. Assim, o presente estudo também visa avaliar os efeitos da MEL-B sobre *L. monocytogenes* CLIST 4165 cultivada em salsicha tipo hot dog (estilo frankfurter). A *Listeria monocytogenes* é uma bactéria gram-positiva, não formadora de esporos e agente causador da listeriose, uma Doença Transmitida por Alimentos (DTA). A ocorrência dessa patologia é devida principalmente à ingestão de alimentos prontos para o consumo contaminados com a bactéria. A atividade antimicrobiana do MEL-B em produtos cárneos foi explorada pela primeira vez neste estudo. Investigou-se como diferentes magnitudes do glicolipídio afetam a *L. monocytogenes* cultivada em salsicha tipo hot dog sob temperaturas abusivas de estocagem.

Objetivos

Esse trabalho tem como objetivo geral avaliar o desempenho do MEL-B como agente antimicrobiano sobre as bactérias Gram-positivas *Cutibacterium acnes* ATCC 6919 e *Listeria monocytogenes* CLIST 4165.

Em relação ao estudo experimental com *C. acnes* foram estabelecidos os seguintes objetivos específicos: (i) avaliar os efeitos da concentração de MEL-B e do tempo de incubação sobre a *C. acnes*; (ii) verificar se o MEL-B é capaz de promover a lise celular da *C. acnes*.

Para os ensaios com *L. monocytogenes* foram definidos como objetivos específicos: (i) avaliar as propriedades antimicrobianas do MEL-B sobre *L. monocytogenes* cultivada em meio de cultura; (ii) investigar a susceptibilidade da *L. monocytogenes* cultivada em salsicha hot dog à diferentes concentrações de MEL-B e temperaturas de incubação; (iii) ajustar um modelo matemático preditivo aos dados de

crescimento de *L. monocytogenes* em salsichas hot dog; (iv) determinar os parâmetros cinéticos de crescimento de *L. monocytogenes* em salsicha hot dog com diferentes concentrações de MEL-B (0,01 e 500 µg/mL) sob 15 e 30 °C.

Metodologia

Os ensaios de atividade antimicrobiana com *C. acnes* foram realizados empregando o método de macrodiluição em caldo, conforme as normas do *Clinical and Laboratory Standards Institute* (CLSI). O inóculo foi preparado a partir de uma cultura de 24 h, a qual foi diluída em solução salina de cloreto de sódio (NaCl) 0,85%, e padronizado em 3-4 log UFC/mL. Em seguida, 100 µL do crescimento bacteriano foi adicionado a tubos contendo 5 mL de Meio Clostridial Reforçado (RCM) com diferentes concentrações de MEL-B (128, 192, 256 e 512 µg/mL). As amostras foram incubadas a 37 °C em anaerobiose por 8 e 16 h. Ao término do tempo de incubação as amostras foram centrifugadas (3.500 rpm/15 min), o sobrenadante foi descartado e a biomassa foi lavada duas vezes com solução salina (0,85% NaCl) para remover o MEL-B residual. Após essa etapa as células foram ressuspendidas em meio RCM, e 100 µL da suspensão foi coletada, diluída e plaqueada pela técnica de profundidade em meio RCM sólido. As placas foram incubadas a 37 °C por 48 h, em anaerobiose. As amostras contendo colônias entre 30 e 300 colônias foram usadas para estimar log UFC/mL. O extravasamento de ácidos nucleicos foi empregado para estimar o dano do MEL-B à membrana celular da cepa. Assim, amostras do cultivo bacteriano após 8 e 16 h foram coletadas, filtradas com membrana de 0,22 µm e empregadas para quantificar a liberação de DNA (fita dupla) e RNA no sobrenadante da cultura. O cultivo bacteriano sem a presença do MEL-B foi estabelecido como controle positivo, enquanto solução de tetraciclina 4 µg/mL foi empregada como controle negativo. A concentração de ácidos nucleicos foi determinada por medição de absorbância (260 nm) usando o espectrofotômetro NanoVue Plus. Os testes de atividade antimicrobiana com *L. monocytogenes* foram primeiramente realizados em caldo Triptona de Soja (TSB) contendo diferentes concentrações de MEL-B - 0, 4, 8, 16, 32, 64, 128, 256, 512, 1000, 2000, 4000, 8000 e 10000 µg/mL. Cem microlitros do inóculo (~ 7 log UFC/mL) foi adicionado aos tubos contendo diferentes concentrações de MEL-B, os mesmos foram incubados sob agitação (210 rpm) a 37 °C. Após 3 e 6 h, 100 µL da cultura de cada tubo foi diluída pela técnica de diluição seriada em água peptonada (0,1%), e então semeada em placas contendo meio Agar Triptona de Soja (TSA), que foram incubadas a 37 °C por 48 h para posterior estimação do número de Unidades Formadoras de Colônias (UFC). No que concerne aos ensaios com salsicha hot dog, foram realizados ensaios preliminares com o intuito de definir as concentrações do glicolipídio (0,01, 0,10, 1,00, 10,0, 100, 500 e 1.000 µg/mL) para os ensaios de cinética microbiana. Para tais experimentos 100 µL de solução de MEL-B foi adicionada à superfície de cada salsicha, seguindo-se homogeneização manual. Posteriormente, 100 µL do inóculo contendo em torno de 5 log UFC/mL de *L. monocytogenes* foi espalhado sobre as salsichas, que foram incubadas a 30 °C. Após 0, 20, 34 e 48 h de incubação, 50 mL de água peptonada (0,1%) foi adicionada às amostras que foram homogeneizadas. Em seguida, uma alíquota de 100 µL foi coletada, diluída pela técnica de diluição seriada em água peptonada (0,1%), e semeada em Agar *Listeria* Ottaviani *&* Agosti (ALOA). As placas de Petri foram incubadas a 37 °C por 48 horas. As colônias bacterianas obtidas nas placas foram contadas, e os resultados foram expressos em UFC/cm^2. A partir dos dados obtidos, duas concentrações de MEL-B foram selecionadas para os experimentos de cinética

microbiana, realizados a 15 e 30 °C. O modelo matemático de Baranyi e Roberts foi ajustado aos dados dos ensaios cinéticos para obtenção dos parâmetros de crescimento – fase lag, estado fisiológico das células, concentração celular máxima e velocidade máxima de crescimento.

Resultados e discussão

Os ensaios com *C. acnes* mostraram que o MEL-B potencializou o crescimento da cepa, alcançando um aumento de 2-3 log UFC/mL em relação às amostras sem o glicolipidio após 8 h (início da fase exponencial) ou 16 h (meio da fase exponencial) de incubação. Os ensaios de extravasamento de material genético evidenciaram que concentração de DNA e RNA para quase todas as condições de estudo excedeu ligeiramente os valores de controle, indicando que o MEL-B não propiciou um dano de mebrana relevante na linhagem. Os ensaios antimicrobianos com *L. monocytogenes* em meio TSB evidenciaram a atividade antimicrobiana do MEL-B. Após 6 h de incubação com concentração do glicolipidio de 4 µg/mL, foi observada uma redução de 4 log UFC/mL comparativamente ao cultivo sem MEL-B. Para as concentrações de 1000, 2000, 4000, 8000 e 10000 µg/mL não se observou o crescimento das colônias em placa. O decréscimo no crescimento da *L. monocytogenes* cultivada em salsicha hot dog variou de 0,5 a 1,5 log UFC/cm^2 quando a cepa foi incubada a 30 °C, por 20 horas, com diferentes concentrações de MEL-B (0,01, 0,10, 1,00, 10,0, 100, 500 e 1000 µg/mL). O crescimento microbiano das amostras incubadas por 34 horas com e sem MEL-B foi similar, em torno de 6,5 UFC/cm^2. As salsichas incubadas por 48 h sem MEL-B apresentaram contagem de aproximadamente 8 UFC/cm^2, enquanto para as amostras com MEL esse valor geralmente ficou em torno de 6 UFC/cm^2. Com base nos dados de inativação de *L. monocytogenes* em salsicha hot dog, foi realizado um estudo cinético para a cepa considerando duas temperaturas de incubação (15 e 30 °C) e duas concentrações de MEL-B (0,01 e 500 µg/mL). O tratamento com soluções MEL-B a 0,01 µg/mL e 500 µg/mL sob 15°C, resultou em uma redução final de aproximadamente 3 log UFC/cm^2 e 1 log UFC/cm^2, respectivamente, comparativamente às amostras sem MEL-B. Com relação aos ensaios a 30 °C, foi observada uma redução máxima de 2 log UFC/cm^2 para ambas as concentrações de MEL-B. Os valores de R^2 (> 0,97) e RMSE (< 0,193) indicam a qualidade do ajuste do modelo matemático preditivo aos dados. As condições experimentais 0,01 µg/mL/15°C, 0,01 µg/mL/30°C e 500 µg/mL/15°C diminuíram os valores dos parâmetros, estado fisiológico das células, fase lag, retardando também a concentração celular máxima e a velocidade específica máxima de crescimento, em comparação com amostras sem MEL-B. Já para a condição 500 µg/mL/30°C houve um aumento expressivo da velocidade específica máxima de crescimento da linhagem.

Considerações Finais

Apesar das conhecidas e amplas aplicações do MEL-B para o setor de cuidados com a pele, esse estudo demonstrou que o glicolídio é capaz de estimular o crescimento da *C. acnes* ATCC 699, um dos agentes etiológicos da acne vulgaris. Assim, supõe-se que o MEL-B seja inadequado para composição de produtos de *skincare* direcionados à pele com tendência a acne. Em relação à *L. monocytogenes*, os ensaios em meio de cultura demonstraram uma notável atividade antimicrobiana do MEL-B. Os valores de CMI e CBM foram 4 e 1000 µg/mL, respectivamente. A atuação do glicolipídio foi menos expressiva quando a *L. monocytogenes* foi cultivado em salsicha hot dog. De maneira geral, os dados

cinéticos mostraram que o tratamento com MEL-B (15 e 30 °C) diminuiu fase lag e a velocidade máxima de crescimento da linhagem em salsicha, em comparação com amostras sem MEL-B. A condição 500 µg/mL/30 °C foi considerada inapropriada para contenção do crescimento da *L. monocytogenes*, uma vez que potencializou a velocidade específica máxima de crescimento da linhagem.

Palavras-chave: acne vulgaris; antimicrobiano; microbiologia preditiva; microbioma da pele, segurança de alimentos.

ABSTRACT

Mannosylerythritol lipids-B (MEL-B) is a glycolipid/biosurfactant mainly produced by the yeast *Pseudozyma tsukubaensis*. This compound has attractive properties, such as emulsifier, antimicrobial, moisturizing, and anti-aging action. Thus, given the properties of this compound for the skincare field, the effect of MEL-B on the growth of *Cutibacterium acnes* ATCC 6919, the etiologic agent of acne vulgaris, was investigated. The activity of the glycolipid on the strain was evaluated by the macro dilution method at concentrations of 128, 192, 256, and 512 µg/mL, incubation of 8 and 16 hours at 37 °C. The membrane damage caused by MEL-B on the bacterium was determined by spectrophotometry (260 nm), quantifying the intracellular genetic material release in the culture medium. The results indicated that MEL-B stimulated *C. acnes* growth by 1 to 3 log CFU/mL compared to samples without MEL-B. Furthermore, the biosurfactant did not cause membrane damage to the strain. Thus, the data suggest the inadequacy of MEL-B in products intended for acne-prone skin care. Another approach of the present research was to investigate the effect of MEL-B on *Listeria monocytogenes* CLIST 4165 grown on hot dog sausage. The antimicrobial activity assays were first performed in trypticase soy broth (TSB), by the macro dilution method, under agitation (210 rpm), for 3 and 6 hours, at 37 °C, and with different concentrations of MEL-B (4 -10,000 µg/mL). Then, similar tests were conducted with hot dog sausage, at 30 °C, under incubation for 20, 34, and 48 hours, and glycolipid concentrations of 0.01, 0.10, 1.00, 10.0, 100, 500, and 1,000 µg /mL. The kinetic parameters of *L. monocytogenes* culture on the hot dog sausage were obtained by fitting the Baranyi and Roberts model to the strain growth data in the presence or absence of MEL-B (0.01 and 500 µg/mL) under 15 and 30 °C. Assays in TSB showed a high susceptibility of the strain to MEL-B, with a minimum inhibitory concentration (MIC) of 4 µg/mL and a minimum bactericidal concentration (MBC) of 1,000 µg/mL. The antimicrobial activity trials with hot dog sausage showed that after 20 hours of incubation, a decrease in the bacterial count (0.5 to 1.5 CFU/mL) was observed for samples treated with MEL-B concerning those without the glycolipid. After 48 hours, the reduction in bacterial count was approximately 2 log CFU/mL for samples with MEL-B compared to those without the compound. It was observed that for the conditions 0.01 µg/mL/15°C, 500 µg/mL/15 °C and 0.01 µg/mL/30 °C, there was a decrease in kinetic parameters, lag phase and specific velocity maximum growth of the lineage. The condition of 500 µg/mL/30 °C, on the other hand, stimulated the development of the strain by reducing the lag phase and increasing the maximum specific growth velocity. It is thus concluded that MEL-B is stimulant and antimicrobial for *C. acnes* and *L. monocytogenes*, respectively. The antimicrobial activity of MEL-B on *L. monocytogenes* grown in hot dog sausage opens possibilities for future studies on the preservative and emulsifying potential of the glycolipid in the food industry, alone or together with other compounds with similar properties.

Keywords: acne vulgaris; antimicrobial, food safety, predictive microbiology; skin microbiome.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND ACRONYMS

AG	Arabic Gum
ALOA	Agar *Listeria* Ottaviani & Agosti
AQP-3	Aquaporin-3
ATP	Adenosine triphosphate
B16	Murine Melanoma
CFU	Colony Forming Units
CLSI	Clinical and Laboratory Standards Institute
CLSM	Confocal Laser Scanning Microscopy
CMC	Critical Micellar Concentration
COX-2	Cyclooxygenase-2
DNA	Deoxyribonucleic Acid
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
DSC	Differential Scanning Calorimetry
EGFR	Epidermal Growth Factor Receptor
ER	Endoplasmic Reticulum
FFEM	Freeze-Fracture Electron Microscopy
FIOCRUZ	Fundação Oswaldo Cruz
GM3	Monosialodihexosylganglioside
GRAS	Generally Considered As Safe
H_2O_2	Hydrogen Peroxide
HepG2	Human Liver Cancer Cell Line
HL-60	Promyeoloblasts Cells
IC50	Half Maximal Inhibitory Concentration
IGFR	Insulin-Like Growth Factor Receptor
K562	Lymphoblast Cells
L_3	Sponge Phase
LLC	Lyotropic Liquid Crystal
L_α	Lamellar Phase
MBC	Minimum Bactericidal Concentration
MDR	Multidrug Resistance
MDR	Multidrug Resistance
MEL-A	Mannosylerythritol Lipids-A

MEL-B	Mannosylerythritol Lipids-B
MEL-C	Mannosylerythritol Lipids-C
MEL-D	Mannosylerythritol Lipids-D
MIC	Minimum Inhibitory Concentration
MSH	Murine Melanoma
NHMs	Primary Human Melanocytes
NIH-3T3	Fibroblast Cell Line
PC12	Rat Pheochromocytoma Cells
PDGFR	Platelet-Derived Growth Factor Receptor
PKC_{α}	Protein Kinase C Alpha
Q_{II}	Bicontinuous Cubic Phase
RCM	Reinforced *Clostridium* Medium
ROS	Reactive Oxygen Species
SAXS	Small-Angle X-ray Scattering
SDS	Dodecyl Sulfate
SEM	Scanning Electron Microscope
siRNA	Small Interfering Ribonucleic Acid
TEM	Transmission Electron Microscope
tRNA	Transfer Ribonucleic Acid
TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth
TSBYE	TSB supplemented with 1% yeast extract
Tyrp-1	Tyrosinase 1
Tyrp-2	Tyrosinase 2
UVA	Ultraviolet-A Rays

SUMMARY

CONCEPTUAL DIAGRAM

Antimicrobial properties of the biosurfactant mannosylerythritol lipids-B against *Cutibacterium acnes* ATCC 6919 and *Listeria monocytogenes* CLIST 4165

Why?

- MEL-B is a natural compound with antimicrobial, moisturizing, antiaging, and emulsifying activity.

What has already been done?

- The anti-aging and moisturizing properties of MEL-B were evidenced under *in vitro* and *in vivo* experiments, but its effects on the skin microbiome are still unknown;
- The antimicrobial effect of MELs was previously observed for vegetative cells and spores, mainly of Gram-positive bacteria;
- Previous studies showed that MEL-A has antimicrobial, preservative, and emulsifying effects in whole/skimmed milk and fresh/frozen bread.

Study hypothesis

- MEL-B has antimicrobial activity against *Cutibacterium acnes* ATCC 6919 and *Listeria monocytogenes* CLIST 4165;
- How the concentration of MEL-B, the incubation time, and temperature affect the antimicrobial performance of glycolipid on the strains.

Experimental methods

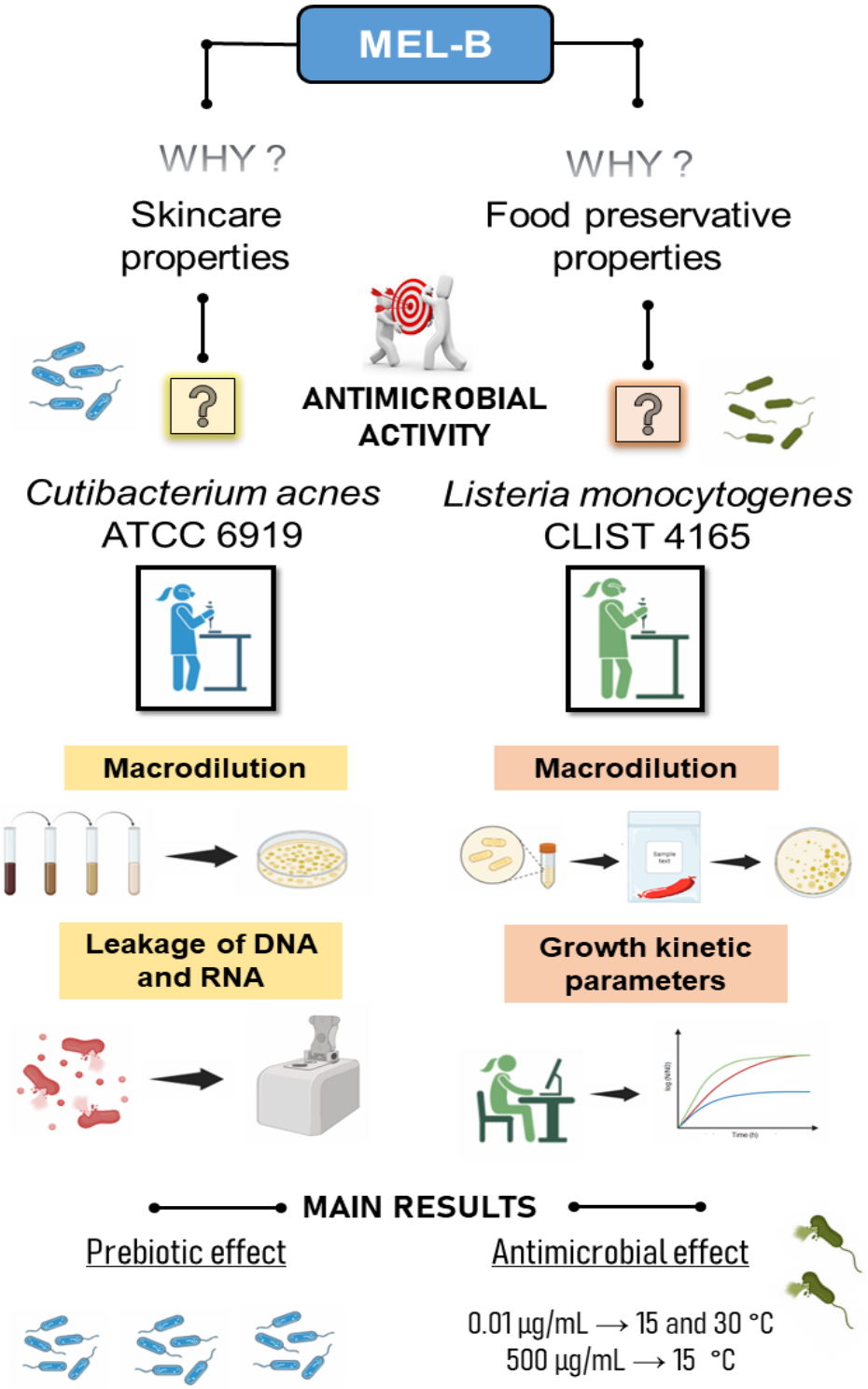
- Evaluate the susceptibility of *C. acnes* ATCC 6919 and *L. monocytogenes* CLIST 4165 to MEL-B using the broth macrodilution method;
- Investigate the membrane damage of MEL-B to the bacteria by quantification of DNA and RNA release;
- Apply a mathematical model to predict the effect of MEL-B on bacterial growth.

Answers

- What is the susceptibility of *Cutibacterium acnes* and *Listeria monocytogenes* to MEL-B?
- Do higher concentrations of MEL-B result in more damage to the strains?
- How does the MEL-B modify the growth kinetic parameters of *L. monocytogenes* cultured in hot dog sausage?

The diagram below represents the graphical abstract of the thesis, with a brief approach to the motivation, methodology, and main results.

SCHEMATIC DIAGRAM OF THE EXPERIMENTAL ASSAYS



1. INTRODUCTION

Mannosylerythritol lipids (MEL) are a class of microbial glycolipids, which traditionally include four counterparts: MEL-A, -B, -C, and -D. These compounds consist basically of 1-O-β-D mannopyranosyl erythritol as a hydrophilic part and two fatty acid chains as a hydrophobic structure, differing as to the presence or absence of acetyl groups at carbons 4 and 6 of the mannose (COELHO et al., 2020[a]; TAKAHASHI et al., 2012).

MEL-B is the main product formed in submerged cultivation of *Pseudozyma tsukubaensis* from vegetable oils (MORITA et al., 2010). Toyobo company produces this glycolipid commercially with a high degree of purity (95% purity).

The antimicrobial activity of MELs was demonstrated previously against vegetative cells and spores of *Bacillus cereus* (SHU et al., 2019), methicillin-resistant *Staphylococcus aureus* (SHU et al., 2021), and *Listeria monocytogenes* (LIU et al., 2020). Okuhira et al. (2020) reported that MELs can inhibit selectively Gram-positive bacterial communities (e.g., *Streptococcus bovis*, *Ruminococci*, and *Fibrobacter succinogenes*) of bovine rumen. This finding suggests that MELs can act as antimicrobial and prebiotic agents.

Regarding the food sector, previous reports investigated the effects of MELs as possible additives and antimicrobial agents. Sun et al. (2021) reported the potential application of glycolipids as antimicrobials for food. One of the reasons for this is the natural and clean label aspects of these compounds, a current consumer demand.

The antimicrobial activity of MEL-A was evidenced in whole and skimmed milk (LIU et al., 2020). The minimum inhibitory concentration (MIC) was 32 μg/mL and 1,024 μg/mL when *L. monocytogenes* wild-type EGD-e (ATCC BAA-679) was cultivated in culture medium (Brain Heart Infusion Broth) and milk, respectively. Shu et al. (2022) showed the bactericidal effect of MEL-A (1.5 v/v) against vegetative cells of *B. cereus* and an inactivation effect on the spores of *B. cereus* and yeast cultured on the fresh and frozen loaf bread.

The surfactant, and emulsifier properties of MEL-A in bread production were investigated by Shu and coworkers (2022). The authors showed that MEL-A (1.5 v/v) improved the rheological properties of frozen dough, namely gas retention, loaf volume, and textural properties.

In another approach, *in vitro* experiments performed with fibroblasts and melanocytes and *in vivo* assays evidenced the effect of MEL-B on the recovery of hydration/moisture of human skin, also acting as a whitening, depigmenting, and anti-aging active agent (YAMAMOTO et al., 2012; BAE et al., 2019[a,b]).

Despite these properties, to our knowledge, there are no reports addressing the antimicrobial effect of MEL-B on the skin microbiota or even a specific strain. The modulation of the skin microbiome is one of the treatments for acne control, and a natural product such as MEL-B could be interesting (CAVALLO et al., 2022; VAN STEENSEL; GOH, 2021).

Cutibacterium acnes is a Gram-positive bacterium associated with acne vulgaris, an inflammatory disease of the skin's sebaceous glands - notably in the face, neck, chest, and upper back. *C. acnes* is the predominant microorganism in the sebaceous glands of acne-prone skin. Indeed, it has been reported that approximately 10^7 viable cells can be found in a single pilosebaceous unit (RAZA et al., 2013; CHOMNAWANG et al., 2007). Moreover, other diseases can be indirectly associated with *C. acnes*, such as sarcoidosis illness, neurodegenerative disorders, and the growth of metastatic cells in the prostate (LEHESTE et al., 2017, ALLHORN et al., 2016).

L. monocytogenes is still a food safety concern since it can be present throughout the production and supply chains. This bacterium has been linked to outbreaks and, recalls due to its diversity, persistence, and easy development in ready-to-eat foods (TOWNSEND et al., 2021).

The literature does not describe the effect of MEL-B on the modulation of skin microbiome or even on a specific strain. Similarly, the antimicrobial performance of MEL-B in food matrix is poorly explored. Thus, the present work aimed to investigate the antimicrobial performance of MEL-B against *Cutibacterium acnes* ATCC 6919 and *Listeria monocytogenes* CLIST 4165.

2. OBJECTIVES

2.1 MAIN OBJECTIVE

This thesis aimed to evaluate the performance of mannosylerythritol lipids-B (MEL-B) as an antimicrobial agent against Gram-positive bacteria *Cutibacterium acnes* ATCC 6919 and *Listeria monocytogenes* CLIST 4165.

2.2 SPECIFIC OBJECTIVES

a) Evaluate the effects of MEL-B concentration and incubation time on the growth of *C. acnes* ATCC 6919;
b) Estimate the membrane damage caused by MEL-B on the *C. acnes*;
c) Investigate the antimicrobial properties of MEL-B against *L. monocytogenes* cultured in a TSB medium;
d) Investigate the antimicrobial properties of MEL-B against *L. monocytogenes* cultured on the hot dog sausage;
e) Evaluate the effects of concentration and temperature (15 and 30 °C) on the antimicrobial activity of MEL-B against *L. monocytogenes* cultured in hot dog sausage at 15 and 30 °C;
f) Adjust the mathematical predictive model to the data of *L. monocytogenes* growth in hot dog sausage at different MEL-B concentrations (0.01 and 500 µg/mL) under 15 and 30 °C;
g) Determine the growth kinetics parameters of *L. monocytogenes* cultured in hot dog sausage at different MEL-B concentrations (0.01 and 500 µg/mL) under 15 and 30 °C.

3. THESIS OUTLINE

The organization of the thesis is presented below:

1. **INTRODUCTION**

2. **OBJECTIVES**

3. **CHAPTER 3:** State of the art on the biological activity of mannosylerythritol lipids-B.

In this chapter, an approach is made to the biological activities of MELs in free form (antimicrobial, skincare, anticancer) and as a structuring agent and enhancer of nanocarriers. An approach to the aggregation structures of MELs is made, emphasizing their influence on the biological properties of this compound. This chapter was published in the Applied Microbiology and Biotechnology Journal.

COELHO, ALS; FEUSER PE; CARCIOFI BAM; DE ANDRADE CJ; DE OLIVEIRA D. Mannosylerythritol lipids: antimicrobial and biomedical properties. **Applied Microbiology and Biotechnology**, v. 104, n. 6, p. 2297-2318, 2020. http://doi.org/10.1007/s00253-020-10354- z.

4. **CHAPTER 4:** Effects of mannosylerythritol-lipids-B on *Cutibacterium acnes* ATCC 6919.

The antimicrobial activity of MEL-B against *C. acnes* was evaluated in this chapter. Two concentrations of MEL-B and two incubation time conditions were used. The damage to the strain's membrane by MEL-B was also investigated by quantification of genetic material extravasated in the culture medium. The data showed that MEL-B potentiates the growth of the lineage, acting as a prebiotic agent. This chapter was submitted to Indian Journal of Microbiology.

5. **CHAPTER 5:** Mannosylerythritol lipids-B as antimicrobial against *Listeria monocytogenes* CLIST 4165 in hot dog sausages.

The antimicrobial activity of MEL-B against *L. monocytogenes* CLIST 4165 cultured in hot dog sausage was evaluated in this section. In general, the data showed that MEL-B reduced the lag phase of the strain and the maximum growth rate under 15 and 30 °C. The use of high glycolipid concentration (500 µg/mL) was unfavorable for strain control at 30 °C. This chapter will be submitted to the Food Microbiology Journal.

4. CHAPTER 1 – STATE OF THE ART ON THE BIOLOGICAL ACTIVITY OF MANNOSYLERYTHRITOL LIPIDS-B

This chapter presents the state of the art of the biological activity of MEL, especially antimicrobial and biomedical activities (Mini-review published in the Applied Microbiology and Biotechnology journal - http://doi.org/10.1007/s00253-020-10354- z). License Number 5616041479926.

Abstract

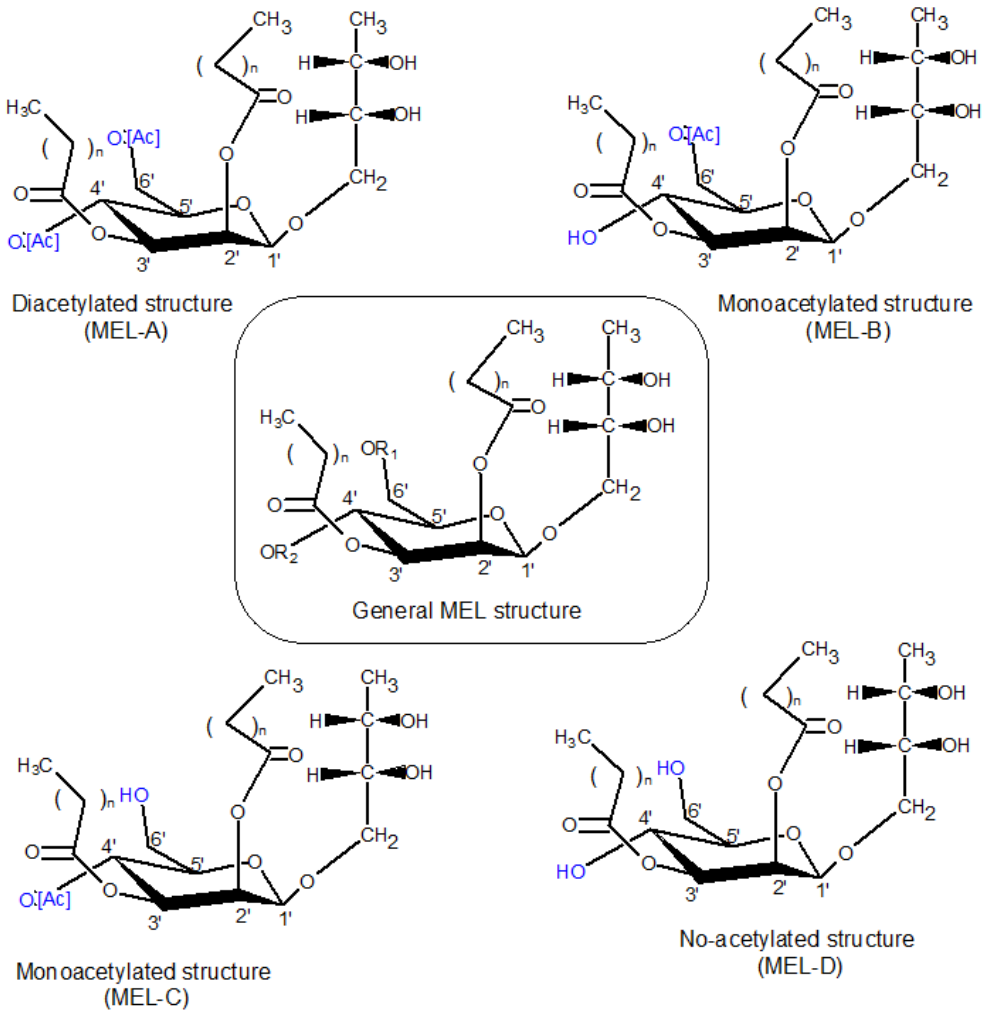
Mannosylerythritol lipids (MELs) have attracted particular interest of medical, pharmaceutical, and cosmetic fields, due to their specific characteristics, including non-toxicity, easy biodegradability, and environmental compatibility. Therefore, this review aims to highlight recent findings on MEL biological properties, focusing on issues related to therapeutic applications. Among the main findings are that MELs can play a fundamental role due to their antimicrobial properties against several nosocomial pathogen microorganisms. Other remarkable biological properties of MELs are related to skincare, as anti-aging (active agent), and in particular on recover of skin cells that were damaged by UV radiation. MEL is also related to the increased efficiency of DNA transfection in liposomes systems. Regarding the health field, these glycolipids seem to be associated with disturbance in the membrane composition of cancerous cells, increasing expression of genes responsible for cytoplasmic stress and apoptosis. Moreover, MELs can be associated with nanoparticles, as a capping agent, also acting to increase the solubility and cytotoxicity of them. Furthermore, the differences in the chemical structure of MEL could improve and expand their biochemical diversity and applications. Such modifications could change their interfacial properties and thus, reduce the surface tension value, enhance the solubility, low critical micelle concentrations, and form unique self-assembly structures. The latest is closely related to molecular recognition and protein stabilization properties of MEL, that is, essential parameters for their effective cosmetical and pharmaceutical effects. Thus, this current research indicates the huge potential of MEL for use in biomedical formulations, either alone or in combination with other molecules.

Keywords: Biosurfactant; Health; Self-assembled structures; Skincare.

4.1 INTRODUCTION

Mannosylerythritol lipids (MELs) are a class of compounds with amphiphilic properties, designated as a glycolipid and also classified as a biosurfactant. Chemically, the four MEL traditional homologues have a similar basic structure (Figure 1), which differ and are classified according to their acetylation degree: MEL-A is diacetylated at the C4 and C6 positions, MEL-B and MEL-C are monoacetylated at C6 or C4, respectively, whereas MEL-D have no acetyl group (FUKUOKA et al., 2007[a]; ANDRADE et al., 2017; JEZIERSKA et al., 2018).

Figure 1 - Chemical structures of mannosylerythritol lipids (MEL).



Source: Coelho et al. 2020[a].

Besides, Saika et al. (2018), point out that the variants of MELs (MEL-A, MEL-B, MEL-C, MEL-D) can be further modified by length of fatty acids chains and their saturation variants, as well as the chirality of mannosylerythritol, which can be conventional or diastereomer.

It is important to note that the biological and interfacial characteristics of MELs are directly related to the chemical structure of the mannosylerythritol lipids, which in turn depend on the lineage employed during the microbial fermentation process, as well as carbon and nitrogen sources present in culture medium (ALIMADADI et al., 2017; JEZIERSKA et al., 2018).

The aforementioned structural diversity confers to MEL exceptional biological properties, which differ significantly from their precursors (INÈS; DHOUHA, 2015). Thus, we explored the following properties of MEL: antitumor effect, amtimicrobial activity, skin-moisturizing effect and enzyme activation/inhibition (FAN et al., 2018; INÈS; DHOUHA, 2015; MNIF; GHRIBI, 2015; GUDIÑA et al., 2013).

Other papers also report biological and surface properties of MELs, as well as their self-assembly structures. However, in the current study we are concerned not only with describing the aggregation structures of MELs, but report them as recognition structures of biological organisms, which can have their surface properties and functions altered as a result of changes in chemical structure. Furthermore, we herein briefly explain how interaction with MEL can modify physics/chemistry properties and the mechanism of action of proteins and polymers.

Finally, the current environmental concerns and stringent regulations create and strengthen market niches that are not approachable for chemical surfactants (ABDEL-MAWGOUD; STHEPHANOPOULOS, 2018; JEZIERSKA et al., 2018; DHIMAN et al., 2016). In this way, the present review focused on MELs' bioactivities, highlighting the recent findings and advances in medical, pharmaceutical, and cosmetic fields as well as in antimicrobial applications.

4.2 ANTIMICROBIAL ACTIVITY

The exact physiological roles of glycolipids over their native producers are not yet fully understood. Generally, they are classified as secondary metabolites, biosynthesized during the late logarithmic or stationary growth phase; that is, glycolipids are not essential for cell viability. Thus, given their antimicrobial properties, theoretically, the primary role of these compounds is to provide safe

environmental niches for their own (ABDEL-MAWGOUD; STHEPHANOPOULOS, 2018; DHIMAN et al., 2016). In this sense, Rodrigues et al. (2015) related that mannosylerythritol lipid exhibit antimicrobial properties, which plays a very important role in colonization and cell-cell competition.

Roca et al. (2015) stated that multidrug resistance (MDR) bacteria become a global concern and a major public health problem. These facts have been reflected in the medical industry's interest in the development of new medicines from known biosurfactants and their application as prophylactic and therapeutic agents (DHIMAN et al., 2016).

Fukuoka et al. (2007)[b] point out that the antimicrobial activity of MELs has been attributed to damage caused in the bilayer cell membranes of microorganisms, and MEL-A and MEL-B patterns are reported to have high antimicrobial activity, particularly against Gram-positive bacteria.

The antimicrobial activity of a new glycolipid, mono-acylated MEL (MEL-M), was studied by Fukuoka et al. (2007)[b], using the conventional agar dilution method. The microorganisms employed were: (i) Gram-positive bacteria (*Bacillus subtilis* NBRC 3134, *Micrococcus luteus* NBRC 13867 and *Staphylococcus aureus* subsp. aureus NBRC 12732); (ii) Gram-negative bacteria (*Escherichia coli* NBRC 3972 and *Pseudomonas aeruginosa* NBRC 13275); (iii) yeast (*Candida albicans* NBRC 1594) and (iv) filamentous fungus (*Aspergillus niger* NBRC 6341). The antimicrobial activity was expressed in terms of minimum inhibitory concentration (MIC), defined as the lowest concentration of the antimicrobial agent, which prevents visible growth of microorganisms. The results tests indicate that MEL-M is less effective (little cell membranes damage) when compared to MEL-A standard, with a MIC of 200 $\mu g.mL^{-1}$ for Gram-positive bacteria, *Micrococcus luteus* NBRC 13867 and a MIC greater than 400 $\mu g.mL^{-1}$ for other bacteria strains, yeasts and filamentous fungi (FUKUOKA et al., 2007)[b].

The influence of alkyl chain length on the antimicrobial performance of MELs was evaluated by Nashida et al. (2018). Chemical synthesis route was used to obtain 20 homologs of MELs, which were tested on different strains: *Micrococcus luteus*, *Enterococci faecalis*, *Enterococci faecium*, vancomycin-susceptible *Enterococcus* and multidrug-resistant strains *Staphylococcus aureus* and *Enterococci.* The results found clearly indicated that the chains containing 8 and 10 carbons exhibited superior

antimicrobial performance, whereas compounds with 6, 12 and 14 carbons - short and long chains – demonstrated quite similar results.

It is worth noting that none of the synthesized compounds demonstrated antibacterial activity against resistant *S. aureus* strain, whereas MEL-D C18 was the only to show high antibacterial activity against vancomycin susceptible *Enterococcus* and vancomycin resistant *Enterococci*, indicating that not only the length of the alkyl radical but also the pattern of acyl groups - diacetylated (MEL -A), monoacetylated (MEL B and C) unacetylated (MEL-D) - is an important aspect for anti-bacterial activity of MELs (NASHIDA et al., 2018).

Shu et al. (2019) investigated the effect of MEL-A, produced by *Pseudozyma aphidis*, as bactericidal and bacteriostatic agents over spores and vegetative cells of *Bacillus cereus*. The authors showed that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of MEL against *B. cereus* cells were 1.25 and 2.50 mg mL^{-1}, respectively. Furthermore, the analysis data of intracellular constituent leakage and cell viability show that MELs caused the unbalance of cellular environments, beyond cell membrane disruption. These results are aligned to the results of bacterial micromorphology performed by using a scanning electron microscope (SEM) and transmission electron microscope (TEM), which confirms that a high concentration of MELs promoted the injury and deterioration of cells. It was also observed that MEL-A has a marked effect in preventing germination of *B. cereus* spores. Thus, Shu et al. (2019) concluded their study indicating the potential of MEL as a safe and efficient preservative in the food industry.

Furthermore, MELs have also pesticide properties. Yoshida et al. (2015), for example, studied the MEL suppressive activity against the early infection behaviors of several phytopathogenic fungal conidia on host leaves of two Gramineae plants (i.e., wheat, rice) and two non-Gramineae plants (i.e., strawberry and mulberry). The results found indicated that necrotic symptoms on leaves, caused by *Blumeria graminis f. sp. tritici* strain T-10, were suppressed by treatment with MEL-A. In conclusion, the authors pointed out that they have a great interest in performing experiments *in situ* conditions to check the actual effectiveness of MEL to suppress powdery mildew of wheat plants.

4.2.1 Enhancers of antimicrobial MELs activity

In general, the metal ions can act as antimicrobial agents due to their ability to cause physical cell's injure or by production of reactive oxygen species (ROS), which alters the typical critical enzyme performance, cell membranes, and genetic material of microorganisms, mainly by the respiratory burst mechanism process (RICE et al., 2019). Precisely, silver nanoparticles can pierce bacterium membranes, promoting extravasation of cytosolic components. Regarding gold nanoparticles, they act unbalancing membrane potential, also inhibit ATP synthase activity as well as tRNA binding to the ribosome (CUI et al., 2012; THOMBRE et al., 2016; RICE et al., 2019).

The mechanism of action of ZnO nanoparticles is associated with the accumulation of these compounds in the outer membrane of bacteria, inducing excessive production of ROS, leading to the overexpression of genes associated with microorganism oxidative stress (THEERTHAGIRI et al., 2019).

Nowadays, the green method for synthesis of nanoparticles has attracted much attention, and the application of biosurfactants for such purpose could minimize or eliminate the hazardous byproducts, compared to their chemical counterparts (BAKUR et al., 2019[a]). Besides, microbial surfactants have some advantages over synthetic ones such as biodegradability, mild production conditions, environmental compatibility, low toxicity, high selectivity, and specific activity at extreme temperatures, pH, and salinities (RODRIGUES et al., 2015; BAKUR et al., 2019[a]). Due to their various potential bioactivities - antimicrobial activity, antioxidant, interfacial, and antitumor properties - MEL has also gained particular attention as reducing and stabilizing agents for the synthesis of nanoparticles.

Mannosylerythritol lipids, produced from *U. maydis* CGMCC 5203, were used by Bakur et al. (2019)[a] as green reducing and stabilizing agents in the synthesis of gold nanoparticles (AuNPs). The antibacterial activity of MEL-AuNPs at concentrations of 25, 50, and 100 $\mu g.mL^{-1}$ was evaluated over Gram-positive bacteria *S. aureus* CCTCC AB91093 and Gram-negative *E. coli* CCTCC AB91112, using well diffusion technique. The data indicate that synthesized materials displayed a noticeable antibacterial effect against *E. coli* and *S. aureus* strains, at the minimum concentration of 25 $\mu g.mL^{-1}$, and the average inhibition zone of MEL-AuNPs against bacterial cells ranged from 12 to 17.6 mm (BAKUR et al., 2019[a]).

Similarly, Bakur et al. (2019[b]) emphasized that an effective and eco-friendly bactericidal agent has also been investigated for the prevention of foodborne diseases - a significant public health problem, especially in underdeveloped countries - caused by microorganisms such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella*, and *Clostridium perfringens*. Thus, Bakur et al. (2019[b]) investigated the antimicrobial effects of eco-friendly nanocomposites MEL@AgNPs, MEL@ZnONPs, and Ag-ZnO/MEL/GA. In the synthesis process, MEL was used as a reducing and stabilizing agent, not to mention their self-assembling properties, which could be utilized in gene transfection and drug delivery. Due to its biocompatible for *in vivo* applications and excellent stabilization of nanomaterials, Arabic gum (AG) was employed as a thickening, emulsifying, and stabilizing agent.

The antimicrobial efficacy - MIC and MBC values - of the synthesized materials were analyzed against *E. coli*, *S. enteric*, *B. cereus*, and *S. aureus,* using a well-diffusion technique. Additionally, treated and untreated *E. coli* (10^8 CFU mL^{-1}) were submitted to scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analysis, in order to reveal the effect of the nanocomposites on microorganism morphology (BAKUR et al., 2019[b]).

The results indicated that MEL@ZnONPs and MEL@AgNPs nanoparticles displayed the same antimicrobial activity against studied microorganisms, with no statistically significant difference ($p > 0.05$) verified between them, except for *E. coli* which were inhibited significantly by MEL@ZnONPs more than MEL@AgNPs. Regarding Ag-ZnO /MEL/GA materials, it was observed that their efficacy was significantly higher compared to monometallic counterparts (BAKUR et al., 2019[b]).

4.3 SKINCARE

Currently, the drive among consumers is the pursuit of green compounds, stimulating the cosmetic industry to seek alternatives for the synthetic components in their formulations (BEZERRA et al., 2018). MELs exhibit antioxidant and protective effects in skin cells, even under the oxidative damage induced by H_2O_2, evidencing their potential as antiaging ingredients for skincare (MORITA et al., 2013). Moreover, the moisturizing effect of MELs over human skin cells damaged with sodium dodecyl sulfate (SDS) was examined. As a result, tests with MELs were able to recover damaged skin cells, showing performance comparable to ceramide-3. Furthermore, as reported in the literature, MEL-B possesses the ability to suppress

the perspiration of skin surface, which reflects an increased in the stratum corneum water content (BHANGALE et al., 2013; MORITA et al., 2013; YAMAMOTO et al., 2013).

Yamamoto et al. (2012) investigated the influence of MEL type and the degree of fatty acid unsaturation of MEL-A on the recovery of cultured human skin model (Testskin, Toyobo, Japan), damaged with SDS (37 °C/5 min). The authors observed that at a concentration of 1% or 5%, MEL-A with 28.7% of unsaturated fatty acids provided viability higher than 90%, with a similar performance of natural ceramide III (94,4% purity). Interestingly, the cells treated with MEL-A containing 42.4% of unsaturation exhibited viability of approximately 60% and 82% at a concentration of 1% and 5%, respectively. Based on those results, it was inferred that the degree of unsaturated fatty acids modified the self-assembling structure of MEL, and thus their biological properties. Regarding other MEL types, MEL-B demonstrated excellent cell viability, more than 80% for both concentrations previously reported, whereas the recovery by MEL-C was approximately 60% and 80% at a concentration of 1 and 5%, respectively (YAMAMOTO et al., 2012).

The mechanism of cell recovery by ceramide is related to an increase of water permeability, and moisture retention of skin, which provide a strengthens of cell structure. All these features were attributed to the arrangement of the lamellar phase, a common parameter for ceramide III and MEL-B, in a wide range of concentrations (YAMAMOTO et al., 2012).

In this sense, MEL-B previously dissolved on 1,3-butylene glycol was employed in an *in vivo* skin recovery experiments, realized with healthy female volunteers in their 30 s. The MEL-B solution was spread on the volunteers' forearm skin (approximately 2 $\mu L.cm^{-2}$). By corneometer measurements, it was observed that after 20 min of solution contact, the water retention of tissue was 147 and 186% for solutions containing 1 and 5% of MEL-B, respectively. Besides, the assays of skin perspiration showed a transepidermal water loss (TEWL) of 86% for MEL-B at 1% and 74% for the solution with MEL-B concentration at 5% (YAMANOTO et al., 2012).

Although ceramide had a performance slightly better than MEL-B, this compound is poorly dissolved in water, a limitation not observed in MEL-B, which due to the configuration of erythritol moiety, presents high hydrophilicity. This feature, together with the effects described above, favors the insertion of MEL-B in skincare products (YAMANOTO et al., 2012).

Besides, the DPPH (1,1-diphenyl-2-picrylhydrazine) radical-scavenging activity of MELs (MEL-A, MEL-B, and MEL-C) was analyzed by Takahashi et al. (2012). The results showed that among glycolipids, only MEL-C achieved satisfactory performance, with scavenging activity of 50.3% at 10 $mg.mL^{-1}$. Also, in this perspective, xanthine/xanthine oxidase tests were performed to determine the superoxide anion-scavenging activity of MELs. The results revealed that MEL-A has a poor antioxidant effect, whereas MEL-B has no antioxidant capacity. On the other hand, MEL-C showed 60% of activity at 2 $mg.mL^{-1}$.

Based on that findings, *in vitro* experiments using cultures of human fibroblasts (NB1RGB cells, from Riken Cell Bank) were performed. *In vitro* studies revealed that MEL-C has the cytoprotective ability, and also attenuate the oxidative stress of NB1RGB cells, in a dose-dependent binding, with the non-toxic effect observed for concentration below 10 $\mu g.mL^{-1}$ (TAKAHASHI et al., 2012).

In order to confirm the previous assumptions, a molecular investigation by western blot analysis was carried out. According to the authors, the oxidative damage by H_2O_2 induces the expression of COX-2 protein, which is related to prostaglandin production. High levels of this compound indicate the organism's response to inflammatory conditions. Thus, by western blot analysis, it was proved that antioxidant and protective effects of MEL-C are due to significant COX-2 repression in treated cells. Thus, studies of Takahashi et al. (2012) demonstrated the potential of MEL-C for application in anti-aging skincare products.

Thus, MEL is safe and sustainable molecules with great potential for application in cosmetics formulation as both active compounds and surfactants (BEZERRA et al., 2018). It is important to note that although MEL does not officially have GRAS (generally considered as safe) status, for all practical purposes, it can be considered as such. This is because microorganisms used in biosurfactant production processes possess GRAS status, and their products are allowed to be incorporated, without problems, in food and pharmaceutical products (AMARAL et al., 2010; MARCELINO et al., 2017).

According to Bae et al. (2019)[a], MEL produced by yeasts generally demonstrated a capacity of moisturizing dry skin, repair damaged hair, activate fibroblasts and papilla cells, and exert antioxidative and protective effects in skin cells, conferring a competitive advantage for use in cosmetics. Nevertheless, very

few of these compounds exhibitsatisfactory anti-melanogenic properties, for cosmetics or medicines.

In this sense, the depigmentary effect of MEL (DKBIO (Daejeon, Korea), with 95% degree of purity, 85% di-acylated MEL-B and 15% tri-acylated MELs) over primary human melanocytes (NHMs) and B16 murine melanoma (MSH) was evaluated by Bae et al. (2019)[b]. Cytotoxicity analysis was performed on NHMs cells, and the test assays showed that MEL did not affect cell viability at concentrations below 5 $\mu g.mL^{-1}$. By using photography, Fontana- Masson (F&M) staining, and two-photon microscopy, the authors demonstrated that MEL inhibits the intracellular melanin content of NHMs in a dose-dependence response, with better results observed at 5 $\mu g\ mL^{-1}$ after five days of incubation. In order to confirm the previous results, the cytotoxicity and anti-melanogenic tests were repeated for α-MSH-stimulated B16 cells, which is a traditional cell used to test *in vitro* depigmenting efficacy. In this case, MEL had no cytotoxicity against cells at concentrations up to 10 $\mu g\ mL^{-1}$, beyond that, the glycolipid dramatically suppressed the extracellular and intracellular melanin contents of α-MSH-stimulated B16 cells. These results led to the conclusion that MEL can significantly inhibit melanin synthesis in melanocytes.

Similarly, Bae et al. (2019)[a] showed that commercial MEL produced by *Pseudozyma* sp. (DKBIO (Daejeon, Korea), with 95% degree of purity, 85% di-acylated MEL-B and 15% tri-acylated MELs) inhibited melanin synthesis in primary normal human melanocytes (NHMs), by ERK/CREB/ gene expression of melanogenic enzymes - tyrosinase Tyrp-1 and Tyrp-2. Additionally, under experimental conditions, MEL is found to have a whitening effect in a human skin equivalent, brightening the tissue color. Clearly, the research of Bae et al. (2019)[a] proved that the MELs potential as an anti-melanogenic additive in skincare products.

A skin whitening formulation containing MEL as an active ingredient was elaborated by Yoo et al. (2017). The developed product was effective for suppressing melanocyte formation, improving skin tone, still providing clean, darkness-relieved, and bright skin.

Bae et al. (2019)[a] showed that MEL-B (with over 95% degree of purity; Daejeon, Korea) could recover aquaporin-3 (AQP3) expression in human keratinocytes, after UVA irradiation-induced damage. Aquaporin-3 (AQP3) is a membrane protein through which water, glycerol, and H_2O_2 are permeated,

being responsible for hydration, elasticity, and resilience of the skin. AQP3 has also been associated with water homeostasis in the epidermis. AQP3 expression in the skin naturally decreases with age, although it could be accelerated by the stimulation of external agents, such as UV irradiation and reactive oxygen species, which drastically decreased the AQP3 expression at mRNA levels. Thus, the effects obtained has proven that MELs are an efficient compound for moisturizing products - since they act as both surfactant and recover the tissue that was damaged by UVA irradiation (BAE et al., 2019)[a].

The synthesis of a skincare melting film, consisting of film-forming polymer, mannosylerythritol lipid, and ceramide, was described in the Choi et al. (2018) patent. The invention provides a broad skin moisturizing effect and promotes remarkably wrinkle reduction. A similar approach can be observed in the Kyoto University patent (2008). Another patent (KIM et al., 2018) reported that MEL was also successfully employed as a stabilizing agent for other compounds with similar properties - anti-aging, anti-wrinkle, antioxidant, and skin bleaching.

Schelges et al. (2017) proposed in their patent the formulation of a prebiotic cleaning agent cosmeticsthat include one or more biosurfactants - rhamnolipids, sophorolipids, and MEL - in combination with one or more anionic surfactants. The bacterial skin flora includes pathogenic (*Propionibacterium acnes*) and non-pathogenic (*Staphylococcus epidermidis*) species. The inflammatory skin diseases begin when, under specific conditions, harmful bacteria proliferate faster than non-harmful ones. The products currently on the market contain non-selective active ingredients that inhibit not only the undesired skin bacteria but also the desired ones, causing the biological imbalance of the skin. The prebiotic cosmetics could overcome the above-said shortcomings, since they directly controlled the growth of pathogenic bacteria, without disturbing the beneficial cutaneous microbial flora.

Thus, Schelges et al. (2017) have found that cleaning prebiotic cosmetic containing biosurfactants in association with an anionic surfactant synergistically increased the efficacy of the previously mentioned product and exhibit satisfactory sustainability and biodegradability requirements.

MELs have been proved to have a favorable emulsifying capacity, which represents one more advantage for their application in skincare products. Specifically, MEL-A exhibited strong potential for application in microemulsions, whereas MEL-B and MEL-C spontaneously form giant unilamellar vesicles, in the

aqueous phase, indicating a superior molecular orientation property and hydrophilic-hydrophobic balance (VARVARESOU; IAKOVOU, 2015).

4.4 DRUGS AND HEALTH

Relevant applications of MELs in the clinical sector include (i) binding system for G and M immunoglobulins, important mediators of human humoral immune response; (ii) gene transfection and gene therapy studies since MELs can be one of the constituents of liposomes, a widely employed system as carriers of genetic material (RODRIGUES et al., 2015).

Dhiman et al. (2016) reported that MEL produced from vegetable oils by *Candida* strains showed several potential biomedical applications due to immunological, antimicrobial, and neurological properties. Moreover, immunosuppressive, immunomodulating, anti-tumor, and anti-inflammatory activity, besides cell stimulation and differentiation, cell-to-cell signaling, self-assembly, interaction with stratum corneum lipids, membrane perturbation and haemolytic activity, have also been emphasized for these compounds which are briefly described below (FRACCHIA et al., 2015).

4.4.1 Liposomes

Sandoval-Altamirano et al. (2017) claim that several biological processes (e.g., signaling, recognition, and adhesion) are mediated by the interaction of glycolipids present on the cells surface. That is why many studies focused on utilizing glycolipids as tools for tailoring the surface of the liposomes, favoring their drug-target interactions with specific cells and tissues.

Liposomes are membrane structures capable of incorporating/encapsulating a wide variety of bioactive agents, which favors theirs as a smart delivery of drugs (DARAEE et al., 2014; SANDOVAL-ALTAMIRANO et al., 2017; WU et al., 2019). Igarashi et al. (2006) state that cationic liposome, for example, is a promising tool for gene delivery due to low immunogenicity and toxicity, simple method of synthesis, and a high potential for active targeting. Nevertheless, compared to the most efficient gene therapy system, (viral vectors), cationic liposomes face some limitations, such as entry into target cells, endosomal/lysosomal escape, avoidance from the degradation of exogenous DNA by nuclease, and transport into the nucleus (VARVARESOU; IAKOVOU, 2015). In this concern, many studies have

demonstrated that association of MEL-A with cationic liposomes increases the effectiveness of genetic material (DNA and siRNA) delivery into the cytoplasm compared to cationic liposomes without MEL-A or than a commercially available liposomal agent.

Naughton et al. (2019) reported that the use of MEL-A in the cationic liposomes' constitution, increased 5-7-fold the efficiency of gene transfection in mammalian cultured cells. Similarly, Inoh et al. (2011) pointed out that MEL-A increases the DNA transfection efficiency of liposomes, inducing membrane fusion between liposomes and the plasma membrane of target cells, promoting more even distribution of the material in the cytosol of them, suppressing protein expression with minimal cytotoxicity.

Other studies disclose the interaction of MEL with other compounds, not only to increase the efficiency of lipose transfection but also to potentiate the effects of drugs' active principle. In this concern, Wu et al. (2019) evaluated the influence of MEL-A on the preparation of liposomes coated with commercial chitosan fungal, for encapsulation of hydrophobic pentacyclic lupane-type triterpenoid betulinic acid. Besides, the authors mentioned the pharmacological potentials of betulinic acid, including antiviral, antibacterial, anti-inflammatory, antimalarial, anti-HIV, and antitumor capacity. Moreover, when betulinic acid enters the human organism via the oral route, it still presents organ protective, antidiabetic, immune-enhancing, and anti-obesity effects (YOGEESWARI et al., 2005; WU et al., 2019). The results obtained by Wu et al. (2019) showed that the association of MEL-A with chitosan in the liposome coating increased the antioxidant activity of betulinic acid.

The effect of pre-condensation of plasmid DNA by a cationic polymer, protamine, on gene transfection by cationic liposome with MEL-A (MEL-A–containing OH liposomes) and without MEL-A (OH liposomes), was evaluated by Inoh et al. (2013). The transfection efficiency of pre-condensed plasmid DNA into NIH-3T3 cells mediated by MEL-A-containing OH liposomes and OH liposomes was determined by luciferase assay. The authors demonstrated a positive effect of protamine and MEL-A association. When OH liposomes were used in transfection experiments, the efficiency of pre-condensed DNA transfection was 2-fold higher than that of non-condensed DNA transfection. On the other hand, MEL-A–containing OH liposomes was 15-fold more active than liposome with non-condensed material. The authors repeated the same experiments for COS7 cells, and in this case, a 10- and 1.5-fold

increase in transfection was obtained for MEL-A-OH liposomes and OH liposomes, respectively. *In vivo* studies were also conducted by Inoh et al. (2013), which examined gene transfection efficiency when the complexes of the cationic liposome/pre-condensed plasmid DNA or non-condensed plasmid DNA was incubated with the target cells in the presence of 10% serum. The MEL-A-containing OH liposomes had betterperformance, approximately ten times higher than non-condensed DNA transfection. Furthermore, the combination of pre-condensed DNA and MEL-A-containing cationic liposomes increase the transfection efficiency by about 20 times than Lipofectamine™ 2000, a commercial delivery reagent.

Interestingly, Inoh et al. (2013) observed by fluorescent-labeled that MEL-A was not present in the intracellular membrane structures of NIH-3T3 cells when it was added to the cells without liposomes complex. On the other hand, when MEL-A-containing OH liposome-plasmid DNA was inserted into NIH-3T3 cells, this glycolipid was detected in the intracellular membranes, mainly, nuclear membrane. Lastly, it was possible to observe that, once associated with the OH liposome, MEL-A may regulate the molecular transport through nuclear pore complexes, accelerating the transport of pre-condensed plasmid DNA across the nuclear membrane.

So far, many studies emphasized MEL-A as a potentiator of the transfection process using cationic liposomes as a vector. However, studies reporting the physiochemical mechanism of the fusion ability of MEL-A are still lacking. In this concern, the zeta potential of cationic liposomes and lipoplexes membranes were evaluated by Ding et al. (2009[a]) in the presence or absence of MEL-A. This stems from the fact that an efficient gene transfection occurs by electrostatic interaction of liposomes and the plasma membrane, and the understanding of this phenomenon paves the way to elucidate the association of both structures.

The results showed that MEL-A did not modify the surface pH of MHAPC-lipoplexes, while OH-Chol-lipoplexes sample showed the opposite behavior, observing the decrease in zeta potential from the range of 50 to 75 mV to a negative value of -14.6 and -21.7 mV in Milli Q and phosphate-buffered saline (pH 7.4, 1/10 PBS), respectively. Therefore, the authors state that MEL-A did not change the protonation of the tertiary amine of MHAPC; but significantly affected the protonation of the secondary amine in OH-Chol. Another important aspect evaluated by Ding et al. (2019[a]), is the liposomal hydration level, which also influences the association with the cell membrane. By incorporation of 6-Dodecanoyl-N,N-dimethyl-2-naphthylamine

(laurdan) into lipids of liposomes, the authors observed that MEL-A hydrated MHAPC-lipoplexes while it dehydrated OH-Chol-lipoplexes as MEL-A concentration increased.

Thus, in order to determine the relation between zeta potentials and hydration levels of vectors over gene transfection, the cellular association of lipoplexes and A549 cells was subsequently evaluated. MHAPC-lipoplexes demonstrated much higher cellular association than OH-Chol-lipoplexes, which can be due to higher zeta potentials and the dried surface of MHAPC-lipoplexes as compared to OH-Chol-lipoplexes (DING et al., 2009[a]).

The effect of chemical (Tween 80) and biological (MEL-A) surfactants on the transfection of cationic liposomes in A549 cells and mouse lung cells was described by Ding et al. (2009[b]). *In vitro* and *in vivo* assays demonstrated that MEL-A successfully favor gene transfection performance and provided the retention of liposome for a prolonged time in the mouse lung, with opposite behavior observed for Tween 80. This fact was justified by the formation of steric barrier around the liposome membrane when Tween 80 was used, making it difficult for the liposome to bind to the target cell membrane receptors (DING et al., 2009[b])

Based on the studies mentioned above, we notice the prevalence of MEL-A in the liposome-mediated transfection process among all MELs types. The papers which discuss the specificity of MELs-liposome interactions are still scarce. However, Ueno et al. (2007)[a] explained the effectiveness of MEL-A before MEL-B and MEL-C over the process highlighted in this section. The data of ethidium intercalation assays demonstrated that MEL-A and MEL-C form closed circular structures, which promote successful liposome encapsulation. Regarding MEL-B, it was observed that it gives rise to an open triangular conformation which results in ineffective encapsulation. Additionally, the fluorescence spectroscopy indicated that only MEL-A and MEL-B exhibited fusogenic activity in liposome system, MEL-C, on the other hand, does not demonstrated the same characteristic. Thus, Ueno and coworkers (2007)[a] proved that MEL-A increases the transfection processes of the DNA capsulation as well as the membrane fusion, which explain their superior performance compared to homologues MEL-B and MEL-C.

Table 1 summarizes the main results of previous studies addressing the influence of MEL on gene transfection when liposomes were employed as a vector.

Table 1 - Literature compilation of MELs effects on the transfection mechanism by cationic liposome.

To be continued

Reference	Liposome constituents	Target cell	Objective	Main results
Inoh et al. (2011)	DOPE (31.6 nmol),OH–cholesterol (21.1 nmol) and MEL-A (10.5 nmol)	B16/BL6 cells	Estimate the efficiency of MEL-A-cationic liposomes for siRNA delivery.	Luciferase protein was suppressed (≈60%) after incubation (30 minutes) of B16/BL6 cells with MEL-A-cationic liposome/ siRNA complexes, demonstrating superior performance compared to a commercial delivery reagent for siRNA (Lipofectamine™ RNAiMax). Bcl-2 expression was significantly affected for system without MEL-A, but not suppressed by MEL-A-containing cationic liposome/siCont complexes.
[b]Ueno et al. (2007)	DOPE (20 nmol), OH-Chol (30 nmol) and MEL-A (10 nmol)	NIH-3T3 cells	Describe the transfection mechanism of cationic liposomes containing MEL-A conjugated with NBD	MEL-A-liposome was able to transfect efficiently (after 1 h) the DNA into the nucleus of target cells, while liposomes without MEL-A showed negligible performance at the same period of time. Wortmannin, an endocytosis inhibitor, significantly suppressed the transfection efficiency mediated by the liposome without MEL-A and had slightly negative effect over MEL-A-liposome transfection.
Igarashi et al. (2006)	DC-Chol, DOPE and MEL-A in a 3:2:2 molar ratio	Human cervix carcinoma cells (HeLa 229)	Investigate the performance and mechanism of gene transfection by cationic liposomes composed of DC-Chol, DOPE and MEL-A, into HeLa cells.	Liposomes containing MEL showed a more effective transfection process than those without MEL or containing transfection reagent Tfx20. MEL-A decreased the aggregation induced by DNA and reduce particles sizes, which improved the distribution of liposome and DNA into the cells cytoplasm. MEL increase a transfection efficiency in serum medium, an important feature for applications for *in vivo* experiments.

				Conclusion
Inoh et al. (2004)	DOPE/OH-Chol (2:3) DOPE/DC-Chol (3:2) MELA, MELB and MELC (5-50nmol)	NIH-3T3, COS-7 and HeLa cells	Investigate the transfection mechanism of MEL-A, MEL-B and MEL-C	The OH-Chol/MEL-A liposome was 6-7-fold more active than liposome without MEL-A. MEL-B and MEL-C were ineffective in the transfection process to target cells. The antisense DNA complex carried by liposome was insert in the plasma membrane of cells, whereas antisense oligonucleotides were detected in nucleus. Mannose and erythritol receptors are not linked to liposomes transfection on the target cells.
Inoh et al. (2001)	DOPE/OH-Chol (2:3) DOPE/DC-Chol (3:2) MELA, MELB and MELC (5-50nmol)	NIH-3T3, COS-7 and HeLa cells	Estimate the effects of MEL-A on gene transfection of cholesterol-containing cationic liposomes.	MEL-A containing cationic liposomes improved the luciferase activity in target cells. Liposome containing cholesterol derivative promote better transfection than that of the DC-Chol. The increased efficiency attributed to MEL-A is due a decrease in particles size, and minimization of DNA aggregation.

Source: Coelho et al. 2020[a].

4.4.2 Cancer treatment

Cancer is one of the major causes of death throughout the world due to the unpredictable nature of its causes and manifestation (DEY et al., 2015; BAKUR et al., 2019[a]). According to Bar-Zeev et al. (2016), multidrug resistance (MDR) is the main impediment for the effectiveness of curative cancer treatments. This situation has led research centers to seek alternative sources of chemotherapeutic agents, which, due to their different manifestations, generate the need for continuous research.

Dey et al. (2015) reported that oncogenic signals of cancer cells are amplified by the overexpression of various membrane-bound receptors such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR) and insulin-like growth factor receptor (IGFR). The glycolipids can interfere over these crucial signaling pathways, by the association of their fatty acids chains with the cancerous cell membrane, suppressing the activities of cells receptors, resulting in the membrane destabilization.

The antitumor activity of MEL seems to be associated with disturbance in the membrane composition, specifically concerning glycolipid GM3 and lactosylceramide, causing induction of cell differentiation and apoptosis (DEY et al., 2015). Additionally, some experimental results shows that MEL-A have the capability of inhibition of phosphorylation of a 60 kDa protein (serine/threonine) in HL-60 cells and 55, 65, 95 and 135 kDa proteins (tyrosine) in K562 cells, while MEL-A and B displayed a cytotoxic effect on HL-60 leukemia cells in the concentration range 5-10 mM (DEY et al., 2015).

More details about MEL interaction with HL-60 membrane are reported by Zhao et al. (2001). It was demonstrated that MEL induces the granulocytic differentiation of cells and also modified their surface composition, specifically glycosphingolipids, which is an important modulator of cellular proliferation, oncogenesis, and differentiation.

Govindarajan (2018) reported the anticancer effect of MEL-A and MEL-B on HL-60 cells when compared to SDS, SE-10, and span-20 chemical surfactants. HL-60 cells had their morphology compromised and were completely inhibited by MEL at a concentration of 10 mM, whereas the chemical surfactants had no effect on tumor cells. Gudiña et al. (2013) also suggest that

MEL has anticancer activity against myelogenous leukemia cell K562, interfering in growth, and differentiation of malignant cells. Similarly, Naughton et al. (2019) reported that MEL-A and -B have the same effect over leukaemia cell HL 60 tumor cells.

Zhao et al. (2001) evaluated the physiological effects of MEL on B16 melanoma cells by quantifying the melanin content in the culture medium and tyrosinase activity. After two days of cultivation, the melanin content of untreated and MEL-treated cell (5 μM) was 0.10 ± 0.02 and 0.18 ± 0.02 μg (μg of protein^{-1}), respectively. The morphology of untreated B16 cells was similar to those observed at the beginning of the trials. On the other hand, due to MEL (5 μM) exposure, the morphology of cells was changed to extended dendrites.

Given these observations, the apoptosis and differentiation of B16 cells induced by MEL were further analyzed by protein kinase C alpha (PKC_{α}) expression. 20 μg of protein extracts were isolated from B16 cells and subjected to treatment with 5 μM of MEL. After 3, 24, and 48 h of incubation, samples were fractionated by SDS-PAGE and then immunoblotted with mouse PKC-specific monoclonal antibodies. A similar blank experiment was also performed. The results showed that at 3 h, the PKC_{α} expression was negligible for both treated and untreated cells, whereas at 24 h, slight protein expression was observed for the two conditions. On the other hand, after 48 h while PKC_{α} expression in untreated cells decreased markedly, whereas the treated cells showed a high protein expression, which was 2.3 times higher than the control (ZHAO et al., 2001). Thus, the experimental data proved that MEL triggers the differentiation of melanoma cells by the PKC signaling pathway.

Wakamatsu and co-workers (2001) conducted a series of molecular tests to precisely define details about the anti-tumoral mechanism of MEL over mousse pheochromocytoma PC12 cells (RIKEN Cell Bank). The authors detected the generation of neurites in approximately 80% of PC12 cells, after treatment with MEL for 72 h. Besides, it was demonstrated an essential role of MEK/ERK kinases expression, together with c-Jun and c-Fos transcription of the MEL differentiation mechanism. The combination of these factors caused the arrest of the cellular cycle, leadingtoanincrease of PC12 cells in the G1

phase (54.8 to 65.4%), and their percentage reduction in the S phase (from 24.3 to 19.7%).

In this sense, a dose and time-dependent cytotoxicity of MEL-A shorter fatty acid chains (C_8-C_{14}), produced by *Pseudozyma aphidis*, over B16 melanoma cells viability were also studied (FAN et al., 2016). It was found that B16 cells treated with MEL-A (15.0 μg mL^{-1} for 24, 48, and 72 h) resulted in damage to cell shape, with the irregular cell membrane and condensed cytoplasm, which is indicative of cellular apoptosis. It can be observed an increase of melanoma cell necrosis and apoptosis with an increase in MEL-A concentration. In addition, melanin was detected in the culture medium, which can be attributed to the changes in the cell cytomembrane permeability caused by MEL-A. Besides, MEL-A had no cytotoxicity in the control cells.

The down-regulation of Bcl-2 expression is one of the first events in B16 apoptosis. Thus, the authors suggested that MEL-A induced the B16 cells apoptosis via the ER stress pathway, combined with the down-regulation of Bcl-2 and the up-regulation of caspase-12, caspase-3, cleaved caspase- 3, CHOP and GRP78, as well as the UPR signaling proteins (FAN et al., 2016).

In this regard, Gudinã et al. (2013) affirmed that the MEL-induced apoptosis process over B16 cells, keeps them in the G0 and G1 growth phases, followed by a sequence of events including chromatin condensation and DNA fragmentation.

Previous studies reported that hepatocellular carcinoma - a sort of liver cancer - is one of the most aggressive tumors around the world. Likewise, nanoparticles consisting of Ag and ZnO were synthesized using MEL as capping, reducing, and stabilizing agent of silver ions. The nanoparticles that had been derived - MEL@AgNPs, MEL@ZnONPs, and Ag–ZnO/MEL/GA - were used for *in vitro* hepatocellular carcinoma and diabetes treatment (BAKUR et al., 2019[b]). Bakur et al. (2019)[b] have proved that at low concentration (5 μg mL^{-1}) none of the particles described previously showed cytotoxicity effect toward human liver cancer (HepG2) cells. However, as concentration levels increased, IC50 concentration achieved 63.25 μg mL^{-1} for MEL@AgNPs, 26.91 μg $mL^{-1}$for MEL@ZnONPs, and 8.97 μg mL^{-1} for Ag–ZnO/MEL/GA. Among the synthesized materials, the best performance of the Ag–ZnO/ MEL/GA sample can be justified on the basis of the synergistic effect of MEL

and gum arabic, since both possesses antitumor features. Besides the role of MELs as a capping agent, they further increase the solubility and cytotoxicity of studied nanoparticles (BAKUR et al., 2019[a]).

Similarly, the potential cytotoxicity of MEL-AuNPs against HepG2 cells was demonstrated in previous studies (BAKUR et al., 2019[a]). For this, different MEL-AuNPs concentrations (10, 25, 50, 75, 100, 125, and 150 μg mL^{-1}) at different exposure times (24 and 48 h) was used. As a result, MEL-AuNPs exhibited a potential inhibition against HepG2 cells, with the IC50 value of 75 and 100 μg mL^{-1} at 24 and 48 h, respectively. The antitumor effects of MEL-AuNPs can be further attributed to interactions between sick cells and particles, and are still related to physicochemical properties of nanoparticles, such as size, the shape of particles, surface charge, types of cells, the MEL-AuNPs concentration and treating time (BAKUR et al., 2019[a]). Thus, Bakur et al. (2019[a]) inferred that the synthesized material exhibited a significant inhibitory effect over cancerous cells showed better performance than other similar materials reported in data available in the literature.

The authors concluded that MEL promotes the synthesis of uniformly sized and shaped particles, and may have a role as a stabilizing agent, preventing nanoparticle aggregation, since no relevant structural alterations were observed for the samples, even after 30 days. Besides, the cytotoxicity of MEL-AuNPs over HepG2 cells was ascribed to the synergic effect of MEL and AuNPs antitumor properties (BAKUR et al., 2019[a]).

4.5 OTHER APPLICATIONS

It has been reported that MEL-A at 0.001 μg mL^{-1} increased the papilla cells' viability around 150%. The stimulating effect of papilla cells opens up a new field of MEL application since papilla cells are a key factor for regulating hair follicle development and growth but are also thought to be a reservoir of multi-potent stem cells. Other applications are related to the recovery of damaged hair, besides its smoothness and flexibility (VARVARESOU; IAKOVOU, 2015).

Foundation is a skin-colored cosmetic applied to the face to improve out the skin tone and softness, as well as to cover spots and flaws. When materials, such as silicone, are used as a coating agent, it is difficult to obtain a

product that possesses moisturizing properties for the skin. On the other hand, MEL has unique self-assembling properties at different interfaces, which increase the skin moisture level (MORITA et al., 2013). Therefore, MELs are used as coating materials for metal oxide particles (titanium dioxide and iron oxides) in cosmetic science, particularly in the development of foundations, since these species' conjunction improves dispersibility and water-resistance as well as hydrophilicity, which represents a bottleneck on traditional cosmetic emulsions. Besides, as mentioned previously, MEL maintains a high level of moisture retention and hydration of the skin (VARVARESOU; IAKOVOU, 2015). The Japanese company, Daito Kasei Kogyo Co., Ltd., s is one of the industries that has explored this niche market by inserting the new powder material in it.

Diabetes mellitus treatments are associated with the administration of a drug cocktail, which contains several compounds, including α-amylase and α-glucosidase intestinal. Postprandial glucose is reduced when both enzymes are inhibited, slowing the absorption of carbohydrates and, consequently, the amount of glucose in the blood. Thus, the research on new inhibitors of these enzymes has increased (HSU et al., 2009).

In this context, Bakur et al. (2019[b]) evaluated the MEL@AgNPs, MEL@ZnONPs, and Ag–ZnO/MEL/GA nanocomposites for α-amylase and α-glucosidase inhibition. The data indicate that all samples exhibited significant inhibition, where the IC_{50} experimental values were 54, 49, and 43 μg mL^{-1} for MEL@AgNPs, MEL@ZnONPs, and Ag–ZnO/MEL/GA, respectively. Regarding α-glucosidase, the inhibitory concentration was 37, 51, and 46 μg mL^{-1} for Ag–ZnO/MEL/GA, MEL@AgNPs, and MEL@ZnONPs, respectively. It is worth noting that Ag–ZnO/MEL/GA showed the highest α-amylase and α-glucosidase inhibition.

Regarding nanoparticle composition, Bakur et al. (2019[b]) reported that postprandial hyperglycemia in humans is minimized when different kinds of gums are used in drug formulation, whereas zinc could act like insulin in the human body, increasing the glucose consumption. The role of MEL in this system is associated with the increase of antidiabetic drug solubility.

It should be noted that, although the previous study includes MEL-nanoparticles cytotoxicity analyzes in their scope, a gap is still evident in relation to genotoxicity damage of synthesized nanoparticles. Singh et al.

(2009) state that the insertion of nanoparticles in the human body can lead to its accumulation in some organs, like the liver, for example. Thus, in the case of using nanoparticles as drug delivery agents, diagnostic tools and therapeutics purpose, one of the important questions that may arise is their cytotoxicity and, at a more intricate level, their genotoxicity. In this regard, Singh et al. (2009) investigated the cytotoxic/genotoxicity effects of sophorolipid-AuNPs and sophorolipid-AgNPs over HepG2 cells. The synthesized nanoparticles showed concentration-dependent cytotoxicity and genotoxicity toward human hepatic cells, with no adverse effects being observed up to concentrations of 100 mM.

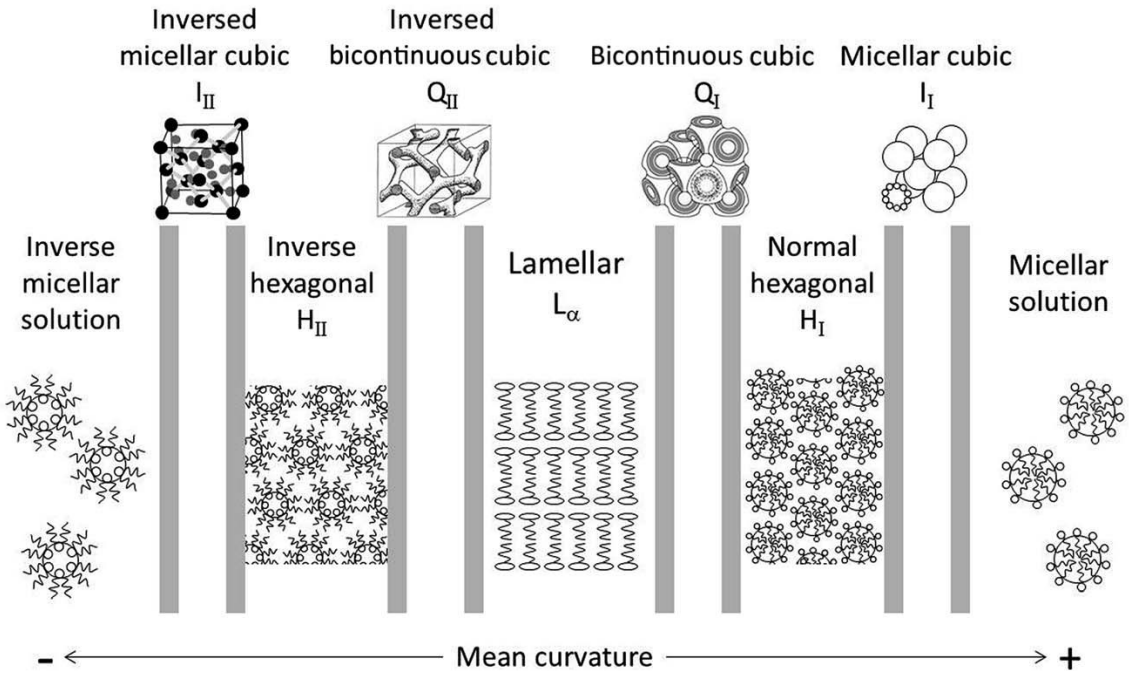
4.6 SURFACE PROPERTIES AND SELF-ASSEMBLED STRUCTURES OF MEL: A NON-CONSENSUAL SUBJECT

It has been established in the literature that glycolipids are associated with several important biological functions in nature, such as membrane formation, mediators of cell–cell-recognitions, and protein stabilization effects (VILL; HASHIM, 2002; IMURA et al., 2007[a]). An important requirement for such molecules to perform their biological roles is their arrangement in particular aggregates forms (GARIDEL et al., 2015).

In general, the biosurfactant aggregation structure is related to the proportion of polar and nonpolar groups in the molecules, composition, hydration, temperature and pressure. Together, these factors are defined by only one parameter, designated as mean curvature (SALIM et al., 2014; RODRIGUES et al., 2015). Thus, Figure 2 depicts the possible LLC arrangements as a function of mean curvature.

MELs are a class of molecules that can assemble into a several forms, including, large unilamellar vesicle, and sponge structure (Figure 3), micelles and lyotropic liquid crystals (LLC) - bicontinuous cubic (Q_{II}), lamellar (L_{α}), micellar cubic (I_I) and hexagonal phases (H_{II}) (MORITA et al., 2009; GARIDEL et al., 2015; RODRIGUES et al., 2015; MEZZENGA et al., 2019). Another relevant aspect of these biosurfactants is that the self-assembly process occurs in hierarchically ordered, which are highly dependent on two parameters, namely, temperature and concentration (MORITA et al., 2009; GARIDEL et al., 2015).

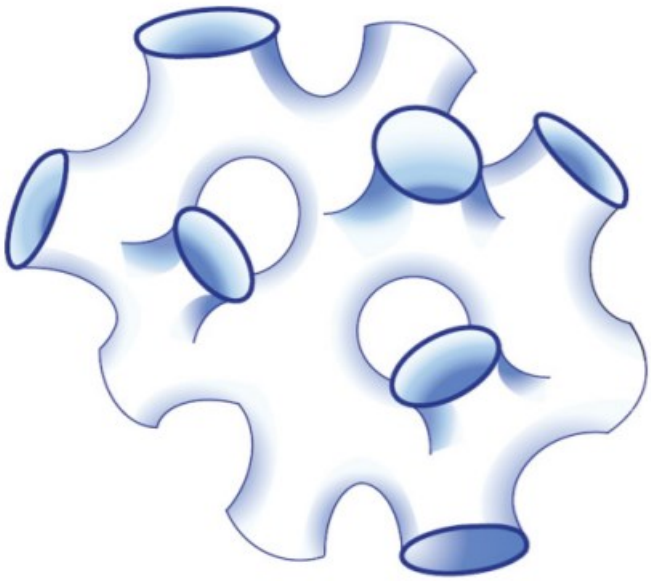
Figure 2 - Self assembled structures of biosurfactants. Figure used with permission of Royal Society of Chemistry, from [Amphiphilic designer nano-carriers for controlled release: from drug delivery to diagnostics, Salim et al. (2014), 5(11) 2014]; permission conveyed through Copyright Clearance Center, Inc.



Source: Coelho et al. 2020[a].

Thus, according to Lukic et al. (2016), the ability of MELs to easily form LLC has been increasingly exploited and represents a promising strategy for improving and expanding their biochemical diversity and applications. In this sense, small-subtle differences in the chemical structure of MEL could change their interfacial properties, surface tension, solubility enhancement, CMC, self-assembly structures, etc., and consequently, their application (FUKUOKA et al., 2012; AKBARI et al., 2018). Thus, it is necessary to understand how these events may change the biological activity of MEL. Thus, Table 2 depicts the diversification of the MEL properties due to chemical structure variants.

Figure 3 - Sponge phase arrangement. Reprinted figure with permission from [Ramón Iñiguez-Palomares, Heriberto Acuña-Campa, and Amir Maldonado, Effect of polymer on the elasticity of surfactant membranes: A light scattering study, 84.1: 011604, 2011] Copyright (2019) by the American Physical Society. doi: 10.1103/PhysRevE.84.011604.



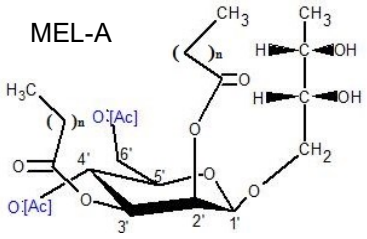
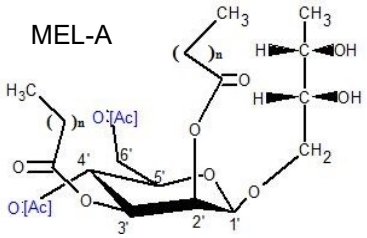
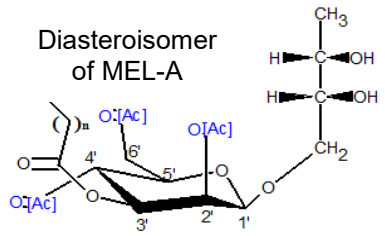
Source: Coelho et al. 2020[a].

According to Imura et al. (2007[a]), several studies about LLC phases of biosurfactants in aqueous media have been undertaken, due to their practical applications in cosmetics, pharmacy, and dispersion technology. Other research (MO et al., 2017) reached the same conclusion and reported that the different LLC structures are successfully employed as a drug carrier since they promote the solubility of the drug in injection solvent and allow the dissolution of bioactive molecules in both, aqueous and oil phases. LLC has also received increased attention due to their potential as nanocarriers for solubilizing nutraceuticals and functional foods (ZOU et al., 2017). Besides, the arrangements mentioned above may also protect the active principle from undesirable oxidation and hydrolysis process. Moreover, Mo et al. (2017) have inferred that lyotropic phases are usually well absorbed by the human body;

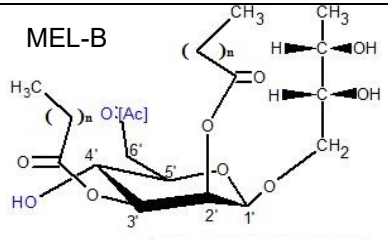
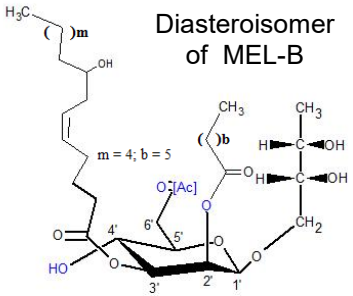
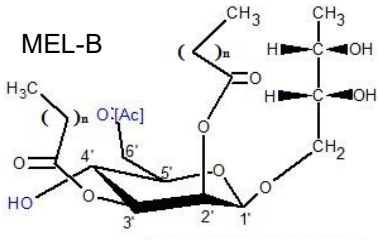
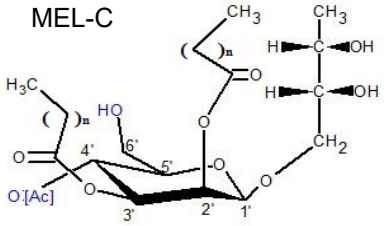
besides they are chemically stable and hardly destabilized by hydrolytic enzymes before entering in the target region.

Table 2 - Overview of chemical structure, applications, surface, and self- assembled properties of MEL.

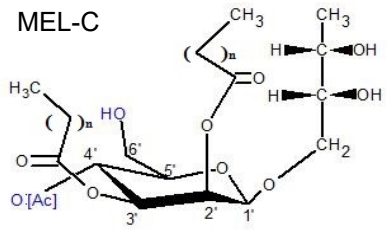
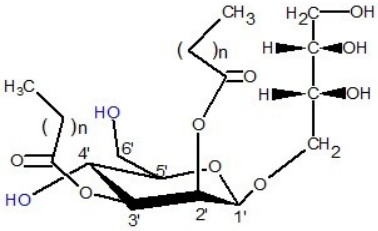
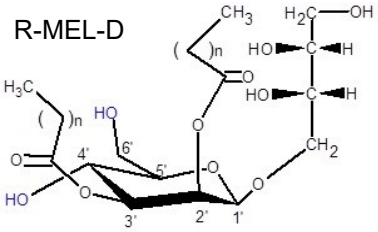
To be continued

MEL structure	CMC (M)	Self-assemble structure	Applications	Microorganism	Production parameters	References
MEL-A 	Not reported	Not reported	Improve liposomes gene transfection efficiency; antioxidant agent	*Pseudozyma aphidis* DSM70725	Culture medium: soybean oil 80 mL.L^{-1}; $MgSO_4 \cdot 7H_2O$ 0.3 g.L^{-1}; KH_2PO_4 0.3 g.L^{-1}; $NaNO_3$ 3 g.L^{-1}; yeast extract 1 g.L^{-1}. Fermentation conditions: 28 °C, 180 rpm for 7 days	Wu et al. (2019)
MEL-A 	Not reported	Not reported	Antibacterial activity against vegetative cells and spores of gram-positive *Bacillus cereus*	*Pseudozyma aphidis*	Culture medium: soybean oil 96.8 mL.L^{-1}; yeast extract 1.5 g.L^{-1}; peptone 1.0 g.L^{-1}; $NaNO_3$ 1.5 g.L^{-1}; $MgSO_4 \cdot 7H_2O$ 0.6 g.L^{-1}; $MnSO_4$ 0.1 g.L^{-1}; $CaCl_2$ 0.03 g.L^{-1}. Fermentation conditions: 28 °C, 180 rpm for 7 days	Shu et al. (2019)
Diasteroisomer of MEL-A 	5.2. 10^{-6}	Sponge (L_3); bicontinuous cubic (Q_{II}); lamellar (L_α); neat surfactant phase (S)	Oil-in-water type emulsions, water-based moisturizers, and washing detergents.	*Pseudozyma crassa* CBS 9959^T	Culture medium: 4% soybean oil; 0.3% $NaNO_3$; 0.03% $MgSO_4$; 0.03% KH_2PO_4; 0.1% yeast extract (pH 6.0). Fermentation conditions: 25 °C, 200 rpm for 7 days	Fukuoka et al. (2008)

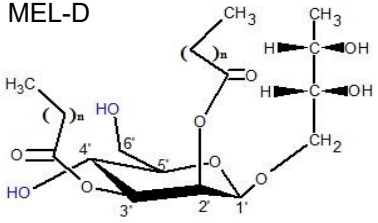
To be continued

MEL-B 	Not reported	Lamellar phase (L_α) Large vesicles (diameter 1-5 µm)	Drug, gene and transdermal delivery systems.	*Pseudozyma tsukubaensis* NBRC 1940	Commercial MEL-B (TOYOBO CO., LTD.)	Worakitkanchanakul et al. (2008)
Diasteroisomer of MEL-B 	$2.2.10^{-5}$	Sponge phase (L_3)	Not report	*Pseudozyma tsukubaensis* NBRC 1940	Culture medium: 10% ($w.v^{-1}$) castor oil; 0.3% ($w.v^{-1}$) $NaNO_3$; 0.03% ($w.v^{-1}$) $MgSO_4.7H_2O$; 0.03% ($w.v^{-1}$) KH_2PO_4; 0.1% ($w.v^{-1}$) yeast extract at pH 6.0 Fermentation conditions: 25 °C, 180 rpm for 7 days	Yamamoto et al. (2015)
MEL-B 	$3.7.10^{-6}$	Lamellar phase (L_α)	Skincare	*Ustilago scitaminea* NBRC 32730	Culture medium: 700 mL of sugarcane juice; 1 $g.L^{-1}$ of urea. Fermentation conditions: 25 °C, 600 rpm, 0.5 vvm of aeration.	Morita et al. (2011)
MEL-C 	4.10^{-6}	Lamellar phase (L_α)	Oil-in-water lype emulsifiers and/or washing detergents	*Pseudozyma graminicola* CBS 10092	Culture medium: 8% $w.v^{-1}$ soybean oil; 0.3% ($w.v^{-1}$) $NaNO_3$; 0.03% ($w.v^{-1}$) $MgSO_4.7H_2O$; 0.03% ($w.v^{-1}$) KH_2PO_4; 0.1% ($w.v^{-1}$) yeast extract at pH 6.0	Morita et al. (2008)

To be continued

MEL-C 	6.10^{-6}	Lamellar phase (L_α)	Washing detergents and oil-in-water-type emulsifiers	*Pseudozyma hubeiensis*	Culture medium: 40 $g.L^{-1}$ soybean oil; 3 $g.L^{-1}$ $NaNO_3$; 0.3 $g.L^{-1}$ $MgSO_4$; 0.3 $g.L^{-1}$ KH_2PO_4; 2.0 $g.L^{-1}$ yeast extract (pH 6.0).	Konishi et al. (2008)
S-MEL-D 	$7.1.10^{-6}$	Lamellar phase (L_α)	Not reported	Enzymatic synthesis (Novozym 435) of MEL-D from MEL-B diastereomers.	MEL-B diastereomers, produced by *Ustilago scitaminea* and *P. tsukubaensis* were hydrolyzed by lipase Novozym 435 (7 days at 60 °C) for removal of acetyl group.	Fukuoka et al. (2012)
R-MEL-D 	$1.2.10^{-5}$	Lamellar phase (L_α)	Not reported	Enzymatic synthesis (Novozym 435) of MEL-D from MEL-B diastereomers.	MEL-B diastereomers, produced by *Ustilago scitaminea* and *P. tsukubaensis* were hydrolyzed by lipase Novozym 435 (7 days at 60 °C) for removal of acetyl group.	Fukuoka et al. (2012)

Conclusion

MEL-D 	$1.2.10^{-5}$	Lamellar phase (L_α)	Drug, gene and transdermal delivery systems	Enzymatic synthesis (Novozym 435) of MEL-D from MEL-B	Reaction medium containing 5 g of MEL-B and 2.5 g of Novozym 435 was dispersed in 90% ethanol (100 mL), and the reaction mixture was stirred for 7 days at 60 °C.	Fukuoka et al. (2011)

Source: Coelho et al. 2020[a].

In this context, the aqueous phase behavior of MEL-A was investigated in the previous study (IMURA et al., 2007[a]) and it was shown that this biosurfactant could self-assembles into a several liquid crystal phase, including sponge (L_3), bicontinuous cubic (Q_{II}), and lamellar (L_α). The results of freeze-fracture electron microscopy (FFEM) and Small-Angle X-ray Scattering (SAXS) analysis showed that the development of the L_3 phase was observed at low MEL-A concentration (< 56 wt %). In the 56-65 wt % range, a highly viscous Q_{II} phase was identified. With the increase of MEL-A concentration (65-87 wt %), the arrangement correspondence to the L_α phase was obtained.

The Q_{II} structure is described by Assenza and Mezzenga (2018), who report it as a single lipid bilayer formed by two interpenetrating and non-communicating periodic channel networks, filled with water. Chen et al. (2014) disclosed the use of this highly ordered structures as a slow-release matrix for drugs composed by active principles with distinct size and polarity. Regarding the L_3 phase, it can be described as a flexible 3D nanostructure, having large aqueous pores. This malleable structure makes the L_3 phase interesting for applications involving drug delivery, protein encapsulation/crystallization, electrochemical biosensors (VALLDEPERAS et al., 2016).

When MEL monomers are in contact, in an aqueous system, they tend to self-organize into an amphiphilic monolayer system (micelles). These classical spherical particles can spontaneously be organized by raising the amphiphile concentration into three LLC structures: (i) hexagonal, (ii) cubic, and (iii) lamellar phases (MO et al., 2017). This last phase is widely studied by their great potential in cosmetic science since it can promote skin hydration, which is due to their structural organization that is quite similar to that observed in the stratum corneum lipidic structure, thus providing the skin hydration. Regarding technological aspects, this structure can serve as emulsion stabilizers (TERESCENCO et al., 2018).

Besides, Tran et al. (2018) highlighted that the transition between L_α and Q_{II} phases is characteristic features of several cellular processes in which there are alterations in membrane topology, such as endocytosis and membrane budding.

MELs also decrease, efficiently, the surface and interfacial tension at the interface between liquids, solids, and gases. Despite the differences in chemical aspects, all variants of MEL have excellent surface properties, although the mono-acylated MELs - MEL-B (CMC 4.5×10^{-6} M) and MEL-C (CMC 4.0×10^{-6} M) - have

lower surface activity compared to the diacylated compounds (CMC $2.7x10^{-6}$ M) (MORITA et al., 2009). Generally, CMC increases as the length of the hydrophobic part of the surfactant increases, exhibiting an opposite behavior when unsaturation and branching are increased. These parameters, jointly with the cohesiveness of the hydrophobic groups in the compound, significantly influence the effectiveness of biosurfactants (MNIF; GHRIBI, 2015).

Kitamoto et al. (1993) investigated the surface (air-water) and interfacial (n-tetradecane-water) properties of MEL-A and MEL-B. The data showed that the CMC value of MEL-A was smaller than that of MEL-B at the surface. This performance indeed was expected since the hydrophobicity of MEL is increased when the acetyl group in the mannose moiety increase. Oppositely, MEL-B CMC value at the interface was smaller than diacetylated MEL-A, which is due to differences in a partition coefficient to n-tetradecane between both MEL structures.

The surface and self-assembly properties of new MEL-C consisted of mainly C_6, C_8, and C_{14} fatty acids were reported by Morita et al. (2008)[a]. The monoacetylated compound showed CMC and surface-tension values of $4.0x10^{-6}$ M and 24.2 mN m^{-1}, respectively. This result was expected and confirms the higher hydrophilicity character of MEL-C compared to MEL-A, which possess the CMC value of $2.7x10^{-6}$ M and surface tension of 28.4 mN m^{-1}. Moreover, the water-penetration scan analysis demonstrated that the new MEL-C efficiently formed the lamellar phase (L_α) at a wide range of concentrations, indicating their potential for application in oil-in-water type emulsifiers and washing detergents.

Interestingly, Niu et al. (2018), demonstrated that MEL-A that was produced (*P. aphidis* DSM 70725) using soybean and waste cooking oil as alternative culture media, presented the same CMC value (20 mg L^{-1}), despite the differences in the length of hydrophobic chains. Soybean oil induced the synthesis of more short-chain fatty acids - C6:0 (23.19%), C10:0 (47.49%), C12:0 (5.94%), C18:1 (7.37%), and C18:2 (9.57%) - in MEL structure, whereas waste cooking oil induced longer chain fatty acids of MEL - C10:0 (6.66%), C16:0 (7.92%), C18:1 (59.78), and C18:2 (13.78%). Thus, an important point regarding the experimental data is that the carbon source induced the formation of different fatty acids in the MEL structure, but they did not influence the surface tension of the obtained compounds.

Studies developed over the past decade have highlighted that the lyotropic liquid crystalline phases based on self-assembly, provides some information about

the molecular recognition and protein stabilization effects of MEL, which is fundamental to their application for cosmetics and pharmaceutical industries (IMURA et al., 2007[a]). According to Morita et al. (2009), the absence or presence of the acetyl group on the head group of MEL causes alterations in their spontaneous curvature and modifies the direction of self-assembly MEL structure. Additionally, Imura et al. (2008) emphasized that the density and orientation of MEL-A assembled monolayer play an essential role in the feasibility of MEL as high-affinity carbohydrate ligand, in several classes of immunoglobulins (M, A, and G).

Kitamoto et al. (2009) presented results indicating that the presence of a single acetyl group in the chemical structure of MEL, that is, MEL-B or MEL-C, induces a spontaneous formation of giant unilamellar vesicles with a diameter greater than 10 μm. When lamellar liquid crystalline is dispersed homogeneously and vigorously in an excess of solvent - usually water - the parallel morphologies of this phase acquire a new conformation, called vesicles or liposomes, which is characterized by a closed ring that isolate the sphere inside of solvent from the continuous part. Such a structure has been capturing the attention of the cosmetic field since they contribute to improving the solubilization of active substances into an aqueous-based ointment without cause tissue injury (FRIBERG, 1990). Furthermore, the vesicles structuration favors their application in biophysical model studies, which aiming to determine the dynamic and structural characteristics of several cellular processes, such as, endocytosis, exocytosis, cell fusion, viral infection, and transport phenomena (KITAMOTO et al., 2009).

Besides, the chemical structure of conventional MEL (A, B, C, and D) and its homologues, give specific phase behavior and distinct self-assembled structures of these compounds in aqueous solutions (FUKUOKA et al., 2008). MEL-A, for example, spontaneously forms an L_3 phase in a wide range of concentrations. However, a change in fatty acid profiles of MEL-A gives rise to a homologous compound, which in turn is highly hygroscopic, compared to conventional one, presenting three or more crystal phases such as L_3, Q_{II}, L_α and the neat surfactant phase (S). As a consequence, alterations in the biological properties of glycolipid were observed, while MEL-A shows a high binding affinity for human immunoglobulin G, the MEL-A diastereomer showed no affinity for this structure (FUKUOKA et al., 2008).

A tri-acetylated derivative (MEL-A2), produced by *P. churashimaensis* from glucose, presented different aqueous phase behaviors compared to conventional MEL-A. The former forms mainly the L_3 together with the Q_{II} and hexagonal (H_{II}) phases, while the latter quickly forms only the L_α phase (MORITA et al., 2011).

The sponge phase, also called coacervates, is a phase that presents a complex synthesis path since their protocol requires complicated multicomponent systems such as surfactants with salt/co-solvent or two oppositely charged polyelectrolytes, which also hinders its characterization. MEL-A is the first natural compound to display the formation of coacervates by only themselves, whereas MEL-B and MEL-C at the same range concentration exhibit formation of vesicles (KITAMOTO et al., 2009). This structure was explored in the work of Zou et al. (2017), as drug loading/release of *Brucea javanica* seed oil (BJO), a traditional Chinese herbal medicine with antitumor activity. It was observed that the L_3 arrangement was successfully employed on the BJO encapsulation process, with efficiencies higher than 90%. Moreover, in vitro cytotoxicity and apoptosis studies demonstrated that the encapsulated drug was about 2.4 times more efficient against A549 human lung carcinoma cell line in comparison to that of the free drug (ZOU et al., 2017).

H_{II} phase can be morphologically described as a structure composed of elongated cylinders arranged in a hexagonal form (LANCELOT et al., 2014). H_{II} liquid crystal structure was explored in the work of Gabr et al. (2017), with two objectives, improve oral absorption of rosuvastatin and potentiate the biopharmaceutical effects of them. The association of H_{II} phase and rosuvastatin provided an increase in the intestinal permeation of the drug. Furthermore, *in vivo* experiments showed that the conjugation of these two compounds promoted a high level of ROS in the plasma for approximately 8 hours, a performance superior to the commercial tablet used as a control condition.

A study exploring the aqueous-phase behavior of MEL-B was conducted by Worakitkanchanakul et al. (2008). The authors proved that MEL-B self-assembly into a lamellar (L_α) phase over remarkably wide concentration and temperature ranges. Moreover, Small-angle X-ray scattering (SAXS) analyses revealed that at low MEL-B concentration (≤ 60 wt %), the interlayer spacing of the sample was constant, with approximately 4.7 nm. At this condition, the L_α phase coexists in equilibrium with the excess water phase (L_α + W). It is also observed that as the MEL-B concentration

increase (> 60 wt %), the interlayer spacing is minimized to 3.1 nm, and only the L_α phase was detected. In a complementary way, by DSC experiments, the authors noted that H-bond formation by water molecules demonstrates an important role in the stability retention of L_α phase after thermal stress tests, conducted in the range of 5 – 95 °C at the rate of 1 °C min^{-1}. Mezzenga and co-workers (2019) mention that a minimal amount of water is needed to stabilize not only the lamellar but also bicontinuous cubic, and inverse hexagonal phases.

Additionally, by confocal laser scanning microscopy (CLSM), Worakitkanchanakul et al. (2008) identified large vesicles (1 – 5 µm) at low MEL-B concentration (< 10 wt.% MEL-B). It is generally difficult to obtain giant vesicles from glycolipids since this structure requires strictly balanced between hydrophobic and hydrophilic groups (Varvaresou and Iakovou 2015). Besides, the glucose dialysis method evidentiated the entrapped volume of MEL-B vesicle, which was around 0.42 µL $µmol^{-1}$. These features make this compound very attractive as active ingredients in skincare smoothing products, as well as applications in drug, gene, and transdermal delivery systems (WORAKITKANCHANAKUL et al., 2008; VARVARESOU; IAKOVOU, 2015).

Previous studies have reported that MEL-C produced by *Pseudozyma siamensis* CBS 9960, exhibits hexagonal and lamellar lyotropic liquid crystalline phases, at different concentrations (MORITA et al., 2008[b]). Konishi et al. (2008) demonstrated that MEL-C synthesized by *Pseudozyma hubeiensis*, from soybean oil, possesses different self-assembling properties, with myelines and lamella (L_α) phases being formed in aqueous solution. The authors also demonstrated that emulsifying index of MEL-C and MEL-A, in soybean oil and a mixture of hydrocarbon (2-methylnaphtarene/n-hexadecane) emulsion, are quite similar, with both glycolipids showing a higher performance as that of conventional surfactants (LAS, Tween80, and SDS). Finally, Konishi et al. (2008) concluded that the characteristics described for MEL-C could be an indication of their potential for application in washing detergents and oil-in-water-type emulsifiers.

Regarding aqueous phase behavior experiments, the results demonstrate that MEL-D is likely to self-assemble into L_α structure under broad concentration ranges. A similar phase structure was reported for MEL-B differing for the concentration boundary (FUKUOKA et al., 2011).

4.7 INTERACTION OF MEL WITH PROTEIN AND POLYMERS

In the previous sections, it was argued that MELs exhibit excellent surface and interfacial activity, beyond their self-assembly and emulsifying abilities, which are relevant parameters for cosmetics and drugs industries. Notwithstanding, these properties vary with MEL structural diversity, concentration, temperature, and also with their molecular association (MADIHALLI et al., 2016; FAN et al., 2018).

Fan et al. (2018), reported the action of MEL as activator/inhibitor of β-glucosidase, noting that this glycolipid also modified the secondary structure and physicochemical properties of mentioned enzyme. When the concentration of MEL was equal or lower than CMC (20.0 ± 5.0 μM) MEL-A enhanced the β-glucosidase activity, acting, however, as an inhibitor at higher concentrations (> 20.0 μM). Moreover, differential scanning calorimetry, circular dichroism spectroscopy and isothermal titration calorimetry analysis, proved that MEL increase the thermal stability of β-glucosidase, since midpoint temperature went from 73.8°C to 76.6°C. Finally, the secondary structure of enzyme was changed when MEL-A was used at CMC. At this specific condition, the percentage of α-helice structure was reduced while β-sheet conformation was increased.

Inoh et al. (2010) studied MEL-A with three different unsaturated fatty acid ratios (9.1%, 21.5%, and 46.3%) were designed, and employed as a cationic liposomes' component. The transfection efficiency followed the order: MEL-A (21.5%) >MEL-A (46.3%) > MEL-A (9.1%), which could be justified by differences in the membrane fusion process and subsequent DNA release. When the fatty acid ratio was 21.5% and 46.3%, the liposomes have similar membrane fusion activities, although, at the 46.3% ratio, the membrane did not deliver DNA into the nucleus of target cells (NIH-3T3). Regarding MEL-A (9.1%)-containing liposomes, the rate of unsaturated fatty acids was so low that liposome could not promote membrane fusion. There is no doubt that the unsaturated fatty acid ratio strongly affects the physicochemical properties of MEL-A, which influences the tumor penetration and efficiency of drugs. As noted, the association of MEL and liposomes, could efficient enhancement both, transfection and selectivity.

Imura et al. (2008) evaluated the effect of the carbon source during the fermentation process on the self-assembling properties and affinity of MEL-A toward human immunoglobulin G. The main fatty acids were C_8 and C_{10} chains for all the three MEL molecules. However, the unsaturated fatty acids content was 0%,

9.1%, and 46.3% when using methyl myristate, olive oil, and soybean oil, respectively.

The analysis of results found indicated that MEL-A with 9.1% of unsaturated fatty acids presented a higher binding affinity for the immunoglobulin G. It may be due to the variation obtained between MELs concerning the ability to form the monolayer form self-assembly structures in the aqueous phase.

According to Rodrigues (2015), the surfactant self-assembly can occur in bulk, as well as at interfaces - gas-liquid and solid-liquid. It is well established that the molecules in monolayers can be self-organized in different ways, depending on the degree of compaction and lateral forces to which they are subjected (RODRIGUES, 2015). Thus, although all MEL-A kinds were adsorbed at the interface, only MEL-A produced from olive oil, forms monolayers, thereby promoting affinity for the immunoglobulin G (IMURA et al., 2008).

This finding was in line with the paper reported by Imura and colleagues (2007)[b] who demonstrated that immunoglobulin G is bound to the carbohydrate moiety of the MEL-A monolayer. Moreover, atomic force microscopy images and surface plasmon resonance analysis revealed that MEL-A are linked to G and M immunoglobulin by mode of multivalent membrane. Based on those previous considerations, it can be inferred that MEL-A plays an essential and efficient role as a carbohydrate ligand, not only for its self-assembling properties but also for its high affinity for glycoproteins of immunoglobulin antibodies.

Moreover, the self-assembling properties of MEL-A appear to stimulate a series of responses that can have detrimental consequences for B16 melanoma, inducing their endoplasmic reticulum (ER) stress and apoptosis (FAN et al., 2016; GOVINDARAJAN, 2018).

In a recent study, Park et al. (2018) prepared polymeric nanovehicles employing poly(ethylene oxide)-b-poly(ε-caprolactone) copolymers (PEO-b-PCL), MEL, TAT peptide (YGRKKRRQRRR-cysteamide) and N,N-Diisopropylethylamine as a precursor. The results showed that the association of asymmetric molecular geometry from two hydrophobic alkyl chains of MEL (Damy Chemical (Korea)) with PEO-b-PC, made the core of synthesized materials more flexible, and promoted a formation of smaller vehicles with a homogeneous phase. By Raman spectroscopy studies of *in vivo* skin permeation, the authors revealed that the features as mentioned above remarkably improved the polymeric nano vehicle cellular uptake

and transdermal delivery. These results are of greater relevance for cosmetics and medical industries, which can apply deformable polymeric nanovehicles in their products.

The effects of MEL-A on the heat-induced aggregation formation of β-lactoglobulin (β-lg) was evaluated by Fan et al. (2019). The finding of this study showed that biosurfactant provide noticeable thermostabilization of the β-lactoglobulin, by minimizing of aggregates commonly formed during thermal treatments process. The authors verified that the aggregation β-lg/whey protein is due to physical interactions, including hydrogen bonding and hydrophobic interactions. Once present, MEL-A acts by unbalancing such mechanism, which is explained by induction of bindings between hydrophobic acetyl or fatty acid chains with the nonpolar groups of protein side chains, which leads to the formation of self-assembling core-shell particles, with β-lactoglobulin integrating the core and MEL-A forming a shell. The complex formed decreased surface tension of β-lgat the water-air interface and improved its emulsifying properties, as well as foam formation and stability. Thus, MEL-A has proven to be a favorable surface additive to improve the functional properties of β-lg (FAN et al., 2019).

4.8 CONCLUSIONS AND FUTURE OUTLOOK

MELs exhibit a vast potential for a new generation of antimicrobial agents. Notably, they have a better performance against Gram-positive bacteria, promoting cell injury and rupture. MEL molecules can either be the main active ingredient or co-adjuvants with other antimicrobial agents and within engineered particles.

There are various health benefits of MEL glycolipid for skincare, including an increased stratum corneum water content, recovery of cells damaged by UV radiation, high whitening pigmented effect of skin spots, and control of acne disease progression.

Also, regarding pharmaceutical science, MELs favor the increase in the transfection efficiency of liposomes, namely, drug-target interactions with specific cells and tissues. Besides, they are associated with cell differentiation, cell cycle arrest, and apoptosis of leukemia HL-60, PC12, and B16 cells.

The product development for the medical, pharmaceutical, and cosmetic purposes needs to be directly linked to the MEL aggregation structure. This structure

is a fundamental characteristic since it is related to the molecular mechanism recognition and can also protect active principle from chemical and enzymatic attack.

MEL has numerous desirable features for future uses in new generations of many pharmaceutical and medical products, as antimicrobial or therapeutic agent, skincare cosmetics and so on. Notably they are produced from renewable materials and safe microorganisms, have chemical structure varieties, are active at low concentrations and have low impact over organismal and environmental, represents a promising material for green technologies.

Besides, the enzymatic modification allows the expansion and creation of new types of MEL structure, which could be engineered according to their desired biological function, increasing their niches of applications.

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5. CHAPTER 2 – EFFECTS OF MANNOSYLERYTHRITOL-LIPIDS-B ON *CUTIBACTERIUM ACNES* ATCC 6919

This chapter reports the effects of MEL-B on the *Cutibacterium acnes* ATCC 6919, an etiologic agent of acne vulgaris. This answer is required to identify the most suitable skin kind – normal, oily, dry, and combination - for MEL-B skincare properties. This chapter was submitted to the Indian Journal of Microbiology.

Abstract

Mannosylerythritol-lipids-B (MEL-B) are microbial-produced glycolipids with skincare properties, notably moisturizing, antimelanogenic, antimicrobial, and antiaging. Thus, there is a potential use of MEL-B in a formulation for treating acne-prone skin. This study investigated the effect of MEL-B against the Gram-positive bacteria *Cutibacterium acnes*. The broth macro dilution method was used to evaluate the growth of *C. acnes* (3-4 log CFU/mL), in the absence (positive control) or presence of MEL-B (128, 192, 256, and 512 µg/mL). Additionally, the leakage of genetic materials was used to determine the potential drug-induced membrane disruption of glycolipids. The amount of DNA and RNA release was quantified spectrophotometrically at 260 nm. Macro dilution technique and membrane integrity experiments showed that MEL-B does not have antimicrobial activity against *C. acnes*. Indeed, MEL-B assisted *C. acnes* growth. Ultimately, MEL-B has been reported as a remarkably active compound for skincare formulations; however, preliminarily, it should be avoided for acneic skin.

Keywords: acne vulgaris; skin microbiome; antimicrobial; glycolipid.

5.1 INTRODUCTION

Mannosylerythritol-lipids (MEL) are a class of glycolipids produced under submerged cultivation using yeasts or fungi (COELHO et al., 2020[a]). According to the degree of acetylation at C4 and C6 on the mannose moiety, MEL homologs can be classified into MEL-A, MEL-B, MEL-C, or MEL-D (COELHO et al., 2020[a,b]). MEL-A is di-acylated, MEL-B is mono-acylated at C6, MEL-C is mono-acylated at C4, and MEL-D has no acetyl group (COELHO et al., 2020[a,b]). These molecules are biosurfactant and present a variety of bioactivities, including biomedical and plant biostimulant potential (COELHO et al., 2020[a,b], MATOSINHOS et al., 2022).

MEL-B subclass has been applied into skincare formulations since it presented remarkable properties, including antimelanogenic and lightening effects, antiaging by modulation of aquaporin-3 expression, moisturizing, and enhancer of delivery transdermal of water-soluble compounds, which may be beneficial to advanced skincare formulation (COELHO et al., 2020[a,b], De ANDRADE et al., 2022). In addition, MEL-B also presented antitumoral activity (FEUSER et al., 2021).

It is worth noting that MEL-B also shows antimicrobial (COELHO et al., 2020[a]) and prebiotic (OKUHIRA et al., 2020) properties. Literature data reported that adverse changes in the skin microbiota could trigger an inflammatory process; consequently, it can culminate in diseases such as acne vulgaris caused by *Cutibacterium acnes* (LEITE; CAMPOS, 2020). In this sense, the aforementioned MEL-B properties raise questions about its viability for acne-prone skin, a condition in which the development of the pathogenic bacteria *C. acnes* is prevalent.

C. acnes is a Gram-positive, facultatively anaerobic, and fastidious bacteria (WASHBURN et al., 2022). Its pathogenicity includes several factors, such as altered follicular keratinization, follicular colonization by these bacterial cells, and inflammation of the pilosebaceous unit (WASHBURN et al., 2022; LEITE; CAMPOS, 2020).

Thus, assuming MEL-B as a promisor part of skincare formula due to moisturizing, antimelanogenic, antimicrobial, and antiaging properties; unprecedently, this study evaluated the effect of MEL-B against human skin pathogenic bacteria acne-prone strain *C. acnes* ATCC 6919.

5.2 MATERIAL AND METHODS

5.2.1 Microorganism strain and chemicals

Cutibacterium acnes ATCC 6919 was purchased from Fundação André Tosello (Brazil). MEL-B (95% purity degree, Toyobo, Japan) was produced from *Pseudozyma tsukubaensis* and purified using liquid-liquid extraction followed by silica chromatography. Other materials used were Reinforced *Clostridium* Medium (RCM, Merck), Agar (Merck), Sodium chloride (99% of purity, Dinamica, Brazil), Anaerobic chamber 2.5 L (Permution, Brazil), Anaerobac® (PROBAC, Brazil), Falcon tube (15 mL), Petri disks (90 x 1.5 mm), and polysulfone membrane 0.22 µm (Millipore).

5.2.2 Bacterial growth curve

The bacterial strain was activated by seeding it in 5 mL of RCM broth. The tube was sealed with 2 mL of liquid agar (~45 °C), as recommended by the manufacturer. Then, the culture was incubated in an anaerobic chamber with Anaerobac® at 37 °C (the optimal temperature for the growth of *C. acnes*) for 24 h.

The inoculum was prepared with the *C. acnes* from that liquid culture after 24 h of growth (early stationary phase), followed by dilution in sodium chloride (NaCl) saline solution 0.85%, reaching around 3-4 log CFU/mL. Then, these standardized inoculants were used for all assays.

In growth kinetics experiments, 100 µL of inoculum was added to tubes containing 5 mL of RCM broth. After that, 100 µL of culture was sampled at different time intervals (0, 3, 6, 9, 10, 12, 14, 18, 24, and 27 h). The samples' optical density at 600 nm was measured in a spectrophotometer (Biospectro SP220). In parallel, the samples were serially diluted with NaCl saline solution (0.85%) and plated by the double-layer poured plate method for colony forming units (CFU) determination. These Petri dishes were incubated under anaerobic conditions at 37 °C for 48 h. Then, the colonies were counted using the colony counter (SHU et al., 2021).

The Baranyi and Roberts (1994) model was fitted to experimental growth data using the *Combase* web platform (www.combase.cc).

5.2.3 Antimicrobial activity of MEL-B against *Cutibacterium acnes* ATCC 6919

The antimicrobial activity of MEL-B was screened by the broth macro dilution method, according to the standards of CLSI (Clinical and Laboratory Standards Institute) for anaerobic and fastidious bacteria.

An initial MEL-B solution (1,024 µg/mL) was prepared using RCM broth as a diluent and used as the stock solution. Then, it was two fold serially diluted to 128, 192, 256, and 512 µg/mL to a final volume of 5 mL.

A volume of 100 µL of *C. acnes* inoculum (around 3-4 log CFU/mL, item 2.2) was added to each tube containing the MEL-B solutions from 128 to 512 µg/mL, followed by incubation at 37 °C for 8 and 16 h in an anaerobic environment.

Then, for counting, samples were centrifuged at 3,500 rpm for 15 min, the supernatant was discarded, and biomass was washed twice with saline solution (0.85% NaCl) to remove residual MEL-B. Subsequently, samples were suspended in an RCM medium and vortexed for 20 s. After that, 100 µL of bacteria suspension was collected, serially diluted into saline solution, and plated in solid RCM, according to the double-layer poured plate method. The plates were incubated under anaerobic conditions at 37 °C for 48 h, and samples containing colonies between 30 and 300 were used to estimate log CFU/mL.

Bacterial suspension in RCM was used as the positive control, while tetracycline solution (4 µg/mL) was used as the negative control (ZHU et al., 2019).

5.2.4 Membrane integrity: DNA and RNA release

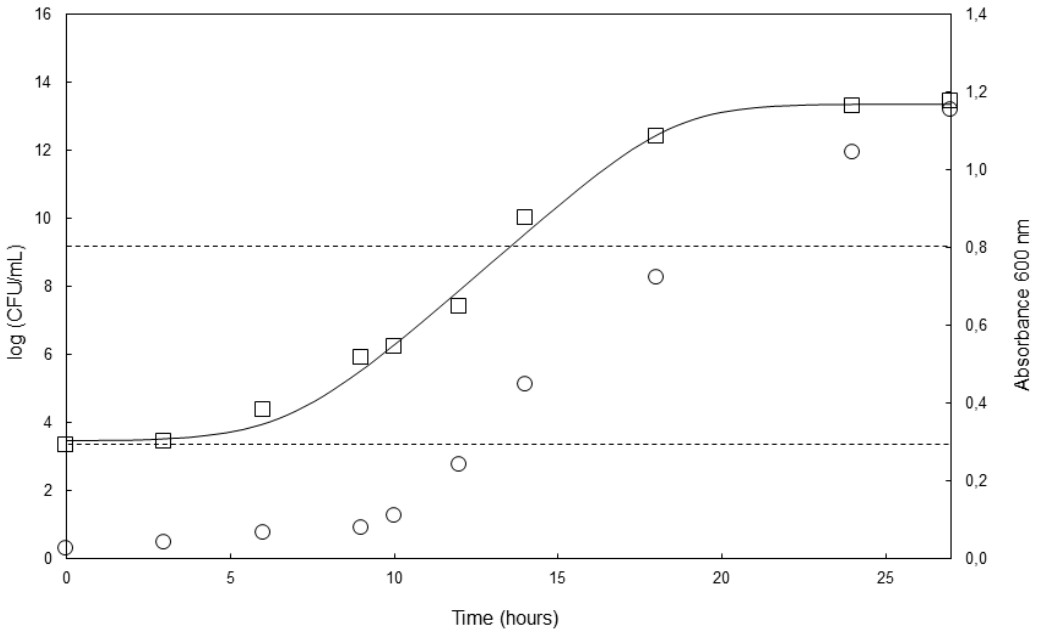
The release of as nucleic acids was used to estimate cell membrane damage (SHU et al., 2019). Thus, suspensions of *C. acnes* cultures after 8 and 16 h exposure to MEL concentrations were taken out, filtrated by a 0.22 µm syringe filter membrane, and used to quantify double-stranded DNA and RNA release in the culture supernatants. The concentration of nucleic acids was determined by absorbance measurement using NanoVue Plus spectrophotometer (260 nm). All experiments were conducted in duplicate.

5.3 RESULTS AND DISCUSSION

The Baranyi and Roberts model was fitted to experimental data of the *C. acnes* growth curve (Fig. 4) with good statistical indexes, presenting a determination coefficient (R^2) of 0.994 for CFU/mL data. Both growth curves – log CFU/mL and optical density measurements – presented a sigmoidal format containing such lag, exponential and stationary phases. However, optical density data are not trustful out of the range 0.3-0.8 (DA SILVA et al., 2019); thus, no model adjustment was used to

those values presented in Figure 4. The lag phase duration was estimated in the solid medium as 6.8 ± 0.8 h and the maximum specific growth rate for the solid culture condition was 0.77 h^{-1} (R^2 0.99). The stationary phase was observed after approximately 24 h of incubation.

Figure 4 - *C. acnes* growth curve in RCM medium by (□) plate count (CFU/mL) and (○) optical density at 600 nm. (-) Baranyi and Roberts model, and (---) optical density minimum and maximum confidence values.

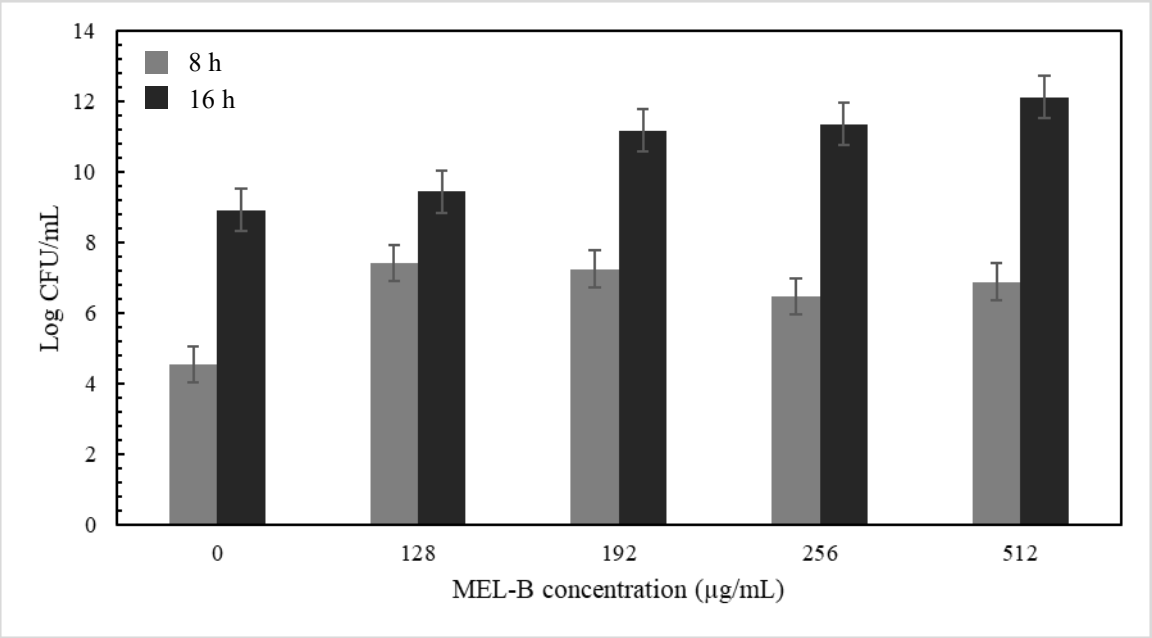


Source: The authors

The results from the treatment of planktonic cells with MEL-B (128, 192, 256, and 512 µg/mL) at the early (8 h) and late exponential phase (16 h) are presented in Figure 5. In the early exponential phase, the increase in the bacterial population occurs gradually, until the cells become capable of dividing at regular times. In the late exponential phase, the cell multiplication rate is maximum, the bacterial population is uniform, both in chemical composition and metabolic activity, as well as in other physiological and morphological characteristics. Thus, at an incubation time of 8 hours, it is possible to investigate the effect of MEL-B on young *C. acnes* cells with a low multiplication rate, while at 16 h, the performance of glycolipid can be observed on the microbial population with maximum growth rate and population uniformity.

The data showed (Figure 5) that MEL-B enhances strain growth, achieving a 2-3 log CFU/mL increase after 8 h or 16 h of incubation.

Figure 5 - *C. acnes* ATCC 6919 counts when exposed to MEL-B concentrations of 128, 192, 256, and 512 µg/mL after 8 and 16 h incubation. Error bars indicate the standard deviation.



Source: The authors.

The disintegration of bacteria membranes (COVE et al., 1983) leads to an increased genetic material release in the culture medium. MEL-B, similarly to antimicrobials such as penicillin and cephalosporins acts on the lysis of the bacterial cell, especially in Gram-positive strains (ENDLY; MILLER, 2017; TOLLENAERE et al., 2022). Tetracycline is used in the systemic treatment of acne. The drug blocks protein synthesis by binding to the site on the 30S subunit of the bacterial ribosome. Thus, tetracyclines are bacteriostatic antibiotics in usual therapeutic concentrations (ZHU et al., 2019).

Membrane integrity assays (Table 3) show that the DNA and RNA concentration for almost all study conditions slightly exceeds the control values. The DNA concentration (double-stranded) for samples treated with MEL-B ranged from

329.0 ± 0.130 to 514.5 ± 0.142 ng/µL, while the RNA concentration observed was between 174.8 ± 0.114 and 281.6 ± 0.062 ng/µL.

Thus, from the data in Figure 5 and Table 4, it can be seen that MEL-B did not cause relevant membrane damage on *C. acnes*. It is assumed that the glycolipid is hydrolyzed in the extracellular environment and, the subsequent contact by receptors, transporters, and signal transduction modulates glycolipid access to the cellular environment (YAMAUCHI et al., 2022).

Table 3 - *C. acnes* ATCC 6919 DNA and RNA release when exposed to MEL-B concentrations of 128, 192, 256, and 512 µg/mL after 8 and 16 h incubation.

MEL-B concentration (µg/mL)	**8 hours of incubation**		**16 hours of incubation**	
	DNA ± CV (ng/µL)	**RNA ± CV (ng/µL)**	**DNA ± CV (ng/µL)**	**RNA ± CV (ng/µL)**
Positive control	362.5 ± 0.080	196.8 ± 0.046	492.0 ± 0.032	267.6 ± 0.070
128	393.5 ± 0.032	191.2 ± 0.022	433.5 ± 0.093	221.6 ± 0.121
192	499.0 ± 0.053	272.8 ± 0.151	425.0 ± 0.178	215.2 ± 0.130
256	392.5 ± 0.069	208.4 ± 0.128	329.5 ± 0.130	174.8 ± 0.114
512	514.5 ± 0.142	281.6 ± 0.062	368.0 ± 0.002	196.8 ± 0.051
Negative control	467.5 ± 0.002	251.6 ± 0.033	470.1 ± 0.036	263.2 ± 0.116

Source: The authors.

From Figure 5, it is observed that MEL-B increases bacterial colonies. In this case, we speculated that glycolipid was in the intracellular space; nevertheless, it was hydrolyzed (especially the fatty acid chains) and then used as a nutrient source (the *β*-oxidation), assisting *C. acnes* growth. Therefore, MEL-B seems to be an unsuitable active compound for acne-prone skin products. According to Platsidaki

and Dessinioti (2018), *C. acnes* represent at least 30% of the facial bacteria population in patients with acne.

Further experiments can deeply elucidate the effect of MEL-B on seboregulation. It is important to notice that oily skin does not necessarily imply acneic skin (ENDLY; MILLER, 2017). According to previous reports, oily skin is a clinical condition that can be observed even in patients without acne and is related to larger facial pores (ENDLY; MILLER, 2017). However, the stimulation of sebum secretion is a key factor in triggering *C. acnes* overgrowth and inflammation mechanisms (TOLLENAERE et al., 2022). In particular, the detection and quantification of porphyrins appear to be another interesting aspect since these compounds are produced by *C. acnes* and indicate pathogenicity severity (PLATSIDAKI; DESSINIOTI, 2018).

The skin microbiome comprises at least 19 bacteria species, predominating *Staphylococci* sp., *Corynebacteria* sp., and *Propionibacteria* sp.. In this sense, several microbial niches exist in the epidermis, and *Propionibacteria* and *Staphylococci* strains are better adapted to the sebaceous microenvironments. Therefore, MEL-B cosmeceutical properties could be directed to dry and moist areas – e.g., elbows, feet, hands, and eye area (MOTTIN; SUYENAGA, 2018).

MEL-B has attractive applications in the skincare field, namely moisturizing, antimelanogenic, and antiaging. However, into the concentrations range of MEL-B between 128 and 512 µg/mL, the pathogenic bacteria *C. acnes* was stimulated to grow. MEL-B increased to 2-3 log CFU/mL in a few hours compared to positive control. Therefore, this research showed the unsuitability of this glycolipid for skincare products aimed at acne-prone skin.

Acknowledgments

This study received financial support from the Brazilian Agencies: the Coordination for the Improvement of Higher Education Personnel (CAPES) and the National Council for Scientific and Technological Development (CNPq).

Conflict interest

The authors declare that they have no conflict of interest.

Data Availability

Data available on request from the authors.

Author contributions

All authors contributed to the paper conception and design. Material preparation and data collection were performed by Ana L S Coelho and Denise A Laroque. Data analysis was performed by all authors. The first draft of the manuscript was written by Ana L S Coelho. All authors commented on previous versions, revised the language, and approved the final manuscript.

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6. CHAPTER 3 - MANNOSYLERYTHRITOL LIPIDS-B AS ANTIMICROBIAL AGAINST *LISTERIA MONOCYTOGENES* CLIST 4165 IN HOT DOG SAUSAGES

This chapter presents the activity of MEL-B against *L. monogytogenes* CLIST 4165 in liquid culture and food matrix, specifically hot dog sausage. This approach evaluates the prospect of MEL-B use as a microbial GRAS preservative to food, a relevant demand of the food industry. This chapter will be submitted to the Food Microbiology Journal.

Abstract

Mannosylerythritol lipids-B (MEL-B) is a glycolipid of microbial origin with antimicrobial properties. Despite the relevance, this is the first report investigating the potential of MEL-B as a food preservative against *L. monocytogenes* in a meat food matrix. The experiments were carried out in Trypticase Soy Broth (TSB) medium (37 °C) and hot dog sausage incubated at 15 and 30 °C. Assays in culture medium with MEL-B showed a Minimum Inhibitory Concentration (MIC) and Minimum Bacteridal Concentration (MBC) of 4 and 1,000 µg/mL, respectively. The mathematical model of Baranyi and Roberts was used to predict the bacteria growth at two temperatures (15 and 30 °C) and concentrations of MEL-B (0.01 and 500 µg/mL). In general, the treatment of hot dog sausages with MEL-B (0.01 µg/mL, 500 µg/mL at 15 °C, and 0.01 µg/mL at 30 °C) decreases the lag phase of *L. monocytogenes*, compared to samples without MEL-B. The maximum specific growth rate of strain was delayed during treatment with 0.01 µg/mL of glycolipid; 15.21 ± 1.15 1/h (15 °C) and 4.20 ± 1.06 1/h (30 °C). The experimental condition of 500 µg/mL MEL-B at 30 °C was unsuitable for delaying the bacteria growth, decreasing the lag phase, and increasing the maximum growth rate (0.436 ± 0.062 1/h). The data suggest that MEL-B could be feasible to attenuate *L. monocytogenes* growth to some extent at abusive temperature storage - a very favorable condition for the development of the strain. We demonstrated that the bacteria were inactivated by MEL-B (a natural compound) even at low concentrations. This fact can contribute to reducing the cost for possible use at a large scale.

Keywords: food safety, foodborne disease, glycolipid, meat products, predictive microbiology.

6.1 INTRODUCTION

Listeria monocytogenes is a Gram-positive, non-spore-forming, and foodborne pathogen bacterium causative agent of listeriosis (LEE et al., 2019). This foodborne disease can be contracted by ingesting contaminated food, including processed meat products (SILVA et al., 2020; DISSON et al., 2021; KOOPMANS et al., 2022). Listeriosis rarely affects healthy individuals, nevertheless symptoms (e.g., septicemia, central nervous system infections) could be severe in the patient risk group such as pregnant women, unborn babies, neonates, elderly and immunocompromised people (QUEREDA et al., 2021; DISSON et al., 2021; FILIPELLO et al., 2020).

In hot dog sausage manufacturing, the potential cross-contamination by *L. monocytogenes* can be related to raw material (slaughtering stage), food handlers (e.g., skin from carrier animals), equipment (e.g., chilling areas), food processing area (e.g., packaging), and distribution (AALITA et al., 2021; SUN et al., 2021; AJOURLOO et al., 2020; INMANEE et al., 2019).

In this regard, it is essential to monitor and control the *L. monocytogenes* to maintain the safety of food products. The control of *L. monocytogenes* development in ready-to-eat foods is still a challenge since this microorganism grows in a wide range of temperatures, and easily adapts to stressful conditions (e.g., acidity, cold temperature, and high salt concentration), which entail cell reprogramming (KOOPMANS et al., 2022; QUEREDA et al., 2021; PARK et al., 2023; RICCI et al., 2022).

MEL-B is a glycolipid of microbial origin, mainly produced by *Pseudozyma tsukubaensis*. In its natural habitat, this microorganism excretes the glycolipid to prevent competition with other microorganisms for food or space (MORITA et al., 2014; COELHO et al., 2020[a]). In this sense, the literature reports the antimicrobial activity of MEL-B for some clinical strains - e.g., *Micrococcus luteus*, multidrug-resistant *Staphylococcus aureus*, and *Enterococci* (MANIGLIA et al., 2019). However, the antimicrobial activity of this glycolipid is rarely reported for spoilage and foodborne pathogenic bacteria (COELHO et al., 2020[b]; DE ANDRADE et al., 2022).

Shu et al. (2019) investigated the antimicrobial activity of MEL-A against vegetative cells and spores of *Bacillus cereus*. The authors observed that glycolipid led to disruption of the cell membrane of vegetative cells and prevented the

germination of spores at a minimum inhibitory concentration (1.25 mg/mL). Liu et al. (2020) evaluated the antimicrobial activity of MEL-A against *L. monocytogenes* in food matrices, namely whole milk and skimmed milk. The finding suggested that 1,024 µg/mL of MEL was needed to achieve an *L. monocytogenes* reduction of 2 log CFU/g at 12 and 24 h. Moreover, it was observed that the fat content of milk does not affect the biological activity of MELs, which is commonly considered an advantage in the use as an antimicrobial in foods.

Thus, the current contribution aimed to evaluate the effects of MEL-B against *L. monocytogenes* cultured in frankfurter-style sausage (hot dog franks). For this, the antimicrobial assays were first carried out in a culture medium to determine the strain's susceptibility to the antimicrobial agent. Then, we investigated whether and how the magnitude of MEL-B concentration affects *L. monocytogenes* cultivated under favorable temperature conditions (30 °C). Finally, a mathematical model was fitted to the growth data of *L. monocytogenes* and estimated the kinetic parameters at two different MEL-B concentrations (0.01 and 500 µg/mL) and temperature incubation (15 and 30 °C).

6.2 MATERIAL AND METHODS

6.2.1 Bacterial strain and growth curve

The *L. monocytogenes* strain (CLIST 4165) used in this study was isolated from human skin and kindly donated by FIOCRUZ (Fundação Oswaldo Cruz, Brazil). Frozen (- 80 °C) stock culture was sub-cultured in Trypticase Soy Broth (TSB) for 6 h (210 rpm, 37 °C) and then plated on Agar Listeria Ottaviani-Agosti (ALOA) (Neogen).

The bacterial growth curve was determined by the count plate method. An isolated colony of *L. monocytogenes* (48 h) was inoculated into 10 mL of sterile TSB (37 °C, 210 rpm). Then, for the count plate method, aliquots were taken out at specific times (0, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420, 480, and 540 min), serially diluted in peptone water (0.1%), and plated on Trypticase Soy Agar (TSA) Petri dishes. Subsequently, the samples were incubated at 37 °C for 48 h. Following incubation, the colony-forming unit (CFU) was counted. The results were expressed in a log (CFU/mL).

6.2.2 Antimicrobial assays in culture medium

The antimicrobial tests were first performed in a growth medium containing different MEL-B concentrations. The antimicrobial solutions of MEL-B at concentrations of 1,000, 2,000, 4,000, 8,000, and 10,000 µg/mL were prepared into sterile Falcon tubes by dissolving a corresponding MEL-B mass in 20 mL of TSB (~50 °C), which was vortex mixed until homogenization. The stock solution (1,000 µg/mL) was diluted to obtain 10 mL of concentrations 0, 4, 8, 16, 32, 64, 128, 256, and 512 µg/mL of MEL-B in TSB.

The samples containing MEL-B were inoculated with 100 µL of *L. monocytogenes* inoculum (around 7 log CFU/mL), incubated under agitation (210 rpm) at 37 °C, and taken at 3 and 6 h. The serial dilutions were realized, and 100 µL of *L. monocytogenes* culture was plated on Trypticase Soy Agar (TSA) and incubated at 37 °C for 48 h, to estimate the numbers of CFUs.

The lowest concentration of MEL-B required to inhibit the visible growth of bacteria was recorded as the Minimum Inhibitory Concentration (MIC). The Minimum Bactericidal Concentration (MBC) was expressed as the lowest concentration that kills bacterial population completely (SHU et al., 2019).

6.2.3 *L. monocytogenes* growth kinetics in hot dog sausages with MEL-B

The hot dog sausages used in this study were purchased at a local market in Florianópolis/SC-Brazil. All packages were sanitized with alcohol at 70% (v/v), opened, and hot dog sausages were poured into sterile plastic trays for homogenization. Afterward, the samples were manually inspected, and material with any cracked or chipped was discarded. Next, the hot dog sausages were gently dried with a paper towel to remove residual liquid and placed in sterile 3M™ plastic bags.

Preliminary tests were conducted to define the MEL-B concentrations for kinetics growth assays. For this, MEL-B solutions (0.01, 0.10, 1.00, 10.0, 100, 500, and 1,000 µg/mL) were prepared as described above (item 6.2.2), differing in terms of diluent, which, in this case, was sterile distilled water. One hundred microliters of MEL-B solution were added to each hot dog sausage surface and manually homogenized.

Then, 100 µL of the *L. monocytogenes* inoculum at around 5 log CFU/mL was spread over the hot dog sausages. The samples were incubated at 30 °C, and

the experiments were performed in duplicate. Samples without MEL-B were used as control. All samples were incubated at 30 °C.

At 0, 20, 34, and 48 h, two samples of each MEL-B concentration and without glycolipid were taken, and 50 mL of peptone water (0.1%) was added to the samples and homogenized. Then, 100 µL were taken, diluted by serial dilution technique, and spread plated on ALOA. The Petri dishes were incubated at 37 °C for 48 h. Finally, bacterial colonies grown on plates were counted. The results were expressed as CFU/cm^2.

From the data obtained, two MEL-B concentrations were selected for kinetics experiments performed at 15 and 30 °C. The kinetics were obtained from two independent experiments performed in duplicate.

The mathematical model of Baranyi and Roberts (1994) (Equation 1) was adjusted to the data from kinetic trials to obtain growth parameters – physiological state of cells (Equation 3), lag time (Equation 4), maximum growth rate, and maximum microbial population. The fit function F(t) is represented in Equation 2.

$$F(t) = t + \frac{1}{\mu_{max}} \, logN_0 + \mu_{max}.F(t) - \log\left(1 + \frac{\exp(\mu_{max}.F(t)) - 1}{\exp(\log\left(\left(\frac{N}{N_0}\right)_{max}\right) - \log(N_0))}\right) \quad (1)$$

$$F(t) = t + \frac{1}{\mu_{max}} \log\left(\frac{\exp(\mu_{max}.t) + q_0}{1 + q_0)}\right) \quad (2)$$

$$h_0 = \ln\left(1 + \frac{1}{q_0}\right) \quad (3)$$

$$\lambda = \frac{h_0}{\mu} \quad (4)$$

Where:

N_0 – initial microbial population;

N - microbial population as a function of time t;

N_{max} - maximum microbial population observed;

μ_{max} - maximum specific growth rate (1/h);

F(t) - adjustment function;

t - time (h);

λ – lag time (h);

q_0 - parameters expressing the physiological state of cells at initial time t_0;

h_0 - express the physiological state of cells at the time of incubation.

The model was fitted to experimental growth data using software R project version 4.2.3 (biogrowth package). The RMSE (root mean squared error) (Equation 5) and R^2 (R-squared) (Equation 6) indices were used to estimate the accuracy between predicted and experimental data.

$$RMSE = \sqrt{\frac{\sum\left(value_{predict} - value_{observed}\right)^2}{n}} \quad (5)$$

$$R^2 = \frac{\sum\left(value_{predict} - value_{medium\ observed}\right)^2}{\sum\left(value_{observed} - value_{medium\ observed}\right)^2} \quad (6)$$

Where:

RMSE – root mean squared error;

$value_{predict}$ – predicted values;

$value_{observed}$ – experimental values;

n – number of observations;

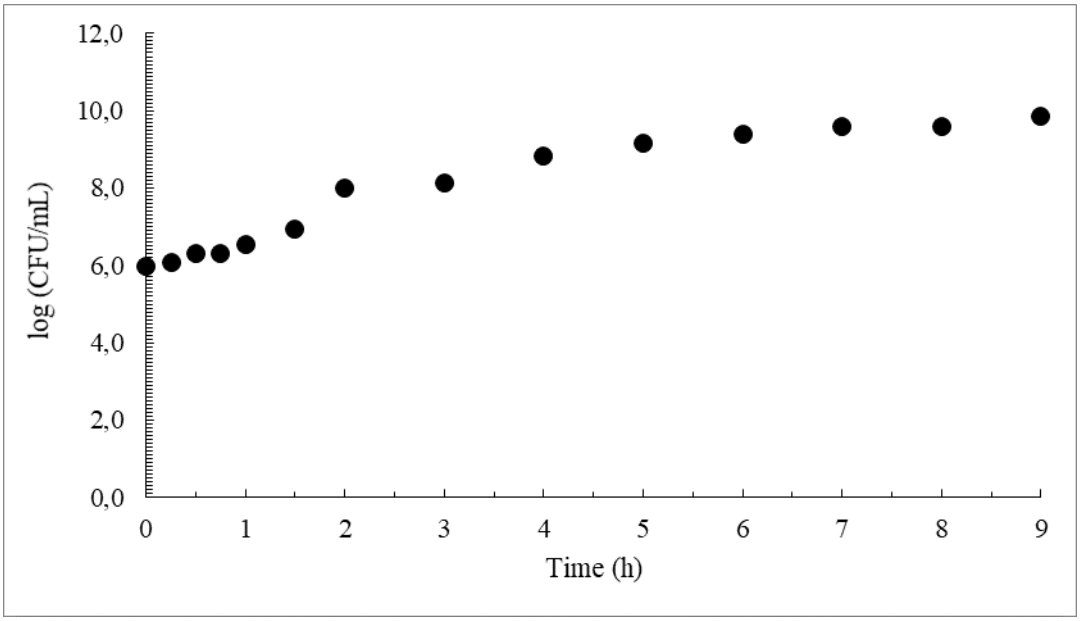
R^2 – R-squared.

6.3 RESULTS AND DISCUSSION

6.3.1 Bacterial strain and growth curve

Figure 6 shows the bacterial growth curve of *L. monocytogenes*, in TSB culture, after 9 hours of incubation (210 rpm) at 37 °C. From the growth curve, the time conditions for subsequent tests were established. The data suggest that at 3 and 6 h, the strain growth is in exponential and stationary phases, respectively.

Figure 6 - *L. monocytogenes* growth curve in TSB culture at 37 °C, determined by (●) count plate method (log CFU/mL).



Source: The authors

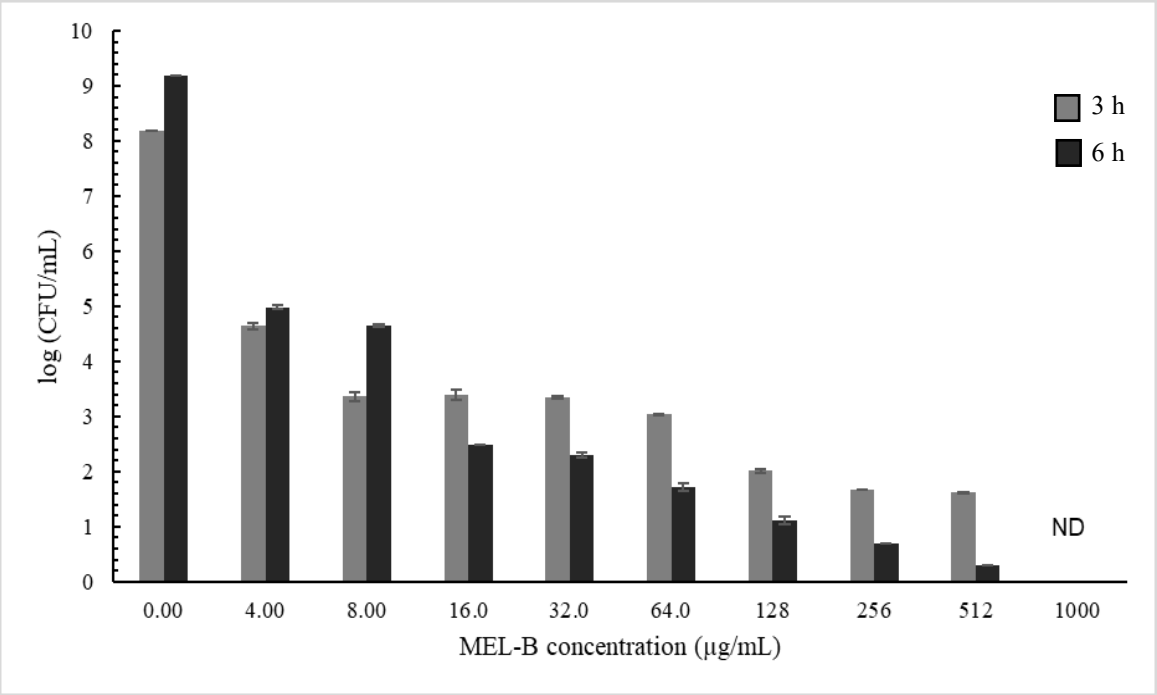
6.3.2 MEL-B antimicrobial effect against *L. monocytogenes* in culture medium

The results of MEL-B antimicrobial assays against *L. monocytogenes* in a liquid culture medium (TSB) are shown in Figure 7. The data analysis showed an antimicrobial activity of MEL-B even at the lowest concentration (4 µg/mL - MIC), achieving a reduction of around 4 log CFU/mL after 6 h of incubation. As the concentration of MEL-B increases, bacterial inactivation increases. After three hours of incubation, *L. monocytogenes* untreated and treated with MEL-B (512 µg/mL) for 3 hours showed a count of 8 log CFU/mL and 1.5 log CFU/mL, respectively, decreasing microbial growth to around 6.5 log CFU/mL. When the strain was incubated with the glycolipid (512 µg/mL) for 6 hours, the microbial growth reduction was approximately 8.5 log CFU/mL. At MEL concentrations of 4 and 8 µg/mL, the *L. monocytogenes* population decreased for both incubation conditions. However, the bacterial count was higher at 6 hours, which is not observed for other MEL concentrations.

Concentration greater than 1,000 µg/mL (MBC) completely inhibited the microorganism growth, evidenced by the null CFU on the TSA plates for both incubation times.

These findings are aligned with previous reports (LIU et al., 2020), where MEL-A (a MEL-B counterpart) presented a MIC of 32 µg/mL against *L. monocytogenes* ATCC BAA-679 in BHI broth, after overnight incubation (37 °C, 180 rpm). Chen et al. (2019) have shown that myristic acid, a compound of hydrophobic nature, has a MIC ranging from 64 to 256 µg/mL for 13 foodborne isolates of *L. monocytogenes* cultured in a TSB medium.

Figure 7 - Antimicrobial activity of MEL-B in liquid culture at 3 e 6 h against *L. monocytogenes* isolated from human skin. ND: bacterial growth not detected in count plates. Error bars indicate the standard deviation.



Source: The authors

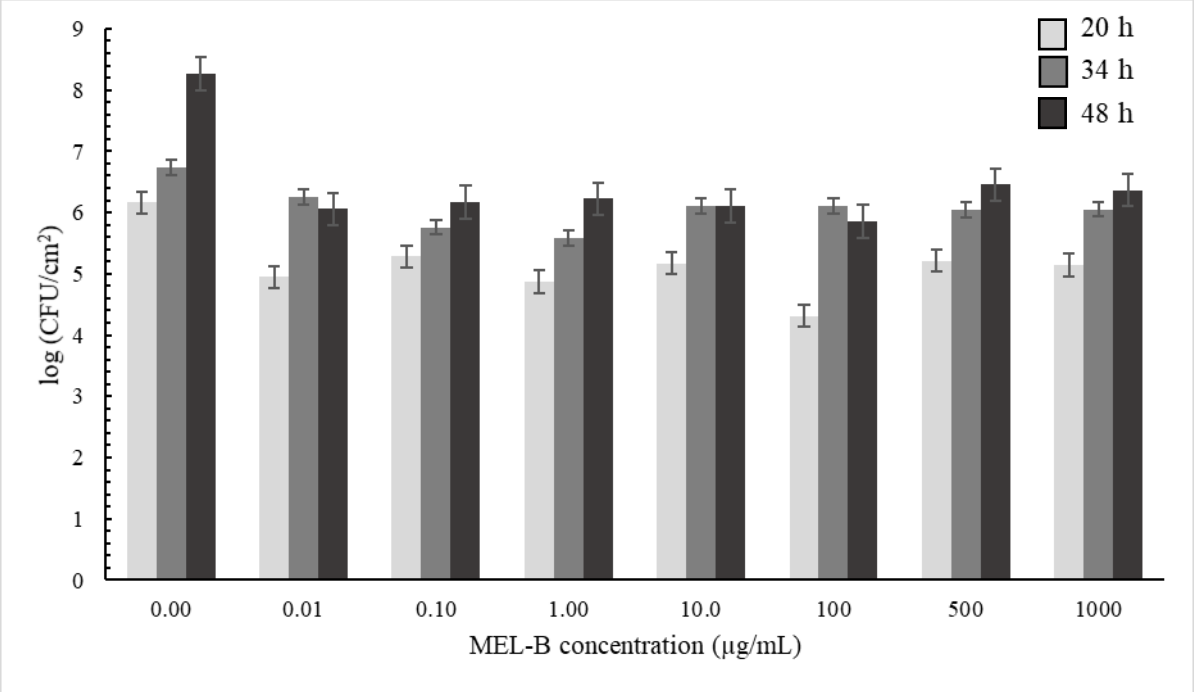
The analysis of data demonstrated the potential of MEL-B compared to other glycolipids. Sun et al. (2021) evaluated the antimicrobial activity of two glycolipids products against *L. monocytogenes* cultivated in a TSBYE (TSB supplemented with 1% yeast extract). The authors identified a MIC of 3.5 and 15 mg/L for Nagardo™ and rhamnolipids, respectively. MBC values of 4.5 g/L for Nagardo™ and 15 mg/L for rhamnolipids were obtained.

6.3.3 *L. monocytogenes* growth kinetics in hot dog sausages with MEL-B

The results of antimicrobial experiments with MEL-B on hot dog sausages (30 °C) can be seen in Figure 8. The reduction of *L. monocytogenes* ranged from 0.5

to 1.5 log CFU/cm^2 when the strain was incubated for 20 h with different MEL-B concentrations. The microbial counts for samples incubated at 34 hours with MEL-B were similar to those without the compound (6.5 log CFU/cm^2). The glycolipid concentration of 1.00 µg/mL results in a better reduction of the bacteria growth, which was approximately 1 log CFU/cm^2. Sausages incubated for 48 h without MEL-B showed a count of around 8 CFU/cm^2, while for samples with MEL, this value was generally around 6 CFU/cm^2.

Figure 8 - Antimicrobial activity of MEL-B against *Listeria monocytogenes* cultured in the hot dog sausage at 20, 34, and 48 h incubation at 30 °C. Error bars indicate the standard deviation.



Source: The authors.

The antimicrobial performance of MEL-B against *L. monocytogenes* was different when the microorganism was cultivated in a culture medium and hot dog sausage (Figures 7 and 8). According to the literature (HAMMOND; HAMMOND, 2001), the liquid culture presents some characteristics that could favor the action of antimicrobial agents. The agitation, for example, keeps the bacterial cells in suspension, minimizing the cell aggregates and potentiating a diffusion of nutrients and, probably, antimicrobial compounds.

It is known that pH, temperature, and salt concentration can affect the surface properties of biosurfactants. Niu et al. (2019) evaluated the effect of temperature (−20°C, 0°C, 25°, 50°C, 65°C, 80°C, and 95°C) for 1 and 2 h, pH (2-12) and the concentration of sodium chloride (0-30% m/v) on the surface properties of MEL synthesized from soybean oil and waste cooking oil. The authors concluded that MELs retained good surface activity, showing constant surface tensions for experimental conditions employed.

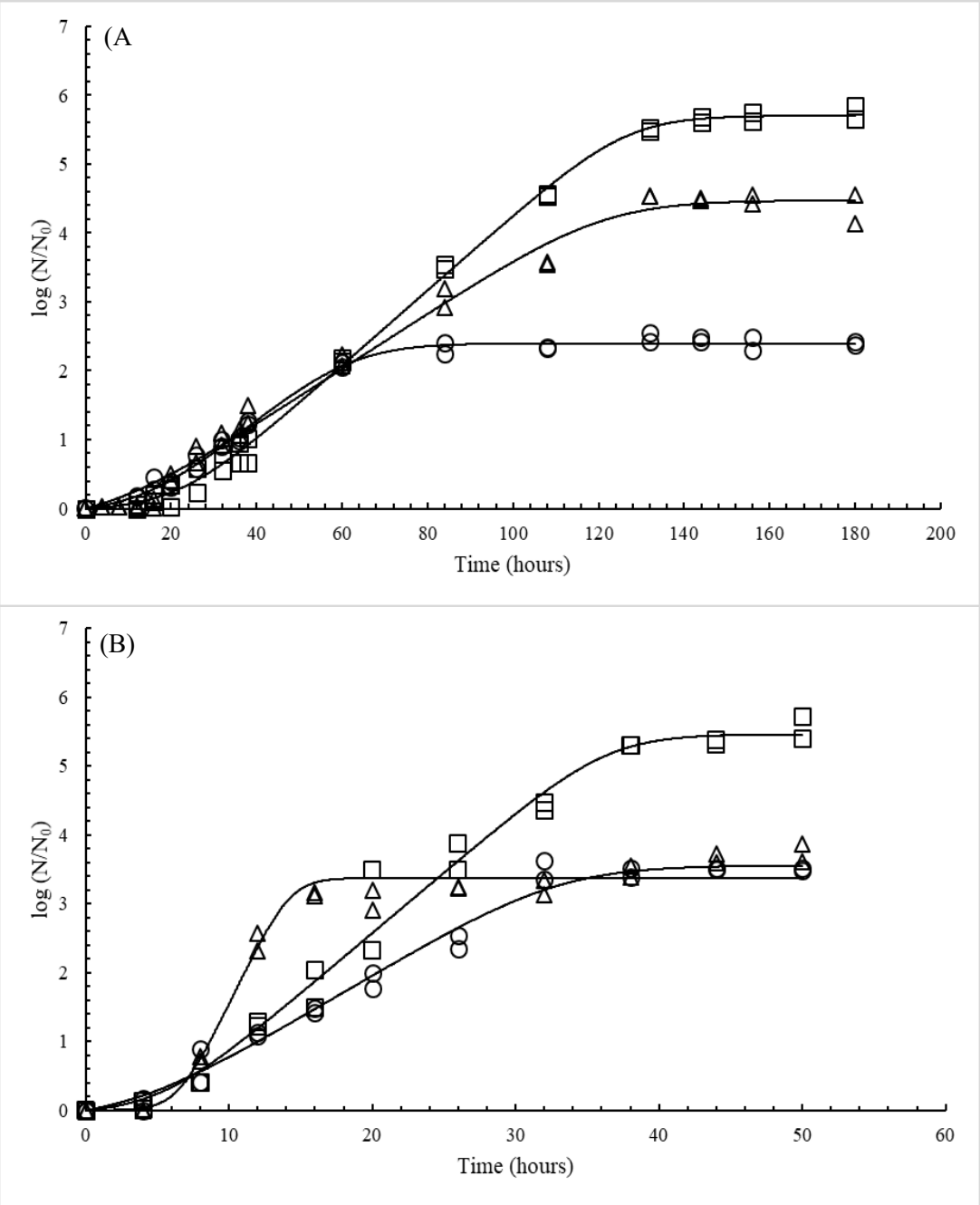
Previous studies by this research group (MASSIA, 2022) reported the physicochemical properties of Frankfurt sausages: pH of 6.36 ± 0.02, water activity of 0.985 ± 0.005, and sodium chloride (NaCl) of 2.64 ± 0.09 (% mass). The culture medium has a pH of 7.3 ± 0.2 (25°C), and a NaCl concentration of 5 g/L, according to the manufacturer's data. Thus, it is observed that the variables that affect the surface properties of MEL do not seem to differ from the conditions of culture medium and sausage. The discrepancies observed on the antimicrobial activity in the culture medium and food matrix can be due to a joint effect of the referred parameters or to some specific characteristic of the food.

In this regard, the rough surface of hot dog sausages can be a factor that contributed to this difference, which leads to preferred growth points of *L. monocytogenes*. Liu et al. (2023) showed that a rough surface of casing sausage configures a key factor for the adhesion and growth of foodborne bacteria. This feature affects the spreadability of cells, presenting different profiles, for example, dense and uniformly distributed, small sparse colonies, small clusters, scarce and uneven distribution, and random adhesion without special attachment preference.

Based on the inactivation data of *L. monocytogenes* observed in hot dog sausage, a kinetic study was carried out for the strain considering two incubation temperatures (15 and 30 °C) and two concentrations of MEL-B (0.01 and 500 µg/mL).

Figure 9A shows the kinetic profile for samples incubated at 15 °C. The treatment with MEL-B solutions of 0.01 µg/mL and 500 µg/mL resulted in a final reduction of approximately 3 log CFU/cm^2 and 1 log CFU/cm^2, respectively, compared to without the MEL-B sample. Regarding the assays at 30 °C (Figure 9B), a maximum 2 log CFU/cm^2 reduction was observed for both MEL-B concentrations at the end of the kinetics experiments.

Figure 9 - Kinetic profile of L. monocytogenes growth curve in hot dog sausage stored at (A) 15 °C and (B) 30 °C, under different MEL-B concentrations: (□) 0.00 µg/mL, (○) 0.01 µg/mL, (∆) 500 µg/mL. The line (−) represent the fit of Baranyi and Roberts model.



Source: The authors

The growth kinetics of *L. monocytogenes* in the hot dog sausage was fitted by Baranyi and Roberts model, and the primary growth parameters are shown in Table 4.

Table 4 - Primary growth kinetic parameters obtained by adjusting Baranyi and Roberts model to the experimental data, for *L. monocytogenes* growth in hot dog sausage at 15 and 30 °C with MEL-B concentrations of 0.01 and 500 µg/mL.

15 °C			
Kinetic Parameters[a]	0.00 µg/mL	0.01 µg/mL	500 µg/mL
$(N/N_0)_{max}$	5.70 ± 0.05[b]	2.40 ± 0.02	4.47 ± 0.08
λ (h)	22.03 ± 1.04	15.21 ± 1.15	9.81 ± 2.33
μ_{max} (1/h)	0.050 ± 0.001	0.052 ± 0.003	0.040 ± 0.002
h_0	1.10	0.79	0.39
30°C			
Kinetic Parameters[a]	0.00 µg/mL	0.01 µg/mL	500 µg/mL
$(N/N_0)_{max}$	5.46 ± 0.13	3.56 ± 0.08	3.38 ± 0.06
λ (h)	5.38 ± 0.98	4.20 ± 1.06	6.42 ± 0.61
μ_{max} (1/h)	0.176 ± 0.010	0.124 ± 0.009	0.436 ± 0.062
h_0	0.95	0.52	2.80

[a]$(N/N_0)_{max}$: maximum cells concentration; λ: lag time; μ_{max}: maximum specific growth rate; h_0:physiological state of cells. [b]Estimated growth parameters ± confidence interval.

Source: The authors

It was noticed that all the adjustments of the model to the data gave R^2 higher than 0.97 and RMSE values lower than 0.193, indicating the goodness of the fit (Table 5).

The kinetic parameters (Table 4) showed that most MEL-B concentrations decrease the h_0 and lag time parameters, delaying the maximum cellular concentration $(N/N_0)_{max}$ and maximum specific growth rate, compared to samples without MEL-B. Previous findings (PAL et al., 2008) showed that *L. monocytogenes* DUP-1044A was able to grow in air-packaged and vacuum-packaged frankfurters sausages processed with antimicrobials sodium diacetate (0.2%) and potassium lactate (2%) and storage at 12 °C. In this case, similarly to that observed with MEL-B,

the antimicrobial performance at abusive temperature was considered more bacteriostatic than bactericidal (PAL et al., 2008).

Table 5 - Statistical indices, RMSE and R^2, obtained from experimental and predicted data of *L. monocytogenes* growth in hot dog sausage at 15 and 30 °C with MEL-B concentrations of 0.01 and 500 µg/mL.

15 °C			
Statistical indice	0.00 µg/mL	0.01 µg/mL	500 µg/mL
R^2	0.99	0.99	0.98
RMSE	0.113	0.061	0.106
30°C			
Statistical indice	0.00 µg/mL	0.01 µg/mL	500 µg/mL
R^2	0.99	0.99	0.99
RMSE	0.119	0.085	0.108

Source: The authors

In addition to the antimicrobial properties of MEL-B, the amphiphilic nature of this compound can modify the bioavailability of water-insoluble substrates, limiting bacterial growth due to the interfacial surface between water and substrate (FUKUOKA et al., 2012; WORAKITKANCHANAKUL et al., 2008). Morita et al. (2014) point out that yeasts of *Pseudozyma* genera (the best MELs producers) in their natural habitat excrete that glycolipid precisely to prevent other microorganisms from adhering to the leaves of plants.

A peculiar behavior was observed when bacteria were treated with 500 µg/mL of MEL-B and incubated at 30 °C. There was an extension of the adaptation phase (lag phase), which went from 5.38 ± 0.98 h to 6.42 ± 0.61 h, μ_{max} increase expressively, from 0.176 ± 0.010 1/h to 0.436 ± 0.062 1/h. Moreover, h_0 value was 2.80 and 0.95 for samples with and without MEL-B, respectively. A similar result was observed in beef frankfurters treated with nisin. The exposure of *L. monocytogenes* to this antimicrobial triggered a stress response, culminating in higher μ_{max}, which was reported as a survival strategy (KUMARI et al., 2020). Furthermore, the genes responsible for the motility of *L. monocytogenes* can be overregulated at 30 °C (ZHANG et al., 2021), which probably facilitated the contact and colonization of the hot dog sausage surface by the strain (PARK et al., 2023).

Thus, it was concluded that MEL-B reduced the population of *L. monocytogenes* cultivated in hot dog sausage. It is important to notice that in realistic environments, adapting bacterial strains from one habitat to another could be a limiting factor for obtaining high cell counts (KALLIPOLITIS et al., 2020; RICCI et al., 2022). For example, Gonzalez-Fandos et al. (2021) mention that the natural *L. monocytogenes* contamination for dry-cured sausages is usually below 2 log CFU/g, with minor occurrences above this value.

This is the first study to address the effect of MEL-B as an antimicrobial agent in hot dog sausage. The investigation of the emulsifying activity of this compound in sausage formulation can be explored in the future jointly with its microbial activity.

6.4 CONCLUSION

Experiments based on culture medium demonstrated a remarkable antimicrobial activity of MEL-B against *L. monocytogenes* in a wide range of concentrations. The MIC and MBC values found were 4 and 1,000 µg/mL, respectively. The antimicrobial performance of glycolipid was less expressive when *L. monocytogenes* was cultured in hot dog sausage. The kinetic parameters revealed that treatment with MEL-B at 15 and 30 °C mainly decreased the lag time of *L. monocytogenes* compared to samples without MEL-B. The maximum specific growth rate of strain was better controlled with MEL-B at 0.01 µg/mL and incubation of 15 °C. The treatment with 500 µg/mL at 30 °C was inappropriate for controlling *L. monocytogenes* growth because it resulted in a more expressive maximum growth rate. Finally, the data indicate that MEL-B can have a valuable application as a food preservative, especially because lower concentrations seem more effective, indicating competitive costs at large-scale applications.

Statements and Declarations

Conflict interest: The authors declare that they have no conflict of interest.

Financial interests: The authors declare that they have no financial interests.

Author contributions: All authors contributed to the paper's conception and design. Material preparation and data collection were performed by Ana L S Coelho, Ana G. Massia, and Denise A Laroque. Data analysis was performed by all authors. The first

draft of the manuscript was written by Ana L S Coelho. All authors commented on previous versions, revised the language, and approved the final manuscript.

Acknowledgments: This study received financial support from the Brazilian Agencies: the Coordination for the Improvement of Higher Education Personnel (CAPES) and the National Council for Scientific and Technological Development (CNPq).

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7. FINAL CONCLUSION

This work evaluated the antimicrobial activity of MEL-B against *C. acnes* ATCC 6919 and *Listeria monocytogenes* CLIST 4165. The experiments showed an opposite behavior of MEL-B on the strains, acting as an antimicrobial and a growth stimulant for Listeria and C. acnes, respectively.

The data showed that MEL-B has no antimicrobial activity against *C. acnes*; thus, no considerable leakage of the genetic material of cells was observed. Indeed, the glycolipid concentrations tested (128, 192, 256, and 512 µg/mL) increased the strain growth by approximately 1-4 CFU/mL after incubation of 8 and 16 h. These results suggest that MEL-B is a growth stimulating compound for *C. acnes*. Thus, it is supposed that glycolipid is unsuitable for acne-prone skincare products, as the imbalance in the skin microflora is one of the causes of the development of the pathology.

L. monocytogenes trials demonstrated a high susceptibility of the strain to MEL-B on the culture medium, with a MIC and MBC of 4 and 1,000 µg/mL, respectively. The assays with hot dog sausage (30 °C) showed similar inhibition of strain under different magnitudes of the glycolipid concentration. The kinetic data (15 and 30 °C) obtained for MEL-B concentrations of 0.01 and 500 µg/mL evidenced a decrease on the lag phase and maximum growth rate for almost all study conditions: 0.01 µg/mL/15 °C, 500 µg/mL/15 °C, 0.01 µg/mL/30 °C. At 500 µg/mL/30 °C, a considerable increase on the maximum growth rate of *L. monocytogenes* was observed; thus, the treatment condition was considered inadequate.

It was concluded in this study that MEL-B can act as a growth stimulant for C. acnes. In this sense, future work could investigate a possible prebiotic activity of MEL-B for the food sector.

This is the first study to address the effect of MEL-B as an antimicrobial agent in hot dog sausage. The study of the antimicrobial mechanism of MEL-B would be interesting, assertively showing its performance. Furthermore, the investigation of the emulsifying property of this compound in the formulation of sausages can be explored in the future, together with its microbial activity.

8. SCIENTIFIC PUBLICATIONS RESULTING FROM THE THESIS

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