Activité antifongique de la réutérine produite par Lactobacillus reuteri ATCC53608 dans les yogourts brassés

Mémoire

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Résumé

L'industrie agroalimentaire est un secteur économique d'envergure mondiale. L'élargissement des marchés a forcé les industries de transformation à développer des méthodes pour augmenter la durée de conservation des aliments. L'augmentation de l'importation et de l'exportation a été possible grâce au développement de méthodes de conservation comme la stérilisation, la congélation, la réfrigération, le séchage, la concentration, les emballages intelligents ainsi que l'utilisation d'agents de conservation. Cependant, les aliments sont sujets aux contaminations microbiennes pouvant causer des maladies d'origine alimentaires et des pertes de produits. Les pertes alimentaires représentent un énorme enjeu économique et environnemental pour l'industrie agroalimentaire mondiale. En 2010, la FAO a réalisé un rapport mondial sur le gaspillage alimentaire ciblant les principales causes et les secteurs les plus touchés. Dans les pays industrialisés comme le Canada, plus de 40 % des pertes alimentaires ont lieu pendant la transformation des aliments. De plus, en Amérique du Nord les produits laitiers représentent le deuxième secteur le plus touché par les pertes après les fruits et légumes. Une des principales causes est la contamination des produits laitiers transformés due à la présence de moisissures et de levures. Ces contaminations occasionnent des pertes alimentaires directement à l'usine, mais elle peut aussi occasionner le rappel d'aliments. Pour une industrie, un rappel peut couter entre 10 et 30 millions de dollars US chaque fois. De plus, la perte de confiance des consommateurs envers les produits lors d'un rappel alimentaire est non négligeable dans le calcul des impacts économiques. Pour limiter l'occurrence des contaminations microbiologiques, les transformateurs utilisent des méthodes de contrôle diversifiées incluant l'utilisation agents antimicrobiens traditionnels comme le sel, les acides organiques et les agents antifongiques comme la natamycine. Cependant, les consommateurs sont de plus en plus réticents à acheter des produits contenant des agents de conservation. Ils demandent des produits naturels qui sont sans risque pour la santé, réduits en sel et bons pour l'environnement. De plus, les agents de conservation sont très limités dans les produits laitiers notamment lors de la production de yogourt. Les yogourts sont surtout sujets aux contaminations par les moisissures et les levures par leur pH acide qui limite la croissance des autres contaminants.

L'utilisation des cultures lactiques bioprotectrices ainsi que leurs métabolites antimicrobiens pour la conservation des aliments est une solution naturelle très prometteuse dans le contexte de l'utilisation d'agents de conservation traditionnels. Cette étude visait à évaluer le potentiel d'utilisation d'un métabolite bactérien antifongique naturel, la réutérine, dans un yogourt brassé pour éliminer la présence de levures et de moisissure. Les résultats ont montré que la réutérine avait un large spectre d'action contre les moisissures et les levures. L'utilisation de la réutérine dans les yogourts brassés s'est avérée plus efficace que la natamycine pour lutter contre les moisissures, mais la réutérine a aussi réduit la population des cultures lactiques utilisées dans la fabrication du yogourt. Des études supplémentaires doivent être réalisées pour limiter l'action de la réutérine sur les ferments lactiques.

Abstract

Food industry is now a worldwide economic sector. Market expansion and the increase of import and export have forced food industries to develop methods to increase shelf life of food products. Freezing, refrigeration, drying, concentration, modified atmosphere packaging and the used of preservatives are examples of technologies used in food industries. However, food is still prone to microbial contamination that can cause foodborne illness and food losses and waste. Food losses and waste represent a huge economic and environmental challenge for the global agri-food market. In 2010, FAO produced a global report reporting the main causes of food losses and the sector most affected by the crisis. In industrialized countries such as Canada, more than 40% of food losses occur during processing. In addition, in North America, dairies are the second most affected sector for losses after fruits and vegetables. One of the main causes of dairy contamination is the presence of yeasts and molds in products. This contamination causes waste directly at the plant, but it can also causes food recalls. For an industry, one recall can cost between $10 M US and $30 M US each time. In addition, the loss of consumer trust in products is significant in the balance of negative impacts. To limit the occurrence of microbiological contamination, processors used diversified control methods including the use of traditional preservatives such as salts and antifungal compounds as natamycin. But tendency showed that customers are more reluctant to buy products containing preservatives. They ask for natural products that are safe for health, reduced in salt and good for the environment. In addition, preservatives are very limited in dairy products, particularly in yogurt. The yogurts are more prone to mold and yeast contamination than other dairy products because of their composition and the presence of potential contaminants in flavoring ingredients and in production environments. The use of bioprotective lactic cultures and their antimicrobial metabolites in food preservation is a very promising natural solution in the context of reducing the use of traditional preservatives and maintaining the quality of food products. This study investigated the use of an antifungal bacterial metabolite, reuterin, in a stirred yogurt to meet the needs of dairy processors for an effective and natural antifungal solution. Results showed that reuterin have a great spectrum of action against both yeasts and molds. The used of reuterin in stirred yogurt was more efficient than natamycin for mold control when used as an antifungal ingredient. However, reuterin have reduced the population of lactic culture used in yogurt processing. Future studies have to be achieved to limit the action of reuterin on lactic culture starters to use it as an antifungal in yogurt. The used of reuterin as an antifungal is promising to reduce potential mold contamination in dairies.

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Liste des abréviations,

AW : Teneur en eau, water activity
FAO : Organisation mondiale de l'agriculture et de l'alimentation, Food agriculture organisation
ST : Solides totaux, Total solid
UFC/ CFU: Unité formatrice de colonie, Colony forming units
HACCP : Hazard analysis of critical control point
ATM : Atmosphère modifiée
PLA: Acide phenyllactique
ADN : Acide désoxyribonucléique
CMI/MIC : Concentration minimale inhibitrice, Minimal inhibition concentration
CMF/ MFC : Concentration minimale fongicide, Minimal fongicidal concentration
LAB: Bactérie lactique, Lactic acid bacteria
ATCC: American Type culture collection
CMBL : Métabiolac collection
LMA : Laboratoire mycologie alimentaire
QC : Québec
CAN: Canada
MRS: DeMan Rogosa Sharp
PD : Potato dextrose
PDA: Potato dextrose agar
YDP: Yeast dextrose peptone
HPLC : Chromatographie haute pression en phase liquide, High pressure liquid chromatographie
GC-MS : Chromatographie en phase gazeuse avec spectrométrie de masse. Gaz chromatography with mass spectroscopy
OD: Densité optique, Optical density
CLSI : Clinical laboratory standard institute
MF: Milk fat
CT: Control
DRBC: Dichloran rose Bengal chloramphenicol.
ANOVA : Analyses de variance à une voie, One-ways analysis of variance
Min : minutes
GSH: Glutathione
Au : Unité arbitraire, Arbitrary units
UHT : Upérisation haute température
GDL : Glucono-delta-lactone
FQ2 : FreshQ2
GRAS : Généralement reconnu comme sécuritaire, Generaly recognized as safe
R : Réutérine, Reuterin
LR: Lactobacillus reuteri
Gly : Glycérol, Glycerol
N : Natamycine, Natamycin
USA : États-Unis d'Amérique, United stated of america

Vis comme si tu devais mourir demain.
Apprends comme si tu devais vivre toujours.
Mahatma Gandhi

La véritable science enseigne, par-dessus tout, à douter et à être ignorant. Miguel de Unamuno

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Avant-propos

Ce mémoire est divisé en trois chapitres. Le premier comprend la revue de l'état des connaissances et les chapitre deux et trois sont rédigés sous la forme d'articles scientifiques.

Les expérimentations du premier article ont principalement été réalisées dans le laboratoire du professeur Ismaïl Fliss de l'Université Laval. J'ai préparé tous les protocoles expérimentaux et réalisé toutes les manipulations. Les personnes ayant contribué scientifiquement à cet article sont M. Benoît Fernandez, M. Ahmed Gomaa et M. Hassan Sabik. J'ai analysé et interprété tous les résultats dans le cadre de mes travaux. J'ai également rédigé l'article en entier. Les travaux ont été supervisés par mon directeur de recherche, M. Ismaïl Fliss, ainsi que mon codirecteur de recherche, M. Daniel St-Gelais.

Les expérimentations du deuxième article ont été réalisées au centre de recherche et de développements de St-Hyacinthe. J'ai réalisé le protocole expérimental, réalisé les expérimentations, analysé les résultats et rédigé l'article. Dans le cadre de ces travaux, j'ai été supervisé par M. Daniel St-Gelais. J'ai également eu la chance d'être supervisée par deux professionnelles de recherche, Mme Sophie Turcot et Mme Annie Caron.

Les deux articles rédigés dans le cadre de mes travaux de maitrise, dont je suis l'auteure principale, seront soumis pour publication. Les co-auteurs du premier article sont Benoit Fernandez, Ahmed Gomaa, Daniel St-Gelais et Ismaïl Fliss. Les co-auteurs du deuxième article sont Daniel St-Gelais et Ismaïl Fliss.

Introduction

Les aliments sont des produits riches en nutriments sujets à des expositions environnementales multiples, de la production des matières premières jusqu'à leur consommation. Les contaminations peuvent être causées par divers microorganismes comme les bactéries, les moisissures, les levures, les protozoaires et les virus (Prescott 2013). Les différents microorganismes peuvent être pathogènes ou non pathogènes. Les microorganismes non pathogènes causent l'altération des produits et donc des défauts au niveau de la couleur, de l'odeur, de la saveur et de la texture des aliments. Certains produits sont plus sujets que d'autres aux contaminations par leur composition (protéines, glucides et lipides) qui sert de nutriments pour les microorganismes (Sperber and Doyle 2009). D'autres facteurs comme le pH, la teneur en eau (activité de l'eau ; Aw) et la transformation des aliments comme les traitements thermiques peuvent influencer la croissance spécifique de certains microorganismes dans les aliments (Sperber and Doyle 2009). La croissance de levures et des moisissures dans les aliments est souvent responsable de l'altération de certains produits (Pitt and Hocking 2009). Les moisissures et les levures ont la capacité de croitre dans des environnements acides, à faible Aw, avec peu d'oxygène et à faible température (Sarah C. Watkinson 2015). Les produits laitiers comme les yogourts sont des produits souvent altérés par les levures et les moisissures (Mataragas, Dimitriou et al. 2011, Garnier, Valence et al. 2017, Gougouli and Koutsoumanis 2017). L'Aw élevée, le faible pH ainsi que la température de conservation font que les yogourts sont favorables à la croissance des levures et des moisissures (Pitt and Hocking 2009). Plusieurs études rapportent l'importance de l'altération des yogourts par les contaminations fongiques ainsi que les conséquences qu'elles peuvent engendrer (Gooch, Feifel et al. 2010, Buzby and Hyman 2012, Garnier, Valence et al. 2017). La contamination des produits laitiers, y compris les yogourts, cause d'importantes pertes économiques pour les transformateurs, mais également d'importantes pertes alimentaires (Caggia, Restuccia et al. 2001, Commission 2015). L'industrie de la transformation canadienne perd 10 à 40 % des produits transformés annuellement. (Gooch, Feifel et al. 2010). Selon un rapport de l'Organisation des Nations unies pour l'alimentation et l'agriculture (Gustavsson, Cederberg et al. 2011), 20 % des produits laitiers produits sont perdus annuellement.

Une des approches pour prévenir et contrôler les pertes alimentaires est d'identifier les sources de contamination (Roodhuyzen, Luning et al. 2017). Dans le cas des levures et des moisissures, leurs présences peuvent provenir de l'environnement de production comme les surfaces, les équipements et l'air. Elles peuvent également provenir des matières premières utilisées lors de la transformation comme le lait ou les produits aromatiques ajoutés aux yogourts comme les fruits, les sirops de fruit, les confitures et les colorants (Garnier, Valence et al. 2017). Plusieurs mesures de contrôle ont été mises en place au cours des années afin de réduire l'incidence des contaminations fongiques dans les yogourts. Elles comprennent des mesures préventives comme les bonnes pratiques de fabrication, le contrôle de la qualité des matières premières, le traitement thermique du lait avant la fermentation et le maintien de la chaine de froid lors de la distribution (Vadillo, Paya et al. 1987, Viljoen, Lourens-Hattingh et al. 2003, Blackburn Clive de 2006). L'emballage sous atmosphère contrôlée d'azote ou de dioxyde de carbone réduit la croissance des moisissures et des levures dans les produits (Sandhya 2010). À l'exception des yogourts aux fruits, aucun agent de conservation n'est présentement autorisé dans les yogourts au Canada. Cependant, l'ajout de probiotiques comme cultures protectrices produisant des métabolites montrant des activités antifongiques est autorisé (Canada 2018). Malgré ces méthodes préventives actuellement employées pour prévenir ou empêcher la croissance des levures et des moisissures dans les yogourts, le problème est toujours d'actualité (Garnier, Valence et al. 2017).

La réutérine est un métabolite bactérien produit par la bactérie probiotique *L. reuteri*. Ce métabolite montre une activité antifongique ainsi qu'un potentiel d'utilisation dans les yogourts comme agent de conservation naturel (Vimont, Fernandez et al. 2018). L'utilisation de la souche directement dans le yogourt a été investiguée (Ortiz-Rivera, Sanchez-Vega et al. 2017). Cependant, cette voie d'utilisation montre des problèmes dans les produits liés à la production de gaz par la souche, à l'ajout non autorisé de glycérol dans les ingrédients ainsi que la haute concentration de la souche productrice nécessaire à la production de réutérine (Langa, Landete et al. 2013, Champagne, Raymond et al. 2015). L'utilisation de la réutérine comme ingrédient antifongique semble une voie plus intéressante pour la conservation des yogourts. Peu d'information est disponible sur l'effet de la molécule sur les yogourts ainsi que l'effet des composants du yogourt sur l'activité antifongique de la réutérine. La présente étude vise à déterminer les conditions d'utilisation de la réutérine pure comme ingrédient

antifongique dans un yogourt brassé et de quantifier son effet inhibiteur sur les espèces fongiques.

Chapitre 1 Revue de littérature

1.1 Le lait

Le lait est l'ingrédient principal du yogourt. Le lait de vache entier est composé de 87,5 % d'eau, 3,3 % de matière grasse, 3,5 % de protéines, dont 2,7 % de caséines et 0,6 % de protéines sériques, 5 % de lactose et 0,7 % de minéraux (Y. and K 2007). Grâce à sa composition protéique particulière. Le lait est utilisé pour la fabrication de produits comme les fromages et les laits fermentés comme le yogourt. Le pH est naturellement situé autour de 6,7. À un pH près de la neutralité, les caséines du lait sont sous forme de micelles. Les micelles sont des structures colloïdales composées de plusieurs caséines associées entre elles par la présence de liens phosphocalciques (Horne 2014). La base de la transformation des produits laitiers implique la déstabilisation des micelles de caséines par des traitements thermiques, l'utilisation d'enzymes ou l'acidification du lait. Le yogourt est le deuxième produit laitier le plus consommé au Canada (Chandan 2013). Il est produit grâce à une acidification lente du lait par des bactéries lactiques. Le procédé de transformation du yogourt brassé est présenté de façon plus détaillée dans la prochaine section.

1.2 La fabrication du yogourt brassé

1.2.1 Définition du yogourt

Le yogourt est lait qui a été fermenté par l'action symbiotique de *Streptococcus thermophilus* et de *Lactobacillus delbrueckii subsp. bulgaricus* provoquant une diminution du pH du lait et la formation d'un gel protéique. Par définition, les bactéries doivent être viables, actives, et en quantité abondante dans les yogourts tout en long de leur durée de conservation (CodexAlimentarius 2011).

1.2.2 Procédé de transformation

La première étape du procédé de transformation du yogourt brassé consiste à préparer un mélange laitier. Les mélanges laitiers utilisés pour la fabrication de yogourt sont composés de lait écrémé standardisé en matière grasse et enrichi en protéines. Les protéines sont normalement ajoutées sous forme de poudre de lait ou de concentré de protéines laitières pour obtenir une teneur finale protéique entre 8 et 8,5 %. La standardisation du contenu

protéique du lait permet d'améliorer la texture des yogourts. Le mélange laitier subit un traitement thermique de 90 à 95 °C pendant 3 à 5 minutes ayant pour but d'éliminer les microorganismes végétatifs présents dans le lait et de dénaturer les protéines du lactosérum pour améliorer leur pouvoir gélifiant (Vasbinder 2002). Suite au traitement thermique, le lait est homogénéisé afin que le gras ainsi que les ingrédients secs ajoutés au lait soient dispersés de manière homogène. Ensuite, le mélange laitier est refroidi à 40 °C dans un échangeur à plaques et transféré dans une cuve de fermentation. Directement dans la cuve, le mélange laitier est ensemencé avec les ferments lactiques *S. thermophilus* et *L. bulgaricus* à des taux d'inoculation entre 1 % et 3 %. Après l'ensemencement, le mélange laitier est maintenu à une température entre 42 °C et 44 °C pour l'acidification. Pendant l'acidification, les ferments lactiques consomment le lactose présent naturellement dans le lait et produisent de l'acide lactique, ce qui fait baisser le pH du lait. La baisse de pH déstabilise de manière graduelle les micelles de caséines initialement de charges négatives en les neutralisant. De plus, la diminution du pH cause la solubilisation des groupements phosphates brisant ainsi les liaisons phosphocalciques entre les caséines, ce qui déstructure les micelles de caséines. Lorsque le pH atteint le point isoélectrique des caséines (4,6), celles-ci s'agglomèrent en sous-unités déminéralisées. Cette désorganisation permet une réorganisation du réseau protéique favorisant les liaisons de faible énergie entre les caséines comme les liens électrostatiques, hydrophobes et Van der walls (Y. and K 2007). La réorganisation des caséines ou la coagulation emprisonne le lactosérum dans le nouveau réseau, ce qui crée le gel du yogourt. L'acidification dure entre 3 à 5 heures dépendamment du taux d'inoculation, de la température et de l'activité du ferment. Le yogourt qui a gélifié dans une cuve, est mécaniquement brassé avec un agitateur à palles, puis pompé dans un système de refroidissement à environ 20 °C puis lissé. Cette étape consiste à faire passer le gel dans une buse de grosseur standardisée. Le passage dans la buse brise les grosses particules de gel en microgel entouré de lactosérum, ce qui donne au yogourt une texture lisse. Après le brassage, des confitures, des mélanges de fruits, des arômes et des colorants naturels et artificiels peuvent être ajoutés au yogourt. Les dernières étapes de la transformation sont la mise en pot des yogourts, l'entreposage et la distribution des produits. Normalement, les yogourts fabriqués dans de bonnes conditions de fabrication devraient avoir une durée de conservation de 30 jours lorsqu'ils sont entreposés entre 4 et 5 °C (Viljoen, Lourens-Hattingh et al. 2003).

De plus, ils devraient contenir moins de 10 spores de moisissures et de levures par mL (Sperber and Doyle 2009). La mise à l'échelle du procédé de transformation des yogourts est source de risque de contamination des produits. Les risques peuvent être liés aux matières premières utilisées, à la contamination des équipements ou à la manipulation par le personnel. Les contaminations les plus courantes sont la contamination par les levures et les moisissures.

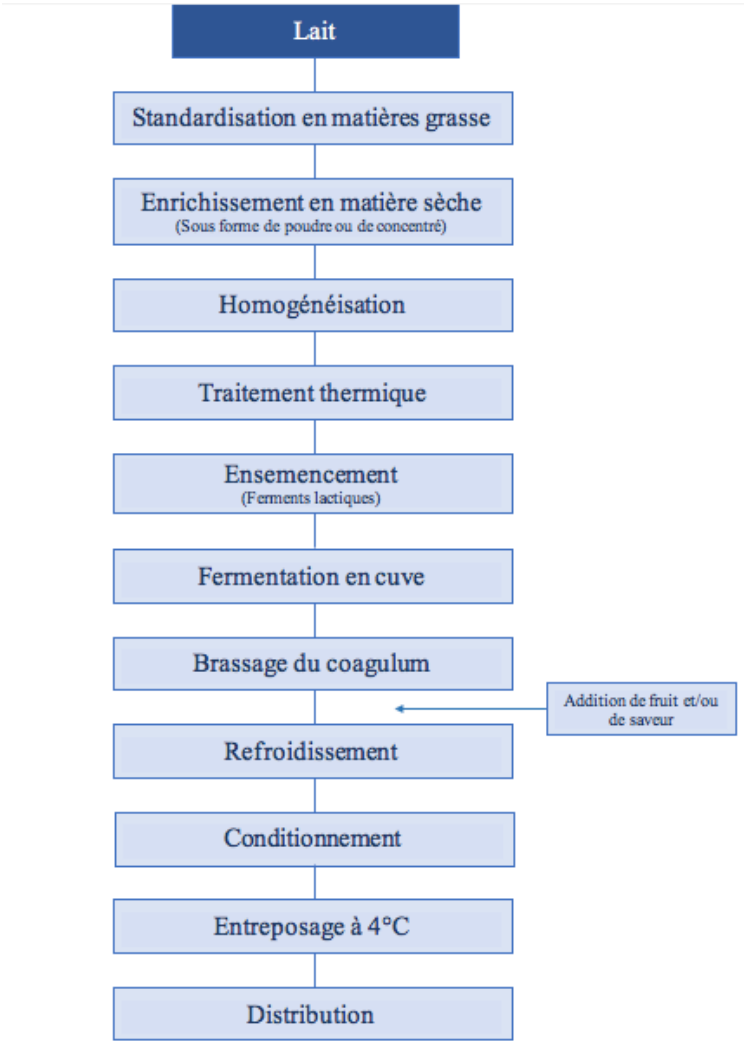

Figure 1-1 Schémas de transformation du yogourt brassé.

1.3 Les contaminants du yogourt

Les yogourts sont principalement sujets à l'altération par des moisissures et les levures (Suriyarachchi and Fleet 1981), puisque les valeurs de pH acides et les basses températures d'entreposage sont favorables à leur croissance (Garnier, Valence et al. 2017). Pour croitre

dans les yogourts, certaines levures et moisissures ont la capacité de produire des enzymes protéolytiques et lipolytiques pouvant dégrader les protéines et les lipides, fermenter le lactose et le sucrose, et assimiler l'acide lactique et citrique, ce qui permet aussi leur croissance (Pitt and Hocking 2009). Les yogourts sont propices à l'altération, car leur pH est plus bas et ils sont souvent aromatisés avec des fruits, ce qui peut augmenter le taux de sucre disponible pour les moisissures (Suriyarachchi and Fleet 1981, Viljoen, Lourens-Hattingh et al. 2003, Davidson, Sofos et al. 2005). Les contaminations peuvent être introduites par les matières premières, les mauvaises méthodes de fabrications, les résidus de lavages ou l'air de l'environnement de production (Caggia, Restuccia et al. 2001). Par exemple, certains genres de moisissures comme *Aspergillus*, *Penicillium* et *Cladosporium* présents dans le lait peuvent être résistants à la pasteurisation (Vadillo, Paya et al. 1987). Également, l'ajout de fruits, de noix et de sucres peut introduire des contaminations (Pitt and Hocking 2009). Le mauvais lavage des équipements de production comme les mélangeurs et les cuves de fermentation peut aussi causer l'introduction de moisissures dans les produits finis (Suriyarachchi and Fleet 1981). Les moisissures contaminantes retrouvées dans les yogourts sont généralement présentes dans l'air des milieux de production, tandis que les levures proviennent des surfaces, des équipements ou des ingrédients (Garnier, Valence et al. 2017). Comme mentionné plus haut, les contaminations peuvent provenir de l'environnement de production et des ingrédients présents dans les yogourts. Les moisissures présentes dans les yogourts peuvent ensuite croitre lentement dans les produits à 4 °C (Davis 1970). Cette croissance est accélérée si les températures augmentent au-delà de 4 °C lors d'un bris de la chaine du froid (Viljoen, Lourens-Hattingh et al. 2003). Les principales levures retrouvées dans les yogourts sont *Atelosaccharomyces pseudotropcalis*, *Candida beverwijkiae, Candida famata, Candida intermedia, Candida lusitaniae, Candida parapsilosis, Candida saitoana, Candida srellata, Candida versatilis, Debaryomyces hansenii, Kluyveromyces marxiianus, Meyerozyma guilliermondii, Peterozyma toletana, Sacharomyces cerevisiae, Torulaspora delbrueckii, Torulopsi sp., Wickerhamomyces anomalus*, *Naganishia albida, Rhodotorula diffluens* et *Rhodotorula mucilaginosa*. Les principales moisissures présententent dans les yogourts sont *Aspergillus sydowii*, *Aspergillus niger*, *Penicillium bialowiezense*, *Penicillium chrysogenum*, *Penicillium aurantiogriseum*, *Penicillium solitum*, *Mucor circinelooides* et *Mucor racemosus* (Mayoral, Martin et al. 2005, Garnier, Valence et al. 2017, Gougouli and

Koutsoumanis 2017). En Ontario, une étude d'un échantillon de 13 manufacturiers a montré que 26,3 % des produits contenaient au-delà de 10^3 cellules/g de moisissures (Arnott, Duitschaever et al. 1974) et donc était impropre à la consommation. La présence de levures et de moisissures dans les yogourts cause plusieurs défauts dans les produits finis.

1.3.1 Impact de la présente de levures et de moisissures dans les yogourts

La présence de croissance de levure et de moisissure dans les yogourts cause plusieurs altérations notables au niveau de la couleur, de l'odeur, de la texture et de la saveur des produits (Suriyarachchi and Fleet 1981, Caggia, Restuccia et al. 2001). Les moisissures et les levures causent ces défauts par la libération d'enzymes, comme des protéases, des lipases et des phospholipases (Sperber and Doyle 2009). Les concentrations en microorganismes viables cités causant l'apparition de défauts dans les yogourts sont de 10^5 et de 10^6 ufc/g (Suriyarachchi and Fleet 1981). La production de gaz comme le CO_2 causée par le développement des moisissures mène dans un premier temps au développement d'odeurs et de gaz. Elle peut également conduire à l'effervescence des produits (Fleet 1990) ainsi qu'à l'écrasement des contenants de yogourt. Une étude sur la contamination des yogourts par *Mucor* et *Penicillium* montre que les moisissures consomment l'oxygène présent dans l'espace de tête au-dessus des yogourts et produisent du CO_2. Ce CO_2 peut être réabsorbé par les moisissures créant un vide et causant l'écrasement des emballages (Foschino, Garzaroli et al. 1993). L'apparition de mycélium de couleur verte, jaune, brune et noire en surface des produits peut être un défaut causé par la croissance de moisissures. Elle est principalement causée par les genres *Penicillium, Aspergillus* et *Cladosporium* (Pitt and Hocking 2009). Les défauts de saveurs peuvent être causés par la présence métabolites produites dues à l'activité lipolytique et protéolytique des levures et des moisissures durant leur croissance. L'activité lipolytique produit des acides gras à courtes chaines et de l'éthanol et cause des goûts fruités et alcooliques indésirables (Caggia, Restuccia et al. 2001, Sperber and Doyle 2009). L'activité protéolytique produit des sulfites et crée des odeurs d'œufs et de terre dans les produits (Sperber and Doyle 2009). Pour contrer ces défauts et la perte des aliments causée par ces microorganismes indésirables, les industriels ont recours à des procédés de conservation physique ainsi qu'à des cultures protectrices pour assurer la qualité et

l'innocuité de leurs produits. Les différentes méthodes utilisées par les industries seront présentées dans la prochaine section.

1.4 Contrôle des moisissures et des levures

La réduction de l'occurrence des contaminations par les moisissures et les levures dans les yogourts tout comme les autres aliments peut être divisée en deux grandes catégories : les méthodes de prévention et les méthodes de contrôle (Figure 1-2).

Les méthodes de prévention concernent principalement l'assurance qualité et le contrôle qualité des installations de production des matières premières et des produits finis. Les principales méthodes préventives utilisées en industrie comprennent l'implantation de mesures d'hygiène de production, par l'application de mesures de lavage et d'assainissement des équipements de production normalisé, par la filtration de l'air environnemental et par des méthodes de gestion des points de contrôle critiques du procédé de transformation : « hazard analysis critical control points » (HACCP). Dans la fabrication des yogourts brassés, les points de contrôle de la fabrication sont établis au niveau de la réception des matières premières, de la pasteurisation, de l'ensemencement, du brassage et de l'incorporation d'ingrédients aromatiques et l'emballage des produits. La plupart des points de contrôle peuvent être gérés par des méthodes de prévention, par l'établissement d'analyses des lots de matières premières ou par la normalisation des méthodes de travail. Les contrôles de la qualité des produits finis sont faits par la mise en place de tests de durée de vie pour chaque lot (Gougouli and Koutsoumanis 2017). Des études ont tenté de modéliser la contamination des yogourts par les microorganismes fongiques considérant plusieurs variables pour créer des algorithmes pouvant prédire la durée de vie des produits (Mataragas, Dimitriou et al. 2011, Gougouli and Koutsoumanis 2017). Cependant, certaines étapes comment la pasteurisation du lait, l'ajout d'ingrédients aromatiques ou l'emballage des produits nécessitent des contrôles supplémentaires assurés par les méthodes de contrôle.

Les méthodes de contrôle peuvent être divisées en deux catégories. Les méthodes qui retardent ou ralentissent la croissance des microorganismes et les méthodes de détruisent physiquement les microorganismes (Figure 1-2). Lors de la fabrication des yogourts, une des principales méthodes de contrôle est le traitement thermique du lait. La pasteurisation est un

traitement thermique visant à détruire la présence de microorganismes végétatifs pathogènes et autres contaminants non pathogènes dans le lait. Cependant, les températures de pasteurisation normalement utilisées pour le mélange à yogourt, soit 90-95 °C pendant 3 à 5 minutes ou 60 °C pendant 30 minutes, sont trop basses pour détruire toutes les spores de moisissures. En effet, certains microorganismes peuvent survivre aux températures utilisées lors des traitements thermiques (Vadillo, Paya et al. 1987). Au niveau de l'ajout d'ingrédients aromatiques comme les fruits, les purées de fruits, les confitures, les sirops ou les solutions aromatiques subissent le même traitement thermique que le lait et peuvent contenir des agents de conservation antifongiques comme l'acide sorbique et l'acide benzoïque ou des sels (sorbate de calcium, benzoate de sodium, benzoate de potassium).

Au niveau de la protection des produits finis, les méthodes utilisées sont principalement des barrières physiques ou l'ajout de culture protectrices puisque l'utilisation d'agents de conservation n'est pas autorisée au niveau règlementaire dans les yogourts (CodexAlimentarius 2011, Canada 2018).

Figure 1-2 Méthode de contrôle et méthode préventive utilisées dans les produits laitiers pour prévenir, inactiver, retarder ou inhiber la croissance des levures moisissures (Garnier, Valence et al. 2017).

Les yogourts contiennent naturellement de l'acide lactique, ce qui permet d'inhiber les microorganismes qui ne supportent pas des valeurs de pH de 4,6 et moins. La présence des ferments permet également une bioprotection contre les contaminants bactériens. De plus, la température d'entreposage des yogourts de 4 °C permet de limiter la croissance des microorganismes mésophiles et thermophiles. Cependant, les barrières microbiologiques ne permettent pas de contrer la croissance des moisissures et des levures puisqu'elles sont psychrophiles et que certaines sont acidotolérantes (Gougouli and Koutsoumanis 2017). Au niveau industriel l'emploi d'emballage sous atmosphère modifiée (ATM) de CO_2 est couramment utilisé dans les produits finis (Devleighere, Vermeiren et al. 2004, Sandhya 2010). La technologie consiste à remplacer l'espace de tête normalement composé d'air, par du CO_2. Comme les moisissures sont des microorganismes aérobies strictes, elles ne peuvent

se multiplier sans oxygène (Pitt and Hocking 2009). Les taux de CO_2 utilisés dans les yogourts sont normalement situés entre 1 % à 50 % (Singh, Wani et al. 2012). Cependant, certaines spores de certaines espèces de moisissures comme *Penicillium ssp*. et *Mucor ssp*. peuvent résister à des concentrations de 0,2 % d'O_2 (Nguyen Van Long, Joly et al. 2016).

L'usage de cultures protectrices peut être employé pour contrer la croissance de contaminants fongiques (V.D. and R. 2016, Garnier, Valence et al. 2017). Les cultures protectrices ont une action antagoniste sur la croissance des microorganismes indésirables, sans modifier les propriétés organoleptiques des produits (Vermeiren et al., 2004). Les cultures protectrices sont utilisées depuis des milliers d'années dans les produits fermentés (Bibek Ray 2013). Les bactéries protectrices les plus étudiées sont les bactéries lactiques caractérisées par leur production d'acide lactique. Cependant, ces bactéries peuvent produire d'autres composés ayant une activité antifongique comme l'acide propionique, l'acide acétique, l'acide phényllactique (PLA et OH-PLA), des dipeptides cycliques et de la réutérine (Schnürer and Magnusson 2005, Yang, Fan et al. 2011, Fernandez, Vimont et al. 2017, Perczak, Golinski et al. 2018). Les souches protectrices de bactéries lactiques les plus étudiées pour leurs effets antifongiques dans les yogourts sont *L. rhamnosus*, *L. casei*, *L. paracasei*, *L. plantarus*, *L. reuteri* (Lavermicocca, Valerio et al. 2000, Rouse, Harnett et al. 2008, Privat and Thonart 2011, Crowley, Mahony et al. 2013). Certains produits commerciaux comme MicroGard™ (Dupont, Danisco, Copenhague, Danemark), HoldBac™ (Dupont, Danisco, Copenhague, Danemark), FreshQ™ (Chr. Hansen, Horsholm, Danemark), BioSafe™ (Chr. Hansen, Horsholm, Danemark), DuraFresh™ (KerryGroup, Tralee, Irlande), Delvo® Cid (DSM, Heerlen, Pays-Bas) et Delvo® Fresh (DSM, Heerlen, Pays-Bas) sont vendus sur le marché comme cultures bioprotectrices destinées à la prévention de la croissance des moisissures et des levures dans les yogourts.

Malgré l'utilisation de méthodes de prévention, de méthodes de contrôle par barrière physique et par l'utilisation de souches protectrices, le problème de croissance fongique dans les yogourts est toujours d'actualité (Garnier, Valence et al. 2017). Une étude réalisée par Fernandez et *al.* (2017) a démontré que l'utilisation de la réutérine, un métabolite bactérien, montrait un potentiel antifongique intéressant dans un objectif de conservation des yogourts. La description de la molécule, sa production, son activité et les récentes applications de la molécule seront présentées dans les prochaines sections.

1.5 La réutérine

1.5.1 La molécule

La réutérine est un produit de dégradation du glycérol connu pour son action antimicrobienne à large spectre. La molécule est un aldéhyde simple composé de trois carbones, d'un groupement alcool et d'un groupement aldéhyde. La réutérine a un faible poids moléculaire, est neutre, soluble dans l'eau et résistante aux enzymes protéolytiques et lipolytiques (Chung, Axelsson et al. 1989). La molécule a été découverte par Axelsson, Chung et al. (1989). Ces chercheurs ont remarqué qu'une souche de *L. reuteri* pouvait produire une substance dite bactéricide, qu'ils ont nommée réutérine. La molécule peut être présente dans un milieu aqueux sous plusieurs formes présentes en équilibre (monomère, monomère hydraté, dimère cyclique, dimère ouvert, trimère et quadrirème) (Vollenweider and Lacroix 2004, Burgé, Flourat et al. 2015). Plusieurs souches du genre *Lactobacillacae* peuvent produire de la réutérine comme certaines *L. coryniformis*, *L. fermentum et L. reuteri* (Magnusson and Schnurer 2001, Schnürer and Magnusson 2005). Par contre, les souches de *Lactobacillus reuteri* sont connues comme étant les seules capables de produire et d'accumuler une grande quantité de réutérine dans leur environnement (Talarico, Casas et al. 1988, Tobajas, Mohedano et al. 2008). *Lb. reuteri* est une bactérie lactique normalement retrouvée dans le tractus digestif animal et humain (Stevens, vollenweider et al. 2010). La production de réutérine par la souche *L. reuteri* sera développée à la prochaine section.

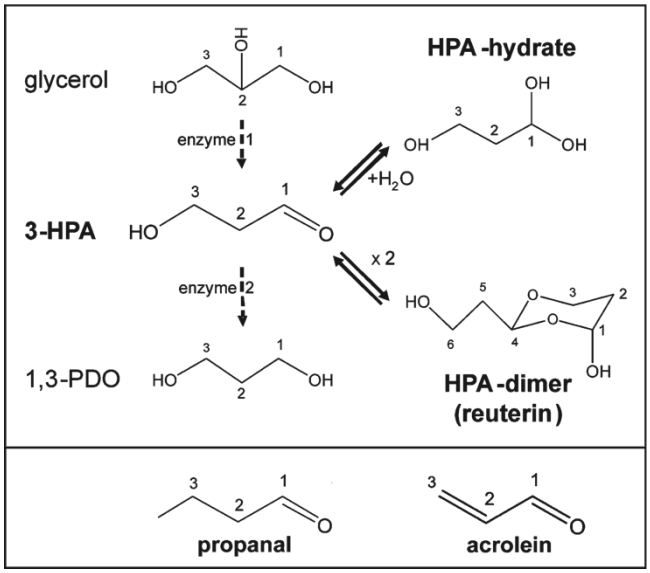

Figure 1-3 Formation of 3-Hydroxypropionaldehyde (3-HPA), HPA hydrate, HPA dimer, and 1,3 propanediol from glycerol and the compound propanal and acrolein. Enzyme 1: coenzyme B12-dependent glycerol dehydratase; enzyme 2, NADH-dependent 1,3-propanediol oxidoreductase (Vollenweider, Grassi et al. 2003)

1.5.2 La production

Certaines conditions sont essentielles à la formation de réutérine. Y. Doleyres et *al*., ont étudié les conditions optimales de production de la réutérine par la souche *L. reuteri* ATCC 53608. La fermentation se produit en deux étapes distinctes, la production de biomasse et la fermentation du glycérol. Suite à une production de biomasse de la souche, la production de réutérine est obtenue sous condition anaérobie en présence de glycérol. La bactérie a la capacité de convertir le glycérol en 3 -Hydroxypropionaldéhyde, par l'action de l'enzyme glycérol déshydrogénase en présence de coenzyme-B12 (Sobolov and Smiley 1959, Vollenweider and Lacroix 2004, Y. Doleyres 2004). Cet aldéhyde est très instable et il est rapidement réduit par la NADH déshydratase sous la forme de 1-3, dipropandiol (1-3, PPO) lorsque la réaction de transformation du glycérol en réutérine n'est pas optimisée. Une étude sur l'optimisation de la production de 3 -HPA montre que la biomasse initiale doit être à une concentration de $1x10^6$ UFC/mL. Cette même étude montre qu'une biomasse trop importante cause une grande production de 3 -HPA et une auto-inhibition des bactéries lors de la phase de fermentation du glycérol (Y. Doleyres 2004). Cependant, un deuxième modèle

de cinétique de production de la réutérine par *L. reuteri* montre que la concentration en biomasse n'a aucun effet sur la production de 3 -HPA et de 1-3 PPO (Tobajas, Mohedano et al. 2008, Dang Vu, Salmieri et al. 2017). Il a été démontré que la production de 3 -HPA augmente avec la concentration de glycérol, par contre elle provoque une production plus rapide de 3-HPA et provoque une l'auto-inhibition de *L. reuteri* à des concentrations élevées en réutérine (Y. Doleyres 2004). La concentration minimale inhibitrice (CMI) de la réutérine pour les souches de *L. reuteri* se situe entre 30 et 50 mM (Cleusix, Lacroix et al. 2007). La température optimale de production de 3 -HPA est de 40 °C. Cependant, le 3 -HPA peut conduire à la production d'acroléine, un composé reconnu comme hautement toxique, par déshydratation au-dessus de 15 °C (Engels, Schwab et al. 2016). D'autres études plus récentes montrent cependant que la réutérine n'aurait pas d'effet négatif sur les cytochromes P450 de cellules hépatiques humaines et que son usage en tant que bioconservateur dans les aliments serait sans danger (Fernandez-Cruz, Martin-Cabrejas et al. 2016).

1.5.3 Le mécanisme d'action

Deux mécanismes d'action de la réutérine ont été proposés au cours des 25 dernières années. Le premier supposait que l'action de la réutérine contre les microorganismes serait due à la similitude de la molécule de 3 HPA dimérique avec le D-ribose, entrant dans la composition de l'ADN. Lors de la première étape de synthèse de l'ADN, la réutérine inhiberait le ribonucléotide réductase et empêcherait la réaction de synthèse (Talarico and Dobrogosz 1990, Ziney and Debevere 1998). Le deuxième modèle propose que le groupement aldéhyde de la réutérine réagisse avec les groupements thiols et les amides primaires présents dans les protéines membranaires microbiennes (Vollenweider, Grassi et al. 2003). En étant fixées par la réutérine, les protéines perdraient leur fonction spécifique comme le transport intermembranaire, ce qui causerait la mort des cellules. En 2010, une étude de (Schaefer, Auchtung et al. 2010) a comparé les deux théories et les résultats montrent que le mode d'action de la réutérine serait mieux décrit par l'action de la molécule sur les groupements thiols.

1.5.4 L'activité antifongique

Axelsson et Chung (1989) a démontré une activité inhibitrice contre *Candida albicans Torulopsis glabrata*, *Saccharomyces cerevisiae*, *Saccharomyces fibuligera*, *Aspergillus flavus* et *Fusarium samfucienum*. Cependant les concentrations de réutérine utilisées dans l'étude n'ont pas clairement été répertoriées. Malgré le potentiel antifongique intéressant de la réutérine, peu d'études se sont intéressées à cet aspect entre les années 1990 et 2000. L'intérêt pour la molécule était plus dirigé vers le potentiel antibactérien (Langa, Landete et al. 2013, Gomez-Torres, Avila et al. 2014). Vers le début des années 2000, plus d'études sur l'effet antifongique ont été publiées. En 2002, Kotoyoshi, Hiroharu et al. (2002) ont démontré le pouvoir inhibiteur de 1 ml de surnageant de réutérine brute contre différentes moisissures. Les principales inhibées étaient le genre *Aspergillus* et les souches *S. cerevisiea*, *Candida krusei, Hasenula anomara, P. chrysogenum. Mucor hiemalis* et *G. candidum*. Cependant, les souches ont été incubées uniquement 24 heures, tandis que les méthodes officielles du compendium des méthodes demandent de 3 à 5 jours d'incubation pour le dénombrement de levures et de moisissures. L'effet antifongique a aussi été démontré in vivo. En effet, une étude a montré une réduction significative des levures *Candida,* une levure causant des infections de la gorge, dans l'environnement buccal d'un groupe de patients traité avec une souche probiotique de *L. reuteri* (Kraft-Bodi, Jorgensen et al. 2015). Les auteurs avancent que la souche aurait pu produire de la réutérine in vivo avec le glycérol issu de la dégradation de gras provenant des aliments et ainsi inhiber les levures pathogènes présentes dans la bouche des patients. Plus récemment, une étude montre le pouvoir antifongique de la réutérine contre *Aspegillus niger*, *Penicillium expensum* et *Fusarium culmorum* Schmidt, Lynch et al. (2018). Les concentrations minimales inhibitrices de ces souches rapportées étaient de 8 mM pour *A. niger* et pour *P. expensum* et de 4mM pour *F. colmorum* (Schmidt, Lynch et al. 2018). Finalement, une étude exhaustive sur le spectre d'activité antifongique a été réalisée sur 10 moisissures filamenteuses et 14 levures (Vimont, Fernandez et al. 2018). Cette étude a démontré que la concentration minimale inhibitrice CMI est plus faible ou égale à 11 mM/L pour toutes les souches et la concentration minimale fongicide est plus faible ou égale à 15,6 mM/L. Cette étude a permis de compléter les connaissances scientifiques sur l'activité antifongique de la réutérine et de connaitre les concentrations à utiliser.

Différentes applications peuvent être envisagées pour l'utilisation de la réutérine. L'une d'entre elles est le contrôle de moisissures et des levures dans les aliments. Comme expliqué précédemment, les produits laitiers et plus particulièrement les yogourts sont propices à la croissance fongique. La prochaine section discutera des différentes applications de la réutérine dans les matrices laitières et dans les laits fermentés.

1.5.5 Utilisation de la réutérine dans les produits laitiers

À ce jour, seules quelques études ont porté sur l'utilisation de la réutérine dans les produits laitiers et encore moins sur l'impact des matrices alimentaire sur l'activité antifongique. En effet, la majorité des études se sont intéressée au contrôle des pathogènes et l'impact des aliments sur l'activité antibactérienne. Les applications de la réutérine ont porté sur différentes matrices laitières comprenant le fromage (EL-Zyney and Debevere 1998, Martin-Cabrejas, Langa et al. 2017), le cuajada (Arques, Rodriguez et al. 2008) et les laits fermentés (Langa, Landete et al. 2013, Ortiz-Rivera, Sanchez-Vega et al. 2017). Dans ces études, l'incorporation de la réutérine a été réalisée selon trois stratégies. La première a été d'ajouter le surnageant de la fermentation anaérobie de *L. reuteri* en présence de glycérol contenant la réutérine et possiblement les autres métabolites issus de la fermentation non purifiée directement à la surface ou dans le produit (EL-Zyney and Debevere 1998, Arques, Rodriguez et al. 2008, Gomez-Torres, Avila et al. 2014). Cette approche a un inconvénient, car elle ne permet pas d'isoler l'effet de la réutérine seule. En effet, lors de la fermentation d'autres molécules comme le 1-3-dipropanediol et les acides organiques produits peuvent aussi avoir un effet antimicrobien ou un effet sur les matrices alimentaires comme un changement de couleur orangé. La deuxième méthode utilisée par Langa, Landete et al. (2013), Martin-Cabrejas, Langa et al. (2017), Ortiz-Rivera, Sanchez-Vega et al. (2017) fut de produire de la réutérine in situ dans les produits laitiers en y ajoutant la souche productrice *L. reuteri* et du glycérol. Tout comme la première méthode, la réutérine peut être accompagnée d'autres métabolites bactériens pouvant masquer son effet réel. De plus, la production de réutérine nécessite une grande concentration de cellules de *L. reuteri* et du glycérol. Ces ajouts aux produits laitiers ne sont pas envisageables dans les aliments puisque le glycérol n'est pas autorisé comme additif et que les souches de *L. reuteri* sont connues pour produire du dioxyde de carbone (CO_2) (Champagne, Raymond et al. 2015). Une

production de CO_2 peut rendre les yogourts effervescents. Une étude récente a proposé d'ajouter directement de la réutérine préalablement purifiée à des yogourts (Vimont, Fernandez et al. 2018). Cette approche semble prometteuse, car elle élimine l'impact de la présence de la souche, du glycérol ainsi que des autres métabolites produits par la souche lors de sa croissance.

Les l'application de la réutérine dans les produits laitiers n'a pas démontré de perte d'activité antibactérienne dans les aliments versus in vitro contre les microorganismes testés. De plus, des études ont montré un effet synergique de la réutérine avec certains acides comme l'acide lactique contre *P. chrysogenum* et *M. racemosus* (Arques, Fernandez et al. 2004) et l'effet a également été observé dans les yogourts (Vimont, Fernandez et al. 2018). De plus, Ziney and Debevere (1998), Brisson, Payken et al. (2010) ont démontré que différents pourcentages de gras n'a pas modifié l'activité de la réutérine contre *Escherichia coli* dans des fromages cottage. Finalement, certaines études ont rapporté que l'ajout de réutérine donnait une teinte orangée aux produits laitiers à de fortes concentrations. Cependant, aucun article ne peut expliquer la cause de cette couleur rose, orangée (Gomez-Torres, Avila et al. 2014, Martin-Cabrejas, Langa et al. 2017, Ortiz-Rivera, Sanchez-Vega et al. 2017). À la lumière de ces résultats, il est clair que des études plus approfondies demeurent nécessaires pour mieux évaluer le potentiel antifongique de la réutérine dans le yogourt.

1.6 Problématique

Les yogourts sont des aliments propices à la contamination par des moisissures responsables d'altérations. Les contaminations sont dispendieuses pour l'industrie de la transformation occasionnant de lourdes pertes de produits. Les transformateurs tentent par plusieurs moyens de limiter la présence de moisissures et de levures dans les environnements de production par des méthodes de lavage rigoureuses et par le contrôle des matières premières. De plus, ils tentent de réduire la croissance de contaminants dans les yogourts par l'usage de produits antimicrobiens chimiques. Malgré les mesures employées par l'industrie, les contaminations et les pertes liées à la croissance fongique dans les yogourts restent d'actualité. Également, les consommateurs demandent de plus en plus des produits sans agents de conservation

chimiques. L'usage de produit antifongiques chimiques tel que les sorbates et la natamycine sont de plus en plus rejetés par les consommateurs et donc pour l'industrie. Pour répondre à la demande des consommateurs, il est nécessaire de trouver une solution naturelle et efficace contre la croissance fongique dans les produits laitiers et plus spécifiquement dans les yogourts brassés. L'utilisation de la réutérine en tant qu'antimicrobien naturel dans les yogourts semble une solution prometteuse. Malgré plusieurs résultats sur son activité antibactérienne, peu d'études ont été consacrées à son activité antifongique dans les yogourts.

1.7 Hypothèse

La réutérine produite par *Lactobacillus reuteri* par fermentation anaérobique inhibe la formation de moisissures dans les yogourts brassés.

1.8 Objectif général de recherche

Produire et évaluer l'activité antifongique de la réutérine contre des isolats d'origine alimentaire de moisissures et valider cette activité dans un yogourt brassé.

1.9 Objectifs secondaires

Objectif spécifique 1 : Produire, purifier et caractériser de la réutérine à partir d'une culture de la souche *Lactobacillus reuteri* ATCC 53608 ;

Objectif spécifique 2 : Étudier et caractériser l'activité antifongique de la réutérine dans une matrice laitière en comparaison avec la Natamycine ;

Objectif spécifique 3 : Valider l'activité antifongique de la réutérine dans le yogourt brassé et évaluer ses effets sur les propriétés physico-chimiques du produit.

Chapitre 2 Bioavailability and Antifungal activity of highly pure reuterin spiked milk and yogurt

2.1 Résumé

Les produits laitiers représentent 16 % des produits alimentaires transformés au Canada. En 2017, la vente représentait 2,1 milliards de dollars canadiens. Les yogourts sont des aliments sujets à la croissance non désirée de contaminants microbiens tels que les moisissures et les levures. Les sources de contamination peuvent être les mauvaises conditions de fabrication ou l'ajout d'ingrédients comme les fruits ou les confitures de fruits. Les contaminations par les levures et les moisissures dans les produits laitiers causent le développement de mauvaises odeurs, couleurs, saveurs et la production de gaz. La contamination des produits laitiers a des conséquences économiques importantes et l'industrie cherche des solutions appropriées pour réduire les pertes de produits alimentaires. L'utilisation de bactéries lactiques (LAB) ou de leurs métabolites est prometteuse pour la conservation du yogourt. La réutérine est un aldéhyde produit naturellement par les espèces de *Lactobacillus*. Elle est décrite comme un composé antimicrobien à large spectre. L'utilisation de la réutérine dans les produits laitiers pour son effet antibactérien a été largement évaluée, mais l'effet antifongique dans le yogourt est peu documenté. Le but de cette étude était de réduire la croissance des levures et moisissures en utilisant différentes concentrations de réutérine purifiée dans un yogourt, en comparaison avec la natamycine. Les résultats ont démontré que la solution de réutérine pure avait un effet fongicide sur *Aspergillus niger*, *Mucor racemosus, Penicillium chrysogenum* et *Rhodotorula mucilaginosa*, tandis que la natamycine avait un effet fongistatique. Dans le yogourt, une concentration de10 mM de réutérine a permis une réduction de la croissance de toutes les espèces testées pendant les 4 semaines d'entreposage à 4 °C. Le résultat le plus notable a été une réduction de 3 log ufc/mL pour *Aspergillus niger* et de 5 log ufc/mL pour *Rhodotorula mucilaginosa*. La quantification de l'activité antifongique de la réutérine utilisée en tant qu'ingrédient a été réalisée pour la première fois dans un yogourt. Cette étude a démontré le potentiel d'utilisation de la réutérine en tant que composé antifongique dans un produit laitier.

2.2 Abstract

Dairy products represent 16% of processed food products in Canada for a total sale of 2.1 billion Canadian dollars in 2017. Poor manufacturing conditions or the addition of sweeteners, fruits or fruit jams can cause mold and yeast contamination. Yogurt contamination with mold and yeasts can result in the development of undesirable odors, flavors, colors and gas production, leading to significant economic losses for dairy industry. Therefore, there is an urgent need for developing new solutions to reduce food losses especially in the current context where the use of chemical additives is increasingly questioned. The use of lactic acid bacteria (LAB) as protective cultures or their metabolites is one of the most promising new approaches for yogurt preservation. Reuterin is an aldehyde naturally produce by *Lactobacilli* species exhibiting a board spectrum of antimicrobial activity. The antibacterial activity of reuterin has been widely documented in the literature. However, only few studies dealing with the antifungal activity have been reported especially in dairy products such as yogurt.

The purpose of this study was to study the behavior, biological activity and efficacy of highly purified reuterin to control the growth of fungi in a commercial yogurt. Results indicated that pure reuterin exhibit significant spectrum fungicidal effect on all the species tested, compared to natamycin which shows a fungistatic effect.

In yogurt, addition of 10 mM of reuterin allows growth reduction of all fungal species tested for the 4 weeks of storage at 4 °C. The most notable result was a reduction of 3 log UFC/mL for *Aspergillus niger* and 5 log UFC/mL for *Rhodotorula mucilaginosa*.

2.3 Introduction

The dairy sector and more specifically fermented dairy products are an important economic sector. Dairy products represent 16% of process food products in Canada, with an annual mean income of 2.1 billion Canadian dollars (EuromonitorInternational 2017). Unfortunately, and as for many other foods, dairy products are susceptible to microbial contamination which leads to the waste of products. It is estimated that 14% of dairy products are altered and lost due to the presence of opportunistic bacterial and fungal organisms in the dairy cow environment (soil, fodder and silage) and therefore in milk and dairy products. The annual cost of these losses is estimated to around $23 billion. Because of their composition and the conditions of their production, milk and dairy products contain a very complex microbial ecosystem. Many microorganisms can be present, multiply and thus affect the quality of the products by the degradation of their constituents (proteins, lipids, lactose) and by the release of undesirable compounds which cause defects of taste, smell, appearance and texture.

The spoilage microorganisms are probably the most difficult to control and the most problematic for the dairy sector. Among them, yeast and molds are involved in large part in alteration of fermented dairy products such as yogurt. Yogurts containing fruity preparations are the most susceptible to yeast damage such as *Debaryomyces hansenii*, as their high fructose and sucrose content promotes the growth of these microorganisms, leading to significant defects in texture, color, taste and appearance.

Poor manufacturing conditions or the addition of sweeteners, fruits or fruit jams can cause mold and yeast contamination of yogurt (Pfaller, Sheehan et al. 2004). Yogurt contamination with mold and yeast can result in the development of undesirable odors, flavor colors and gas production (Foschino, Garzaroli et al. 1993) leading to significant economic consequences for dairy processors.

The control of microbial contamination of dairy products is usually achieved by using chemical food additives and salt. Natamycin is currently allowed to be used as an antifungal compound on the surface of cheese in many countries including Canada (Dalhoff and Levy 2015). Although very effective, these traditional microbiological barriers are increasingly challenged because of their possible negative impacts on consumer health as increase of cancer occurrence or allergic reaction (Garnier, Valence et al. 2017). The latter

consider that the presence of “chemical” ingredients in food is more problematic than the possible presence of contamination (Evens G 2010, Tobler, Visschers et al. 2011). In this context and in addition to the growing popularity of the concept of clean label, consumers are looking for fresh, natural and minimally processed food products (Roman, Sanchez-Siles et al. 2017). The search for more natural alternatives become therefore an urgent need for the food sector.

One of the most promising solution relates to the use of protective cultures. Their efficacy in enhancing food quality and safety is now well established by the public and the scientific community (Yadav and Shukla 2017).

The use of lactic acid bacteria (LAB) or their metabolites has attracted a lot of attention in recent years as a new alternative to prevent contamination in yogurt (Crowley, Mahony et al. 2013). The 3- hydoxy-propionaldehyde (3-HPA) also called reuterin is an aldehyde naturally produced by *Lactobacilli* species (Vollenweider and Lacroix 2004). It was first discovered by Chung, Axelsson et al. (1989) and it is described as a broad spectrum antimicrobial compound.

The molecule can be produced by glycerol transformation by *Lactobacillus reuteri* isolates or by chemical synthesis. The molecule is known as a complex of 4 different forms of monomer, dimers, tetramers and quadrimers (Vollenweider, Grassi et al. 2003). According to Schaefer et al. (2010), reuterin is acting on thiol groups and cause an oxidative stress on microorganisms. Reuterin has been reported as active against gram-negative, gram-positive bacteria, molds, yeast and protozoa (Chung, Axelsson et al. 1989, EL-Zyney and Debevere 1998, Vimont, Fernandez et al. 2018).

Reuterin can be added to food products as a purified ingredient or can be produced in situ when the producing strain is added. (Schnürer and Magnusson 2005). Addition of reuterin-producing strains in dairy products to control the growth of pathogens has been reported in many studies (EL-Zyney and Debevere 1998, Arques, Rodriguez et al. 2008, Martin-Cabrejas, Langa et al. 2017, Ortiz-Rivera, Sanchez-Vega et al. 2017). Although the efficiency of this strategy has been demonstrated in some studies, its use at a routine basis to the milk industry still limited by the fact that *L. reuteri* do not grow well in milk and that its addition at high concentration in yogurt can cause non-desired gas production problems (Champagne, Raymond et al. 2015). Moreover, since reuterin is produced by bioconversion of glycerol,

which should be added in milk, but it is not allowed in several fermented milk products including yogurt.

To overcome limitations related to the use of reuterin producing strains, addition of pure reuterin has been proposed. Vimont et al. (2018) evaluated the antifungal activity of reuterin in artificially surface spiked commercial yogurt and showed visual reduce of mold growth.

Many other studies have shown a significant inhibition of several food pathogens by reuterin in different food matrices (Arques, Rodriguez et al. 2008, Langa, Landete et al. 2013, Vimont, Fernandez et al. 2018). Most of these studies focused on antibacterial activity of reuterin. However, data on the antifungal potential of reuterin are still missing. Moreover, the bioavailability and the inhibition activity of reuterin in a complex food matrix have rarely been studied. For example, in milk and milk products, it is important to evaluate the interaction of reuterin with milk compounds such as proteins and lipids and its impact on reuterin bioavailability. The reuterin may interact with thiol groups present on milk protein as β-Lactoglubuline and lose its activity (Vasbinder 2002, Schaefer, Auchtung et al. 2010).

The application of purified reuterin for food preservation implies the development of effective methods for its large-scale production and purification. Production of reuterin is usually achieved through glycerol biotransformation in anaerobic condition by a strain of *Lactobacillus reuteri* (Doleyres, Beck et al. 2005). The process results in a supernatant containing reuterin, residual glycerol as well as other bacterial metabolites including acrolein (Vollenweider, Grassi et al. 2003). There is a reversal in the scientific community of whether the inhibitory activity is due to reuterin or the presence of acrolein in *L. reuteri* culture supernatant (Engels, Schwab et al. 2016). The acrolein is a toxic aldehyde that can be created when reuterin degradation occurs. However, some toxicity studies one human liver cells microsome showed that reuterin is much less toxic than acrolein and it can be used as a preservative (Fernandez-Cruz, Martin-Cabrejas et al. 2016). It is therefore very important to use a reuterin free of acrolein.

The purpose of this study was to study the bioavailability and the inhibition activity of highly purified reuterin in milk and steered yogurt and to compare this antifungal activity to natamycin.

2.4 Material and method

2.4.1 Strains

The stock culture of *L. reuteri* ATCC® 53608TM (American Type Culture Collection, Manasa, VA, USA), *A. niger* CMBL-121 (Metabiolac Collection, Laval University, Québec, QC, CAN), *M. racemosus* LMA 722 (Laboratoire Mycologie alimentaire, Laval université, Québec, QC, CAN), *P. chrysogenum* CMBL 114 (Metabiolac Collection, Laval University, Québec, QC, CAN) and *R. mucilaginosa* CMBL-112 (Metabiolac Collection, Laval University, Québec, QC, CAN) were kept frozen at -80 °C. The *L. reuteri* ATCC 53608 strain was selected among five other because of its high reuterin production and easy propagation. The bacteria were stored in Deman Rogosa Sharpe broth (MRS., Nutri-bact, Quebec, Canada) medium with 20% glycerol. Molds were stored in Potato Dextrose broth (PD, BD-Difco, Sparks, MD, USA) medium with 50% glycerol. Yeast was stored in 10 g/L yeast extract (BD-Difco), 20 g/L Dextrose (Thermo Fisher Scientific Inc., Ontario, Canada) and 10 g/L Peptone (BD-Difco) medium (YDP).

2.4.2 Production of Reuterin

Reuterin was produced by the double fermentation method first described by (Doleyres, Beck et al. 2005) with some modifications. Based on Doleyres results, the glycerol aqueous solution used was 300 mM/L, cell concentration was $1x10^{10}$ and the bioconversion time was set at 45 min. Each bioconversion was done at 21 °C in anaerobic chamber (Forma scientific anaerobic system, 1025 model, Waltham, MA, U.S.A.).

2.4.3 Purification and Quantification of Reuterin

A calibration curve was generated using a standard solution of reuterin with concentrations ranging from 0.01 mM to 1 M of pure reuterin (Vimont, Fernandez et al. 2018).

Purification of reuterin was done on three productions carried separately. The purification was done by HPLC on a preparative HPLC equipment (Nexera x2, Shimadzu, Nakagyo-ku, Japan) equipped with ultraviolet visible detectors (SPD-20A/20 OF, Shumadzu, Nakagyo-Ku, Japan) used at 190 nm wave length. The separation was done on an Ion-300 semi Prep column (ICE-99-6350, Concise separation, San Jose, CA, USA) in isocratic phase with a

mobile phase made of sulfuric acid aqueous solution (MilliQpore Sigma, MO, USA ; H2SO4, 10mm, Sigma Aldrich, Saint-Louis, MO, USA) and a flow rate of 4 mL/min. The column was kept at 40 °C and the running duration was 40 minutes. The collected fractions of reuterin of each production were pooled together and concentrated by vacuum concentrators for 8 hours at, 65 °C, 14 torr (SpeedVac, ThermoFischer scientific, Waltham, Mo, USA).

Quantification and purity analyses were done by HPLC. Analysis were performed on an analytical HPLC (HP serie 1100, Agilent, Santa Clara, CA, USA). The separation was done with a Coregel, Ions-300 column (ICE-99-9850, Concise separation, San Jose, CA, USA). The mobile phase was a sulfuric acid aqueous solution (MilliQpore Sigma, MO, USA ; H2SO4, 10mm, Sigma Aldrich, Saint-Louis, MO, USA). The flow rate was 0.4 mL/min and the column temperature was 40 °C. The detection was performed using a refractive index detector (Waters 410 differential refractometer, Milford, MA, USA) and the reuterin peak was identified at 25.4 min on the HPLC chromatograms in preliminary tests with the same running condition. The production yield was calculated from the ratio of reuterin concentration on the initial amount of glycerol of the solution. The purification yield was calculated from the ratio of the total reuterin amount (mM) after concentration, on the supernatant reuterin amount (mM). The reuterin amount was obtained by multiplying volume (mL) by the concentration (mM/mL). Purity of the reuterin molecule was calculated from the relative abundance on the integrated HPLC chromatogram.

2.4.4 GC-MS Analysis

The characterization of reuterin purified solution was determined by a gas chromatography with mass spectra analysis (GC-MS) as described by (S. Lamothe, C. Guérette et al. 2018) with some modifications. Pure reuterin (100 µL) diluted 75 times were used instead of MDA solution. Samples were run on a DB-5 column (Agilent, technologies, Palo, Alto, CA., USA.) in the same condition as Lamothe et al. 2018. Fragment ions between 50 and 600 and m/z were monitored in a full scan mode of the mass hunter. The main GC peak was used to characterize the form of the reuterin present in the pure solution. The mass weights of the degradation fragment ions detected by MS in the main GC chromatogram peak were compared to the theoretical reuterin mass weight of the monomer, dimer, trimer or quadrimer proposed by (Burgé, Flourat et al. 2015) added with the derivatives molecule mass weight.

Using, the fragment's mass weight of the main product, the native chemical formula was reconstructed by assemblage.

2.4.5 Fungal Spore Suspension Preparation

Frozen stocks of *A. niger*, *P. chrysogenum* and *M. racemosus* were plated on a PDA medium at 25 °C in a dark environment. After 5-day incubation period, a piece of mold was transferred to a fresh PDA (Potato Dextrose Agar, BD-Difco, Sparks, MD, USA) plate and set to grow again in the same condition for 5 more days. Obtained molds were then transferred to a final growth of 12 days on a fresh PDA plates to get conidia formation. Mold conidia were recovered with sterile foam tip in 2 mL solution. The RPMI 140 medium (with glutamine and without sodium bicarbonate, MultiCell media, Wisent Inc., Montreal, Québec, Canada) supplemented with 0.164 M of 3-N-morpholino propane-Sulfonic acid (MOPS, Oakville, ON, Canada) was used for minimal inhibitory concentration reducing 90% of mol growth (MIC) and minimal fungicidal concentration (MFC) determination. Peptone water 100g/L (BD-Difco, Sparks, MD, USA) with tween 20, 10 g/L (SIGMA, Saint-Louis, MO. USA) was used for milk and yogurt assays. *R. mucilaginosa* was propagated in YPD broth twice 48 hours at 30 °C then yeast cells were diluted 100 folds (100 µL/10 mL) in solution. As for molds, RPMI 1640 medium was used for MIC and MFC tests, but sterile saline (9 g/L NaCl) was used for the test in milk and yogurt. Yeast and mold concentration in the respective solution were determined by hemocytometers (Prescott 2013) and diluted in the same solution there prepared in order to obtain an inocula concentration of 1×10^6 conidia or cells/mL.

2.4.6 Minimal Inhibitory Concentration and Minimal Fungicidal Concentration Assay

Minimal inhibitory concentration 90 (MIC90) was determined by using the microplate CLSI-M27A official method (Clinical and Laboratory Standard Institute 2008) for Yeast and CLSI-M38 (Clinical and Laboratory Standard Institute 2017) for filamentous molds. Two fold serial dilutions of Reuterin and natamycin were prepared in pure distilled water from a stock solution with concentration of 60 mM and 5 mM respectively. Optical density (OD) of the plate was read after 48 h of incubation at 25 °C at 595 nm with a microplate reader (infinite®

F200 PRO, Tecan Inc., Durham, NC, USA). MIC corresponds to the first well when total growth inhibition was observed. The minimal fungicidal concentration (MFC) is defined by the minimal antifungal concentration reducing 99.99% of the initial inoculum. The MFC was determined by using official method (CLSI, 2017). A PDA petri was separated into quarters and 10 µL aliquot of each well that showed the same OD as the medium control was spotted in different quarters of the PDA plate. The plates were incubated 48 to 72 h at 25 °C. The lowest concentration with no growth was considered as the MFC. According to (Pfaller, Sheehan et al. 2004) ratio of MFC/MIC ≤ 4 can be considered as a fungicidal effect and a ratio > 4 as a fungistatic effect.

2.4.7 Antifungal Activity Assays in Milk

The antifungal activity of reuterin and natamycin in milk was determined by the CLSIM44-A2 disk diffusion methods (Clinical and laboratory standard institute 2009) and M51-A(Clinical and laboratory standard institute 2010) with some modifications. Serial dilutions of reuterin and natamycin were prepared in 2 mL Eppendorf tube (Thermo Fisher Scientific, Hampton, N.H. USA), containing 500 µl of milk 3.25% M.F. (Agropur, Québec, Canada). Reuterin was then tested at final concentrations of 250, 125, 62, 31, 15 and 7.5 mM. Natamycin was tested at 0.006, 0.003, 0.0015 ηm. A sample of reuterin (100 mM) and natamycin (0.005 mM) both diluted in water were used as a control. PDA soft agar plates (11.25 g/L agar) inoculated with 1% of *A. niger*, *P. chysogenum, M. racemosus* or *R. mucilaginosa* were prepared to get a final concentration of 1×10^4 spores/mL. The inoculated soft agar was poured in a Petri dish until solidification. Wells were done with the big side of a 5 mL pipette in the cold PDA plates. Then, 80 µl of each reuterin or natamycin solutions were added in the wells. The plates kept at 4 °C right side up for 30 min then incubated for 48 h at 25 °C and the presence of inhibition zone was noted.

2.4.8 Antifungal Activity Assays in Commercial Yogurt

The antifungal activity of different concentrations of reuterin in yogurt was tested against the three filamentous fungal and a yeast species by using a commercial plain non-fat yogurt (Ultima, Quebec, Canada). A modified protocol described by (Fernandez, Vimont et al. 2017) was used. Five grams of yogurt were placed in wells of a 6-well plate (353046, FALCON, Canada). Molds and yeast suspension were prepared as described previously. Then, 500 µL

was added to yogurt samples, and mixed with a sterile loop for 30 seconds to obtain a final concentration of 10^4 spores/mL. Different concentrations of reuterin (0 mM (CT), 1 mM, 2.5 mM, 5mM and 10 mM) and natamycin (5 ppm) were then added randomly to one of the 6 wells of the plate. Plates were stored at 4 °C and fungal growth were monitored for 4 weeks. Four plates were done for each fungal species and the experiment was done in duplicate. For each sample, mold and yeast counts were determined by agar plate enumeration on Dichlororan Rose Bengal Chloramphenicol Agar (DRBC, Difco BD-Difco, Sparks, MD, USA) medium (Official testing method MFHPB-22; (Douey and Wilson 2004). The pH was determined with a portative pH meter (Symphony pH meter, model SP7P, VWR, Radnor, PEN, USA) in all the yogurt samples.

2.4.9 Statistical Analysis

All statistical analyses were performed using the SAS®Studio Software (SAS institute Inc. Cary, NC, USA). The experiment was carried out in split plot design to consider the effect of analysis over time. All experiments were done in duplicate. All the comparisons were performed using the one-way analysis of variance (ANOVA) general linear model (LS-Mean) test. For all analysis, $P \leq 0.05$ was considered statistically significant.

2.5 Results

2.5.1 Production and Purification of Reuterin

The biotransformation results are presented in table 2-1 and the purity are presented in figure 2-1. A final reuterin solution with a concentration of 56 mM/L was obtained with a biotransformation rate of 28%. After purification, 154 mL of pure reuterin was obtained which corresponds to a purification yield of 73%. The pure solution was concentrated 50 times to reach a final pure reuterin solution at a concentration of 2420 mM/L corresponding to a recovery of 62% of the initial reuterin produced in the crude supernatant. The HPLC profile of reuterin preparation before and after purification is presented in Figure 1. At the end of the biotransformation step, the supernatant contains three main products (Figure 2-1 a.), 66% of glycerol at retention time of 23.3 min., 24% of reuterin eluted at 25.4 min and 2% of propanediol at the retention time of 29.2 min. After purification, only the pic of reuterin (99.99%) was detected (Figure 2-1b.) at 25.4 min. Chemical analysis by GC-MS

showed that the major product was 3-HPA in the monomer form. The mass ion spectra of the highest peak on the GC chromatogram (data not shown) are presented in Figure 2-2 a. The chemical structure suggests by the ions spectra fragments interpretation with the derivatization molecule O-PFB and BSTFA is presented in Figure 2-2 b. The different molecular mass fragments presented by the number on the spectra correspond to the mass weight of the full molecule when ions are removed one at a time. The reuterin chemical structure without the derivatization molecule is presented in Figure 2-3. The different molecular mass fragments are also presented by the number on Figure 3b and those ions molecular mass are the same as shown on the ions mass weight spectra. The only form matching with the ion's fragment spectra was the monomer form.

2.5.2 Antifungal Assays (MIC, MFC)

The MIC90 values and the MFC/MIC ratios obtained for the four fungal strains in presence of reuterin and natamycin are presented in Table 2-2. The MIC90 values of reuterin vary significantly ($P \leq 0.05$) from 1.89 mM for *A. niger* was to 7.58 mM for *R. mucilaginosa*. For natamycin, MIC90 values for all the microorganisms was significantly ($P \leq 0.05$) lower than those obtained with reuterin. *A, niger*, *P. chrysogenum* and *M. racemosus* showed similar MIC90 values between 0.005 mM and 0.08mM. Highest MIC90 value (0.16 mM) was obtained with *R. mucilaginosa* whereas *M. racemosus* was shown to be the most sensitive with a MIC90 value of 0.005mM. Based on MFC/MIC ratios, reuterin seems to exhibit a fungicidal effect, while natamycin seems to be more fungistatic with MFC/MIC values higher than 4.

2.5.3 Antifungal Assays in Milk

The antifungal activity of reuterin and natamycin in milk is presented in figure 2-4. Reuterin remains active in milk against all the species tested. This activity is concentration dependent as shown by the decrease in the diameter of the inhibition when lower concentrations were used. As observed with pure reuterin diluted in water, the inhibition activity of reuterin diluted in milk was also strain dependent. The same test for natamycin, result in a steady size

of the radius of the inhibition zone for all the concentrations tested mixed in milk or in water. Inhibition zones were also similar for all fungal species.

2.5.4 Antifungal Assays in Commercial Yogurt

The significative changes in population of the different fungal species in commercial yogurt in presence of different concentrations of reuterin or 5 ppm of natamycin are presented in Figure 2-5. The mold population change was dependent of the species and the reuterin concentration. For *A. niger* strain (Figures 2-5 a.), 10 mM of reuterin reduced significantly ($P \leq 0.05$) the growth during the storage. The gradual reduction led to a final reduction of 3 log UFC/mL after four weeks, compared with the control without reuterin. The other treatments including natamycin did not significantly reduce *A. niger* growth compared to the control. In the case of the *P. chrysogenum* strain (Figure 2-5b.), 10 mM reuterin showed a significant reduction of 0.5 log cfu/mL to 0.7 log cfu/mL compared with the control without reuterin between the weeks two and three of storage. After four weeks, the control also showed a mold growth reduction of 0.5 log cfu/mL, ending at the same population as the 10 mM treatment. Other treatments did not show a significant reduction of *P. chrysogenum* growth. For *M. racemosus* strain (Figures 2-5 c), a concentration 5 mM of reuterin reduced significantly its population of 0.5 log cfu/mL to 1 log between weeks two and four. A concentration of 10 mM reduced the number of *M. racemosus* of 1.5 log cfu/mL after four weeks of storage compared with control treatment. The use of natamycin reduced the population of 3 log cfu/mL during the first 3 weeks, but after, the strain regrowth to reach a final population of 1.2 log cfu/mL. The growth of the strain *R. mucilaginosa* (Figure 2-5d), after 1 week of storage was inhibited for 3 weeks with a concentration of 10 mM of reuterin. However, the yeast regrows after 3 weeks. For the other reuterin concentrations the population of yeast continually increased during the 4 weeks of storage to reach populations close to 6 log cfu/mL. The presence of natamycin reduced the population of *R. mucilaginosa* of 4 log cfu/mL during the 4 weeks of storage. The pH results were not significantly different between all the treatments (data not shown). Base on visual growth (Figures 2-6), 2.5 mM reuterin reduced visually *R. mucilaginosa* growth compared with the control without treatment.

2.6 Discussion

In the current context of reducing food losses combined to limit the use of traditional food preservatives such as natamycin, the discovery of new natural compound is an urgent need for the food sector. Reuterin, an aldehyde produced by bioconversion of glycerol by *Lactobacillus* strains, is one of the most promising alternatives, mainly because of its large spectrum of inhibition activity including significant antifungal activity.

Antifungal activity of reuterin producing strains in cheese and yogurt was already reported in other studies (Martin-Cabrejas, Langa et al. 2017, Ortiz-Rivera, Sanchez-Vega et al. 2017). Recently, we have shown the potential of using *L. reuteri* culture supernatant containing reuterin to control the growth of mold and fungi in milk products (Vimont, Fernandez et al. 2018).

A require of using reuterin as an antifungal additive in food is to produce pure reuterin free of glycerol, 1–3 propanediol, acrolein or other contaminants. Studies carried out with non-purified culture supernatants (Engels, Schwab et al. 2016, Asare, Greppi et al. 2018) raised the doubt that the antibacterial and antifungal effect is due to the pre-occurrence of acrolein and not of reuterin. In this study, a depth characterization was carried out by GC-MS analysis and confirmed that the main reuterin form present in the pure aqueous working solution was constituted of monomers. These results are in agreement with those reported by (Burgé, Flourat et al. 2015) who have used the same method of purification. High purity reuterin solution used in this study demonstrated a broad spectrum of inhibition activity by reducing the growth of both yeast and filamentous molds tested.

The antifungal activity of reuterin was evaluated in yogurt preparation by determining the minimal inhibitory concentration (CMI) and the CMI/CMF ratio of each species. CMI values have shown that reuterin was active against *A. niger*, *M. racemosus*, *P. chrysogenum* and *R. mucilaginosa*. This broad spectrum of activity can be explained by the mode of action of reuterin as already shown by Vimont (2018) and which involve an oxidative stress on the microorganism membrane. More specifically, the aldehyde function of reuterin reacts with thiol groups of small molecules as glutathione (glutamylcysteinylglycine, GSH) and small proteins (Engels, Schwab et al. 2016). CMI and the CMI/CMF ratio values are slightly different from those of Vimont (2018) but still in the same range. These differences can be

explained by the differences in strain characteristics including growth and tolerance to chemical stresses. These results were already reported by (Chung, Axelsson et al. 1989, Vimont, Fernandez et al. 2018) who showed different inhibition activities of reuterin cell-free supernatant and pure reuterin of different species in commercial medium. Our results have also shown different sensitivity profiles to reuterin among fungal species. *M. racemosus* and *R. mucilaginosa* seem to be less sensitive to reuterin than *A. niger*. *P. chrysogenum*. This is in agreement with results reported by Vimont, (2018). The analysis of the MIC/MFC values bring useful indication about the mechanism of action of reuterin toward fungal strains. Here, the ratio values obtained strongly suggest that reuterin has a fungicidal activity since all ratio values were ≤ 4. This fungicidal activity is crucial for food preservation since even very low concentration of yeast may induce significant food spoilage (Garnier, Valence et al. 2017).

The results obtained with natamycin are significantly different from those obtained with reuterin. Indeed, for most yeast strains, the CMI are similar and are close to 0.16 mM/L Moreover, the CMI/CMF values are significantly higher than 4, indicating that natamycin has a fungistatic activity. The results shown in this study are in agreement with those reported by Vimont (2018). The differences in the antifungal activity between reuterin and natamycin can be explained by the differences in the mechanism of actions of these two molecules. The natamycin is a polyene antifungal acting on the ergosterol forming channel in fungal cell causing cells leaking. The mechanism of action of natamycin also seems to be concentration dependent. At low concentrations, natamycin acts by altering the membrane which induce a perturbation of the growth without killing the cell. Consequently the ratio CMI/CMF is higher than 4 and the activity is fungistatic (Resa, Gerschenson et al. 2013).

In this study, we have also investigated the influence of milk on the inhibition activity of reuterin. The inhibition activity of non-purified reuterin or the reuterin-producing *L. reuteri* strain was already investigated in previous studies. It was shown that reuterin produced by an *L. reuteri* strain at a concentration of 150 AU/mL (1 AU/mL=0.11mM) (Cleusix, Lacroix et al. 2008), reduced the growth of *Listeria monocytogenes* and *Escherichia coli* in UHT milk containing 0.5% to 3% fat (Ziney and Debevere 1998). According to Ziney (1998), fat does not impact reuterin activity. Another study has shown antifungal activity of reuterin against *Penicillium expandidum* strain at a concentration of 33.97 mM in fermented milk (Ortiz-

Rivera, Sanchez-Vega et al. 2017). In our study, we tested qualitatively the antifungal activity of reuterin in milk against four different mold and yeast species in comparison with natamycin. Contrarily to natamycin, inhibition zones obtained with reuterin are concentration dependent. This is probably due to the fungicidal effect of reuterin. For natamycin, and because of the fungistatic mechanism of action, all the concentrations induce similar inhibition zones on the agar plates. It should be noted that these results did not give any indication about the stability and the availability of both reuterin and natamycin in milk. Even if our results are not quantitative, it brings new information to the literature regarding the activity of pure reuterin in milk against four different mold and yeast species. It suggests the high potential of the application of reuterin as a new antifungal compound for milk and milk product applications.

Yogurt composition differs from milk by the acidic pH, the complex structure and the composition because it's a fermented milk product. According to our results, reuterin and natamycin kept their antifungal activity in yogurt as observed in milk. The antifungal activity of the two molecules depended on the mold species in yogurt as well as in commercial medium and in milk. In this study, reuterin concentration between 5mM and 10 mM was active against all the species tested. These concentrations are the lowest reuterin concentration reported showing a decrease in mold and yeast counts for four weeks of storage at 4 °C. Natamycin 5 ppm was not active against *P. chrysogenum* and *A. niger.* The different sensibility to reuterin of *A. niger*, *P. chrysogenum* and *M. racemosus* can be first related to the nature resistance of the species. Some species such as *Penicillium* are more resistant to chemical and external stress than other species like *Aspergillus* (Sarah C. Watkinson (2015). This is also supported by the fact that the *P. chrysogenum* strain was also more resistant to reuterin in the condition tested because reuterin causes an oxidative stress to microorganisms. Secondly, sensibility of the species can also be explained by the mode of action of the molecule. As previously mentioned, reuterin induces oxidative stress on cells. Oxidative stress in living organisms causes the production of defense molecules such as glutathione (GSH) (Grant (2001). Engels, Schwab et al. (2016) has shown that at a temperature of 4 °C, the presence of reuterin does not cause the production of GSH in *E. coli*, whereas at 37 °C the reuterin create the production of GSH. As at 4 °C the growth of microorganisms is slower, they interact much slower with their environments and therefore may react less quickly with

the presence of reuterin. The growth rate of mold and yeast species can therefore have an impact on the action of the reuterin. *P. chrysogenum* grows slower than *A. niger*, and the latter were more highly inhibited by reuterin (Prescott (2013), Sarah C. Watkinson (2015). For natamycin, the difference of sensibility of *P. chrysogenum* and *A. niger* can be explained by the resistance of the species to the polyene antifungal class (Kanafani and Perfect 2008). Regrowth was observed with natamycin and reuterin treatment respectively for *M. racemosus* and *R. mucilaginosa*. The regrowth of *M. racemosus* treated with natamycin can be explained by its fungistatic effect. Because the reuterin has shown a fungicidal effect in commercial and milk medium, we suggest that the regrowth of *R. mucilaginosa* is due to the spores initially added to the yogurt which may not have been completely killed. Reuterin as to be in contact with the mold or the yeast to inhibit the antifungal activity. Yogurt is a gel created by a protein network of casein. Because reuterin is not mobile in the yogurt's gel, the yeast may not have been in contact with the molecule and growth.

The reuterin antifungal activity results complement those obtained by (Vimont, Fernandez et al. 2018), which were able to reduce visual growth of *M. racemosus* and *P. chrysogenum* in a commercial yogurt for 21 days with a concentration of 6.9 mM of pure reuterin. The concentrations used in this study were the lowest reuterin concentrations reported reducing fungal growth in a milk product for 4 weeks storage. It is also the first time that antifungal reuterin activity is evaluated quantitively against four species in a yogurt, in the normal storage condition.

2.7 Conclusion

In this study, supernatant containing reuterin was produced by fermentation with the strain *L. reuteri*. Reuterin was purified and tested for its inhibition activity against various fungal strains in milk. Results are compared to those obtained with natamycin. Significant fungicidal activity was observed with pure reuterin when a fungistatic activity was observed with natamycin. We have also shown that reuterin kept its antifungal activity in milk and in commercial yogurt against all the fungal species tested. A significant growth reduction of the four tested fungal species was observed when reuterin was used at a final concentration of 10 mM in commercial yogurt for a period of four weeks. This study suggests a great potential

of reuterin as an antifungal compound in dairy products and other food product subject to fungal contamination.

2.8 Tables

Table 2-1 Purification of reuterin produced by *L. reuteri* ATCC 53608

	Volume (mL)	reuterin concentration* (mM/L)	Recovery (%)
Culture supernatant	210	56	100
Purified reuterin	154	55	73
Concentrated reuterin	3	2420	62

*HPLC assay, recovery : remaining reuterin as % of the initial concentration

Table 2-2 In vitro MIC90 and MFC ranges of reuterin and natamycin against 3 filamentous molds and yeast as determined by CLSI-M35 official method.

Strains	MIC90* reuterin (mM/L)	MIC90* natamycin (mM/L)	MFC†/MIC reuterin	MFC†/MIC natamycin
Aspergillus niger	1.89^{c}	0.08^{de}	1^{ef}	16^{de}
Rhotorula mucilaginosa	7.58^{a}	0.16^{d}	1^{d}	16^{b}
Penicillium chrysogenum	3.79^{b}	0.08^{de}	1^{de}	32^{c}
Mucor racemosus	7.58^{a}	0.005^{e}	2^{f}	143.8^{b}

*MIC90 = minimal concentration inhibiting 90% of the fungal population.
†MFC = minimal concentration inhibiting 100% of the fungal population.
Values in the same column with different letter are significantly different ($P \leq 0.05$).

2.8 Figures

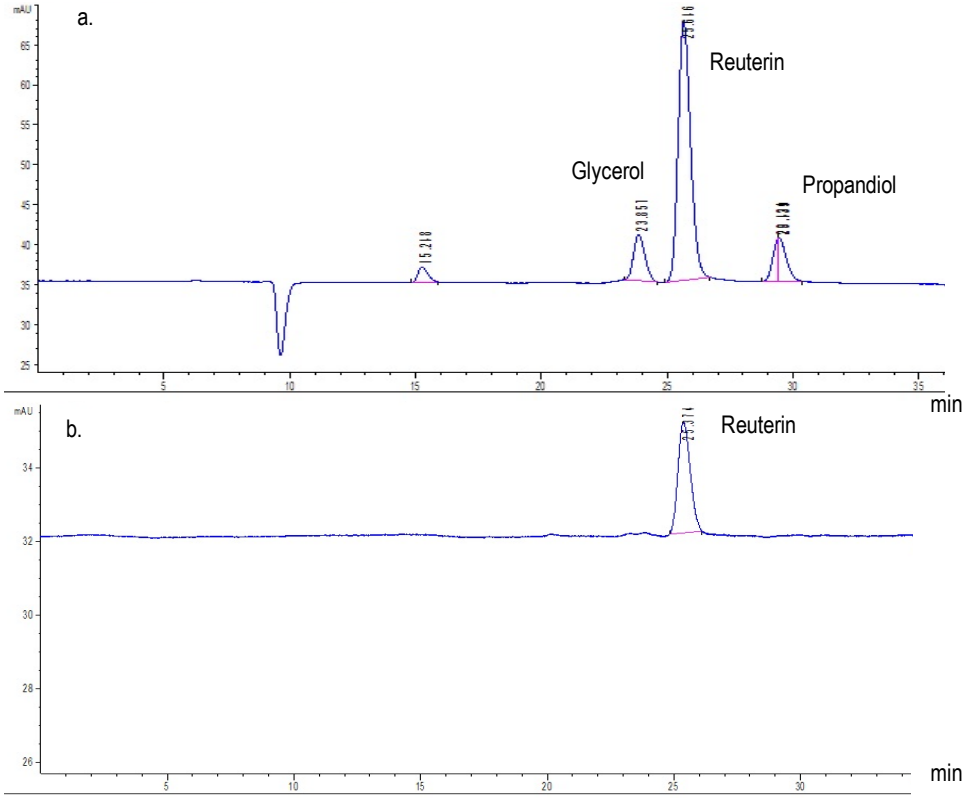

Figure 2-1 Chromatogram of non-purified (A) and purified reuterin (B) by HPLC isocratic phase.

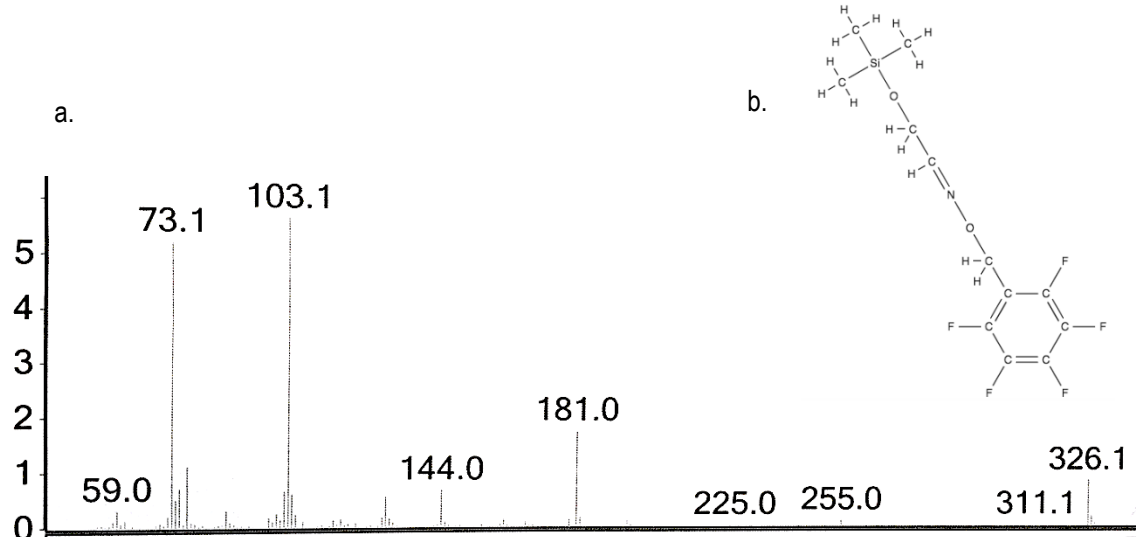

Figure 2-2 MS spectrum of 3-HPA (80 ng mL 1) in MDA. m/z values of 73, 103, 144, 181, 225, 255, 311, 326 and 341 correspond to 3-HPA and its derivatives of (CH3)Si and $C_6F_5CH_2ON$ adducts.

Figure 2-3 3-HPA (a) (80 ng mL-1) without derivatives adducts in the monomer form.

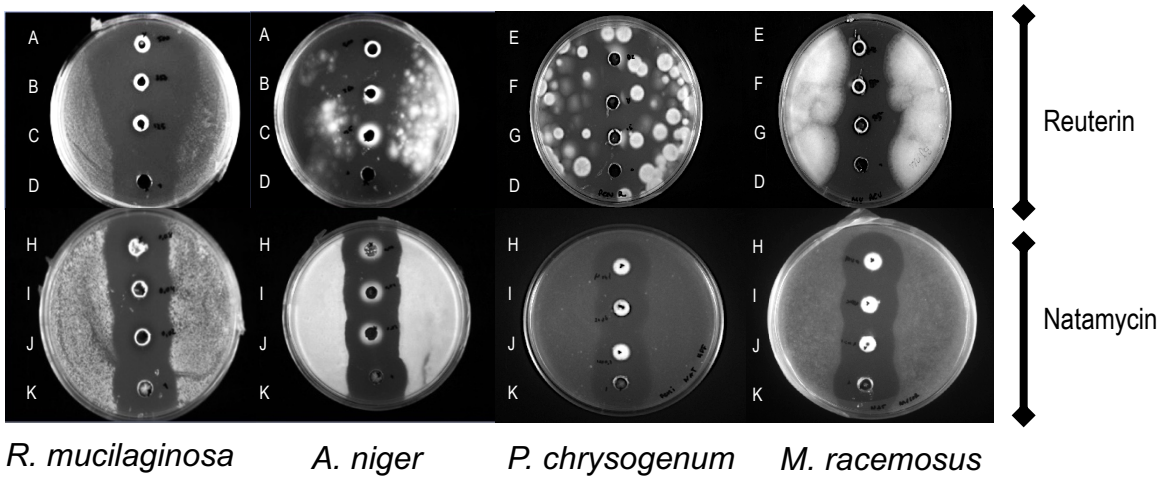

Figure 2-4 Antifungal activity of reuterin and natamycin diluted in 3.25% M.F. milk (3 first wells on each plate) compared to a control (CT; last well on each plate) mixed in water, against *R. mucilaginosa*, *A. niger*, *P. chrysogenum* and *M. racemosus* by agar diffusion method. Reuterin concentration: A, 250 mM in milk; B, 125 mM in milk; C, 62 mM in milk; D, 100mM in water; E, 31 mM in milk; F, 15 mM in milk; G, 7 mM in milk. Natamycin concentration: H, 0,008 mM in milk; I, 0,004 in milk; J, 0.002 in milk; K, 0.005 in water.

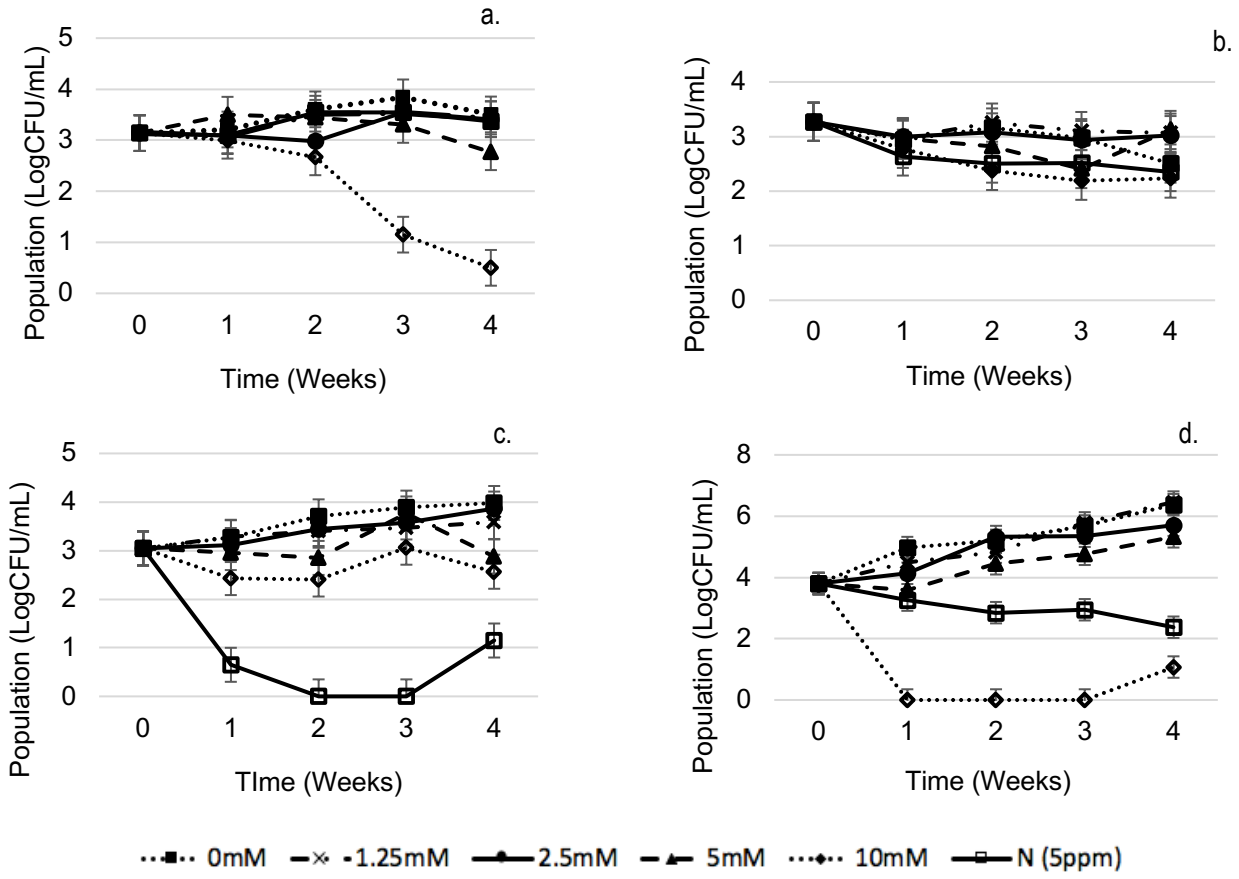

Figure 2-5 Change in population of *A. niger* (a.), *P. chrysogenum* (b.), *M. racemosus* (c.) and *R. mucilaginosa* (d.), during 4 weeks of storage at 4 °C in presence of 0, 1.25, 2.5, 5 and 10 mM of reuterin and 5 ppm of Natamycin in commercial yogurt. Error bar represent standard error on the mean (SEM).

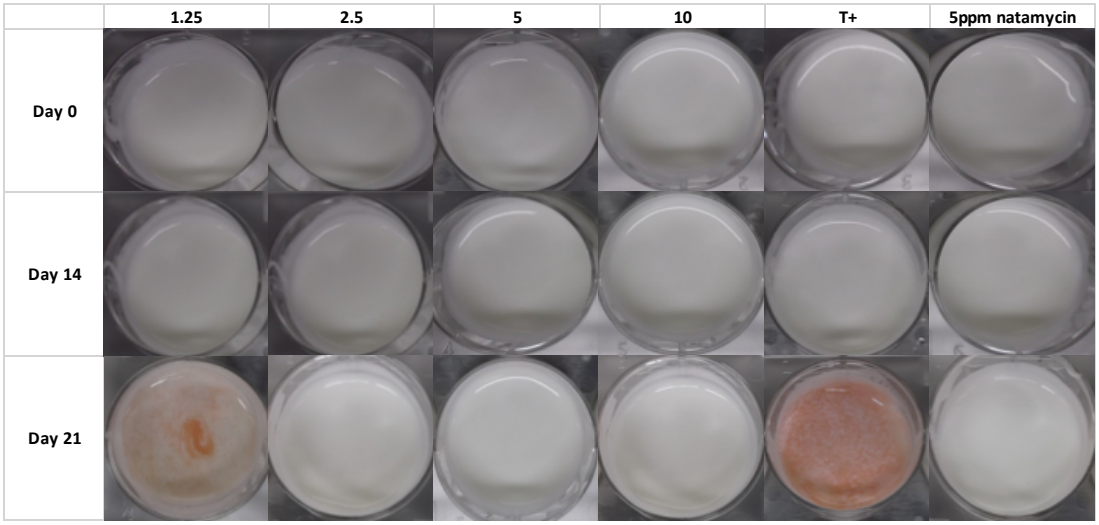

Figure 2-6 Growth of *R. mucilaginosa* in commercial yogurt treated with different reuterin concentration (1.25, 2.5, 5, 10 mM), natamycin 5ppm and control of sterile water (T+) and stored at 4 °C for 21 days

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Chapitre 3 Reuterin use as an antifungal ingredient in a stirred yogurt process

3.1 Résumé

Les yogourts sont des produits laitiers très populaires au Canada. Les yogourts se distinguent des autres produits laitiers par leur contenu élevé en eau et leur acidité. Ces deux particularités font des yogourts des produits qui sont sujets à la contamination par les moisissures et les levures. Ces contaminations causent des défauts de saveur, d'odeurs et de couleurs qui les rendent impropres à la consommation. Au niveau industriel, les contaminations sont la principale cause de pertes pour les transformateurs. Au Canada, l'utilisation d'agents de conservation reconnus pour leur activité antifongique n'est pas permise dans les yogourts. Par conséquent, les industriels sont à la recherche d'autres solutions pour réduire la croissance des moisissures et des levures dans les yogourts. La réutérine est une molécule produite par la biotransformation bactérienne du glycérol par des souches *L. reuteri*. Cette molécule est connue pour son activité antifongique à large spectre. L'activité antifongique de la réutérine est à ce jour bien caractérisée. Cependant, l'impact de la réutérine utilisée comme un ingrédient antifongique dans une matrice complexe comme le yogourt n'a été que peu étudié. L'effet de la réutérine pure sur les ferments *L. bulgaricus* et *S. thermophilus* ainsi que la stabilité de la réutérine pure dans un yogourt ne sont pas connus. Le but de cette étude était d'étudier l'impact de la réutérine pure sur la viabilité du ferment lactique à yogourt, d'étudier la stabilité de la réutérine dans le yogourt et de comparer l'activité antifongique de la réutérine à celle d'antifongiques commerciaux tels que Nataseen et FQ_2 contre *A. niger* pendant un entreposage de 15 jours. La réutérine pure, la natamycine et le FQ_2 ont été testés dans un modèle de yogourt brassé inoculé avec *A. niger* et acidifié avec un ferment lactique constitué d'une souche de *L. bulgaricus* et *S. thermophilus* ou avec de la Delta- Gluconolactone (GDL). Les principaux résultats ont démontré que la réutérine pure utilisée comme ingrédient après l'étape de brassage a permis d'inhiber complètement *A. niger* pendant les 15 jours d'entreposage indépendamment de la méthode d'acidification. La réutérine s'est aussi montrée plus efficace contre *A. niger* que la natamycine et que le FQ_2. Cependant, la réutérine a réduit la viabilité des deux bactéries lactiques *L. bulgaricus* et *S.*

thermophilus. De plus, la concentration de réutérine a diminué, passant de 5 mM à 0 mM au cours de l'entreposage pour l'acidification chimique au GDL et l'acidification avec les ferments thermophiles, suggérant une interaction avec les composantes de yogourts et non un effet des ferments. Pour la première fois, l'activité antifongique de la réutérine pure utilisée comme ingrédient a été comparée avec des agents antifongiques commerciaux. Cette étude a démontré le potentiel d'utilisation de la réutérine comme agent antifongique dans un yogourt. Cependant, d'autres études doivent être effectuées pour limiter l'impact de la molécule sur la viabilité des ferments lactiques utilisés dans la production de yogourt.

3.2 Abstract

Yogurt is a dairy product widely consumed in Canada. Yogurt differs from other dairy products in their high-water content and acidity. These two features make yogurts more prone to fungal contamination. Contamination of yogurts by molds and yeast causes defects in flavor, texture and color, making them unsuitable for consumption. The contamination causes food losses and it has significant economic impacts on yogurt processors. In Canada, the preservatives known as antifungal are not allowed in the yogurts. Therefore, yogurt processors are looking for solutions to reduce mold growth in yogurt. Reuterin is a molecule that has shown antifungal activity and is produced by the bioconversion of glycerol by *L. reuteri* strains. The antifungal activity of reuterin against mold and yeast is now better characterized. However, the impact of reuterin used as an ingredient in yogurt ferments as well as the stability of the pure molecule in yogurt has been little studied. The purpose of this study was to show the impact of pure reuterin on yogurt starter viability, the stability of pure reuterin in a yogurt matrix and to compare reuterin antifungal activity with commercial antifungals such as Nataseen and FQ_2 against *A. niger*. Reuterin, natamycin and FQ_2 were tested in a stirred yogurt inoculated with *A. niger* and acidified with *L. bulgaricus* and *S. thermophilus* or with GDL. The main result of this study was that reuterin used as an ingredient after stirring step of yogurt production shown a complete inhibition of *A. niger* for 15 days storage independently of the acidification method. The reuterin treatment was more efficient for mold inhibition than natamycin and FQ_2 treatment. However, pure reuterin have affected negatively the viability of the yogurt's starter *L. bulgaricus* and *S. thermophillus* in

contrary of the FQ2 treatment that protected the starters. Also, the reuterin concentration in yogurt during storage decreased from 5 mM to 0 mM after 15 days showing and interaction with yogurt matrix for both acidification methods. For the first time, reuterin was compared with commercial antifungals in a yogurt. It also showed that reuterin may interact with yogurt components because we saw a decrease of the concentration of reuterin in yogurt during storage. Reuterin still have a great potential for a use as an antifungal compound in dairy products. However, more studies are needed to determine how the activity of reuterin on starters can be minimalized.

3.3 Introduction

Yogurts are dairy products widely consumed in Canada and in the world (Chandan 2013). They differ from other dairy products in their high-water content and acidity. These two features make yogurts more prone to fungal contamination (Pitt and Hocking 2009). The main production steps of yogurt are the milk thermic treatment, the inoculation with a lactic acid starter constituted with strains of *L. bulgaricus* and *S. thermophilus*, the bacterial acidification at 42 °C, the packaging in containers and the distribution (CodexAlimentarius 2011). Yogurt is subdivided in two categories, stirred and set style yogurt. The main difference is that for set-style yogurts, fermentation is carried out directly in retail container while for stirred yogurts acidification occurs in a yogurt vat and some stirring operations are performed before the yogurt is packed into retail containers (Y. and K 2007).

Mold often comes from the production environment or aromatic agents like fruits added to yogurt (Blackburn Clive de 2006). Stirred yogurt are more prone to mold and yeast contamination than firm yogurt because of the additional stirring operations (Suriyarachchi and Fleet 1981, Guenard-Lampron, St-Gelais et al. 2019). Contamination of yogurts by molds and yeast causes defects in flavor, texture and color, making them unsuitable for consumption (Pitt and Hocking 2009). The contamination causes food losses and it has significant economic impacts on the yogurt processors (Arnott, Duitschaever et al. 1974, Blackburn Clive de 2006, Abdulla, Martin et al. 2013). In Canada, no preservatives are allowed in the yogurts. Therefore, yogurt processors are looking for solutions to reduce mold growth in yogurt. To counter this issue, some companies producing additives propose to use natamycin, which is generally recognized as a safe (GRAS) antifungal preservative (D.S.M.

2014). Natamycin is an antifungal used as preservative in grated cheese but its use in yogurt is not allowed (Hekmat and Reid 2007, Dalhoff and Levy 2015). The protective strains like *L. casei* and *L. rhamnosus* can also be used to reduce mold and yeast growth (Leyva Salas, Thierry et al. 2018). Protective strains have the ability to produce metabolites such as organic acids, cyclic dipeptides and other molecules that may have antifungal effects (K., J. et al. 2002, V.D. and R. 2016). Reuterin is a molecule that has shown antifungal activity and is produced by the bioconversion of glycerol by *L. reuteri* strains (Vimont, Fernandez et al. 2018). Studies have shown that it is possible to produce reuterin directly in yogurt by using reuterin productive strains *L. reuteri* (Langa, Landete et al. 2013, Gomez-Torres, Avila et al. 2014, Martin-Cabrejas, Langa et al. 2017, Ortiz-Rivera, Sanchez-Vega et al. 2017). However, the presence of *L. reuteri* strain in yogurts may cause gas production (Champagne, Raymond et al. 2015).

Vimont, Fernandez et al. (2018) have shown that the use of purified reuterin in yogurts can reduce the visual growth of weathering mold for 28 days at 4 °C. The production of purified reuterin by HPLC as well as the characterization of the antifungal activity against four mold and yeast strains was carried out in our research. The results present in the first article of this document (Chapter 2) show that pure reuterin is active in a synthetic medium and in milk against all the tested species. In addition, we have shown that the use of reuterin in commercial yogurt contaminated with the four strains of fungal species allowed reducing significantly the growth of three species. If the antifungal activity of pure reuterin against molds was better characterized, its impact as an ingredient in yogurt has only been little studied. The activity of reuterin is attributable to the fixation of the reuterin to the thiol group present on the protein of fungal cells. When the reuterin is fixed to proteins, the molecule may lose its activity. The efficiency is attributable to non-fix to protein that is free to react with fungal membrane proteins. Yogurt composition is high in proteins. Reuterin may fix to yogurt's proteins and reduce reuterin free in the matrix, necessary for antifungal activity. The aim of this study was to see the impact of yogurt matrix on reuterin concentration in yogurt and the impact of reuterin on yogurt lactic acid starter when reuterin is used as an ingredient. In addition, the antifungal activity of reuterin against *A. niger* was compared to natamycin and a protective strain of *L. rhamnosus* in a stirred yogurt produced in the laboratory.

3.4 Materials and methods

3.4.1 Strains

The stock culture of *L. reuteri* ATCC® 53608TM (American Type Culture Collection, Manasa, VA, USA) and *A. niger* CMBL-121 (Metabiolac collection, Laval University, Québec, QC, CAN) were stored in the same conditions described in section 2.3.1. (chapter 2). The stock cultures of *L. delbrueckii subsp. Bulgaricus* 210R (Gist Brocades, Millville, UT, USA), *S. thermophilus* HC15 (Chr. Hansen, Mississauga, ON, Canada), *L. rhamnosus* FQ2 (Chr. Hansen, Mississauga, ON, Canada), were kept frozen at -80 °C. *L. bulgaricus*. And *S. thermophilus* were stored in skim milk (20% total solids, wt/wt; Crino low heat, 309150723-02, Agropur, Saint-Hyacinthe, QC, Canada) with a supplement of 5% sucrose (Thermo Fisher Scientific, Hampton, N.H. USA) and 0.35% of ascorbic acid (Sigma-Aldrich, Saint-Louis, MO, USA). *L. rhamnosus* was used as lyophilized culture.

3.4.2 Chemicals and Antimicrobials

Natamycin commercial brand Nataseen® was purchased from Siveele (Breda, Netherlands). Glucono-delta-lactone (GDL), vancomycin and tetracycline were purchased from Sigma-Aldrich (Saint-Louis, MO, USA).

3.4.3 Reuterin production and purification

Reuterin has been produced and purified as described in chapter 2.

3.4.4 Strains activation

Bacterial strains *L. bulgaricus*, *S. thermophilus* and *L. reuteri* were activated in 12% (total solid (wt/wt) reconstituted skim milk with deionized water, previously sterilized at 110 °C for 10 min. For the growth of *L. reuteri*, reconstituted skim milk was supplemented with 0.3% (wt/wt) of yeast extract. All the bacterial strains were inoculated at a rate of 10% (1 mL/10 mL). The strains were incubated at 36 °C for 16h. *L. bulgaricus* and *S. thermophilus* were transferred at 1% (100 µL/10 mL) rate in 14% (total solids; wt/wt) skim milk reconstituted with deionized water. *L. reuteri* was transferred at 1% in milk 14% total solids with 0.3% (wt/wt) of yeast extract. The strains were incubated at 37 °C until pH dropped to 4.6 pH unit. The strains were then stored at 4 °C until production day. The mold

strains was activated as described in section 2.3.5 (chapter 2). One hour before the production, the mold spore suspension (10^5 spores/mL) was prepared in peptone water as described in section 2.3.6.(chapter 2) and kept at 4 °C until yogurt mix inoculation. *L. rhamnosus* (FQ2) was added directly in yogurt as recommended by the company specifications.

3.4.5 Yogurt milk

Yogurt milk (0.5 kg) was made with low heat skim milk powder (Crino 309150723-02, Agropur, Saint-Hubert, QC, Canada) reconstituted with 500 mL deionized water at a concentration of 14% total solids (wt/wt). The powder and the water were homogenized on a magnetic stirring plate (Isotemp, Thermo Fisher Scientific, Hampton, N.H. USA), for 3 hours at 500 rpm at room temperature. After preparation, yogurt milk was stored at 4 °C overnight for mineral equilibrium. The day of production, a thermic treatment of 10 min at 110 °C was applied on yogurt milk. After the thermic treatment, the yogurt was rapidly cooled at 42 °C in ice. The yogurt milk was contaminated with *A. niger* at a rate of 1% (v/v) to reach a final spore concentration of 1×10^4 spores/mL.

3.4.6 Yogurt Production

Yogurt production was done in an aseptic environment under a scientific biological cabinet (Thermo Scientific™ 1300 series class II, Waltham, Massachusetts, USA). Five contaminated yogurts milk were acidified with thermophilic starters, *L. bulgaricus* and *S. thermophiles* and one acidified with GDL (Table 3-1). For yogurt produced with GDL, 60 mL of yogurt milk was poured into 90 mL sterile plastic bottles (C566-60AQSECO, Simport, Beloeil, CAN) in which 1.5% of GDL was added. The yogurt acidified with GDL **(R-GDL)** was treated with reuterin at a concentration of 5 mM. The rest of the yogurt milk was inoculated with *L. bulgaricus* and *S. thermophilus* at a rate of 1.5% (v/v) for each strain for a final ratio of (1 :1). After, 60 mL of inoculated milk was poured in 90 mL sterile plastic bottles and treated with different treatments. The first treatment was 5 mM of reuterin **(R)**, the second was 5 ppm of Natamycin **(N)**, the third was the *L. reuteri* strain at 1% and 50mM glycerol **(LR-GLY)**, the fourth was a commercial protective culture of *L. rhamnosus* **(FQ_2)**, and the last not treated and consider as control **(CT)**.

The yogurt sample containing active bacteria **LR-GLY and FQ_2** were formulated before incubation to allow the growth of bacteria present in the treatment. In LR-Gly treatment, 1% (v/v) of the *L. reuteri* fresh culture and 50 mM (w/v) of glycerol was added to the sterile bottle with yogurt. For FQ2 treatment, 0.5 mg (1×10^6 UFC/mL) of *L. rhamnosus* lyophilized culture was added to the bottle with yogurt. All the yogurt treatments were vortexes for 30 seconds before the acidification step. Then all yogurts samples were incubated at 42 °C in an incubator (Fischer scientific, Hampton, N.H. USA), until the pH dropped to 4.6.

After acidification, yogurts were immediately cooled to 25 °C in an iced bath. Natamycin 5ppm and reuterin 5 mM were added to yogurt samples after acidification and cooling step to complete the **R, N** and **R-GDL** treatments. The reason why these treatments were applied after acidification is not to disrupt starters. Pre-test data (not presented) showed that reuterin negatively affect the acidification step. All the yogurt samples were mixed for 30 seconds with a sterile inoculation loop before stirring steps.

Each fermented yogurt was transferred into 60 mL syringe (BD, Franklin-Lakes, N.J., USA) and stirred as described by (Michalski, Calzada et al. 2008). After that, each stirred yogurt was distributed into six 15 mL falcon tubes (Falcon Corning, N.Y., USA), containing 10 mL each. The falcon tubes were stored at 21 °C for analysis after 0, 1, 5 and 15 days. The high storage temperature allowed promoting the growth of molds and better quantify the fungal growth reduction of different treatments. R treatment and CT treatment were also stored at 4 °C to see if the reuterin treatment is usable in a real conservation temperature.

3.4.7 Microbial Counts

Microbial counts were done at 0, 1, 5 and 15 days of storage. Serial dilutions of yogurt samples were done in 0.5% (w/v) peptone water (BD-Difco, Sparks, MD, USA) in corning glass bottles (Corning, N.Y. USA). *Streptococcus thermophilus, L. bulgaricus*, *L. rhamnosus* and *L. reuteri* population were determined by pour plate counts. M17 (BD-Difco, Sparks, MD, USA) medium supplemented with 0.5% lactose (Sigma-Aldrich, Saint-Louis, MO, USA) and 15 g/L agar (BD-Difco, Sparks, MD, USA) was used for *S. thermophilus* and De Man Rogosa Sharp (MRS, BD-Difco, Sparks, MD, USA) medium acidified with 0.1% acetic acid (Sigma-Aldrich, Saint-Louis, MO, USA) used for *Lactobacilli ssp.* Acidified MRS supplemented with vancomycin (1 mg/L) was used for *L. rhamnosus* and tetracycline

(9 mg/L) for *L. reuteri*. *A. niger* population was determined by spread plate counts on PDA medium acidified with 1% (v/v) tartaric acid (8371-16, RICCA, Arlington, TX, USA). The thermophilic bacteria were incubated in anaerobic jars at 37 °C for 18 hours before reading. Mold plates were incubated in dark area at 25 °C for 3 days before reading. Enumeration of all species was done in duplicates.

3.4.8 Reuterin Quantification

Reuterin quantification in CT, R-GDL, R and LR-Gly yogurt was done after 0, 1, 5 and 15 days of storage. Two milliliters of each sample were centrifuged in 2.5 mL tubes (Thermo Fisher Scientific, Hampton, N.H. USA), at 17 *g* for 20 min at 4 °C in an Eppendorf centrifuge (5418 R, Eppendorf, Hambourg, GER). The supernatant was recovered and filtered on 13 mm diameter 0.22 μm InnoSep-SF13 syringe filter (Chrom4, Thüringen, GER) directly into 2 mL glass vial with PTFE septa 12 mm cap (Agilent, Santa Clata, CA, USA). Samples were analyzed by high pressure liquid chromatograph (HPLC) device (1290 infinity, Agilent, Santa Clara, CA, USA) equipped with refractive index detectors (RID). The yogurt supernatant samples were injected on an ions-300 HPLC column (ICE-99-9850, Concise separation, San Jose, CA, USA) at 40 °C in a mobile phase of 10 mM H_2SO_4 (Sigma-Aldrich, Saint-Louis, MO, USA). The injection volume was 100 μL, the flow rate of the mobile phase was 0.4 mL/min and sample run duration was 40 minutes. The calibration curve was done with serial dilution in water of pure reuterin (12.75 M/L) as described in the literature (Vimont, Fernandez et al. 2018). Samples were run on the HPLC immediately after preparation.

3.4.9 pH and acidity determination

pH and acidity analysis were done by titration with NaOH 0.1N (Sigma-Aldrich, Saint-Louis, MO, USA) by using an automatic titrator model Titralab TIM850 (Hach, Loveland, CO, USA). pH was taken before acidity titration with the titrator. Titration was done on 10 mL of each yogurt samples mixed with 10 mL distilled water in a plastic cup until to reach of pH of 8.6. The amount of NaOH used to titrate samples was multiplied by 10 to get the acidity of each sample.

3.4.10 Statistical Analysis

The 6 treatments were applied randomly to 6 yogurts samples and analyzed over time. The experiments were in a split plot design with the treatment as the mean factor and time as the second factor. The experiment was done in triplicate starting from the different frozen stock. All statistical analyses were performed using the SAS® Studio Software (SAS institute Inc. Cary, NC, USA) using the same statistical analysis and significant *P*-values describe in section 2.4.12. The treatment acidified with GDL was removed from the bacterial count statistical analysis because the treatment was made without bacteria and remove from the comparaison. The *L. rhamnosus* and *L. reuteri* viability counts were removed from statistical analysis because only two treatments were inoculated with these strains.

3.5 Results

3.5.1 Reuterin Quantification

The reuterin concentration in R, R-GDL and LR-GLY yogurts during storage is presented in Table 3-2. A significant interaction between types of yogurt and storage time was observed ($P \leq 0.05$). The test for in situ production of reuterin in LR-GLY yogurt in presence of the *L. reuteri* strain and glycerol did not show significant reuterin production during the 15-day storage. For R and R-GDL yogurts, in which reuterin was added as an ingredient, the concentration of reuterin decreased significantly during the 15-day storage period. After 1 day of storage, the reuterin concentrations in R and GDL-R yogurts were respectively 3.4 mM and 4.1 mM comparatively to 5 mM added at the beginning of storage. After five days of storage, the reuterin content in R and GDL-R yogurts was 0.8 mM. At the end of the storage period, the reuterin concentration in R and GDL-R treatment was 0.3 mM.

3.5.2 *L. reuteri* and *L. rhamnosus, L. bulgaricus* and *S. thermophilus*, viability

The strain *L. reuteri* did not grow in fermented milk and the bacteria was not detectable in the treatment LR-Gly. The strain was detectable after inoculation, but not after incubation of the yogurt. The *L. rhamnosus* population was 7.51 cfu/mL before acidification and was

between 8.9 and 8.0 Log (cfu/mL) for the 15 days of the storage without significant difference. The changes of population of thermophilic starter *L. bulgaricus* and *S. thermophilus* during storage of CT, FQ2, LR-GLY, N and R yogurts are presented respectively in Figures 3-1 and 3-2. A significant interaction between storage time and types of yogurt was observed ($P \leq 0.05$). The population of *L. bulgaricus* was between 7.08 and 7.97 log (cfu/mL) and the population of *S. thermophilus* before acidification was between 7.08 and 7.67 log (cfu/mL) for all yogurts. No significative differences were detected between treatment for thermophilic starter. After acidification, the *L. bulgaricus* and *S. thermophilus* in all yogurt milk reached respectively, 9.13 log and 8.9 (cfu/mL).

After 1 day of storage, the *L. bulgaricus* and *S. thermophilus* the population of all treatments were the same as after acidification except for yogurts with R yogurts. In the R yogurts, the *S. thermophilus* population was stable, but that of *L. bulgaricus* population decreased of 5.44 log (cfu/mL) compared to CT yogurts.

After 5 days of storage, the *L. bulgaricus* population in all yogurts was stable, but the bacteria were dead in the yogurt with R yogurts. The *S. thermophilus* population in R yogurts was 4.89 log (cfu/mL) less than the CT yogurts. The other treatments did not show significant difference compared with the CT yogurts.

After 15 days storage, the *L. bulgaricus* population was stable for allyogurts, but no viable *L. bulgaricus* bacteria were found in the yogurt with R yogurts. The *S. thermophilus* population in CT and LR-GLY yogurts showed, respectively, a reduction of 5 log CFU/mL compared to day 5. The N and R yogurts showed a reduction of respectively 6.5 and 1.7 Log CFU/mL compared to day 5. The final population of *S. thermophilus* for CT and LR-GLY yogurts was 3 log CFU/mL and was 1 log CFU/mL for N and R yogurts. No reduction of *S. thermophilus* population was observed in FQ_2 yogurt. *L. bulgaricus* population for all yogurts after 15 days storage was not significantly different when compared with day 5, except for R yogurt where its population was completely inhibited. The population after 15 days storage was in the range of 7.5 and 8 log CFU/mL for all yogurts except for R yogurt.

3.5.3 pH & acidity

Changes in pH values during storage of all yogurts are presented in Figure 3-3. The pH results observed depended both on storage time and on treatment. Before acidification (day 0) there was no significant difference of pH between the yogurts acidified with bacteria with a pH value of 6.3 for all yogurts. The yogurt acidified with GDL (GDL-R) showed a pH significantly lower than the other yogurts before acidification with a pH of 5. After the acidification step (data not shown in the figure 3-3), all the yogurts were at the same pH values with a pH of 4.6. After one day and for the full time of storage, the pH dropped non-significantly from 4.00 to 3.6 for all the yogurts (CT, FQ2, LR-GY-N), except for both with reuterin. The two yogurts with reuterin differ statistically from the other yogurts and the CT yogurt without treatment with a pH higher pH than 4.6.

Changes in acidity during storage of all yogurts are presented in Figure 3-4. The acidity, as for the pH, depends on storage time in treatment. Before acidification (day 0), the acidity of all yogurt was not statistically different between yogurts with acidity between 23 and 38 °D, except for GDL-R yogurts that was statistically different with the acidity of 75 °D. After the acidification step of yogurt process, the acidity of all yogurts dropped. As for the pH, results after one day and for the rest of the storage period were significantly different for both yogurts treated with reuterin (R and GDL-R). The acidity increased between 80 and 102 °D for yogurts without reuterin. The acidity was significantly lower for R and GDL-R with the acidity of 48 and 58. After five days of storage, no differences were observed between FQ2, N and CT yogurts. For these three yogurts, the acidity increased significantly between days one and day 5 of storage to reach levels between 145 and 151 °D. The acidity of FQ2, N and CT increased non-significantly between day five and day 15 of storage. For LR-GLY yogurts the same pattern of acidification was observed as for the other yogurts without reuterin but was significantly lower. The acidity of LR-GLY after five days stay constant until day 15. For both yogurts with reuterin (R and R-GDL) the acidity did not change significantly between five and 15 days of storage.

3.5.4 Mold Reduction

Changes in population of *A. niger* in all yogurts during storage at 21 °C are presented in figure 3-5. For all storage periods, a complete inhibition of the growth of *A. niger* in yogurts R and GDL-R was observed. For the other types of yogurts, the population of *A. niger*

decreased slowly between day one and day five and was significantly similar to CT yogurt except for N yogurt at day 15. For this later, the number of *A. niger* was significantly lower than CT yogurt. The effect on mold reduction of reuterin in yogurt was also tested at 4 °C during a storage of 15 days (data not presented). The results obtained showed the same tendency as observed at 21 °C storage, but the reduction of mold population in yogurt was slower at 4 °C. After one day, the yogurt R showed a reduction of 3 log cfu/mL compared with yogurt CT. Complete inhibition of mold was observed after 5 days of storage at 4°C instead of 1 day, when yogurts were stored at 21 °C (Figure 3-5).

3.6 Discussion

In this study, the effect of purified reuterin used as an ingredient and in situ reuterin production with a *L. reuteri* strain was compared with a commercial protective culture and with natamycin for fungal control in stirred yogurt.

First, no reuterin was detected in the yogurt treatment with the *L. reuteri* strain and 50 mM glycerol (LR-GLY). These results were differing from literature, in which some research have shown the antibacterial activity of reuterin produced in situ in the dairy food matrix like fermented milk and in cheese against a large spectrum of bacteria (Langa, Landete et al. 2013, Martin-Cabrejas, Langa et al. 2017, Ortiz-Rivera, Sanchez-Vega et al. 2017). One of these studies showed antifungal activity of reuterin produced in situ in fermented milk against *Penicillium expansum* (Ortiz-Rivera, Sanchez-Vega et al. 2017). All these studies have all used high *L. reuteri* inoculum to induce reuterin production in food. Ortiz-Rivera (2017) has used 5.1×10^9 cfu/mL, Langa (2013) used 5 Log cfu/mL and Martin-Cabrejas (2017) used $1x10^6$ cfu/mL. The strain of *L. reuteri* used in this study did not grow in fermented milk and the population was not detectable in yogurt after acidification. The low population of the strain in yogurt can explain why no reuterin was produced in the yogurt. Another difference between the studies that were successful in producing reuterin in situ in dairy products is that the *L. reuteri* bacteria were used in the stationary growth phase (Langa, Landete et al. 2013, Gomez-Torres, Avila et al. 2014, Martin-Cabrejas, Langa et al. 2017, Ortiz-Rivera, Sanchez-Vega et al. 2017). It was shown that the *L. reuteri* strain produce more reuterin in a high concentration of bacteria and in the stationary phase (Doleyres, Beck et al. 2005, Tobajas, Mohedano et al. 2007).

The reuterin quantification in yogurt matrix over time showed that the molecule decreased during storage for yogurts acidified with thermophilic starters and also acidified only with the GDL. These results suggest that decrease of reuterin was probably due to the interaction of reuterin molecules with some yogurt components but not to the presence of the thermophilic starter. Arques, Rodriguez et al. (2008), Langa, Landete et al. (2013) have observed the same phenomena in cheese model and in yogurt. Arques, Rodriguez et al. (2008) proposed that the decrease may be due to interaction with milk components. The decrease of reuterin in a cheese model was observed by Langa, Landete et al. (2013) after 25 days of storage. In their study, the *L. reuteri* strain was added to a cheese model supplemented with glycerol to induce in situ reuterin production. Before 25 days, they showed that the *L. reuteri* strain produced reuterin steadily in cheese, but after 20 days the population of *L. reuteri* strain dropped and the reuterin in cheese also decreased. This result is also in accordance with the mechanism of action of the reuterin. It is possible that reuterin reacts with free thiol groups of proteins as denatured β-lactoglobulin present in yogurt (Vasbinder 2002, Cleusix, Lacroix et al. 2007). Reuterin may also create Schiff base in yogurt. The Schiff base results of a reaction of an aldehyde like reuterin with a primary amine, which are present in yogurt (Silva, Silva et al. 2011). The chemical reaction of reuterin with yogurt components may have caused the decrease quantification by HPLC. The HPLC method only detects free molecules present in yogurt. If reuterin was fixed to protein, the detection of reuterin was not possible by HPLC and a decrease could be observed.

It is known that reuterin has a broad spectrum of activity against many microorganisms. The results obtained in this study have shown that the presence of reuterin in yogurt decreased the population *S. thermophilus* and *L. bulgaricus*. According to several standards, yogurt must have a minimum of 1×10^6 CFU viable *S. thermophilus* and *L. bulgaricus* per mL to be considered as a yogurt. Hence, the presence of reuterin could cause a problem because the number of thermophilic starter decreased lower than 1×10^6 cfu/mL. However, this result is in discordance from results obtained by Langa, Landete et al. (2013). These authors observed that the reuterin did not affect the counts of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* in their yogurt models. However, they used a concentration of 1.5 mM comparatively to 5 mM used in our study. Therefore, it is possible that the concentration of

reuterin used in their yogurt models (1.5 nM) was too low to observe bacterial mortality. Moreover, the same authors have also tested a reuterin concentration of 4 mM in cheese and they observed a diminution of cheese starter population.

The pH and acidity changes of the yogurt samples during storage were related to starter viability. Also, all yogurts were stored at 21 °C to allow a better growth of *A. niger*. The thermophilic bacteria population dropped during storage, but the bacteria continued to produce lactic acid. The acid production was observed by the decrease of the pH and the increase of titrable acidity in all samples without reuterin (Cruz, Walter et al. 2010). A decrease of viability of the *S. thermophillus* population was noted after 15 days of storage and the mortality is related to the decrease of pH and the increase of the acidity. Lactic acid production by lactic acid bacteria can occur in bacterial stationary growth phase. This phenomenon is called decoupling. It is well known that *S. thermophilus* is more affected by acidic pH than *L. bulgaricus*. During storage at 21 °C, the *L. bulgaricus* strain may have produced lactic acid and have killed the *S. thermophilu*s bacteria and reduce *L. bulgaricus* population (boudjema, fazouane-naimi et al. 2009). Three yogurts stood out. Yogurt R with 5 mM of reuterin, yogurt R-GDL also with 5 mM of reuterin but acidified with GDL and yogurt FQ_2 without reuterin but with the presence of *L. rhamnosus* protective culture. The pH and acidity values of the R-GDL yogurt stayed constant during storage. Because this yogurt was acidified with GDL, no thermophilic starter was present. The pH and acidity values for yogurt R also stayed stable during storage probably because the population of lactobacilli dropped at day one and the population of streptococci after day five. The yogurt FQ_2 was different. Even though the pH dropped and lactic acid content increased during storage, populations of streptococci stayed relatively high comparatively to other yogurts, especially after day 15. This result seems to show that the presence of *L. rhamnosus* protects the strain *S. thermophilus* from low pH condition. It is possible that the *L. rhamnosus* strain has produced exopolysaccharides (EPS) that have created a protective coating around the *S. thermophilus* strain. To support this idea, Boke, Aslim et al. (2010) have found a positive correlation between ESP production strain and acid tolerance for *L. bulgaricus* and *S. thermophilus* strain.

Reuterin added as an ingredient totally inhibited *A. niger* in R and R-GDL yogurts from day one to day 15 of the storage. These results confirm those obtained by Vimont, Fernandez et al. (2018) and Langa, Landete et al. (2013) in yogurt. These authors observed that the addition of 3 mM reuterin in commercial yogurt inhibits the visually the growth of *P. chrysogenum* and *M. racemosus* for 28 days at 4 °C. However, this study is the first to show a total inhibition by plate count using a concentration of 5 mM of reuterin.

As observed in the reuterin quantification results, the concentration of reuterin in yogurt decreased over time. However, the fungi did not grow for the 15 days of storage. Reuterin is known as a molecule having activity fungicidal (chap 2), so the concentration used in this study may have killed *A. niger* on the first days and it did not show regrowth even if reuterin concentration decrease. It may also be because reuterin might still keep it antifungal activity even if it created a Schiff base in yogurt. Research has shown that schiff base form of antifungal as nystatin increase the reactivity of the antifungal (Silva, Silva et al. 2011).

In this study FQ_2 and Natamycin commercial antifungal products were compared with reuterin. The FQ_2 product is constituted of a protective culture (*L. rhamnosus*) sold as an antifungal. *L. rhamnosus* strains are known as an antifungal compound producer including phenylacetic acid, acetic acid and propionic acid (Cortes-Zavaleta, Lopez-Malo et al. 2014). In this study, FQ_2 yogurt containing the *L. rhamnosus* strain did not demonstrate an antifungal activity against *A. niger* as effective as reuterin. The absence of antifungal activity of the strain of *L. rhamnosus* against *A. niger* was not due to a low population of the strain in yogurt. Protective culture can be specific to certain mold and yeast strains and some strain can be resistant. In Fernandez and Vimont 2017, two *L. rhamnosus* strains have shown different inhibition of a *P. chrysogenum* strain in cottage cheese. In this case, the *A. niger* has been resistant to the *L. rhamnosus* strain of the FQ_2 treatment.

The natamycin concentration used in this study was 5 ppm. A previous experiment has shown a reduction of 0.5 log cfu/mL of molds and a population below 50 cfu/ml for yeast in yogurt stored at 4 °C for a period off 6 weeks in presence of 5 ppm of natamycin compared with a yogurt without preservatives (D.S.M. 2014). In our study, natamycin used at 5 ppm showed a gradual decrease for the full-storage period and lead to a decrease of 1.5 log cfu/mL after

15 days. First, the difference observed can be related to the different formulation of the natamycin product. We used a different a commercial product (Nataseen) composed of 95% natamycin and salt increasing natamycin solubility in yogurt. The product used in the other study was a natamycin solution, which was less soluble due to the non-presence of the salt in the formulation. Second, the storage temperature was higher in our study (21 °C comparatively to 4 °C), which can promote the growth of molds and make them more sensitive to natamycin present in yogurt. When compared to reuterin, natamycin was less active against *A. niger* during all periods of storage. Reuterin inhibited mold growth at the first day, while natamycin reduced growth gradually at the concentration tested. Reuterin has a fungicidal effect whereas natamycin has a fungistatic effect (Chapter 2). The drastic decrease in mold population in yogurt with reuterin is in concordance with fungicidal effect. Unlike reuterin, the effect of natamycin was gradual, suggesting a fungistatic effect on molds. Both commercial products tested in this study to reduce fungal growth at 21 °C were less effective than 5 mM reuterin.

3.7 Conclusion

In this study purified reuterin was used as an antifungal ingredient in stirred yogurt before stirring step. Despite the decline in detection of reuterin concentration in the yogurts, no growth of *A. niger* was observed throughout the 15 days of yogurt storage of yogurt at 21 °C. Also, reuterin was more effective than other commercial antifungal ingredients tested. However, the reuterin used at a concentration 5 mM also reduced the viability of thermophilic starters, especially *S. thermophilus*. This mortality of thermophilic strains had an impact on the pH for yogurt treated with reuterin by keeping the pH value constant during storage. Instead, in the yogurt treated with other agents, strains continued to acidify the samples and the values of pH dropped. The presence of commercial antifungal protective strain of *L. rhamnosus* in yogurt, although it did not reduce the number of mold, protected the population of thermophilic starters, especially *S. thermophilus* during storage.

Purified reuterin used as an ingredient has a great potential to be added as an antifungal preservative in stirred yogurt. However, to improve it use, additional experiments should be done to find strains of *L. bulgaricus* and *S. thermophilus* that show more resistance to reuterin than those used in this study. Other areas that further studies should focus on are: the

combined use of *L. rhamnosus* protective strain with reuterin in yogurt to protect thermophilic strain against reuterin, to evaluate the encapsulation of the reuterin, as in alginate beads, to protect the molecule against yogurt matrix which would allow to reduce the concentration to be used in the products and reduce the impact on the lactic acid starter. Finally, before reuterin could be used as an antifungal preservative in other types of food, it will be important to determine its cytoxicity.

3.8 Tables

Table 3-1 Treatments[1] used with stirred yogurts.

	GDL-R	R	LR-GLY	FQ2	N	CT
Acidification agent	1.5% GDL	1.5% of *L. bulgaricus* + 1.5% of *S. thermophilus culture*				
Treatment applied before acidification			50 mM glycerol+1% of *L. reuteri* culture	$2x10^6$ cfu/mL of *L. rhamnosus* culture		
Treatment applied after acidification	5mM Reuterin				5 ppm Nataseen	

[1] CT= Control, FQ2=*L. rhamnosus*, LR-GLY= *L. reuteri* + glycerol, N =Nataseen 5ppm, R= Reuterin 5 mM and GDL-R = Reuterin 5ppm yogurt acidify with GDL .

Table 3-2 Changes in reuterin concentration (mM/L) in yogurts during a storage of 15 days at 21°C.

	Time (days)				
Yogurt[1]	0	1	5	15	Standard Errors of Mean (SEM)
R	5^{d}	3.4^{b}	0.8^{c}	0.3^{d}	0.13
GDL-R	5^{d}	4.1^{a}	0.8^{c}	0.3^{d}	0.13
LR-GLY	0^{d}	0^{d}	0.01^{d}	0.01^{d}	0.13

[1] LR-GLY =*L. reuteri* + glycerol, R=Reuterin 5 mM and GDL-R =Reuterin 5ppm yogurt acidify with GDL

[abcd]Means in the same column and the same line with different superscripts are significantly different ($P \leq 0.05$).

3.9 Figures

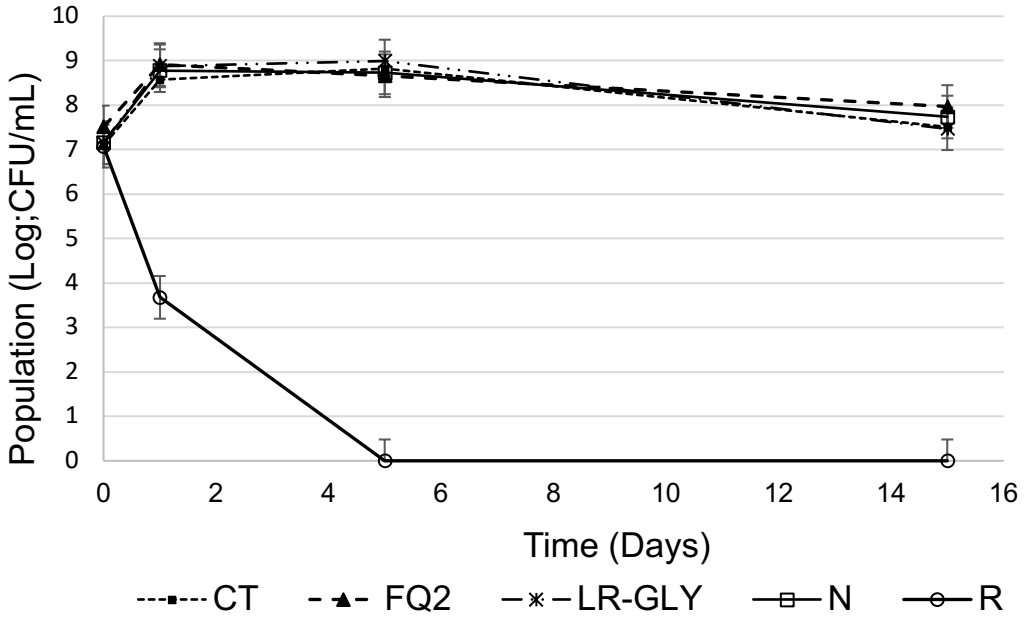

Figure 3-1 Change in population of *L. bulgaricus* during storage at 21 °C in different yogurts: control (CT), *L. rhamnosus* (FQ2) *L. reuteri* + glycerol (LR-GLY), Nataseen 5 ppm (N) and Reuterin 5 mM (R). Error bar represent standard error on the mean (SEM)

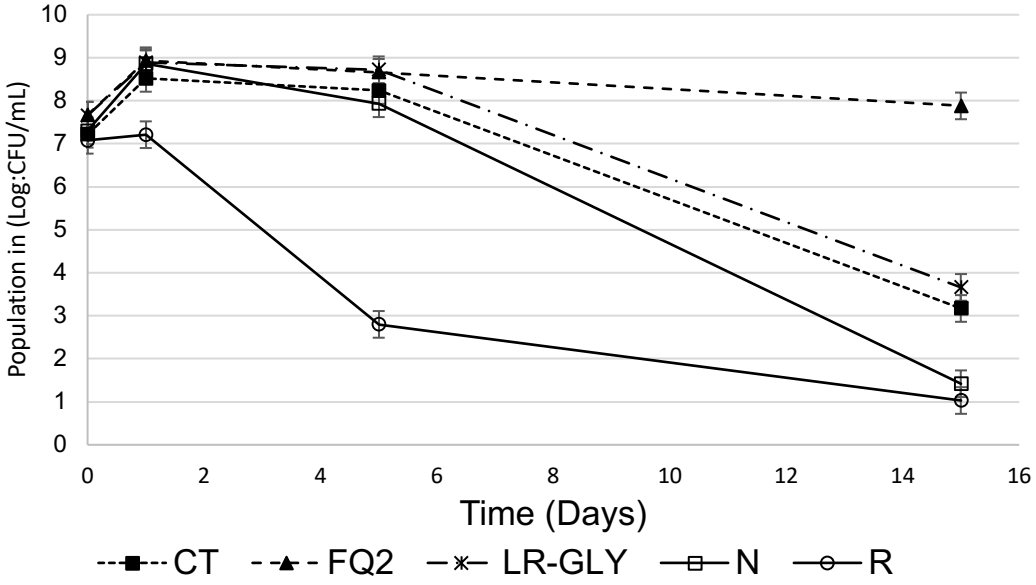

Figure 3-2 Changes in population of *S. thermophilus* during storage at 21 °C in different yogurts: control (CT), *L. rhamnosus* (FQ2), *L. reuteri* + glycerol (LR-GLY), Nataseen 5ppm (N) and Reuterin 5 mM (R). Error bar represent standard error on the mean (SEM)

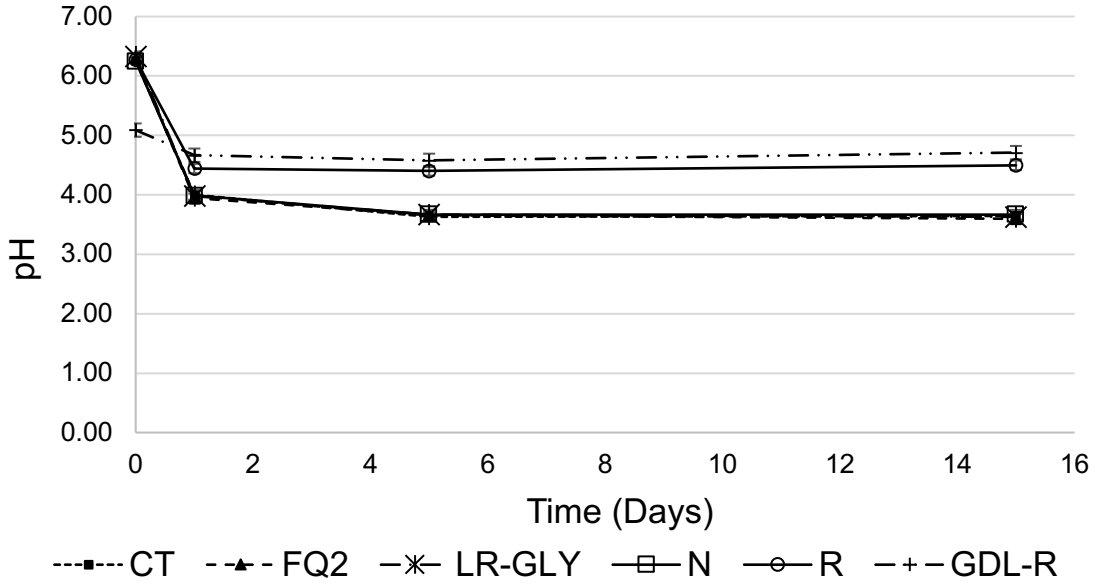

Figure 3-3 pH variation during storage at 21°C in different yogurts: control (CT), *L. rhamnosus* (FQ2), *L. reuteri* + glycerol (LR-GLY), Nataseen 5ppm (N), Reuterin 5 mM (R) and Reuterin 5ppm yogurt acidify with GDL (GDL-R). Error bar represent standard error on the mean (SEM)

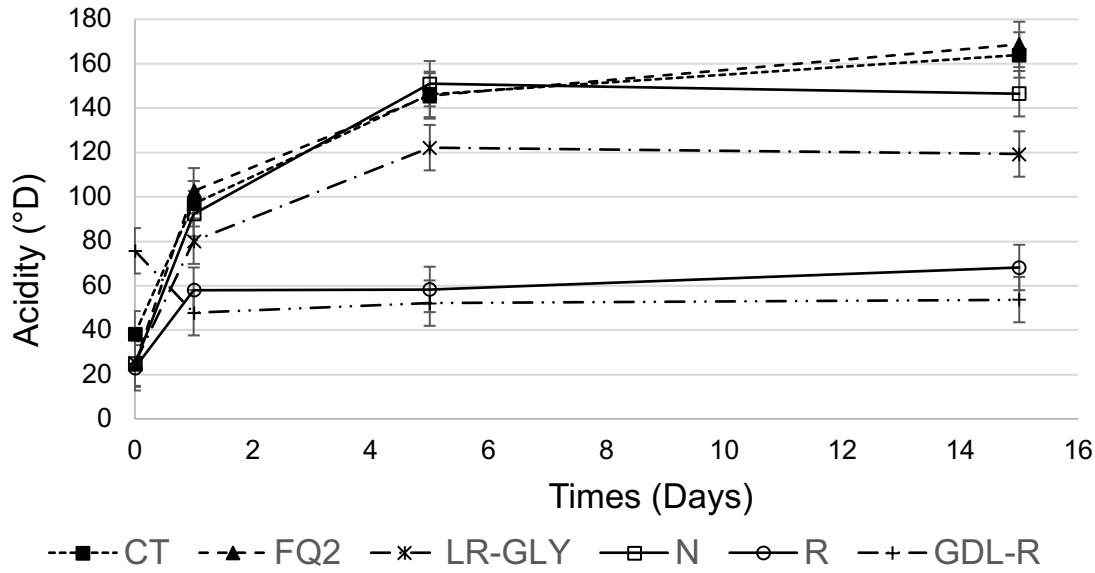

Figure 3-4 Acidity variation during storage at 21°C in different yogurts: control (CT), *L. rhamnosus* (FQ2), *L. reuteri* + glycerol (LR-GLY), Nataseen 5ppm (N), Reuterin 5 mM (R) and Reuterin 5ppm yogurt acidify with GDL (GDL-R). Error bar represent standard error on the mean (SEM)

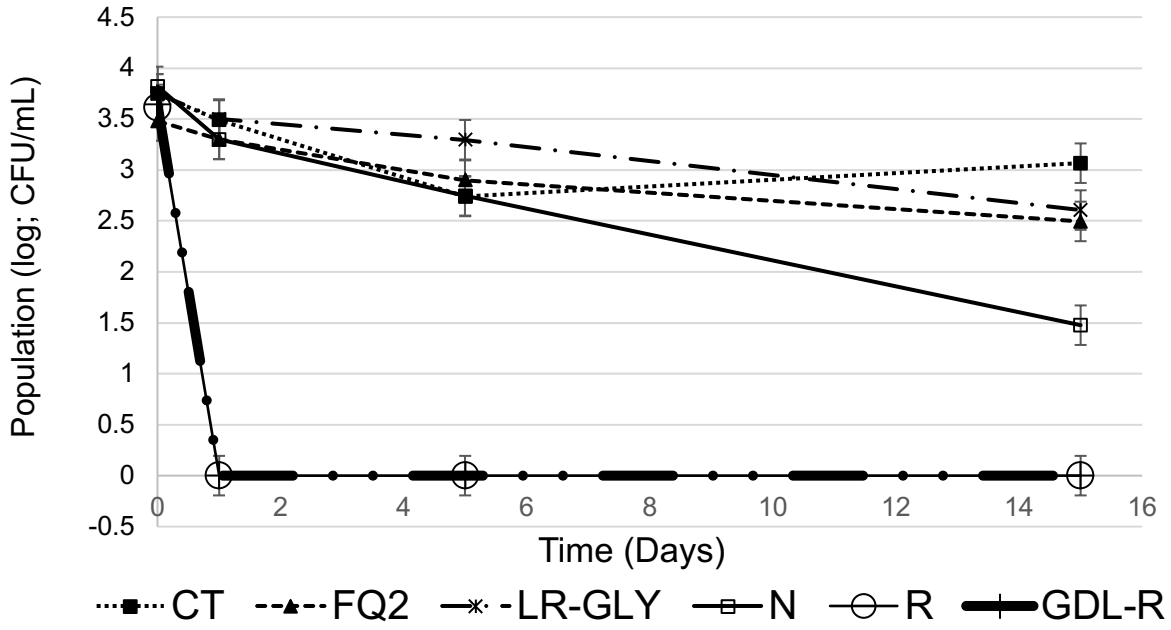

Figure 3-5 Changes in population of *A. niger* in different yogurts: control (CT), *L. rhamnosus* (FQ2), *L. reuteri* + glycerol (LR-GLY), Nataseen 5ppm (N), Reuterin 5 mM (R) and Reuterin 5ppm yogurt acidify with GDL (GDL-R). Error bar represent standard error on the mean (SEM)

Conclusion générale

L'objectif général de cette étude était de produire et d'évaluer l'activité antifongique de la réutérine contre des isolats d'origine alimentaire de moisissures et valider cette activité dans un yogourt brassé. La prémisse de l'étude était que la réutérine produite par *Lactobacillus reuteri* utilisée comme ingrédient inhibe la formation de moisissures dans les yogourts brassés.

De la réutérine pure a été produite et son activité antifongique a été évaluée contre quatre espèces fongiques isolées d'environnement laitier. La méthode de détermination des CMI et des CMF a permis de valider que la réutérine avait une activité fongicide contre toutes les espèces fongiques testées. Cependant, les CMI n'étaient pas les mêmes pour chaque espèce. Différemment de l'activité de la réutérine, les CMI et de CMF observée pour la natamycine montrent un effet fongistatique contre les souches testées lors de cette étude.

Le lait est l'ingrédient principal du yogourt, conséquemment il était nécessaire que la réutérine conserve une activité antifongique dans le lait pour l'intégrer aux yogourts. Le mélange de la réutérine et de la natamycine avec le lait a montré que la réutérine ainsi que la natamycine conservent leurs effets antifongiques en présence de lait contre toutes les espèces testées.

Le yogourt est différent du lait de par son pH, sa composition et son aspect physicochimique. L'activité antifongique de la réutérine était conservée dans le lait, mais elle devait aussi être conservée dans le yogourt. L'utilisation de différentes concentrations de réutérine dans un yogourt à permis d'observer une réduction de la croissance d'*A. niger*, *P. Chrysogenum*, *M. racemosus* et de *R. mucilaginosa* en utilisant une concentration de 10mM.

Suite aux résultats probants de l'utilisation de la réutérine comme antifongique dans un yogourt brassé commercial, la réutérine a été intégrée à des yogourts brassés lors de productions en laboratoire. L'effet des traitements sur la croissance d'*Aspergillus niger* a montré que la réutérine a permis de l'inhiber complètement sur une durée de 15 jours à 21 °C. La natamycine a réduit la croissance fongique à partir du jour 5 et les autres traitements n'ont montré aucune réduction comparée au témoin sans agent antifongique. L'effet des traitements sur les populations des ferments thermophiles montre que la réutérine diminue la vialibilité de *S. thermophillus* et *L. bulgaricus*. Contrairement à la réutérine, *L. rhamnosus*

semble augmenter la viabilité des ferments dans les conditions testées lors de cette étude. Finalement, la quantification de la réutérine au cours de l'entreposage a montré que la concentration en réutérine diminuait en fonction du temps. Ces résultats suggèrent une interaction avec les composantes protéiques présentes dans le yogourt.

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