

**INHIBITION ACTIVITY OF LACTIC ACID BACTERIA ISOLATED
FROM FERMENTED FOODS AGAINST VEGETATIVE CELLS AND
SPORE GERMINATION OF *BACILLUS* SPP.**

WASIM S. M. QADI

(Matric NO. 3150007)

Thesis submitted fulfillment for the degree of Master of Science
(FOOD BIOTECHNOLOGY)

Faculty of Science and Technology

UNIVERSITI SAIN ISLAM MALAYSIA

NILAI

July 2017

DEDICATION

TO

My dead father and My mother

Suhail mohmed qadi

And

Fathia mohmed hoor

My brother and sister for the inspiration:

Baseem, Mohmed, Odai, Qosai, jamela, Braa

all my uncle and my friend

Ayman hoor

Dr. Ahmed B. Abed

The martyrs of Palestine especially sourif martyrs

Mahmmmod Gnemate

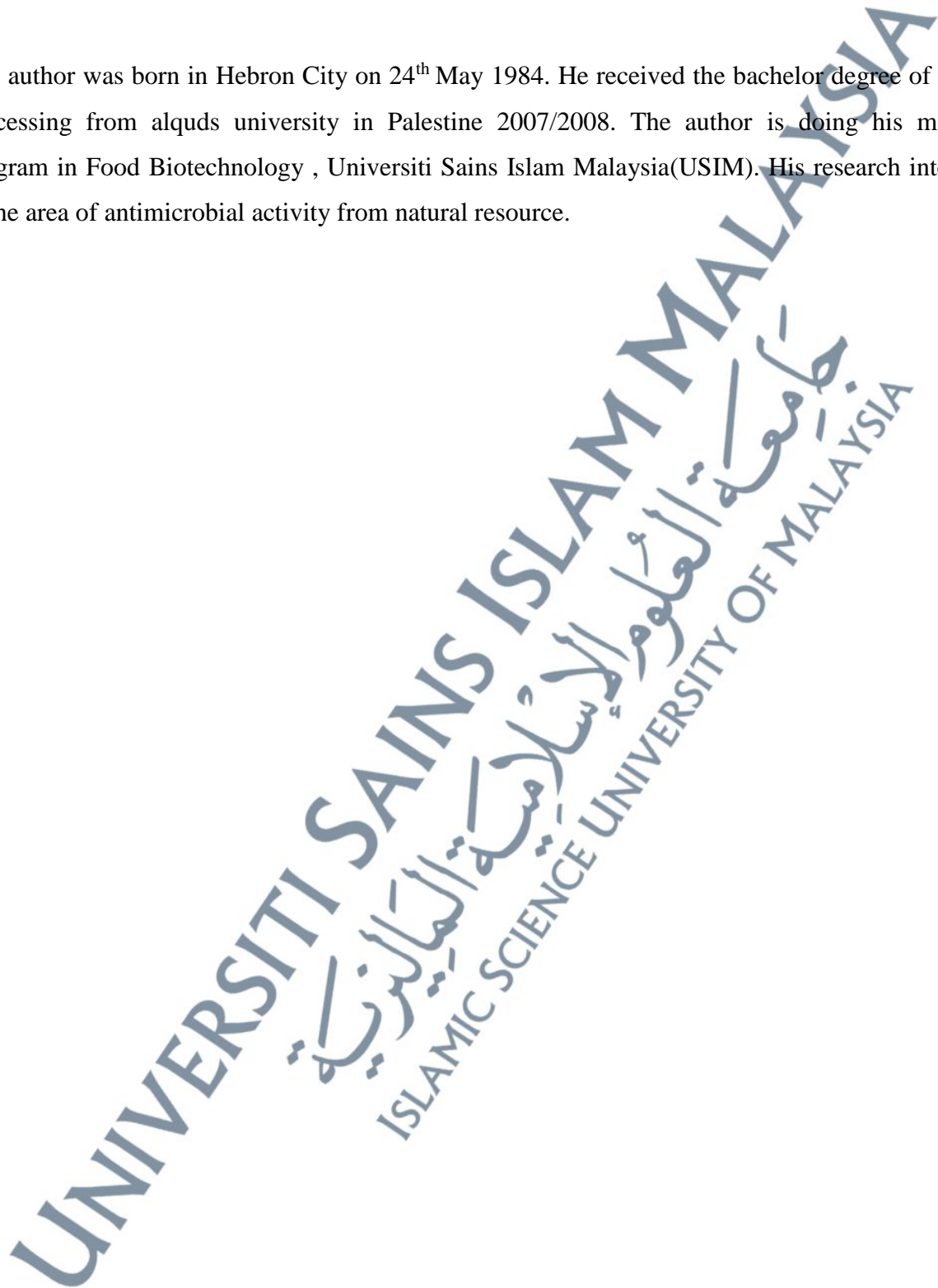
The prisoners of Palestine especially Prisoners Sourif

Jamal abed El ftah hoor

Ahmed abu fara

BIODATA OF THE AUTHOR

The author was born in Hebron City on 24th May 1984. He received the bachelor degree of food processing from alquds university in Palestine 2007/2008. The author is doing his master program in Food Biotechnology , Universiti Sains Islam Malaysia(USIM). His research interest in the area of antimicrobial activity from natural resource.



APPROVAL SHEETS

This thesis “**Inhibition activity of lactic acid bacteria isolated from fermented foods against vegetative cells and spore germination of *Bacillus* spp.**” submitted to the Faculty of Science and Technology (FST), Universiti Sains Islam Malaysia(USIM) and was accepted as fulfillment of the requirement for the degree of master of science.

ZAITON HASSAN, PHD.D,

Associate professor

Faculty of science and technology (FST)

Universiti Sains Islam Malaysia(USIM)

Date:

UNIVERSITI SAINS ISLAM MALAYSIA
جامعة العلوم الإسلامية
ISLAMIC SCIENCE UNIVERSITY OF MALAYSIA

ACKNOWLEDGEMENTS

In the name of Allah, The most Graceful, The most merciful.

All praise is to Allah, lord of the universe, for giving me the strength in the journey to complete this thesis.

First and foremost, I would to express my deepest gratitude and appreciation to my supervisor, Associate professor Dr. Zaiton Hassan for her attention, kindness, patience and most of all, for her faith towards me, also for her professional guidance, helpful comments and generous advices throughout preparing this project and thesis, I consider myself very lucky to be given this honor to work with her.

I want to thank the University of Universiti Sains Islam Malaysia represented Prof. Dr. Musa Ahmed And faculty members In the College of Science.

Next, I would like to thank my family especially my mother fathia, my brother Baseem, Mohmed, Odai, Qosai and my sister Jamela, Braa and all my uncle. And I would like to thank my friend Dr. Ahmed B. Abed for A lot of advice and assistance during my studies, and so much thank for my friend Dr. Samer Samara my old brother in foreign land.

Finally , Send thanks to the unknown soldiers who are a big reason to end my studies

ABSTRAK

Aktiviti Perencatan Bakteria Asid Laktik Diasingkan Daripada Makanan Diperam Terhadap Sel-sel Vegetatif dan Percambahan Spora *Bacillus* spp.

Bacillus spp. menghasilkan spora dan juga sejenis bakteria gram positif yang boleh menyebabkan kerosakan makanan dan penyakit bawaan makanan. Bakteria asid laktik (LAB) terkenal dengan keupayaan mereka untuk mensintesis kompaun yang boleh menghalang pertumbuhan sel-sel vegetatif dan percambahan spora *Bacillus* spp. Tujuan kajian ini adalah untuk menyaring LAB daripada yogurt, pes udang yang telah ditapai ataupun dikenali sebagai "belacan" dan durian yang telah ditapai yang dikenali sebagai "tempoyak" dengan bertujuan untuk melihat aktiviti antimikrob terhadap pertumbuhan sel vegetatif dan percambahan spora oleh *Bacillus cereus*, *B. subtilis* dan *B. spizizenii*. Keputusan spot assay agar menunjukkan bahawa daripada 45 sel LAB yang telah disaring, 98% daripadanya dapat menghalang dengan kuat pertumbuhan *B. cereus*, 78% dapat menghalang *B. spizizenii* dan 51% dapat menghalang *B. subtilis*. Supernatan LAB menunjukkan aktiviti perencatan yang kuat terhadap *B. cereus* iaitu sebanyak 58%, diikuti oleh *B. spizizenii* sebanyak 44.5% dan *B. subtilis* sebanyak 31%. Pelbagai aktiviti perencatan terhadap sel-sel dan percambahan spora diperhatikan apabila pH supernatan telah diselaraskan kepada pH 4, 5 dan 6. Supernatan daripada kesemua LAB yang telah disaring menunjukkan aktiviti perencatan yang bagus terhadap sel-sel vegetatif iaitu sebanyak 46.5% terhadap *B. cereus*, 31% terhadap *B. subtilis* dan 37.8% terhadap *B. spizizenii* pada pH 4. LAB yang diasingkan daripada belacan dan tempoyak menunjukkan aktiviti pada pH 5 dan 6 di mana 15.5% terhadap *B. subtilis*, 20% terhadap *B. cereus* dan 2% terhadap *B. spizizenii*. Supernatan daripada LAB yang diasingkan daripada yogurt kehilangan aktiviti perencatan pada pH 5 dan 6. Percambahan spora *Bacillus* spp. terjejas disebabkan oleh sel-sel LAB dan supernatant seperti yang telah dinilai daripada keputusan assay spot, sel LAB yang telah diasingkan daripada yogurt, belacan dan tempoyak merencat dengan kuat percambahan spora oleh *B. subtilis* (89%), *B. cereus* (75.5%) dan *B. spizizenii* (73.3%). Sebaliknya, supernatan menunjukkan aktiviti perencatan yang berbagai terhadap percambahan spora di mana 24.4% terhadap *B. subtilis*, 29% terhadap *B. cereus* dan 15.5% terhadap *B. spizizenii*. Percambahan spora telah kuat direncatkan oleh pH supernatan di mana perencatan yang lebih besar berlaku pada pH 4 berbanding pH 5 dan 6. Supernatan daripada LAB yang telah diasingkan daripada belacan dan durian dapat merencatkan percambahan spora pada pH 5 dan 6. Sementara itu, supernatan daripada LAB yang diasingkan daripada yogurt kehilangan aktiviti perencatan terhadap percambahan spora pada pH 5 dan 6. Aktiviti antimikrob oleh supernatan LAB (BP2, BK4, BT1, D2) telah berkurangan apabila diuji dengan proteinase k dan RNaseI. LAB yang diasingkan daripada belacan dan tempoyak adalah mudah terkesan kepada Penicillin G (PI), gentamicin (CN), Chloramphenicol (C), Tetracycline (TE), tetapi resisten kepada vancomycin dan Streptomycin (S). Kajian ini menunjukkan kehadiran kompaun di dalam supernatants LAB yang mempunyai aktiviti perencatan terhadap sel-sel vegetatif dan

percambahan spora pada pH 5 dan 6. Kompaun ini mungkin mempunyai potensi untuk penggunaan biopreservatif terhadap kerosakan makanan dan penyakit bawaan makanan disebabkan oleh *Bacillus* spp.

Kata kunci :*Bacillus cereus*, *B. subtilis*, *B. spizizenii*, bakteria asid laktik, percambahan spora, aktiviti antimikrobial.



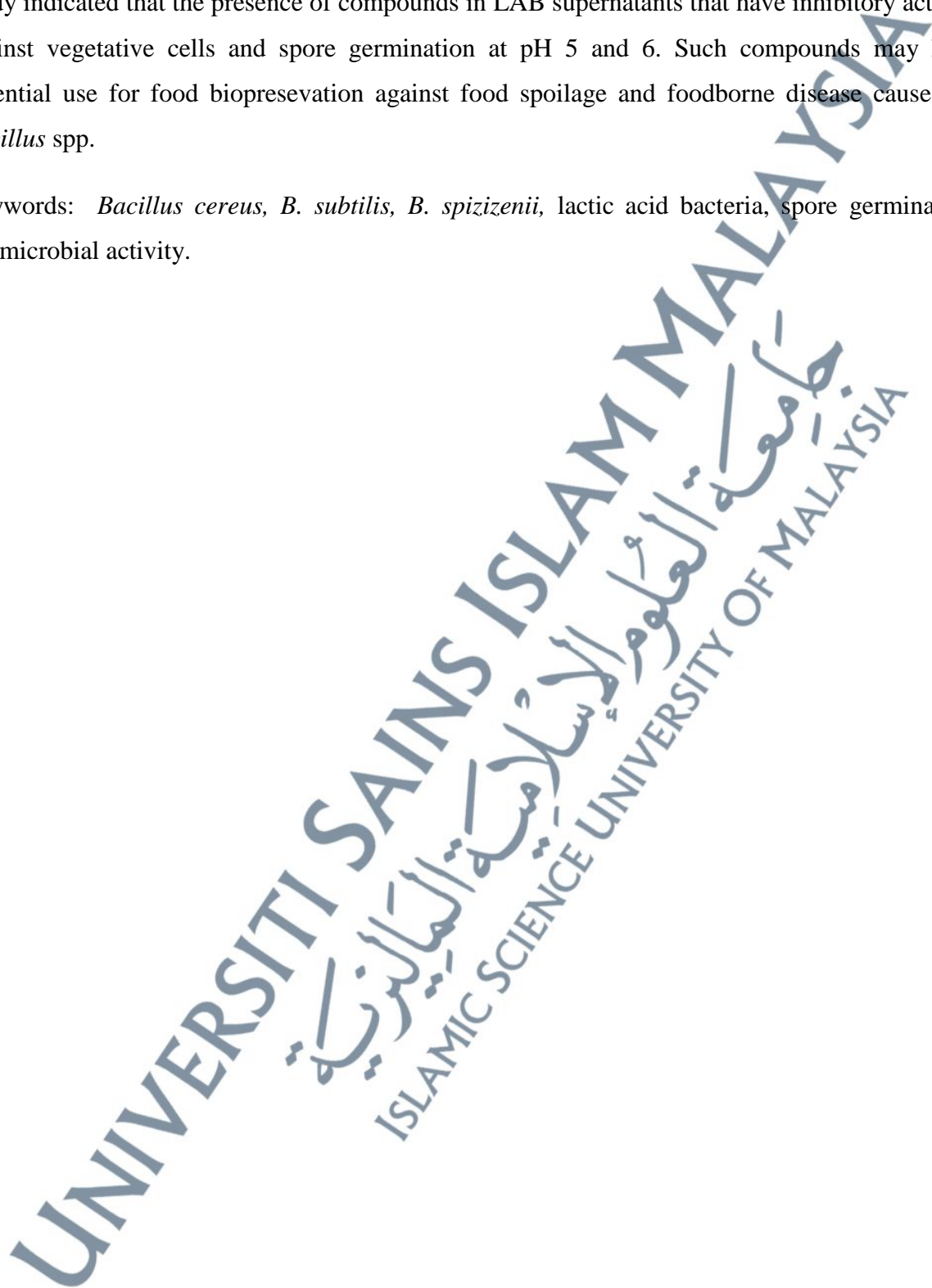
ABSTRACT

Inhibition Activity of Lactic Acid Bacteria Isolated From Fermented Foods Against Vegetative Cells And Spore Germination of *Bacillus* spp.

Bacillus spp. are endospore-forming, Gram-positive bacteria able to cause food spoilage and foodborne diseases. Lactic acid bacteria (LAB) are known for their ability to synthesize compounds that can inhibit growth of vegetative cells and spore germination of *Bacillus* spp. The aim of this study was to screen LAB isolated from yogurt, fermented shrimp paste “belacan” and fermented durian “tempoyak” for antimicrobial activity on vegetative cell growth and spore germination of *Bacillus cereus*, *B. subtilis* and *B. spizizenii*. Results of agar spot assay showed that from the 45 LAB cells 98% inhibited strongly growth of *B. cereus* compared to 78% against *B. spizizenii* and 51% against *B. subtilis*. The LAB supernatant showed strong inhibitory activity against *B. cereus* 58%, followed by *B. spizizenii* 44.5% and *B. subtilis* 31%. Variable inhibitory activity against vegetative cells and spore germination was observed when the pH of supernatants was adjusted to pH 4, 5 and 6. The supernatants of all LAB isolates showed good inhibitory activity against vegetative cells 46.5 % against *B. cereus*, 31% against *B. subtilis* and 37.8 % against *B. spizizenii* at pH 4. LAB isolated from belacan and fermented durian showed activity at pH 5 and 6: 15,5% against *B. subtilis*, 20% against *B. cereus* and 2% against *B. spizizenii*. Supernatant of LAB from yogurt lost its inhibitory activity at pH 5 and 6. Spore germination of the *Bacillus* spp. was affected by LAB cells and supernatants. LAB cells isolated from yogurt, belacan and fermented durian inhibited strongly spore germination of *B. subtilis* (89%) than *B. cereus* (75.5%) and *B. spizizenii* (73.3%) as evaluated by spot assay. In contrast, the supernatant demonstrated variable inhibitory activity against spore germination as indicated by 24.4% for *B. subtilis*, 29% for *B. cereus* and 15.5% against *B. spizizenii* as evaluated by well method. Spore germination was strongly inhibited by pH of supernatant, greater inhibition at pH 4 compared to pH 5 and 6. The supernatant of LAB isolates from belacan and durian inhibited spore germination at pH 5 and 6. While Supernatant of LAB from yogurt lost its inhibitory activity against spore germination at pH 5 and 6. Antimicrobial activity of supernatant LAB (BP2, BK4, BT1, D2) were diminished when treated with proteinase k, RNase I. LAB isolates from “belacan” and fermented durian were susceptible to Penicillin G (PI), Gentamicin(CN),

Chloramphenicol(C), Tetracycline(TE) but resistant to vancomycin and Streptomycin (S). This study indicated that the presence of compounds in LAB supernatants that have inhibitory activity against vegetative cells and spore germination at pH 5 and 6. Such compounds may have potential use for food biopreservation against food spoilage and foodborne disease caused by *Bacillus* spp.

Keywords: *Bacillus cereus*, *B. subtilis*, *B. spizizenii*, lactic acid bacteria, spore germination, antimicrobial activity.



ملخص البحث

نشاط بكتيريا حمض الاكتيك المعزوله من الطعام ضد نمو خلايا وابواغ البكتيريا العصيه

البكتيريا العصيه هي بكتيريا قادره على تشكيل الابواغ القادره على التسبب في فساد الطعام والتسبب في الامراض للانسان. بكتيريا حمض الاكتيك قادره على انتاج بعض المركبات لها القدره على تثبيط نمو الخلايا والابواغ البكتيريا العصويه, وكان الهدف من هذه الدراسه عزل بكتيريا حمض الاكتيك من بعض المواد الغذائيه كالدوربان واللبن والبيكان والتأكد من فعاليه هذه البكتيريا ضد انواع مختلفه من البكتيريا العصويه وهي الباسيلوس سيروس, الباسيلوس سبتلوس والباسيلوس سبايزيني باستخدام طريقة السبوت والويل. اظهرت النتائج عن طريقه السبوت ان 45 من عزلة البكتيريا اظهرت نشاطا ضد نو خلايا البكتيريا العصويه حيث ان 98% من البكتيريا الاكتيك اظهرت نشاط قوي ضد بكتيريا الباسيلوس سيروس و 78% ضد بكتيريا الباسيلوس سبايزيني و 51% ضد بكتيريا الباسيلوس السبتلوس, كما ان الراشح البكتيري اظهر نشاط ضد نمو خلايا البكتيريا العصيه عن طريق طريقة الويل حيث اظهرت النتائج أن 58% من البكتيريا لها نشاط قوي ضد بكتيريا الباسيلوس سيروس و 44% ضد الباسيلوس سبايزيني مقارنة ب 31% ضد الباسيلوس سبتلوس, نشاطات مختلفه اظهرتها البكتيريا ضد نمو خلايا وابواغ بكتيريا الباسيلوس عند تغير درجة الحموضه ل 4,5,6.5 حيث لوحظ أن الراشح البكتيري له تاثير على نشاط ونمو خلايا الباسيلوس 46.5% ضد الباسيلوس سيروس و 31% ضد الباسيلوس سبتلوس و 37.8% ضد الباسيلوس سبايزيني عند ضبط درجة الحموضه على 4. عزلات البكتيريا من الدوربان والبيكان اظهرت نشاطاً قويا ضد بكتيريا الباسيلوس عند درجة حموضه 5,6,5 : 15.5% ضد الباسيلوس سبتلوس و 20% ضد الباسيلوس سيروس و 2% ضد الباسيلوس سبايزيني في حين أن الراشح البكتيري من عزلات البكتيريا التي مصدرها اللبن تفقد قدرتها على النشاط عند ضبط درجة الحموضه عند 5,6,5. ابواغ بكتيريا الباسيلوس تأثرت بواسطة خلايا والراشح البكتيري لبكتيريا حمض الاكتيك, عزلات بكتيريا حمض الاكتيك المعزولة من اللبن والدوربان والبيكان اظهرت نشاطا قويا ضد ابواغ بكتيريا الباسيلوس حيث أن 89% ضد الباسيلوس سبتلوس و 75.5% ضد الباسيلوس سيروس و 73.3% ضد الباسيلوس سبايزيني عن طريق طريقة السبوت. على العكس من ذلك اظهر الراشح البكتيري نشاطات مختلفه ضد ابواغ بكتيريا الباسيلوس حيث أن النتائج كانت كالآتي أن 24% ضد الباسيلوس سبتلوس و 29% ضد الباسيلوس سيروس و 15.5% ضد الباسيلوس سبايزيني. أظهر الراشح البكتيري نشاطا قويا ضد ابواغ الباسيلوس عند ضبط درجة الحموضه عند 4 مقارنة بدرجة الحموضه عند 5 و 6.5. حيث أن العزلات المعزولة من الدوربان والبيكان اظهرت نشاطا ضد ابواغ الباسيلوس عند ضبط درجة الحموضه على 5 و 6.5 في حين أن العزلات المعزولة من اللبن لم تظهر أي نشاط ضد ابواغ الباسيلوس عند ضبط درجة الحموضه عند 5 و 6.5. نشاط العزلات أنخفض عندما اختبرت بواسطة أنزيم بروتييناز ك و أرانيز 1. عزلات بكتيريا حمض الاكتيك المعزوله من الدوربان والبيكان كانت عرضة وتتاثر لبعض المضادات الحيوي للالبنسلين, جيتاميسين, الكلورامفينيكول, النتراسيكلين في حين أظهرت مقاومة لفانكوميسين و ستربتومايسين. اشارة هذ الدراسه الى وجود بعض المركبات في الراشح البكتيري لديها القدره على تثبيط نمو خلايا وأبواغ الباسيلوس ويمكن أستخدامها في الصناعات الغذائيه كمضافات طبيعيه.

CONTENT PAGE

TABLE OF CONTENT

CONTENT	Page
AUTHOR DECLARATION	ii
DEDICATION	iii
BIODATA OF THE AUTHOR	iv
ACKNOWLEDGEMENTS	vi
ABSTRAK	vii
ABSTRACT	ix
ملخص البحث	xi
TABLE OF CONTENT	xii
LIST OF TABLES	xiv
LIST OF FIGURES	xv
LIST OF ABBREVIATIONS	xvi
CHAPTER I: INTRODUCTION	1
CHAPTER II: LITERATURE REVIEW	4
2.1 Lactic acid bacteria	4
2.2 Antimicrobial activity and antispore formation of lactic acid bacteria	8
2.3 Antimicrobial compound produce by LAB	11
2.3.1 Organic acid	11
2.3.2 Hydrogen peroxide	12
2.3.3 Bacteriocins	13
2.3.4 Reuterin	16
2.4 mechanism of the antimicrobial activity of compound produce by lactic acid bacteria	18
2.5 <i>Bacillus</i> spp.	20
2.5.1 Spore germination of <i>bacillus</i> spp.	21
2.5.2 Food spoilage by <i>Bacillus</i> spp.	23
CHAPTER III: METHODOLOGY	25
3.1 Material and method	25
3.1.1 Sample	25
3.1.2 Isolation and purification of lactic acid bacteria from fermented food	25
3.1.3 Preparation of spore suspensions	26
3.1.4. Screening for antimicrobial activity of isolated LAB against <i>Bacillus</i> spp.	26
3.1.4.1 LAB cells spot method	26

3.1.4.2 Well method	26
3.1.5 Effect of pH on antibacterial activity of LAB supernatant	27
3.1.6 Effect of enzymes on LAB supernatant against <i>Bacillus</i> spp.	27
3.1.7 Detection of protein hydrolysis	28
3.1.7.1 Preparation of skim milk agar and cultures	28
3.1.7.2 Measurements of protein hydrolysis	28
3.1.8 Determination of antibiotic resistance of the isolates	28
3.1.9 Statistical analysis	29
3.2 Result	30
3.2.1 Isolation of LAB from food samples	30
3.2.2 Antimicrobial activity of LAB against growth of vegetative <i>Bacillus</i> spp. as determined by agar spot assay and well-diffusion method	32
3.2.2.1 Agar spot assay	32
3.2.2.2 Well-diffusion method	34
3.2.3 Antimicrobial activity of LAB cells and supernatant on <i>Bacillus</i> spp. spore germination by agar spot assay and well-diffusion method	36
3.2.3.1 Agar spot assay	36
3.2.3.2 Well-diffusion method	38
3.2.4 Effect of pH of LAB cell free supernatants on growth inhibitory activity of <i>Bacillus</i> spp. vegetative cells and spore germination	40
3.2.5 Detection of proteolytic activity of selected LAB isolates	43
3.2.6 Effect of enzymes treated LAB cell free supernatant on antimicrobial activity	44
3.2.7 Antibiotic susceptibility of LAB isolates	48
3.3 Discussion	50
3.4 Conclusion	54
3.5 Recommendation	55
Reference	56

LIST OF TABLES

TABLES		Page
Table 1:	The main lactic acid bacteria associated with milk and milk product fermentation	5
Table 2:	health benefits when milk is fermented	6
Table 3:	Gram stain and catalase test of LAB isolated from different food samples	31
Table 4:	Antimicrobial activity of LAB against vegetative cell <i>Bacillus spp.</i> using agar spot assay	33
Table 5:	Antimicrobial activity of LAB supernatant against vegetative cell of <i>Bacillus spp.</i> using well diffusion method	35
Table 6:	Antimicrobial activity of LAB against spore germination of <i>Bacillus spp.</i> using agar spot assay	37
Table 7:	Antimicrobial activity of lactic acid bacteria supernatant against spore germination of <i>Bacillus spp.</i> using well diffusion method	39
Table 8:	Antimicrobial activity of LAB cell free supernatants against vegetative cell of <i>Bacillus spp.</i> after pH adjusted as determined by well-diffusion method incubated at 37°C for 24 h	41
Table 9:	Antimicrobial activity of pH adjusted LAB cell free supernatants against vegetative cell of <i>Bacillus spp.</i> as determined by well-diffusion method incubated at 37°C for 24 h	42
Table 10:	Protoylytic activity of LAB isolates on skim milk agar	43
Table 11:	Growth percentage of <i>Bacillus spp.</i> with LAB supernatant after treatment with Proteinase K measured in microtiter plate incubated at 30 °C for 48h	47
Table 12:	Growth percentage of <i>Bacillus spp.</i> with LAB supernatant after treatment with RNase I measured in microtiter plate incubated at 30 °C for 48h	47
Table 13:	Antibiotic resistance of the LAB isolates to different antibiotics as measured by diameter of inhibition zones	49

LIST OF FIGURES

Figures	Page
Figure1: Generalized scheme for the fermentation of glucose in lactic acid bacteria	7
Figure 2: Fermentation pathway for the production of reuterin in <i>Lactobacillus reuterin</i>	17
Figure 3: Schematic of main mechanisms by which Lactic Acid kills bacteria	19
Figure.4: The sporulation cycle of spore-forming bacteria	22
Figure 5 : Schematic representation of the internal structure of a bacterial spore	22
Figure 6: Growth of LAB isolates on modified MRS with 0.8% CaCO ₃ showing clear zones around the colonies	30
Figure 7: Antimicrobial activity of LAB against vegetative cell <i>Bacillus spp.</i> using agar spot assay	32
Figure 8: Plate showing growth inhibition of vegetative cell <i>Bacillus spp.</i> By CFS of LAB using well-diffusion method incubate at 37°C for 24 h	34
Figure 9 Antimicrobial activity of LAB cells against spore germination of <i>Bacillus spp.</i> using agar spot assay	36
Figure10: Plate showing inhibition zone of <i>Bacillus spp.</i> spore germination by well-diffusion method incubate at 37° C for 24 h	38
Figure 11: Clear zone surrounding the colonies indicate proteolysis activity	43
Figure 12: Effect of enzymes on LAB supernatant against <i>Bacillus spp.</i>	44
Figure 13: Growth of <i>Bacillus spp.</i> in treated supernatant with proteinase k as measured by optical density 560 nm in 96 wells titer plates	45
Figure 14: Growth of <i>Bacillus spp.</i> in treated supernatant with RNase as measured by optical density 560 nm in 96 wells titer plates	46
Figure 15: Antibiotic susceptibility assay of the LAB isolates using antibiotic disc method	48

LIST OF ABBREVIATIONS

BHI	brain heart infusion Broth
BLIS	Bactriocin-like inhibitory substance
CaCO ₃	Calcium carbonate
CFS	Cell free supernatant
°C	Celsius
CFU	Colony Forming Unit
CO ₂	Carbon dioxide
G	Gram
h	Hour
HCL	Hydrochloric acid
H ₂ O ₂	Hydrogen peroxide
LAB	Lactic acid bacteria
µg	Microgram
Mg	Milligram
ml	milliliter
µl	Microliter
Mm	Millimeter
µm	Micrometer
MRS	de Man Rogosa Sharpe
NaCl	Sodium chloride
NAOH	Sodium Hydroxide
Nm	Nanometer
OD	Optical density
O ₂ -	Superoxide
OH-	Hydroxyl
PLA	phenyllactic acid
rpm	Revolution per minute
S.D	standard deviation
Spp.	Species
TGE	Tryptone Glucose Extract broth
U	Unite
UHP	Ultra high Pressure
UTI	Urinary tract infection