DEVELOPING
A GUIDE OF BIOINFORMATIC DATABASE FOR PROBIOTIC PRODUCTS

Melike YILMAZ

İzmir Institute of Technology
July, 2019

DEVELOPING
A GUIDE OF BIOINFORMATIC DATABASE FOR PROBIOTIC PRODUCTS

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Biotechnology

by
Melike YILMAZ

July 2019
İZMİR

We approve the thesis of **Melike YILMAZ**

Examining Committee Members:

Prof. Dr. Hayriye Şebnem HARSA
Department of Food Engineering, İzmir Institute of Technology

Assoc. Prof. Dr. Efe SEZGİN
Department of Food Engineering, İzmir Institute of Technology

Asst. Prof. Dr. Şükrü GÜLEÇ
Departments of Food Engineering, İzmir Institute of Technology

Assoc. Prof. Dr. Ayşe NALBANTSOY
Departments of Bioengineering, Ege University

Assoc. Prof. Dr. Çağatay CEYLAN
Departments of Food Engineering, İzmir Institute of Technology

5 July 2019

Prof. Dr. Hayriye Şebnem HARSA
Supervisor, Department of Food Engineering
İzmir Institute of Technology

Assoc. Prof. Dr. Efe SEZGİN
Co-Supervisor, Department of Food Engineering
İzmir Institute of Technology

Assoc. Prof. Dr. Engin ÖZÇİVİCİ
Head of the Programme of Biotechnology and Bioengineering

Prof. Dr. Aysun SOFUOĞLU
Dean of the Graduate School of Engineering and Sciences

ACKNOWLEDGMENTS

First of all, I would like to specially thank my supervisors Professor *Şebnem Harsa* and Associate Professor *Efe Sezgin* at İzmir Institute of Technology (IZTECH), for their support and sharing valuable information with me throughout my graduate studies, and also for their supporting in applying the Erasmus programme to develop my thesis at Instituto Superior Técnico (IST) in Portugal.

I also would like to thank my supervisors Associate Professor *Miguel Nobre Parreira Cacho Teixeira* and Assistant Professor *Pedro Tiago Gonçalves Monteiro* for giving me the opportunity to develop, perform and write some sections of my thesis at IST during ERASMUS period. Thank you for all the times to help me whenever I asked my questions to them, their contribution to me with all the guidance. I learned a lot of things and gained their experiences during my thesis work. I also want to thank Professor *Nuno Mira* for his help to find my supervisors at IST, and to Professor *Isabel Sá-Correia*, for giving me the opportunity to join the Biological Sciences Research Group.

Besides, I would like to thank MSc. *Mafalda Cavalheiro* and MSc *Pedro Pais*, helping to me performing my experiments in the laboratory, contribution to me their knowledge and, their patient for my a lot of questions and amazing friendship.

Also, my friends from laboratory *Ana Vila-Santa, Mónica Galocha, Diana Pereira* thank you for the helping during the development of my thesis, and their friendship during the Erasmus period.

I would like to thank my family, specially my father, *Hasan Yılmaz*, and my mother, *Emine Yılmaz*, my sister, *Mülkiye Yüksel*, and my brother, *Oktay Yılmaz*, for supporting and believing me all my life. Specially, my boyfriend, *Özgür Altın*, thank to him so much for supporting, believing, being patient and friendly to me and always being with me. Also, I would like to thank my stand-in parent, *Ayşe Başkanoğlu and Müge Başkanoğlu*, supporting and believing to me my university education life.

Finally, a special thank you for all my friends who have been the people who support me no matter what and always are with me whatever I want, and Erasmus friends in Portugal, thank you for all to perfectly spend time together during Erasmus period.

A case study of *S. boulardii* work was financially supported by Fundação para a Ciência e Tecnologia (FCT), contracts PTDC/BBB-BIO/4004/2014 and PTDC/BII-

BIO/28216/2017. Funding received by iBB – Institute for Bioengineering and Biosciences from FCT (grant IUD/BIO/04565/2013) and Programa Operacional Regional de Lisboa 2020 (project number 007317) is acknowledged.

I would like to *Dr. Sinem Candemir* as Medical advisor from Bicodex Global Pharmaceutical Company and also *MSc. Cansu Yılmaz* as Medical manager from Global Sanofi Pharmaceutical Company in Turkey for sharing literature their probiotic products and contributing data with this MSc Thesis.

ABSTRACT

DEVELOPING A GUIDE OF BIOINFORMATIC DATABASE FOR PROBIOTIC PRODUCTS

Recently, probiotic use has rapidly expanded, as they have potential health effects for microbiota to protect homeostasis in the human body. Bioinformatics is generally defined as collecting and analysing biological data. Establishing a bioinformatic system for probiotics, would have a potential to emphasize the beneficial impacts for human health, while enabling cross examination on diseases and products.

In this study, new information has been collected about probiotics based on *in vitro*, *in vivo*, clinical trials and meta-analysis to develop a comprehensive guide. Meta-analyses of sixteen and seventeen randomized, controlled trials of *S. boulardii* (*Sb*) against diarrhea reported pooled relative risks of 0.51 (95% CI [0.40-0.64]) in adults and 0.55 (95% CI [0.42-0.72]) in children, respectively. These results demonstrated that *Sb* was effective for preventing and treating different types of diarrhea in adult and children patients.

An *in silico* gene expression study conducted in Tecnico Lisboa* comparing *Sb* probiotic and non-probiotic *Saccharomyces cerevisiae* (*Sc*) strains showed transcription regulation differences in 26 genes.

An *in silico* pipeline that was used as the basis for a new query in the ProBioYeastract database was developed. A cross-strain promoter analysis, comparing *Sb* CNCM I-745 and Unique28 strains with *Sc* S288C strain showed that the expression of 26 probiotic-related genes was predicted to be controlled by different transcription factors in probiotic vs non-probiotic strains. Among the evaluated six selected genes, a gene involved in biofilm formation, aggregation, and adhesion, *EFG1*, was found to be up-regulated in *Sb* CNCM I-745 compared to *Sc* BY4741.

*Supervisors; Assoc. Prof. Miguel Teixeira and Asst. Prof. Pedro Monteiro

ÖZET

PROBİYOTİK ÜRÜNLERİN BİOİNFORMATİK VERİTABANI İÇİN BİR REHBER GELİŞTİRİLMESİ

Son zamanlarda, probiyotik kullanımı mikrobiyota üzerinde potansiyel sağlık etkilerine sahip olduklarından dolayı insan vücudundaki homeostazı korumaya yönelik olarak hızla artmaktadır. Biyoinformatik genel olarak biyolojik verilerin toplanması ve analiz edilmesi olarak tanımlanmaktadır. Bu bağlamda, probiyotikler için biyoinformatik bir sistem kurulması, hastalıklar ve ürünler üzerinde sorgu yapılmasını sağlarken, insan sağlığı için yararlı etkileri vurgulanarak öneminin üstünde durulması hedeflenmektedir.

Bu çalışmada, kapsamlı bir kılavuz geliştirmek için *in vitro*, *in vivo*, klinik ve meta-analize dayalı çalışmalar baz alınarak probiyotikler hakkında yeni bilgiler toplanmıştır.

S. boulardii'nin (*Sb*) ishalin önlenmesine karşı yapılan randomize kontrollü çalışmaları içeren meta-analizlerde yetişkinlerde 0,51 (% 95 CI [0,40-0,64]) ve çocuklarda 0,55 (% 95 CI [0,42-0,72]) göreceli riskleri bulunmuştur. Bu sonuçlar, *Sb*'nin yetişkin ve çocuk hastalarda farklı ishal tiplerinin önlenmesinde ve tedavisinde etkili olduğunu göstermiştir.

Tecnico Lisboa'da* *Sb* probiyotik ve probiyotik olmayan *Saccharomyces cerevisiae* (*Sc*) suşlarını karşılaştıran *in silico* gen anlatım çalışmasında 26 gende transkripsiyon düzenleme farklılıkları olduğu gösterilmiştir.

ProBioYeastract isimli özgün bir veritabanına katkıda bulunmak üzere *in-silico* "pipeline" yaklaşımıyla bir tasarım geliştirildi. *Sb* CNCM I-745 ve Unique28 suşlarını *Sc* S288C suşu ile karşılaştıran bir şuslar arası promotör karşılaştırma analizi, 26 probiyotik özellik ile ilişkili gen anlatımının, probiyotik olan ve probiyotik olmayan suşlarda farklı transkripsiyon faktörleri tarafından kontrol edilebileceği tahmin edildi. *Sb* CNCM I-745'te, seçilen altı gen arasından *EFG1* geni biyofilm oluşumu, kümelenmesi ve adezyon testleri ile yüksek bulunup aynı zamanda gen tanımlanmasında *Sc* BY4741'e kıyasla yukarı regüle edildiği gözlendi.

*Danışmanlar; Doç. Dr. Miguel Teixeira, Yrd. Doç. Dr. Pedro Monteiro

TABLE OF CONTENTS

LIST OF FIGURES x

LIST OF TABLES xiii

ABBREVIATIONS xv

CHAPTER 1. INTRODUCTION 1

1.1. Overview of Microbiota, Probiotics, and Prebiotics 1

1.2. *In vitro*, *In vivo*, Clinical Studies 5

1.3. Mode of Actions of Probiotics 6

1.4. Safety and Risk Assessment of Probiotics 7

1.5. Importance of Bioinformatics Databases 10

1.6. Commonly used Probiotics 12

1.6.1. *Lactobacillus* as a probiotic 12

1.6.2. *Bifidobacterium* as a probiotic 13

1.6.3. *Bacillus* as a probiotic 14

1.6.4. *Saccharomyces* as a probiotic 15

1.7. Probiotics against Diseases caused by Diarrhea 16

1.7.1. Against Antibiotic-Associated Diarrhea (AAD) 16

1.7.1.1. Against Rotavirus Infection (in children) 17

1.7.1.2. Against *Clostridium difficile* Infection (CDI) 17

1.7.1.3. Against Diarrhea caused by *Helicobacter pylori* 18

1.7.2. Against Irritable Bowel Syndrome (IBS) & Inflammatory Bowel Disease (IBD) 19

1.7.3. Against Metabolic Diseases: lactose and gluten intolerance and obesity 20

1.7.4. Against Gluten Intolerance or Celiac Disease 22

1.7.5. Against Lactose intolerance or malabsorption 23

1.7.6. Against Obesity & Type II diabetes 24

1.7.7. Against Cancer Diseases 24

1.8. The importance of molecular basis of probiotics: A case study of probiotic, *S. boulardii* and compared to non-probiotic strain, *S.cerevisiae* 25
1.8.1. Mode of Action for *S. boulardii* as Probiotic..... 27
1.8.1.1. Luminal Action 28
1.8.1.2. Mucosal Action 29
1.8.1.3. Trophic Action 34
1.9. Aim of the Study 35

CHAPTER 2. METHODOLOGY 38
2.1. Study Design of Probiotic Research 38
2.1.1. Literature Survey 39
2.1.2. Market Research 39
2.2. Data Integration 39
2.3. Data Collection and Process 40
2.4. Meta-analysis of *S. boulardii* in children and adults 40
2.5. A Case study of *S.boulardii* 42
2.5.1. Cross-strain promoter analysis: *Sc* vs *Sb* 43
2.5.2. Gene Expression Analysis 44
2.5.2.1. Yeast strains and Growth Conditions 44
2.5.2.2. RNA Extraction 49
2.5.2.3. Real-Time PCR (RT-PCR) 49
2.5.3. Aggregation Assessment 52
2.5.4. Adhesion to human epithelium cells 52
2.5.5. Biofilm Quantification 52
2.5.6. Statistical Analysis 53

CHAPTER 3. RESULTS AND DISCUSSION 54
3.1. *In vitro*, *In vivo*, Clinical and Meta-Analyses Studies for Probiotics 54
3.1.1. *Lactobacillus spp.* as Probiotics 54
3.1.2. *Bifidobacterium spp.* as Probiotic 55
3.1.3. *Bacillus spp.* as Probiotic 61
3.1.4. *Saccharomyces spp.* as Probiotic 66
3.2. Probiotic use in Turkey and Their Regulations 67
3.3. A meta-analysis of *S. boulardii* 83
3.3.1. In Adults 83

3.3.2. In children 86
3.4. Contribution to the development of the ProBioYeastract Database..... 88
3.5. New clues on the probiotic activity of *Sb*, when compared to *Sc* 88
3.5.1. Cross-strain promotor comparison of putative probiotic gene regulation..... 89
3.5.2. Differential gene expression of selected genes: *Sb* vs *Sc* 89
3.6. *Sb* exhibits higher aggregation, adhesion to human epithelial cells and biofilm formation than *Sc* 103

CHAPTER 4. CONCLUSION 106

REFERENCES 108

APPENDICES
APPENDIX A. R CODES 127
A. 1. R codes for Meta-analysis *S. boulardii* in adult 127
A. 2. R codes for Meta-analysis *S. boulardii* in children 129
APPENDIX B. PROJECT COVER..... 132

LIST OF FIGURES

Figure **Page**

Figure 1.1. Schematic illustration of Relationship between Human Host and Microbiota 1

Figure 1.2. Representation of the healthy balance between intestinal epithelial cells and the microbiota (homeostasis) and disease consequences on this balance (dysbiosis) 2

Figure 1.3. Major benefits of probiotics for human health 3

Figure 1.4. Summary of good probiotic features 4

Figure 1.5. Schematic diagram of *in vivo* models and clinical studies used to support probiotic health claims 5

Figure 1.6. Mechanisms of action for probiotics in the gastrointestinal tract 6

Figure 1.7. Illustration of the holistic approach of beneficial effects of probiotics exerted on human health 7

Figure 1.8. QPS status of microorganisms to assess risk analyses 9

Figure 1.9. The example of the clinical database about commercial probiotics in Canada 10

Figure 1.10. The example of a bioinformatic database website about probiotics, called PROBIO 11

Figure 1.11. The example of bioinformatic database about searching of transcription factors of yeast called YEASTRACT (Yeast Search for Transcriptional Regulators And Consensus Tracking) 12

Figure 1.12. Illustration of formation and risk factor of *C. difficile* infection. 18

Figure 1.13. The mechanism of Action of *S. boulardii* against the consequences of *C. difficile* infection 18

Figure 1.14. The mode of action of probiotic for Inflammatory bowel disease 20

Figure 1.15. Summary of digestive enzymes stimulated or produced by *S. boulardii* ... 22

Figure 1.16. Mechanism of celiac disease 23

Figure 1.17. Schematic pathway representation of galactose utilization and its related genes in *S. boulardii* 24

Figure 1.18. Fatty acid metabolism in *S. cerevisiae* 25

Figure 1.19. Schematic representation of the possible mode of action for *S. boulardii*. 28

Figure 1.20. Schematic representation of the intestinal epithelium barrier 28

Figure **Page**

Figure 1.21. Schematic representation of SCFA formation microbial metabolic pathways in the human gut 30

Figure 1.22. Bacterial adhesion and biofilm formation 31

Figure 1.23. The mode of action *Sb* according to bacterial infections 32

Figure 1.24. Mode of Action of *Sb* against *E. coli*, *C. difficile* and *Salmonella* infections 32

Figure 2.1. The overall design of probiotic research diagram 38

Figure 2.2. The pipeline of cross-strain promoter comparison using YEASTRACT bioinformatics tools. 43

Figure 2.3. Scheme of experimental methodology to determine the expression of the selected genes. 44

Figure 2.4. Scheme of experiments of the growth of cells carried out to RNA extraction 48

Figure 2.5. Schematic representation of the experimental design of 96-well polystyrene titter plates (Greiner) for biofilm determination. 53

Figure 3.1. Forest plot of meta-analysis of 16 randomized controlled trials based on treatment with *S. boulardii* or placebo for diarrhea in adults. The x-axis depicts effect size, with solid black squares denoting the relative risk and the line indicating 95% CI. The size of the black squares is proportional o study size. (I^2= 15.33%, P<0.0001 ****) 84

Figure 3.2. Baujat plot to identify studies contributing to heterogeneity for *S.boulardii* meta-analysis in adults, The X-axis and Y-axis show the contribution probiotic-treated group and influencing control group of the trial on the overall heterogeneity, respectively. 85

Figure 3.3. Funnel plot to indicate publication bias by using mixed-effects meta-regression model 85

Figure 3.4. Forest plot of meta-analysis of 17 randomized controlled trials based on treatment with *S. boulardii* or placebo for diarrhea in children. The x-axis represents effect size, with solid black squares denoting the relative risk and the line indicating 95% CI. The size of the black squares is proportional to study size (I^2 = 81.31 %, P<0.0001 ****). 86

Figure 3.5. Baujat plot to identify studies contributing to heterogeneity for *S. boulardii* meta-analysis in children, the x-axis and y-axis show the contribution probiotic-treated group and influencing control group of the trial on the overall heterogeneity, respectively. 87

Figure 3.6. Illustration of Funnel plot in children to indicate publication bias by using mixed-effects meta-regression model 87

Figure **Page**

Figure 3.7. Distribution of gene expression level of selected genes by using YPD medium in *Sb* and *Sc* was taken into account as a reference value, identified by RT-PCR analysis to be related to the regulation of genes. The genes found as *EFG1* and *IMA1* (up-regulated) and *FLO5*, *TGL4*, *YDC1* and *SPE2* (down-regulated). Error bars represent the corresponding standard deviations. ***** P<0,00001; **** P<0,0001, ***P<0.001; ** P<0,01; * P<0,05 101

Figure 3.8. Distribution of gene expression level of selected genes by using YPD+Sodium cholate that mimics human gastrointestinal system, in *Sb*, and *Sc* was taken into account as a reference value, identified by RT-PCR analysis to be related to the regulation of genes. The genes found as *EFG1* (up-regulated) and *FLO5*, *TGL4*, *YDC1* and *SPE2* (down-regulated). Error bars represent the corresponding standard deviations. **** P<0,0001;* P<0,05 101

Figure 3.9. Distribution of the putative TF binding sites in the promoter regions of the *EFG1* genes in *S. boulardii* Biocodex (ORF KO01_01677) and Unique28 (ORF AB282_01893) strains, as obtained in the "Search TF" query of the ProBioYeastract database. 102

Figure 3.10. Distribution of the putative TF binding sites in the promoter regions of the *IMA1* genes in *S. boulardii* Biocodex (ORF KO01_01662) and Unique28 (ORF AB282_01878) strains, as obtained in the "Search TF" query of the ProBioYeastract database. 103

Figure 3.11. The percentage of aggregation in *Sb* and *Sc* cells, *Sb* is formed more aggregation when grown in YPD medium, compared to *Sc* under the same conditions. Standard deviation being represented by the error bars. ***** P<0,00001. 104

Figure 3.12. Percentage of Adhesion in *Sb* and *Sc* cells, *Sb* is formed more adhesion when grown in YPD medium, compared to *Sc* under the same conditions. Error bar represents the corresponding standard deviation, * P<0,05. 104

Figure 3.13. Biofilm formation followed by Presto Blue Cell Viability Assay and measurements of absorbance at 570 nm and 600 nm (reference) for the *Sc* and *Sb*, when compared to different medium (YPD, SDB, RPMI both pH=4 and pH=7). Error bars represent the corresponding standard deviations. ***** P<0,00001; **** P<0,0001; * P<0,05. 105

LIST OF TABLES

Table **Page**

Table 1.1. Mostly used probiotic microorganisms in the pharmaceutical and food industry 12

Table 1.2. Genes predicted to underlie *Sb* mode of action as a probiotic agent. 36

Table 2.1. Clinical data for probiotic and control groups in adults 41

Table 2.2. Clinical data for probiotic and control group in children 42

Table 2.3. An example of a cross-strains promoter analysis, using ProBioYeastract and YEASTRACT database 45

Table 2.4. Summary of selected genes for experimental evaluation after cross-strain promotor analysis..... 48

Table 2.5. PCR reaction mixture components and their volumes 50

Table 2.6. Thermal cycling parameters for the first step of the real-time RT-PCR 50

Table 2.7. RT- PCR reaction components and their volumes for each sample 50

Table 2.8. Primers used in screening and quantitative Real-Time PCR……..51

Table 3.1. Summary of the major findings on the probiotic efficacy of *Lactobacillus spp.*, according to *in vitro*, *in vivo*, clinical and meta-analysis studies for children and adult patients. EHEC- Enterohemorrhagic *Escherichia coli*, IL-8 Interleukin 8, IL-6 Interleukin 6, IL-1β Interleukin 1 β, TNF-α Tumor Necrosis Factor-alpha, IFN-γ Interferon Gamma, CDI- C. difficile infection, AAD Antibiotic-Associated Diarrhea, FAP- Functional Abdominal Pain,PC-Placebo controlled, PG- Parallel-group, R- randomized, MC-Multicentre, RCT-Randomized control trials, Ab-antibiotic, NA-No Available, wk-week, mo-month, yr-year. 56

Table 3.2. Summary of the major findings on the probiotic efficacy of *Bifidobacterium infantis subsp.*, according to *in vitro*, *in vivo*, clinical and meta-analysis studies for children and adult patients. IECs-Intestinal Epithelial cell, IL-8 Interleukin 8, IL-10 Interleukin 10, T_H1-T helper cells1,T_H17- T helper cells 17, IBS-Irritable Bowel Syndrome, IBD Inflammatory Bowel Disease, PC-Placebo controlled, PG- Parallel-group, R- randomized, Ab-antibiotic, NA-No Available, wk-week, mo-month..... 62

Table **Page**

Table 3.3. Summary of the major findings on the probiotic effectiveness of *Bacillus*, according to *in vitro*, *in vivo*, clinical and meta-analysis studies for children and adult patients. EHEC- Enterohemorrhagic *Escherichia coli*, IL-8 Interleukin 8, IL-6 Interleukin 6, IL-1β Interleukin 1 β, TNF-α Tumor Necrosis Factor-alpha, IFN-γ Interferon Gamma, CDI- *C. difficile* infection, AAD Antibiotic-Associated Diarrhea, TD Traveler's diarrhea PC-Placebo controlled, ORT- oral rehydration therapy, PG- Parallel-group, P-Prospective R- randomized,SC-Single-centre Ab-antibiotic, NA-No Available. 64

Table 3.4. Summary of the major findings on the probiotic efficacy of *S. boulardii*, according to *in vitro*, *in vivo*, clinical and meta-analysis studies for children and adult patients. EHEC- Enterohemorrhagic *Escherichia coli*, IL-8 Interleukin 8, IL-6 Interleukin 6, IL-1β Interleukin 1 β, TNF-α Tumor Necrosis Factor-alpha, IFN-γ Interferon Gamma, BBM -Brush Border Membrane, IgA-Immunoglobulin A, CDI- *C. difficile* infection, CDD-*C. difficile* disease, AAD-Antibiotic-Associated Diarrhea, TD-Traveler's diarrhea PC-Placebo controlled, PG- Parallel-group, R- randomized, DB-Double Blind, DM-Double Mask, MC-Multicentre, Ab-antibiotic, NA-No Available, yr-year, mo-month, wk-week, d-day. 69

Table 3.5. Review of Probiotic market in Turkey 76

Table 3.6. Design of Cross-strain promoter comparison table to help the building of ProBioYeastract Database 90

ABBREVIATIONS

WHO: World Health Organization

FDA: American Food and Drug Administration

EFSA: European Food Safety Authority

FOSHU: Foods for Specified Health Uses

CFDA: China Food and Drug Administration

TGA: Therapeutic Good Administration

CFIA: Canadian Food Inspection Agency

AAD: Antibiotic-Associated Diarrhea

CDI: *Clostridium difficile* infection

TD: Traveler's diarrhea

LPS: Lipopolysaccharide

IBS: Irritable bowel syndrome

CD: Celiac disease

ST: Salmonella typhimurium

MAPK: Mitogen-activated protein kinase

YPD: Yeast-extract-peptone-dextrose

SDB: Sabouraud's dextrose broth

RPMI: Roswell Park Memorial Institute

CFU: Colony forming units

cDNA: complementary DNA

RT-qPCR: quantitative real-time polymerase chain reaction

DEPC: diethylpyrocarbonate

EDTA: Ethylenediamine tetraacetic acid

Sb: *Saccharomyces boulardii*

Sc**:** *Saccharomyces cerevisiae* S288C

YEASTRACT: Yeast Search for Transcriptional Regulators And Consensus Tracking

SI: Sucrose-Isomaltase

MGA: Maltase-glucoamylase

ANP: Aminopeptidase N

LPH: Lactase-phlorizin hydrolase

IAP: Intestinal Alkaline Phosphatase

TFBS: Transcription Factor Binding Sites

BBM: Brush Border Membrane

TGF-β: Transforming Growth Factor beta

CHAPTER 1

INTRODUCTION

1.1. Overview of Microbiota, Probiotics, and Prebiotics

Microbiota is generally defined as all microorganisms such as bacteria, yeasts, fungi, virus living in a specific environment. It is briefly defined as the microbial taxa associated with humans. Human microbiota consists primarily of bacteria, viruses, fungi, and many eukaryotic microorganisms. The microbiome is defined as the catalogue of microbes on the microbiota and their genes. The microbiome plays a pivotal role in the improvement and functionality of the innate and adaptive immune responses (Di Cerbo et al., 2016). The majority of this microbial population has been colonized in the skin, genitourinary system and respiratory system, primarily the gastrointestinal tract (Figure 1.1) (Collison et al., 2012).

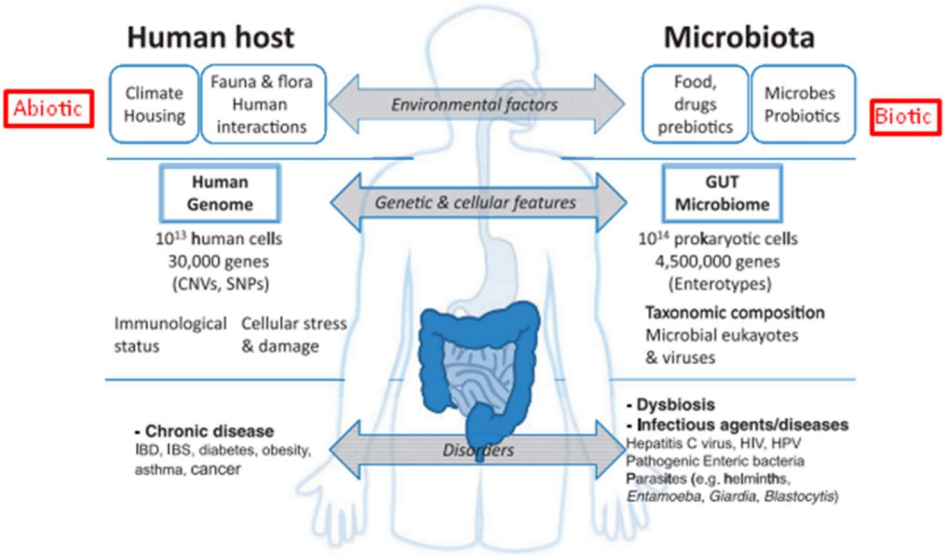


Figure 1.1. Schematic illustration of relationship between Human Host and Microbiota (Source: Collison et al., 2012)

Homeostasis is defined as the maintenance of steady states on the human body by coordinate physiologic mechanisms. On the other hand, **dysbiosis** is an imbalance in

the microbiota associated with harmful results for the host and briefly can be said altered microbiota (Figure 1.2).

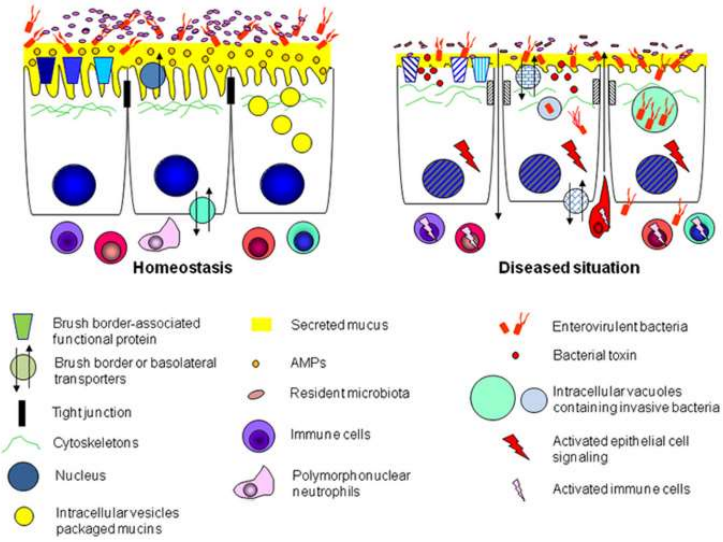


Figure 1.2. Representation of the healthy balance between intestinal epithelial cells and the microbiota (homeostasis) and disease consequences on this balance (dysbiosis)(Source: Moal and Servin, 2013)

The word **Probiotic** comes from a Latin/Greek root and means literally "for life". In 1857, Pasteur discovered lactic acid bacteria for the first time. However, it was Élie Metchnikoff that became known as the father of probiotics since he asserted that lactic acid bacteria induced lower pH in the colon due to the breaking down of lactose, thus inhibiting the growth of proteolytic bacteria (Ozen and Dinleyici, 2015). The word Probiotic was coined by Fuller and defined as a "live non-pathogenic microbial feed or food supplement which beneficially affects the host by improving its intestinal microbial balance" (Czerucka and Rampal, 2002).

Nowadays, according to the World Health Organization (WHO), Probiotics are defined as "Live microorganisms which when administered in adequate amounts confer a health benefit to the host." (Olveira and González-Molero, 2016; Hunt et al., 2017).

Based on this premise, there has been an increasing trend of using probiotic organisms worldwide to contribute to human health, particularly as co-adjuvants in the treatment and prevention of gastrointestinal disorders such as antibiotic-associated-diarrhea, irritable bowel syndrome and inflammatory bowel diseases, Infections caused by pathogenic bacteria, and so on, as well as allergies, obesity and intolerances. Furthermore, decreasing cholesterol levels and lowering of blood pressure have been

associated with probiotic usage (Figure 1.3). Many of these probiotic organisms contribute by either substituting or aiding the re-establishment of the natural gastrointestinal flora, or microbiota (Table 1.1). Probiotics are strain-specific and it is important to know which probiotics are isolated from the main source for the protective effect on the human diseases (Tuo et al., 2018).

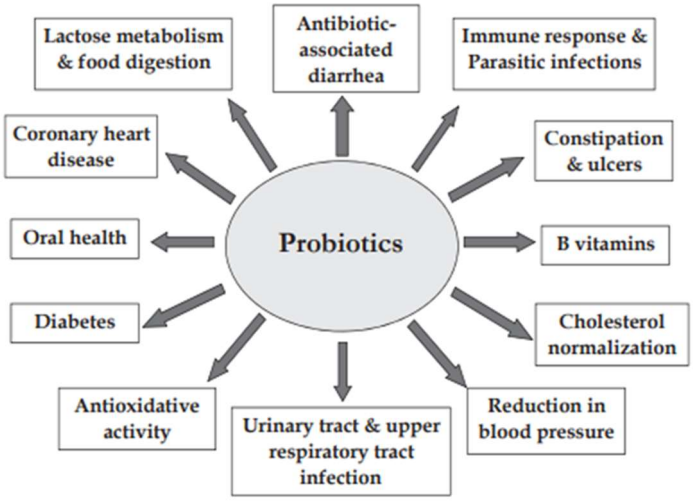


Figure 1.3. Major benefits of probiotics for human health
(Source: Nagpal et al., 2012)

Some of the conditions that need to be met in order for a specific microbial strain to be called Probiotic are the following (Vandenplas et al., 2015);

**In vitro* studies: to show potential probiotic activity

*Assessment of safety: to indicate that the strain carries no human or environmental toxicity

**In vivo* studies: to indicate probiotic activity, that is a positive health impact in the target host

*Clinical studies: to prove probiotic activity in human health in terms of dose, efficacy, and effectiveness

*Meta-analysis: to show clinical evidence performed statistical methods

*Good probiotic properties (Daliri and Lee, 2015), which may include (Figure 1.4.) :

- Resistance to pancreatic enzymes, low pH and bile which provides survival during passage through the intestinal tract, an important property for oral administration
- Adhesion to the intestinal mucosa; pathogen exclusion, preventing its adhesion and colonisation; enhancing damaged mucosa recovery; prolonged transient colonization (Kechagia et al., 2013)
- Having human origin which means being a natural human commensal
- Proven, through clinical evidence, to induce positive health effects
- Having good technological features for industrial manufacturing, which include strain stability; oxygen tolerance, and short generation time (Fietto et al., 2004)
- Production of antimicrobial compounds, active against pathogens, such as organic acids, hydrogen peroxide and bacteriocins (Gut et al., 2018)

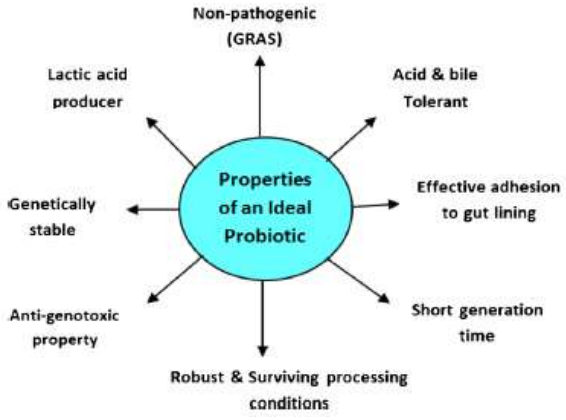


Figure 1.4. Summary of good probiotic features
(Source: Pandey et al., 2015)

Probiotic products are available in different forms in over the world, for instance, capsules of freeze-dried or lyophilized cultures, heat-dried culture supernatants, mixed in dairy foods such as yoghurts, cheese, milk, or ice cream, or other foods such as kefir, chocolate, wafers. The kinds of probiotic products are also regulated under different guidelines according to their use such as food, dietary supplement, or prescription (McFarland, 2017).

Prebiotics refer to non-digestible fibers in the diet that selectively stimulate the growth of beneficial colon bacteria in the host's microbiota (Pandey et al., 2015).

Commonly known and used commercially prebiotics following by;

*Oligofructose

*Inulin

*Galacto-oligosaccharides

*Lactulose

*Breast milk oligosaccharides etc.

A good prebiotic should be 1) resistant to the actions of acids in the stomach, bile salts and other hydrolyzing enzymes in the intestine 2) not be absorbed in the upper gastrointestinal tract 3) easily fermentable by the beneficial intestinal microflora.

Synbiotic is defined as including both probiotic and prebiotic. They were developed to overcome possible survival difficulties for probiotics such as pH, H_2O_2, organic acids, oxygen, moisture stress, etc. (Pandey et al, 2015) Synbiotic formulations include the probiotic strains like *Lactobacilli*, *Bifidobacteria spp*, *S. boulardii*, *B. coagulans,* etc., and the addition of the important prebiotics are used formulation of oligosaccharides like fructooligosaccharide (FOS), galactooligosaccharides (GOS) and xylooligosaccharides (XOS), inulin, etc.

1.2. *In vitro*, *In vivo*, Clinical Studies

In vitro studies of probiotics was a preferable choice because of the simplicity and the low cost of such approaches. Therefore, it is more economical and provides more rapidly results. Moreover, suitable *in vitro* tests have been adopted to choose strains based on their ability to survive transit through the different compartments of the gastrointestinal system such as harsh pH values in the stomach.

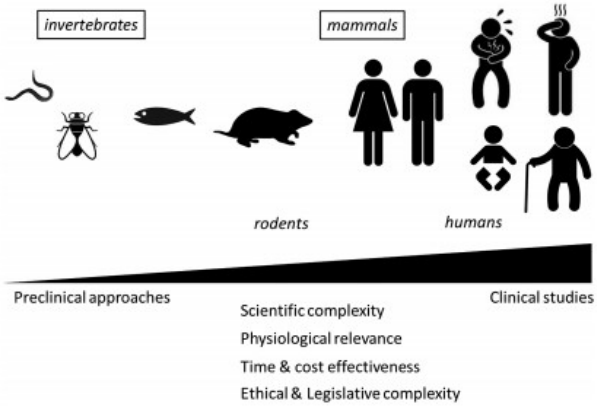


Figure 1.5. Schematic diagram of *in vivo* models and clinical studies used to support probiotic health claims (Source: Papadimitriou et al., 2015)

In vivo assays has preferred less by scientific studies due to the increased cost, ethical reasons and taking a long time; but, in most cases, it may be more suitable. Hence, *in vitro* studies will be an indispensable part of the discovery of new probiotics and determining their effectiveness. After performing *in vivo* studies, clinical trials have continued to support the efficacy of probiotics as shown in Figure 1.5.

1.3. Mode of Actions of Probiotics

The mode of action of probiotics is still totally not understood but they may act as surrogate normal microflora following antibiotic therapy until recovery is achieved (McFarland, 2010). Mechanism of probiotic activity has mainly immunologic and nonimmunologic benefits on human health as shown in Figure 1.6.

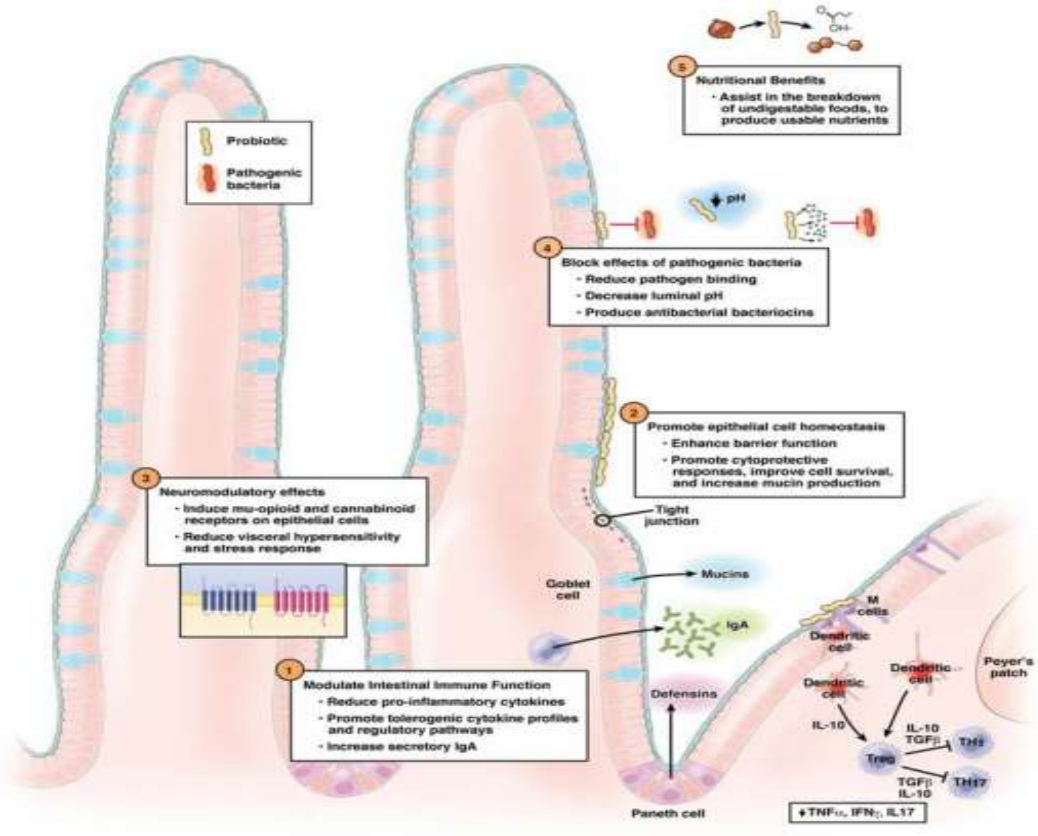


Figure 1.6. Mechanisms of action for probiotics in the gastrointestinal tract (Source: Ciorba, 2012)

There is a new approach of beneficial effects of probiotic, including considering of mode of action such as bile tolerance, low pH adaptation and specially adhesion of

human gut. These features provide preventing and treating gastrointestinal diseases, food allergies and intolerances, at the same time, they might be responsible for the reduction of cholesterol levels and blood pressure as illustrated in Figure 1.7.

Immunologic benefits are followed by; modulating cytokines, inducing tolerance to food antigens, activating local macrophages, increasing antigens and secretory immunoglobulin A (IgA) production. However, when looking at the nonimmunologic benefits; helping digestion of food, competing for pathogens, producing bacteriocins to inhibit pathogens, modifying pathogen-derived toxins, stimulating epithelial mucin production, enhancing intestinal barrier functions (Papadimitriou et al., 2015). For example, the mechanisms of action of one of the well-studied probiotics are *S. boulardii* that includes luminal action (anti-toxic effect, antimicrobial activity), trophic action (enzymatic activity, increased IgA) and mucosal anti-inflammatory signaling effects (decreased synthesis of inflammatory cytokines) (McFarland, 2010). These features mentioned as section 1.8 in detail.

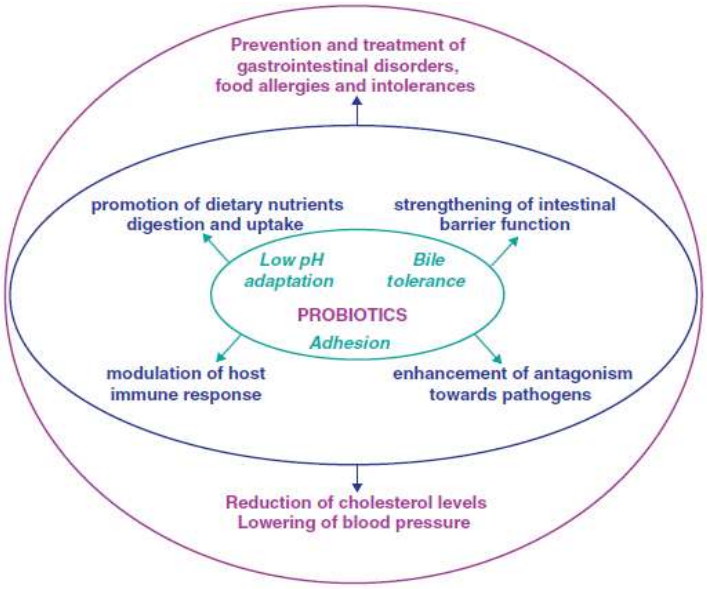


Figure 1.7. Illustration of the holistic approach of beneficial effects of probiotics exerted on human health (Source: Siciliano and Mazzeo, 2012)

1.4. Safety and Risk Assessment of Probiotics

Probiotics should be clinically validated and documented health effects of minimum effective dosage in products should be available. They should also be classified

as Generally Recognised As Safe ‘GRAS’, In America, an American Food and Drug Administration (FDA) responsible for designation for chemicals or food additives considered safe by experts. This will imply a previous ‘history of safe use’ and safety in food. In Europe, risk assessment of probiotics are responsible for European Food Safety Authority (EFSA), was established in 2002, to address the increasingly important and complex scientific and technical issues in relation to food and feed safety. Nevertheless, the exact regulatory model for approval of a novel probiotic microbe has remained ambiguous (Sanders et al., 2010).

Lactobacillus species, *Bifidobacterium* species, *Streptococcus* and *Enterococcus* species, and should be following Qualified Presumptions of Safety (QPS) considering by the EFSA. For example, members of the genera *Lactobacillus* are most commonly given generally-recognised-as-safe (GRAS).

According to FAO/WHO guidelines, Probiotics could be used as a global standard for evaluating criteria as following by;

*” Strain identification.

*Functional characterization of the strain(s) for safety and probiotic attributes.

*Validation of health benefits in human studies.

* Honest, not misleading labeling of efficacy claims and content for the entire shelf life.” (Pandey et al., 2015)

The QPS concept is defined to microorganisms either used as viable cells in the food chain, as illustrated in Figure 1.8 or to produce enzymes, metabolites, dead biomass or other specific end products that are not expected to contain live microbial cells.

Microorganisms which are not on the QPS list, are not necessarily considered to be unsafe.

Safety concerns according to the QPS list;

*“ Virulence/pathogenicity to humans

*Virulence/pathogenicity to vertebrate animals

*Antimicrobial resistance

*Environmental safety” (Ricci et al., 2017)

For the probiotic strain *Lactobacillus helveticus* MTCC5463,

If the safety-related genes classified into resistome (all the antibiotic resistance genes) heavy metals, adverse metabolic genes, virulence-related genes, and stress-related

genes, the presence of genes in these categories should be checked the safety assessment of this strain. Moreover, they should be non-invasive, non-carcinogenic and non-pathogenic to human (Gut et al., 2018; Kechagia et al., 2013).

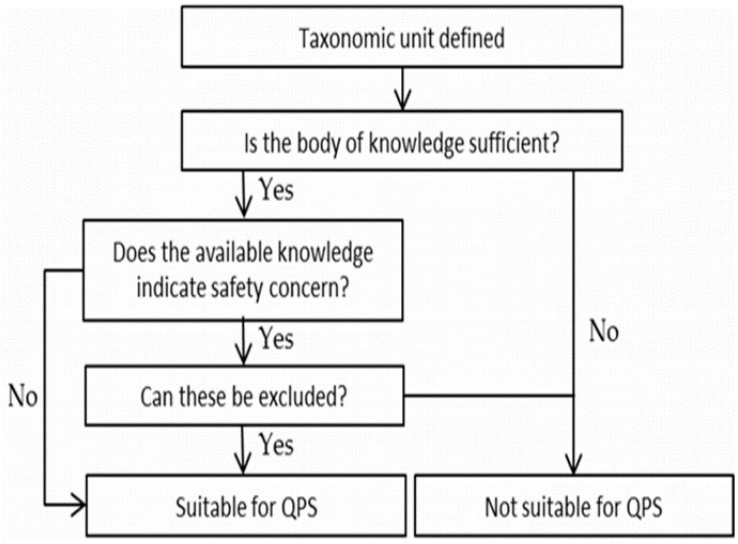


Figure 1.8. QPS status of microorganisms to assess risk analyses
(Source: Laulund et al. 2017)

"Probiotics are safe" easily cannot say due to fact that the assessment of safety of probiotics should be taken into consideration of a variety of factors following by; (Sanders et al., 2010) "Record isolation history and taxonomic classification of the candidate probiotic following by;

- Manufacturing controls that eliminate contamination (including cross-contamination between batches) of the probiotic with microbes or other substances.
- Absence of association of the probiotic with infectivity or toxicity, assessed at the strain level.
- Absence of transferable antibiotic resistance genes
- Physiological status of the consuming population. Special consideration must be made for use in vulnerable populations, including newborn infants and the critically ill.
- Dose administered
- Method of administration (oral or otherwise)
- Absences of allergenic material (for example, dairy proteins) for products targeted for allergic populations."

1.5. Importance of Bioinformatics Databases

Bioinformatics is defined as "Research, development, or application of computational tools and approaches for expanding the use of biological, medical, behavioral, health and nutrition data, including those to acquire, store, organize, archive, analyze, visualize or build biological knowledge from very large and traditionally unrelated sources." (Desiere et al., 2001)

Recently, an online guideline to probiotics has being launched in Canada. (http://www.probioticchart.ca/). This is an unique application so far on probiotics area and also being used by the USA. The website includes bypasses to seek clinical applications for commercially available probiotic products. All the information in this guide is based on clinical research on the probiotic products already been in the market. The search engine of the database allows their users to access information for probiotics about their benefits for human health with respect to indication, age/gender and brand name as indicated in Figure 1.9.

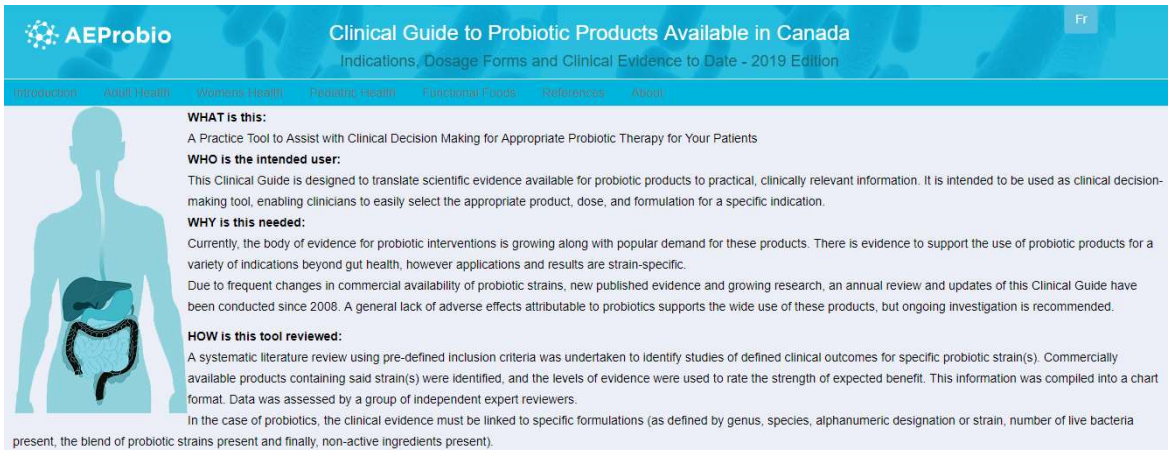


Figure 1.9. The example of the clinical database about commercial probiotics in Canada (Source: Probiotic chart)

To give another example of bioinformatic database about probiotics, called PROBIO, was developed according to 448 marketed, 167 clinical trial/field trial, and 382 research probiotics for use in human, animals, and plants as shown in Figure 1.10 (http://bidd2.nus.edu.sg/probio/homepage.htm). Marketed Probiotic Products and Constituent Probiotic Species named of the table includes;

*name of the manufacturer and their product names

*beneficial impacts claimed by the manufacturer

*constituent probiotic types/ strains and formulation

Clinical studies of the marketed probiotics studied by next-generation sequencing, metabolomic or genetic analysis include;

*Probiotic species or strains

*Probiotic functions

*Association of gut microbiota with a related disease

*Information on clinical trial cohort

*Analysis method of gut microbiota

*Observed outcomes

Modes of probiotic action, molecules, and species

* Relation between specific probiotic action and probiotic strain

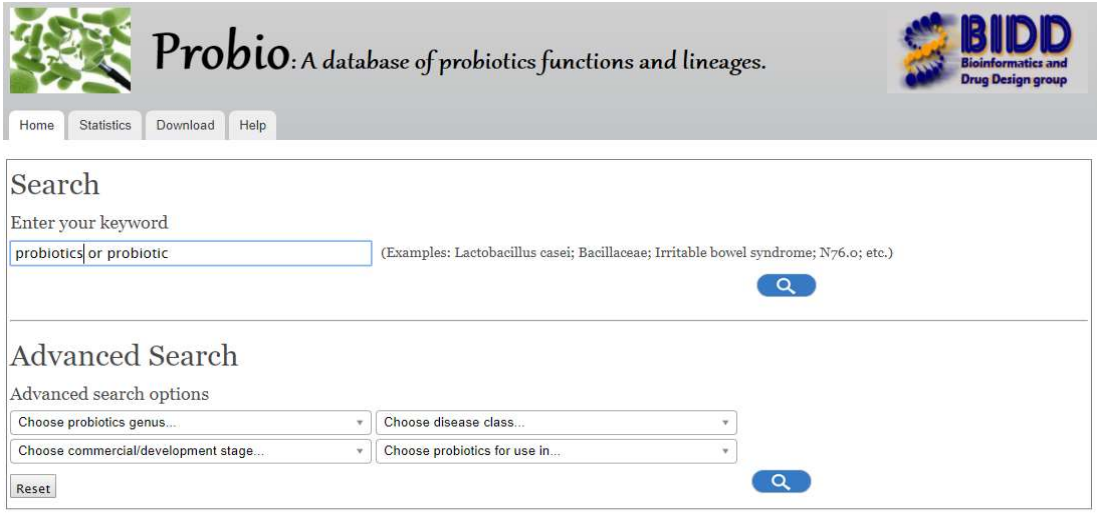


Figure 1.10. The example of a bioinformatic database website about probiotics, called PROBIO (Source: Tao et al., 2017)

The YEASTRACT (Yeast Search for Transcriptional Regulators and Consensus Tracking) database is a repository of curated published transcriptional associations that offers tools for transcription regulation analysis in yeast as shown in Figure 1.11 (http://www.yeastract.com/). In its first release, it focused solely on the model yeast *S. cerevisiae* (Teixeira et al., 2006; Teixeira et al., 2018) but more recently, it was expanded to pathogenic yeasts of the *Candida* genus in the form of the PathoYeastract database (Monteiro et al., 2017).

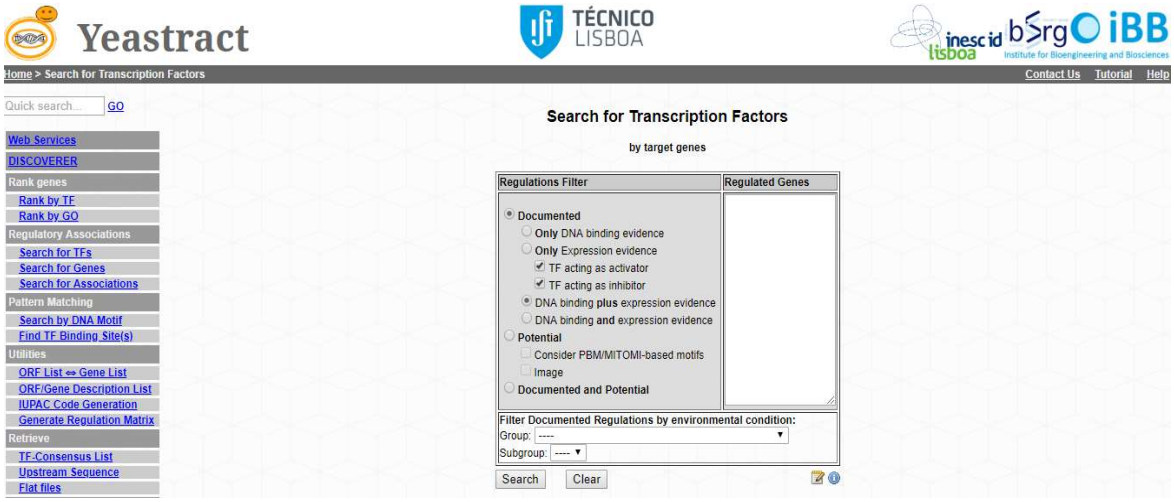


Figure 1.11. The example of bioinformatic database about searching of transcription factors of yeast called YEASTRACT (Yeast Search for Transcriptional Regulators And Consensus Tracking) (Source: Teixeira et al., 2006; Teixeira et al., 2018)

1.6. Commonly used Probiotics

Recent reports commonly informed probiotics as follows: bacteria in the genus (1) *Lactobacillus*, including *Lactobacillus acidophilus, Clostridium butyricum*, *L. reuteri*, *L. bulgaricus, L. casei*, *L. paracasei, L. rhamnosus*, *L. salivarius*, and *L. plantarum*; *Bifidobacterium*, including *Bifidobacterium infantis*, *B. adolescentis*, *B. animalis*, *B. longum*, *B. breve*, and ovary double Bacteroides; (2) Gram-positive cocci, such as *Streptococcus thermophilus*, *S. faecalis*, and *Lactococcus*; (3) spores, such as *Bacillus subtilus*, *Bacillus clausii* , and (4) yeast, such as *S. boulardii*., commercially available probiotic products include probiotic amended yogurt, encapsulated live bacteria, bacteria powder, oral liquids, and various preparations of single strains. Recently, commonly used probiotics in the industry are summarized in Table 1.1.

1.6.1. *Lactobacillus* as a probiotic

Lactobacilli are small, slender, nonmotile, gram-positive on-spore-forming anaerobes or facultative anaerobes that ferments glucose into lactose, and they colonize the gastrointestinal and urinary tracts of humans' especially female genital tract, thus,

they are an important part of the microbial flora. Some species of *Lactobacillus* are used naturally as probiotics.

Table 1.1. Mostly used probiotic microorganisms in the pharmaceutical and for industry (Source: Holzapfel et al., 2001)

Bacteria		***Spores***	***Yeast***
Lactobacilli spp.	***Bifidobacterium spp.***	***Bacilli spp.***	***Saccharomyces spp.***
Lactobacillus rhamnosus	*Bifidobacterium animalis/lactis*	*Bacillus coagulans*	*Saccharomyces boulardii*
Lactobacillus acidophilus	*Bifidobacterium bifidum*	*Bacillus subtilus*	
Lactobacillus casei	*Bifidobacterium longum*	*Bacillus clausii*	
Lactobacillus bulgaricus	*Bifidobacterium infantis*		
Lactobacillus plantarum	*Bifidobacterium breve*		

This section focuses on new insights into the well-studied probiotic bacterium *Lactobacillus spp* such as *Lactobacillus rhomnosus* GG (LGG), *Lactobacillus reuteri* etc. As an example, LGG is a commonly known gram-positive bacterium originally isolated from the healthy human intestine. It has been widely used in the manufacturing of yogurt as a food supplement. It is one of the most studied among *Lactobacillus* probiotics strain types in clinical trials.

1.6.2. *Bifidobacterium* as a probiotic

A *Bifidobacterium* was originally isolated by Henry Tissier (of the Pasteur Institute) from a breast-fed infant, and he named the bacterium *Bacillus bifidus communis*. Tissier claimed that bifidobacteria would displace the proteolytic bacteria that cause diarrhea and recommended the administration of Bifidobacteria to infants suffering from this symptom.

Konieczna et al. (2012) reported that *Bifidobacterium infantis* 35624 isolated from resected human healthy gastrointestinal tissue and has been shown to be nonpathogenic,

acid and bile resistant, and it survives passage through the gastrointestinal tract. This specific strain of probiotic has been demonstrated to regulate intestinal and systemic inflammation in both animal and human studies, subsequently resulting in symptomatic relief of IBS symptoms. Furthermore, *B. infantis* positively affects epithelial cells, dendritic cells and lymphocytes to treat autoimmune disorders.

O'Mahony and colleagues showed that consumption of *B. infantis* could change inflammatory cytokine profiles while paralleling development in IBS symptoms (O'Mahony et al., 2005)

In vitro studies showed that *B. infantis* have anti-inflammatory effects in murine models of infection, arthritis and respiratory inflammation.

Recently, Lyseng-Williamson (2017) have reported that *B. infantis* as a dietary supplement provides a stable, convenient, beneficial and well-tolerated so as to promote and maintain for the digestive system in the gastrointestinal tract.

1.6.3. *Bacillus* as a probiotic

Bacillus clausii commonly used as probiotics in human health. It is a gram-positive, endospore-forming, aerobic and facultative alkaliphilic rod bacterium, germination and outgrowth of spores in the intestine. The genus *Bacillus* is generally designated as a group of soil inhabitants (Elshaghabee et al., 2017). *Bacillus* probiotics are commonly known as *B. subtilis*, *B. clausii*, *B. cereus*, *B. coagulans* and B. *licheniformis*. Spore formers have some important advantages compared to non-spore formers e.g *Lactobacillus*. In this regard, the benefits of using spores as probiotics are following by ; (Huynh et al., 2005; Jayantini and Sudha, 2015)

•They are heat stable

•They can be stored at room temperature that is unaffected their viability

•They are also resistant to acidic conditions of the stomach (low pH) and so can survive the transit to reach the intestine.

•*In vitro* studies suggest that both *B. clausii* spores and cells can adhere to the bowel wall and colonize the mucosa

•As *B. clausii* is extremely stable to acidic conditions, the entire dose of ingested bacteria reaches the small intestine intact.

•They provide more shelf-life

Bacillus probiotics have commonly preferred the cure of diarrhea and in the prevention of infectious diseases (Urdaci et al., 2004).

In an earlier study reported that spores of *Bacillus* sp. prevent gastrointestinal side effects on human due to oral antibiotic therapy because they are resistant to antibiotics. The possible impacts of spores are to restore an intestinal flora following the destruction of commensals by antibiotics, immunostimulation, and raised the secretion of IgA (Bozdogan et al. 2003). In this regard, Studies have been reported to show antimicrobial, anti-oxidative and immune-modulatory activity by different kinds of *Bacillus* strains in the host. Furthermore, *Bacillus* species can produce a large number of antimicrobials.

Recently, an *in vivo* study by Elshaghabee et al. (2017) revealed that the spores of *B. clausii* (Enterogermina®) were shown to enhance the production of IFN-γ in murine spleen cells, rabbits and mice animal models.

One a recent clinical trial by Tewari et al. (2015), double-blind, randomized and placebo control in 244 preterm neonates, has indicated that there is no significant difference in the incidence of late-onset sepsis (LOS), however, full feeds were achieved significantly faster by treating probiotic group of *B. clausii*. Another study, twenty healthy subjects were administered orally with Enterogermina®, containing spores of four strains of *Bacillus*, has been demonstrated successfully to survive transit through the human GIT, during which germination, out-growth, and multiplication could happen (Ghelardi et al., 2015).

1.6.4. *Saccharomyces* as a probiotic

Saccharomyces boulardii also called *Saccharomyces cerevisiae var. boulardii*, was isolated by the French scientist Henri Boulard in 1920 from the skin of lychee and mangosteen in Indochina, during a cholera outbreak (Edwards-Ingram et al., 2007; Batista et al., 2014).

S. boulardii is a well-studied probiotic yeast known as a therapeutic agent for the prevention of recurrence of several gastrointestinal diseases, which are mainly grouped into acute and chronic. Acute diseases include Antibiotic-associated diarrhea (AAD), *Clostridium difficile* infection (CDI), and Acute diarrhea, including that caused by Rotavirus infection in children, Persistent diarrhea, Enteral nutrition-related diarrhea,

Traveler's diarrhea (TD), and *Helicobacter pylori* infection. On the other hand, chronic diseases include Crohn's disease, Ulcerative colitis and Irritable bowel syndrome (IBS) (Kelesidis and Pothoulakis, 2012).

Compared to bacterial probiotics, *S. boulardii* is naturally resistant against all kinds of antibiotics, given its eukaryotic nature (Czerucka et al., 2007; Graff et al., 2008; Kelesidis and Pothoulakis, 2012).

A number of studies, conducted *in vitro*, *in vivo*, or as clinical or meta-analysis, have shown that *S. boulardii* is a probiotic, having a positive impact in the treatment and prevention of several diseases of the gastrointestinal tract.

1.7. Probiotics against Diseases caused by Diarrhea

Diarrhea is a widespread health problem all over the world. It generally is diagnosed when observing mushy or watery stool, per-day stool weight of >200 g, or stool frequency of more than three per day (Högenauer et al., 1998). There are a lot of *in vitro*, *in vivo*, clinical studies indicating the efficacy of probiotics to reduce acute diarrhea. Most of the studies reported that species of *L. rhamnosus* GG, *L. reuterii*, *L.acidophilus*, *S.boulardii* and also *Bacillus* spores were also to diminish the symptoms of acute diarrhea without causing any adverse effects to human health (Sudha et al., 2013).

1.7.1. Against Antibiotic-Associated Diarrhea (AAD)

Antibiotic-associated diarrhea is defined as "otherwise unexplained diarrhea that occurs in association with the administration of antibiotics" (Varankovich et al., 2015). Antibiotics prescription has been increasing currently worldwide. Their use leads to additional disturbances in the gut flora resulting in a lot of symptoms at the clinical level. For instance, it leads to osmotic diarrhea, caused by suppression of anaerobic bacteria, a decrease in carbohydrate metabolism, disruption of protective effects of commensal bacteria and alleviation of colonic mucosal resistance to pathogenic bacteria, finally resulting in dysbiosis (altered microbiota).

S. boulardii is a well-known kind of probiotic yeast that can mostly relieve antibiotic-associated diarrhea (Surawicz et al., 1989; Kotowska et al., 2005; Szajewska and Kołodziej, 2015).

The causes of AAD can be rotavirus infection in children (Kurugöl and Koturoğlu, 2005), *C. difficile, Candida spp* and *Salmonella spp* infection. Associated to AAD, disturbance caused by allergic and toxic effects of antibiotics on intestinal mucosa has also been registered (Högenauer et al., 1998).

One clinic study conducted single-center, randomized, double-blind, placebo-controlled dose-ranging by Gao et al. (2010) shown that combination of two *Lactobacillus* probiotic strains (*Lactobacillus acidophilus* CL1285® + *Lactobacillus casei LBC80R*® Bio-K + CL1285) was well tolerated and effective for decreasing risk of AAD in hospitalized patients on antibiotics. Furthermore, Probiotic prophylaxis might be based on a dose-ranging effect that was indicated with 100 billion cfu, reaching more successful results and fewer gastrointestinal events compared to 50 billion cfu.

1.7.1.1. Against Rotavirus Infection (in children)

Acute Rotavirus infection is AAD that targets mostly children. Kurugöl and Koturoğlu (2005) suggested in a double-blind placebo-controlled study that *S. boulardii* has a significant effect on the duration of acute diarrhea, and hospital stays in children.

1.7.1.2. Against *Clostridium difficile* Infection (CDI)

Clostridium difficile is a known spore-forming, anaerobe, and gram-positive bacterium. (Khan and Elzouki, 2014; Seekatz and Young, 2014). Its spores are very resistant to severe environmental conditions. This type of infection typically causes antibiotic-associated diarrhea and pseudomembranous colitis (Peniche et al., 2013). The main risk factors of CDI include intensive usage of antibiotics, old age, multiple co-morbid conditions, long stays in hospitals, etc., as summarized in Figure 1.12 (McFarland, 2006; Predrag, 2016).

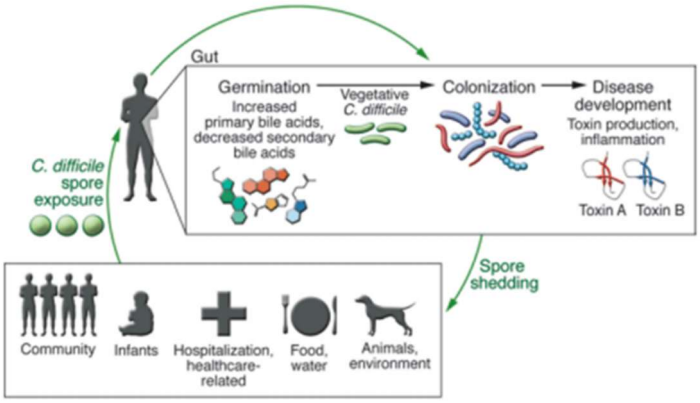


Figure 1.12. Illustration of formation and risk factor of *C. difficile* infection (Source: Seekatz and Young, 2014)

C. difficile produces 2 main toxins, Toxins A (enterotoxin) and B (cytotoxin) (Pothoulakis, 2009). These toxins are responsible for *C. difficile* pathogenesis that leads to increasing Regulatory T cells (Tregs). Toxin release leads to the production of secretory IgA (sIgA), inflammatory cytokines and neutrophils in the gut to maintain homeostasis as summarized in Figure 1.13.

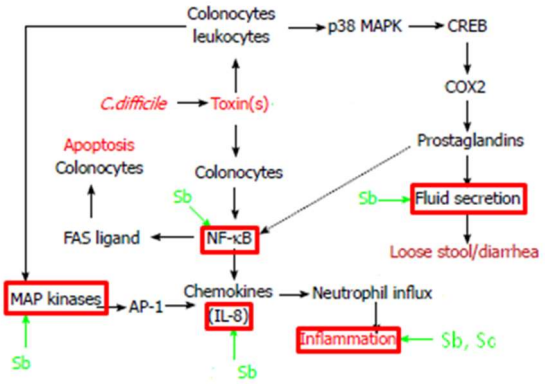


Figure 1.13. The mechanism of Action of *S. boulardii* against the consequences of *C. difficile* infection (Adapted from Fitzpatrick, 2013)

1.7.1.3. Against Diarrhea caused by *Helicobacter pylori*

Helicobacter pylori is a spiral, microaerophilic, gram-negative bacterium with flagella (Kamboj et al., 2017) and the causative agent of gastric and duodenal ulcers, being a risk factor for gastric malignancies. *Helicobacter pylori* use urease to gain accessto epithelial cells by increasing the pH which in turn lowers mucus viscosity

allowing the organism to propel itself through the mucus layer that coats the stomach wall.

Homan and Orel (2015) reported that *S. boulardii* possess neuraminidase activity. This activity by *S. boulardii* removed from the surface α (2-3)- linked sialic acid, the ligand for the sialic acid-binding *H. pylori* adhesion. One randomized clinical study has shown that *S. boulardii* decreases the overall side effect rate, and there is no difference observed in efficacy on the treatment of *H. pylori* infection (Zhu et al., 2017). Supporting these studies, one meta-analysis indicated that *S. boulardii* with triple antibiotic therapy for treatment of *H. pylori*, recovery rate was found for 9 RCT (adult, n = 1708) and 2 RCT (children, n = 330) with supplementation probiotic: 80.0% (95% CI = 77–82), no probiotic: 71.0% (95% CI = 68–74), RR = 1.11, 95% CI = 1.06–1.17 and with supplementation probiotic 87.5%, no probiotic:77.2% RR = 1.13, 95% CI = 1.03–1.25, respectively (Szajewska et al., 2015).

A Randomized, prospective and open study with a mixture of probiotics among 100 subjects (*L. acidophilus* + *B. bifidum*) has been shown that recovery rate of *H. pylori* infection reached the values as treating with probiotic: 83.7% and no probiotics: 64.4 % (Wang and Huang, 2014).

1.7.2. Against Irritable Bowel Syndrome (IBS) & Inflammatory Bowel Disease (IBD)

Irritable bowel syndrome (IBS) is a chronic gastrointestinal problem, associated with chronic abdominal pain and discomfort. Other symptoms of IBS are abdominal distension, bloating and flatulence, straining, and urgency (Distrutti et al., 2016; Spiller et al., 2007).

There is not enough data in clinical studies to confirm the efficacy of *S. boulardii* against this syndrome, but *in vivo* and *in vitro* studies suggest as much (Sivananthan and Petersen, 2018). In a randomized clinical trial study for irritable bowel syndrome, the group treated with *S. boulardii* exhibited higher levels of pro-inflammatory cytokines interleukin-8 (IL-8), tumor necrosis factor-α, anti-inflammatory cytokines IL-10 and IL-10/IL-12 ratio, and significantly lower levels of human blood and tissue cells (Distrutti et al., 2016). *S. boulardii* regenerates lymphocytes such as B lymphocytes, NK cells, and T cells in a model of chronic IBD. This effect is illustrated in Figure 1.14.

Moreover, the production of high levels of NO leads to inflammatory effects in IBD because NO is released through the conversion pathway of L-arginine to NO and L-citrulline. Three isoforms of the nitric oxide synthase (NOS) catalyze these reactions. These are Neuronal NOS, endothelial NOS and inducible NOS (iNOS) (Zanello et al., 2009). One *in vivo* study showed that *S. boulardii* inhibits iNOS activity in the rat castor oil-induced diarrhea model (Girard et al., 2005).

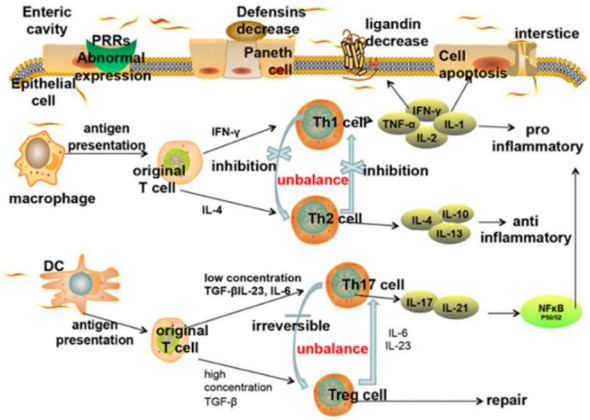


Figure 1.14. The mode of action of probiotic for Inflammatory bowel disease
(Source: Huang and Chen, 2016)

1.7.3. Against Metabolic Diseases: lactose and gluten intolerance and obesity

Recent studies have shown that probiotics play an important role in the regulation of energy homeostasis, metabolic inflammation, lipid metabolism, and glucose metabolism as shown in Figure 1.15 (as reviewed in Moré and Vandenplas 2018). *Sb* probiotic activity against metabolic diseases, such as lactose and gluten intolerance, is thought to be due to the supply of digestive enzymes or induction of their expression by epithelial cells as followed:

Sucrose-Isomaltase (SI), SI displays α-glucosidase activity, hydrolyzing oligomers with (1→6)-α-d- glucosidic linkages including sucrose (Bernasconi et al. 1986). A recent study reported that *Sb* leads to the upregulation of sucrase-isomaltase expression in intestinal cells. *Sb* also produces SIs, encoded by the *IMP1*, *IMP2*, and *IMP5* genes, influencing palatinose metabolism. Amongst these iso-maltases, Imp1 and

Imp2 possess a high affinity to palatinose. Interestingly, unlike *Sc*, *Sb* has no *IMP3* and *IMP4* genes, and in some strains not even *IMP2* (Khatri et al., 2013; Khatri et al., 2017).

Maltase-glucoamylase (MGA), the expression of α-glucosidase, containing 2 domains with differing substrate specificity on maltose/starch and glucose oligomers with α(1→4) bonds, is up-regulated upon exposure to *Sb* (Bernasconi et al., 1986; Zaouche et al., 2000).

Aminopeptidase N (ANP) - known as alanyl aminopeptidase, or neutral brush border aminopeptidase, these enzymes digest peptides generated from hydrolysis of proteins by gastric and pancreatic proteases. The expression of these host proteins is stimulated upon exposure to *Sb* (Khatri et al., 2017; Zaouche et al., 2000; Moré and Vandenplas, 2018).

Lactase-phlorizin hydrolase (LPH) - known as a digestive enzyme, LPH has two domains, one splitting, among others, lactose, cellobiose o-nitrophenyl- β-glucopyranoside, and o-nitrophenyl- β-galactopyranoside, and the other splitting, among others, phlorizin, β-glycopyranosylceramides, and m-nitrophenyl-β-glucopyranoside. The expression of these host proteins is stimulated upon exposure to *S. boulardii* (Bernasconi et al., 1986).

Intestinal Alkaline Phosphatase (IAP) – IAP is a protein expressed by the intestinal epithelium to protect gut homeostasis. IAP dephosphorylates lipopolysaccharides derived from the cell wall of gram-negative bacteria, preventing transmigration of bacteria across the epithelium; it also dephosphorylates other potentially pro-inflammatory ligands.

In addition, its functions are detoxification of bacterial endotoxins, dephosphorylation of Tri- and Di-Phosphorylated nucleotides, regulation of the intestinal microbiome and lipid absorption. When IAP expression is decreased, it leads to increased intestinal inflammation, dysbiosis, bacterial translocation and subsequently systemic inflammation (Moré and Vandenplas, 2018).

IAP plays an important role in the intestine encompasses both protection from systemic infections and chronic inflammatory diseases (Estaki et al., 2014).

Interestingly, *S. boulardii* stimulates intestinal alkaline phosphatase (IAP) expression, offering treatment for gluten intolerance, and obesity (Moré and Vandenplas, 2018).

1.7.4. Against Gluten Intolerance or Celiac Disease

Celiac disease (CD) is a malabsorptive enteropathy, triggered by an inappropriate T cell-mediated immune response to dietary gluten proteins. After ingestion of glutens, transforming into gliadin peptides reach the subepithelial region of the intestinal mucosa.

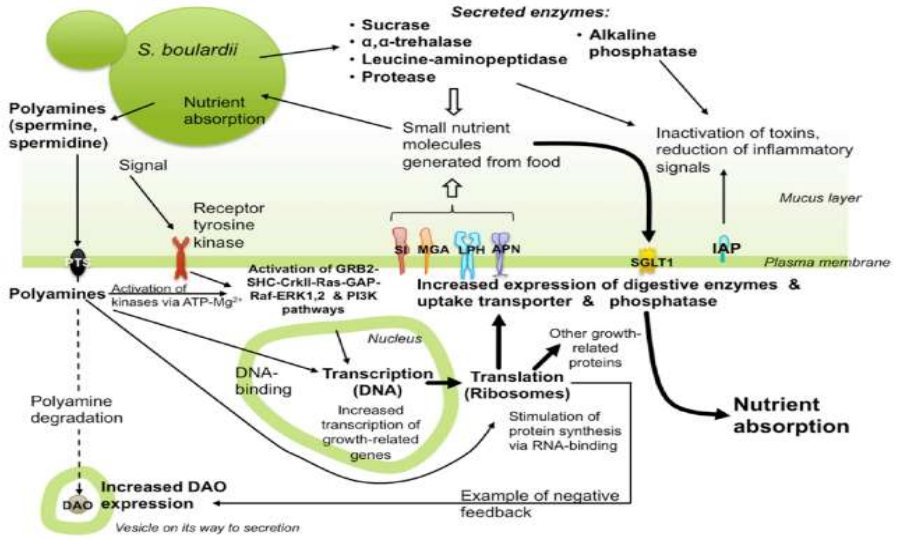


Figure 1.15. Summary of digestive enzymes stimulated or produced by *S. boulardii* (Source: Moré and Vandenplas, 2018)

Glutenins are also involved in T cell response and trigger an inappropriate T cell-mediated immune response, which might result in intestinal mucosal inflammation and extraintestinal manifestations. In addition, Gliadins and glutenins include a high content of proline (15%), hydrophobic amino acids (19%), and glutamine (35%), so they are named prolamins. Because of this glutamine- and proline-rich structure, gluten proteins are resistant to complete digestion by pancreatic and brush border proteases (Caputo et al., 2010). As a result of this, the proline mechanism plays a key role in balancing microbiota.

There are two important pathways for celiac disease: one is the direct impact on the epithelium that includes the innate immune response, the other involves the adaptive immune response involving CD4+ T cells in the lamina propria that recognize processed gluten epitopes. High levels of pro-inflammatory cytokines are produced by activated gliadin-specific CD4+ T cells. Therefore, stimulating a Th1 response results in mucosal remodeling and villous atrophy. When IgA-deficiency is determined in patients, they are

generally asymptomatic, this situation leading to the development of gastrointestinal disorders such as celiac disease (CD) and allergies (Mantis et al., 2011) as shown in Figure 1.16.

Cristofori et al. (2018) reported that *S. boulardii* KK1 strain might be able to hydrolyze the gliadin toxic peptides, and its consumption was followed by improved enteropathy and a decrease of histological damage and pro-inflammatory cytokine production, in a model of gluten sensitivity in BALB/c mice.

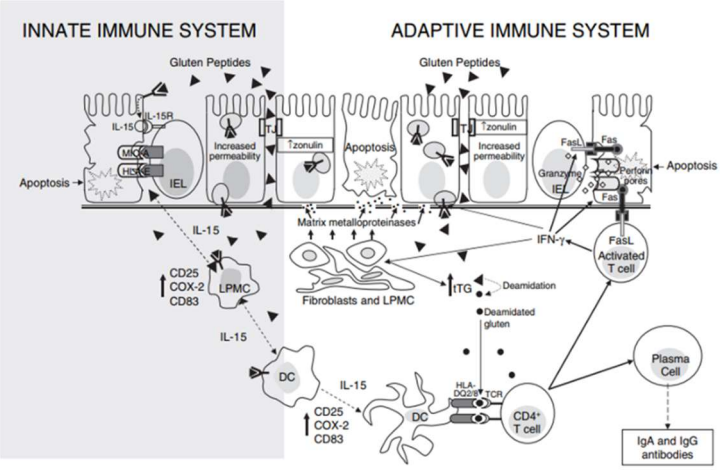


Figure 1.16. Mechanism of celiac disease
(Source: Ciccocioppo et al. 2005)

1.7.5. Against Lactose intolerance or malabsorption

Lactose intolerance or lactose malabsorption is a disorder related to the lack of enzymes required to degrade lactose, including lactases or beta-galactosidases, into glucose and galactose as indicated in Figure 1.17. Within this context, Oak and Jha (2018) reported that *S. boulardii* increases the activity of intestinal enzymes, for example, disaccharidases, α-glucosidases, alkaline phosphatases, and aminopeptidases.

Primary lactase deficiency occurs when the body produces considerably less lactase, and it can only break down smaller amounts of lactose.

Secondary lactase deficiency occurs when less lactase is produced as an indirect consequence of bowel problems or chronic inflammation, such as those associated with gluten intolerance or Crohn's disease (Oak and Jha, 2018).

Bacterial enzymes degrade undigested lactose in the large bowel, leading to osmotic diarrhea. Other symptoms of lactose deficiency are bloating, feeling full, pain and abdominal discomfort, flatulence, and a condition called irritable bowel syndrome (IBS).

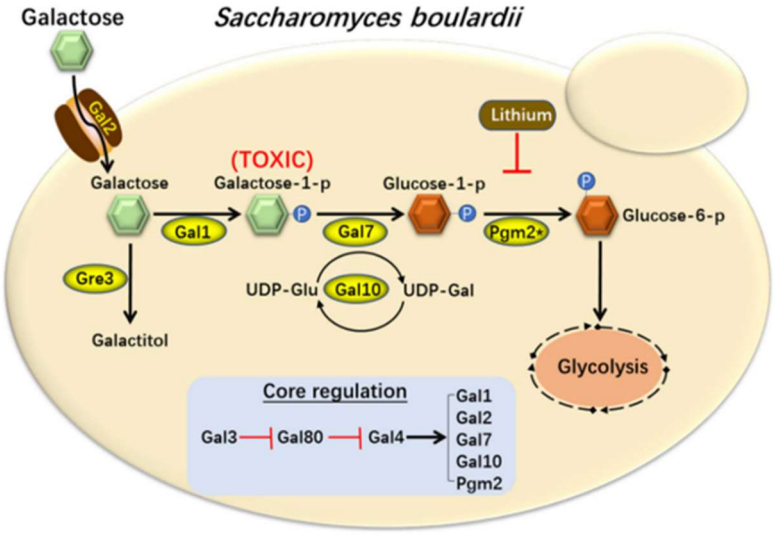


Figure 1.17. Schematic pathway representation of galactose utilization and its related genes in *S. boulardii* (Source: Liu et al.,2018)

S. boulardii rises the intestinal absorption of D-glucose that might enhance the uptake of water and electrolytes during diarrhea (Oak and Jha, 2018).

1.7.6. Against Obesity & Type II diabetes

Obesity is defined as abnormal or excessive fat distribution (Kobyliak et al., 2016). In this metabolic disease, lipid metabolism plays an important role. Interestingly, an *in vivo* study showed that when *S. boulardii* was administered daily by oral gavage in obese and type II diabetic mice during 4 weeks, mice displayed decreased body weight gain and fat mass. It also decreased the hepatic steatosis or fatty liver, which means buildup fat in the liver, and total liver lipids content, systemic inflammation and plasma cytokine concentrations of IL-6, IL-4, IL-1β, and TNF-α. in obese mice (Everard et al., 2014). According to this study, *S. boulardii* might be a favourable co-adjuvant to treat obesity and Type II diabetes.

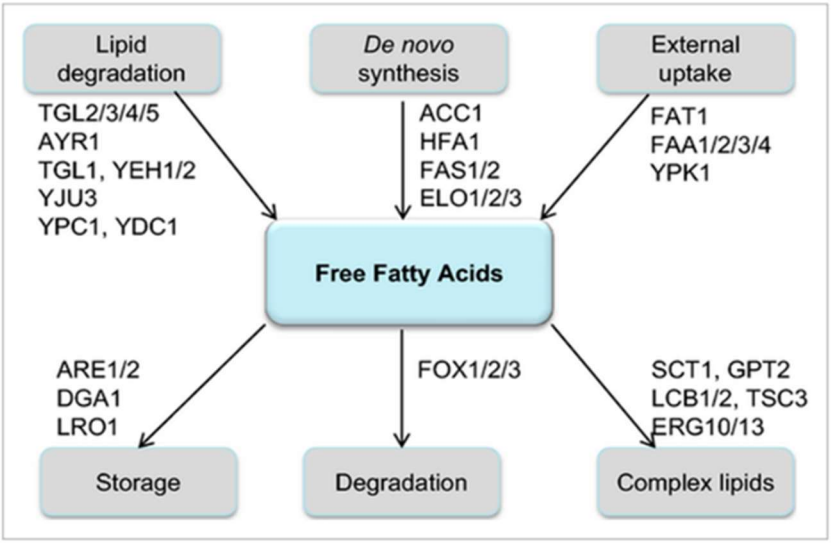


Figure 1.18. Fatty acid metabolism in *S. cerevisiae*
(Source: Klug, 2014)

Fatty acids are building blocks for the synthesis of membrane lipids (phospholipids, sphingolipids) and storage lipids (triacylglycerols, steryl esters). Moreover, phospholipids, sterols, and sphingolipids are essential components of cellular membranes. Intestinal short-chain fatty acids (SCFAs) and their link provide healthy microbiota in the human body. Thus, secreting the SCFAs by *S. boulardii*, is believed to be linked to obesity diseases. Fatty acid metabolism-related genes in yeast *S. cerevisiae* as indicated in Figure 1.18.

1.7.7. Against Cancer Diseases

Cancer is defined as a progressive aggregation of mutations in genetic material of the cell. It leads to uncontrolled proliferation of cells, insensibility of growth factors, and capacity to infect surrounding tissues observed in most cancer patient. Probiotics have many positive effects on health for cancer prevention and tumor suppression. Several studies have been reported that probiotics have antiproliferative or proapoptotic activities on a wide range of cancer cells such as colon, stomach, breast, cervix, and myeloid leukaemia cells. Due to the fact that probiotics are often used as an adjuvant during anticancer chemotherapy and radiotherapy treatments (Nazir et al., 2018).

Several mechanism of probiotics in cancer prevention and treatment are reported following by; modulation of gut microbiota, enhancement of gut barrier functions,

degradation of potential carcinogens and protection effect of DNA damage of intestinal epithelium, and enhancement of immune and inflammatory system in the body (Nazir et al., 2018).

2-dimethylhydrazine (DMH) and N-nitrosodimethylamine (NDMA) are known as carcinogens substances. They can lead to changes in the DNA sequence which may cause tumorigenesis. Thus, probiotics may degrate these carcinogens (Nazir et al., 2018).

Akazad et al. (2002) showed that lactic acid bacteria decreased the risk of bladder cancer when 200 g of yogurt was regulary consumed for 10 weeks among 180 patient and 445 population-based controls.

One *in vivo* study of breast cancer case indicated that when *L. acidophilus* and cyclophosphamide given orally to the mouse, tumor development and their effects on the immune system were observed. In addition, IFN-γ, IL-4, TGF-β and lymphocytes are increased and the amount of tumor size is decreased (Maroof and Hassan 2012).

As a result, the potential mechanisms of probiotics in preventing, treating, and reducing the progression of cancer are still poorly understood and need to be further elucidated of exact mechanism by which local actions of probiotics affect the systemic immune responses against transformed cells.

1.8. The importance of molecular basis of probiotics: A case study of probiotic, *S. boulardii* and compared to non-probiotic strain, *S.cerevisiae*

S. boulardii survives transit through the GI tract both *in vitro* and *in vivo* and inhibits the growth of a number of microbial pathogens. Indeed, *S. boulardii* can live longer in the gut than *S. cerevisiae* (Łukaszewicz, 2012; Liu et al., 2016). In this context, it is interesting to observe that while *S. cerevisiae* strains grow and metabolize at an optimal temperature of 30ºC, *S. boulardii* grows optimally at human body temperature, 37º C. Additionally, *S. boulardii* grows more rapidly than *S. cerevisiae* and is more tolerant to low pH and bile acids. Possibly due to these characteristics, *S. boulardii* has been shown to be more resistant than *S. cerevisiae* to gastric conditions (Fietto et al., 2004). The gastric environment has extremely low pH which is generally ~2.0. At this pH, *S. boulardii* proteins continue to be positively charged, thus remaining able to

establish electrostatic interactions with negatively charged components of the cell wall of gut bacteria, a requirement for its probiotic activity (Urdaci, 2008).

There are main discriminatory metabolites between *Sb* and *Sc* which are trehalose, myo-inositol, lactic acid, fumaric acid and glycerol 3-phosphate (Łukaszewicz, 2012).

Mackenzie et al. (2008) determined that non-medical *Sc* strains have the capability of producing lactic acid, valine, fumaric acid, malic acid, glycerol-3-phosphate and TCA cycle intermediates such as fumaric, citric, isocitric, succinic and malic acids. On the other hand, 4-Hydroxyphenylethanol related to tyrosine metabolism, 2,3,4-Trihydroxybutanal, Pentonic acid 1,4-lactone, myo-inositol are synthesized by *Sb* (Mackenzie et al., 2008).

Despite the observed phenotypic differences, a study focused on the analysis of the genome sequences of five *Sb* strains used commercially as probiotics, has shown that the genome of *Sb* is 99% similar to that of *Sc* (Edwards-Ingram et al., 2007; Khatri et al., 2017). The surprising observation that *Sc* and *Sb* are very similar in terms of their genomic sequence, raises the question of what are the features that make *Sb* a probiotic, while *Sc* is not.

1.8.1. Mode of Action for *S. boulardii* as Probiotic

The possible mechanisms of probiotic activity in intestinal inflammatory diseases for therapeutic agents include the following properties (Figure 1.19);

- Antagonism against enteric pathogens (antimicrobial effect, toxin deactivation, etc)
- Enhancement of the gut mucosal barrier (digestibility, nutritional value, secreting of SCFAs, etc)
- Inhibition or enhancing of local secretion of inflammatory mediators (anti-inflammatory and pro-inflammatory)
- Stabilization of local immunological activity (Immunomodulation effect, etc)
- Quorum sensing (Adhesion, aggregation, biofilm formation, etc)

The mode of action of *S. boulardii* can be mainly categorized as having luminal action, trophic action and mucosal action (McFarland, 2010).

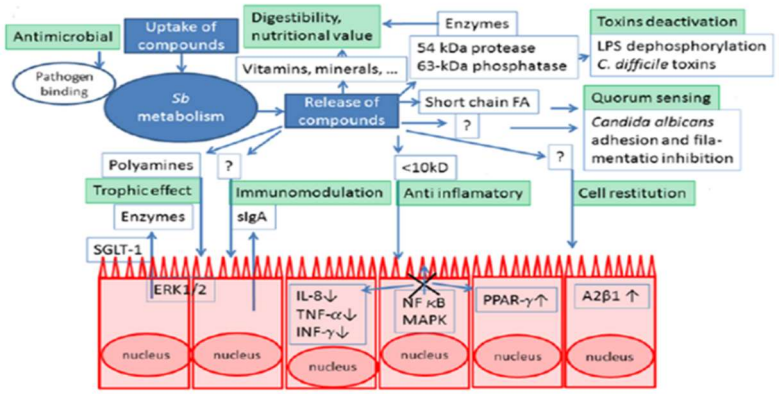


Figure 1.19. Schematic representation of the possible mode of action for *S. boulardii* (Adapted from Łukaszewicz, 2012)

1.8.1.1. Luminal Action

The intestinal epithelial cells are generally classified as enterocytes, paneth cells and goblet cells as shown in Figure 1.20. The main functions of the epithelium are to form a selective barrier in the intestine walls and to support nutrient and water transport while protecting from microbial contamination of the interstitial tissues.

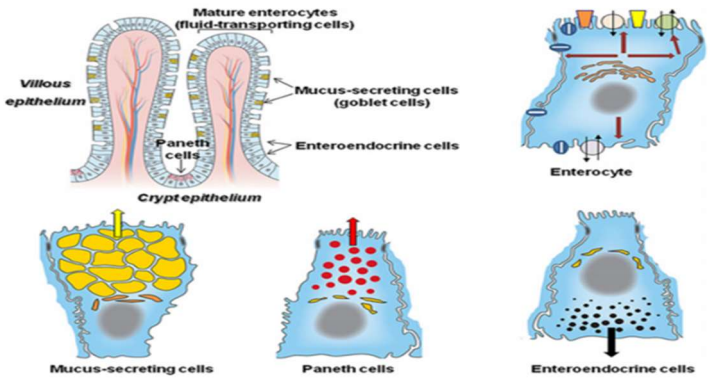


Figure 1. 20. Schematic representation of the intestinal epithelium barrier (Source: Moal and Servin, 2013)

Disruption of the intestinal epithelial barrier associated with intestinal diseases is often caused by the deterioration of normal regulatory mechanisms that control gene expression, tight junction structure, and cytoskeletal signaling. Disruption of epithelial barrier integrity gives rise to several gastrointestinal diseases, including infection by pathogens, obesity and diabetes, necrotizing enterocolitis, irritable bowel syndrome and

inflammatory bowel disease (Bron et al., 2017). In addition to these, it might give rise to allergic diseases (Muñoz-quezada and Gil, 2012).

***Sb* preserves tight junction functions:** The tight junction is a component of the apical junctional complex that seals the paracellular space between epithelial cells. It is composed of transmembrane proteins, cytoplasmic adaptors, and the actin cytoskeleton. *Sb* maintains tight junction by exerting a multifactorial effect which includes the inhibition of pro-inflammatory cytokines, such as IL-8, and preventing the activation of MAP kinases Erk1/2 and JNK/SAPK (Wang et al., 2004). Furthermore, Bioactive factors released by *Sb* trigger activation of various cell signaling pathways that give rise to the strengthening of tight junctions and the barrier function. Related genes include *STE11*, *STE7*, *FUS3*, *KSS1*, *SSK2/22*, *PBS2*, *HOG1*, *BCK1*, and *SMK1*.

1.8.1.2. Mucosal Action

The mucus layer includes an inner and an outer layer. Inner mucus protects the apical epithelium whereas the outer mucus layer includes a large number of bacteria.

SCFA production: Short chain fatty acids (SCFAs) are known as volatile fatty acids produced by the gut microbiota in the large bowel as bacterial fermentation products from food components that are unabsorbed/undigested in the small intestine (Ríos-covián et al., 2016).

SCFA production in the colon is dependent on how rapidly carbohydrates are fermented. They are related to colonic absorption of water and electrolytes which effects the controlling of AAD. *Sb* enhances the normal level of SCFAs, producing acetic acid (C2), propionic acid (C3) and butyric acid (C4), important metabolites produced by the anaerobic flora, representing 90–95% of the SCFA present in the colon. Acetic acid is found mostly in the colon and makes up more than half of the total SCFA detected in feces.

Butyrate is the main energy source for intestinal epithelial cells (Figure 1.21). It influences epithelial cell proliferation, cell differentiation, mucus secretion, and barrier function in the large intestine. It has anti-inflammatory and antioxidant potential (Patel and Dupont, 2015). In addition, it inhibits NfkB activation (Distrutti et al., 2016).

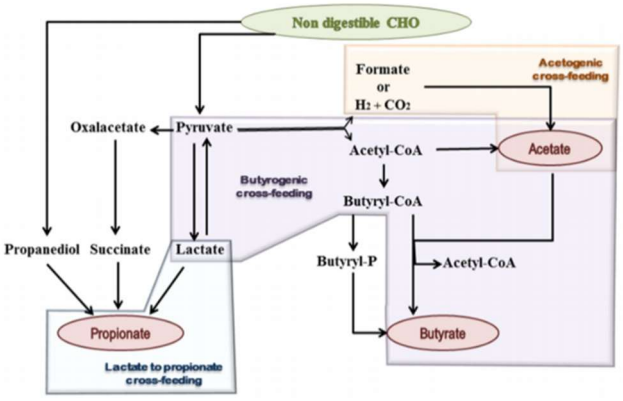


Figure 1.21. Schematic representation of SCFA formation microbial metabolic pathways in the human gut (Source: Ríos-covián et al., 2016)

Butyrate decreases bacterial translocation and improves the organization of tight junctions. It induces the production of mucin, a glycoprotein preserving the integrity of the intestinal epithelium.

Epithelial goblet cells secrete mucins. Mucins can be found bound to the brush border membrane or packaged within large intracellular vesicles, as shown in Figure 1.20 as yellow vesicles that upon exocytosis into the luminal compartment form a thick mucus layer overlying the epithelium. Butyrate specifically regulates MUC gene expression in intestinal epithelial goblet cells deprived of glucose. Recent studies have demonstrated that mucin secretion is promoted by SCFA produced during carbohydrate fermentation. Hence, the metabolism of butyrate in colonocytes is closely linked to some of its gene-regulating effects (Gaudier et al., 2004).

Interestingly, *Sb* has been indicated to induce the secretion of mucins and defensins from the host (Vandenplas et al., 2009). A clinical study also has shown that increase fecal SCFA concentrations especially butyrate concentration in patients. They hypothesized that increase of fecal SCFA concentrations especially butyrate, *Sb* may have preventive the effects of treating on TEN-induced diarrhea (Schneider et al., 2005). In addition, fatty acids or their monoglyceride derivatives have long been known as antimicrobial agents that kill Gram-positive and Gram-negative bacteria. They also show antiviral and antifungal activity.

Prevention of microbial pathogen adherence: Recent studies have suggested that *Sb* has a protective effect against *Escherichia coli*, *Vibrio cholerae*, *Salmonella sp*., *Candida albicans*, *C. difficile* and *H. pylori* infections (Khatri et al., 2017; Kareem et al., 2018). Two of the ways in which *Sb* is predicted to exert its anti-bacterial activity is

through the production of adhesion proteins and through the inhibition of pro-inflammatory cytokine production (Figure 1.22).

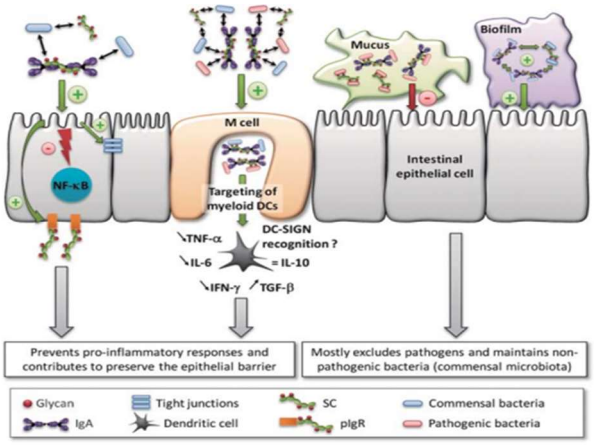


Figure 1.22. Bacterial adhesion and biofilm formation
(Source: Mantis et al., 2011)

Adhesion & Flocculation : Adhesion to intestinal mucosa plays a key role for colonization and is important for the interaction between *Sb* and the host, modulation of the immune system and antagonism against pathogens (Muñoz-quezada and Gil, 2012). *Sb* acts by producing adhesion proteins, such as flocculins, that favor the adhesion of *Sb* cells to pathogenic bacteria cells, thus inhibiting their interaction with intestinal receptors and subsequent host invasion (Khatri et al., 2017; Tiago et al., 2012). Flocculation genes in *Sb* include *FLO1*, *FLO5*, *FLO8*, *FLO9*, *FLO10*, *FLO11*, *FIG2*, *EFG1* and *AGA1* (Khatri et al., 2017).

Antimicrobial effects of 63-kDa Phosphatase LPS: *EHEC* infection leads to inflammation and disruption of the epithelial barrier. Dahan et al. (2003) showed that EHEC infection induced TNF-α synthesis that is implicated in apoptosis of T84 cells. *Sb* was found to stimulate a decrease in TNF-α and related apoptosis in *EHEC*-infected T84 cells. It blocks nuclear factor (NF)-κB activation, IL-8 gene expression, IL-8 production, TNF-α gene expression and secretion by lymphoid and non-lymphoid cells. This effect has been linked to the activity of a 63-kDa *Sb* protein phosphatase, that inhibits the toxicity of *E. coli* surface endotoxins (Buts et al., 2006). This phosphatase may be encoded by the *PHO8*, *PRP3*, *JIP4*, *SNF1*, *SNM1*, *PEX29*, *CWC21*, *VPS52*, *VPS72*, *VP60*, *RIB3* or *PAC11* genes (Khatri et al., 2017). *Sb* displays high dephosphorylation activity (Buts and Keyser, 2006) and was observed to decrease the inflammatory profile of LPS-activated dendritic cells and to block T-cell proliferation (Thomas et al., 2009)

Moreover, *Sb* CNCM I-745 was found to secrete an alkaline phosphatase, with the capability of inactivating *E. coli* lipopolysaccharide by dephosphorylation (Moré and Vandenplas, 2018).

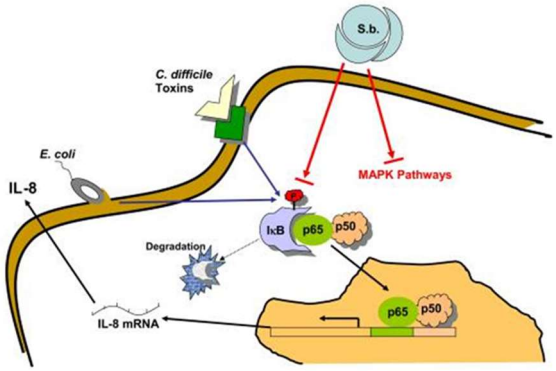


Figure 1.23. The mode of action *Sb* according to bacterial infections (Source: Pothoulakis, 2009)

Antimicrobial effects of 54 kDa Serine Protease: *S. boulardii* expresses a 54-kDa serine protease that exhibits the ability to degrade *C. difficile* toxin A (Figure 1.23). One study showed that *Sb* significantly decreases the liquid secretion and permeability caused by toxin A in rat ileum. Secreted serine protease by *Sb* decreases the ability of toxins A and B to bind human brush border membrane and inhibits the pathogenic impact of both toxins on colonic epithelial cells (Czerucka and Rampal, 2002; Vandenplas et al., 2009). *Sb* might additionally behave in the intestinal lumen by blocking the toxin receptor (Pontier-bres et al., 2014; Graff et al., 2008).

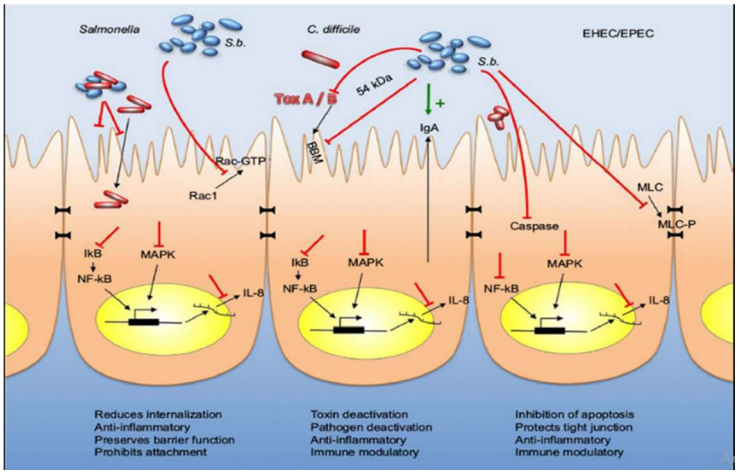


Figure 1.24. Mode of Action of *Sb* against *E. coli*, *C. difficile* and *Salmonella* infections (Source: Stier, 2016)

Antimicrobial effects of 120-kDa protein: *In vivo* studies show that *Sb* administration leads to inhibition of *Vibrio cholerae* or *Enterotoxic Escherichia coli*

(ETEC) pathogenesis. This effect was linked to diminished sodium and water secretion in intestinal loops, as well as decreased *V. cholerae* toxin-induced cAMP levels in rat intestinal cells (Kindenplas, 1999; Khatri et al., 2017; Pontier-bres et al., 2015). This protective effect was found to be related to a 120 kDa protein (Czerucka et al., 1994) (Figure 1.24). 120 kDa secreted proteins in *Sb* are encoded by *KIN1*, *MAD1*, *TFC4*, *VAS1*, *KAP120*, *PIK1*, *NMD5*, *JSN1*, *PUF2*, *RGC1*, *ENA5*, *KCS1*, *SEG2*, *NUP120* and *MSH3* genes (Khatri et al., 2013).

Toxin-related signalling: cAMP is known as a potent second messenger that is associated with various stimulatory processes in many cell types. *Cholera* toxin is an enterotoxin that stimulates cells by increasing the synthesis cAMP. Vasoactive intestinal polypeptide (VIP) and prostaglandins (PGE2), mediators in cholera pathogenesis, recognize receptors coupled to adenylate cyclase. These mediators induce Cl- secretion by a cAMP-dependent signal transduction pathway.

Apparently, *Sb* may interfere with the adenylate cyclase-cAMP transduction pathway and Cl- secretion. In addition, Czerucka and Rampal (1999) determined that *Sb*, through the activity of a 120 kDa protein, exerts inhibitory influence on *Cholera*-induced cAMP concentration an 125I- efflux in T84 monolayers, inhibits receptor-mediated and non receptor-mediated cAMP-induced secretion, reduces 125I- efflux but not 1,4,5-triphosphate in carbachol-treated cells, and stimulates 1,4,5-triphosphate synthesis in T84 cells.

Sb decreases the activation of Erk1/2 mitogen-activated protein kinase and, consequently, IL-8 secretion induced by *C. difficile* toxin A. *Sb* further reverses the drop in intestinal permeability after exposure to *C. difficile* toxins A and B (Pontier-bres et al., 2015).

An animal study showed that *Sb* decreases the levels of inflammatory cytokines and activates mitogen-activated protein kinases (p38, JNK, and ERK1/2), phospho-IkB, p65-RelA, phospho-jun and c-fos in the colon, belonging to signaling pathways involved in the activation of inflammation stimulated by *Salmonella typhimurium* (ST). When *ST* binds to *Sb*, this action reduces *ST* translocation which results in diminished activation of inflammation-associated and signaling pathways, leading to reduced intestinal inflammation in a murine model of typhoid fever (Martins et al., 2013).

Immunomodulation effect of increased secretion of immunoglobulin SIgA levels: Lamina propria is defined as the connective tissue that underlies the epithelium of the mucosa and contains various myeloid and lymphoid cells, including macrophages, dendritic cells, T cells, and B cells. Dendritic cells (DC) are involved in the control of T cell activation, by inducing the activation of naive T cells (Thomas et al., 2009).

Recent studies have reported that when *Sb* attaches to dendritic cells (DCs) and increases the secretion of immunoglobulins A and M and of cytokines, including interleukin (IL)1β, IL-12, IL-6, TNF-α and IL-10 (Stier, 2016).

Secretory IgA (SIgA) is the first line of defense against enteric toxins and pathogenic microorganisms (Mantis et al., 2011). *Sb* exposure leads to the increased intestinal secretion of immunoglobulins, including immunoglobulin A (IgA), leading to improved defence against pathogens.

But and colleagues observed on rat small intestine that *Sb* dramatically enhances the secretion of immunoglobulin A (IgA) (Buts et al., 1990). When *Sb* leads to increased IgA secretion, it enhances the immunologic barrier produced by the intestinal mucosa (Peniche et al., 2013; Seekatz and Young, 2014).

1.8.1.3. Trophic Action

When *Sb* is orally administrated, it upgrades intestinal functions by three important mechanisms which are:

•Endoluminal secretion of substantial amounts of spermine and spermidine which after absorption increase the intracellular pool of polyamines and the synthesis of brush border membrane (BBM) glycoproteins, enzymes, and carriers;

•Endoluminal secretion of enzymes by the yeast cells itself;

•Activation of messengers which transduce mitogenic and metabolic signals from the apical membrane to the nucleus using the (di) phosphorylation of intracellular serine, threonine and tyrosine kinases.

Enzymatic activity: *Sb* secretes, during its intestinal transit, enzymes such as lactase, maltase α-glucosidase, sucrase-isomaltase, maltase-glucoamylase, α,α trehalase, a zinc-metalloprotease acting as a leucine aminopeptidase and alkaline phosphatase able to dephosphorylate bacterial endotoxins (Border et al., 2010).

Sb stimulates the expression of disaccharidases, sucrases, production of glycoproteins in the microvilli. One *in vivo* study showed that *Sb*, when administered orally to rats, upgrades endoluminal N-terminal hydrolysis of oligopeptides allowing aminopeptidase to move within the lumen. This action could be important on inhibiting reactions to food antigens, while mucosal permeability is enhanced (Buts et al., 2002).

Polyamines secreted by *Sb* also induce protein synthesis via RNA binding and stabilization, resulting in an increase in growth-related and differentiation-related proteins, including digestive enzymes such as lactase, maltase, sucrase, which will be inserted into the brush border membrane (Vandenplas et al., 2009; Moré and Vandenplas, 2018). Polyamines further protect lipids from oxidation and increase the activity of short-chain fatty acids (SCFA) (Kareem et al., 2018).

1.9. Aim of the Study

The main objective of this study is to develop a new comprehensive guide that includes; *in vitro, in vivo*, clinical research and meta-analysis data on probiotics to be used by pharmacists, hospitals and pharmaceutical industry; also for well-being purposes for consumers.

Besides, goals of this study include dissemination of knowledge for consumer awareness together with collection of scientific evidence based on the probiotic products. Within this context, such a database may rapidly provide answers, such that, which probiotic product could be used for which illness in specific.

To support this hypothesis, conducting meta-analyses with *S. boulardii*, a well-documented probiotic, was aimed.

In addition, a case study conducted in Tecnico Lisboa was also designed on the onset of this thesis. In this part of the study, a *S. boulardii* probiotic strain was selected to determine the difference in probiotic activity observed in *S. boulardii* compared with *S. cerevisiae*. To evaluate that in a systematic way, an analysis of transcription regulation in *S. boulardii* is required, which prompted us to start building the ProBioYeastract database and develop additional strain-comparison tools.

Table 1.2. Genes predicted to underlie *Sb* mode of action as a probiotic agent.

***Sb* probiotic effect**	**Related disease**	**Related Pathways**	***Sb* mode of action**	**Genes underlying *Sb* mode of action**	**Reference**
Anti-toxin effect	Diarrhea and colitis caused by *C.difficile* infection	x	Production of a 54 kDa serine protease, protecting against toxin A and B	(Serine protease) *PRC1, GLN3 GAT3, RRT12 YSP3*	(Khatri et al., 2017, Khatri et al., 2013 ; Pothoulakis, 1993; Castagliuolo et al., 1996;)
Anti-toxin effect	Diarrhea caused by *E. coli*	NF-κB and Mitogen-Activated Protein Kinase (MAPK) signaling pathways	Secretion of a 63 kDa protein phosphatase which inhibits *E. coli* toxins	(Phosphatase) *PHO8, PRP3 JIP4, SNF1 SNM1, PEX29 CWC21, VPS52 VPS72, RIB3 PAC11*	(Khatri et al., 2017; Khatri et al., 2013)
Anti-toxin effect	Diarrhea caused by *Vibrio cholera*	cAMP pathway	Secretion of a 120 kDa protein that neutralizes cholera toxin by decreasing cAMP levels in the intestinal cells	*KIN1, MAD1 TFC4,VAS1 KAP120, PIK1 NMD5, JSN1 PUF2, RGC1 ENA5, KCS1 SEG2,NUP120 MSH3*	(Khatri et al., 2013)
Anti-microbial effect	Irritable Bowel Diseases (IBD)	Butyrate metabolism	SCFA production, especially butyrate	*ACC1, HFA1*	(Klug, 2014)
Anti-microbial effect	AAD related to pathogen infection	Protein secretory pathway	Increased adhesion protein production	*FLO5*, *FLO8 FLO9*, *FLO10*	(Khatri et al., 2017)

(cont. on next page)

Table 1.2. (cont.)

				FLO11,FIG2 EFG1, AGA2 (SAG1)	
Trophic Effect	Lactose intolerance	Galactose metabolism & Leloir pathway	Lactase overexpression for degrading lactose	*MIG1, PGM1 GAL7, GAL10 GAL1, CYC8 GAL2, GAL4 GAL80, PGM2 GAL3, TUP1*	(Khatri et al., 2017)
Trophic Effect	Obesity Type 2 diabetes	Phosphatidate biosynthesis I (dihydroxyacetone pathway)	Activation of lipid degradation in dendritic (DCs) cell	*TGL2/3/4/5 AYR1, TGL1 YJU3,YPC1 YDC1*	(Klug, 2014)
Prevention of tight junction distribution	Irritable bowel syndrome (IBS),Gluten intolerance, gastroenteritis, and *H. pylori* infections	MAP kinases Erk1/2 and JNK/SAPK.	Inhibition of MAP kinases Erk1/2 and JNK/SAPK	*STE11,STE7 FUS3, KSS1 SSK2, PBS2 HOG1,BCK1 SMK1*	(Schaeffer and Weber ,1999)
Increased immune defense in the gut	Allergic diseases	Arginine and proline metabolism	Polyamines secretion	*SPE2,SPE3 CAR1, CAR2 PUT2, PUT1 PRO1, PRO2 PRO3*	(Khatri et al., 2013)
Immunomodulation effect	Gluten intolerance & Celiac Disease	Palatinose metabolism	Increased production of immunoglobulin IgA by epithelial cells Upregulation of palatinose uptake and metabolism	(Isomaltase) *IMA1*	(Khatri et al., 2017)

CHAPTER 2

METHODOLOGY

2.1. Study Design of Probiotic Research

Many factors may affect the efficacy of probiotics in prophylaxis, therefore, this study was mainly conducted as extracted information from *in vitro*, *in vivo* and clinical trials and in providing as much information as possible about them. Published studies mainly have reported characteristics of the probiotic (e.g strain, dose, and duration of treatment), of the antibiotic (e.g type of antibiotic, duration of the therapy) of the patients (e.g age group like adult or children). This study was preliminary designed about these factors shown in Figure 2.1.

Figure 2.1. The overall design of the probiotic research diagram

In addition, outcomes were important for this study so as to correctly evaluate the probiotic efficacy for different diagnosis mainly based on probiotic strain type. For this reason, results from different *in vitro*, *in vivo* and clinical trials were assessed. At the same time, adverse effects and safety consideration of probiotics will be integrated most important databases for regulation (e.g FDA, EFSA, FOSHU, etc) when transforming into bioinformatics online platform. By forming *in vitro, in vivo* and clinical studies tables

based on different kinds of probiotics (e.g. *S. boulardii*, *B. clausii*), activity and effectivity scale were performed according to the efficacy of probiotics on diseases.

Overall, this study will be preliminary study design sections to form bioinformatics online database.

2.1.1. Literature Survey

The current literature about probiotics has been reviewed and a search has been conducted the following databases: Pubmed, Medline, ScienceDirect, Google Scholar, etc.

The search terms used were: probiotic or probiotics, prebiotics, symbiotics, *S. boulardii*, *Bacillus clausii*, *Bacillus, Lactobacillus*, *Bifidobacterium*, *Bifidobacterium infantis*, Irritable Bowel Syndrome or IBS, Inflammatory Bowel Disease or IBD, diarrhea or antibiotic associated-diarrhea or diarrhea caused by pathogens such as *H. pylori*, *C. difficile* etc, obesity, lactose intolerance, gluten intolerance or celiac disease, mode of action of probiotics etc. These papers were examined and their abstracts were reviewed for relevance. Moreover, all related references in these papers were checked to make sure all potentially relevant papers were retrieved in this study.

2.1.2. Market Research

Uncontrolled use of probiotics has become an interesting way popular in Turkey. Hence, market research in Turkey (e.g. Reflor produced by Biocodex, Enterogermina manufactured by Sanofi, etc.) about the commonly used probiotic products were collected related papers, labels or internet search for their products.

2.2. Data Integration

Data integration will be aimed to construct on bioinformatic online platform as following by;

Taxonomy of Strains (NCBI Taxonomy Database)

https://www.ncbi.nlm.nih.gov/taxonomy/

For Genetics (NCBI Genomic Database, Yeastract Database, etc)

https://www.ncbi.nlm.nih.gov/genome

http://www.yeastract.com/

For Toxicology of strains (Toxicology Database)

https://toxnet.nlm.nih.gov/

For Probiotics safety, specifications and regulations directed to follow these websites ;

In Europe (EFSA, European Food Safety Authority)

https://www.efsa.europa.eu/en/search/site/probiotic

In the USA (FDA, Food and Drug Administration)

https://www.fda.gov/Food/DietarySupplements/

In Japan (FOSHU, Food for Specified Health Uses)

http://www.mhlw.go.jp/english/topics/foodsafety/fhc/02.html

In Canada (CFIA, Canadian Food Inspection Agency)

http://www.inspection.gc.ca

In China (CFDA, China Food and Drug Administration)

http://eng.sfda.gov.cn/WS03/CL0758/

In Australia (TGA, Therapeutic Good Administration)

https://search.tga.gov.au

2.3. Data Collection and Process

One of the most important steps of this study was data collection from *in vitro*, *in vivo*, clinical studies. Collected data was used to form data tables and perform a meta-analysis.

2.4. Meta-analysis of *S. boulardii* in children and adults

As meta-analysis is known a statistical integration of evidence from multiple studies that address common research questions. They are used to be guide clinical practice, and health policy for consumers. Moreover, they provide better understand the

findings of clinical studies. In this regard, meta-analyses were performed by Rstudio programming using the function of metafor that based on a random effect model. In this section, clinical studies data (placebo control, *Sb*- and administrated probiotic, *Sb*+) was collected from literature separately related to *S. boulardii* in adults (Table 2.1) and children (Table 2.2)

Forest, Funnel (publication bias), and Baujat plot were performed by developing Rstudio codes (Appendix A). Afterwards, values of heterogeneity, p values, and the sample size were calculated based on control and probiotic group.

Table 2.1. Clinical data for probiotic and control groups in adults

			Probiotic Group		**Control Group**	
Study_id	**Authors and Year**	**Sample Size**	**Event**	**Total**	**Event**	**Total**
1	Adam et al. 1977	388	9	199	33	189
2	Monteiro et al. 1981	240	19	121	33	119
3	Surawicz et al. 1989	180	11	116	14	64
4	McFarland et al. 1994	193	7	97	14	96
5	Lewis et al. 1998	69	7	33	5	36
6	Surawicz et al. 2000	82	3	32	7	50
7	Cremonini et al. 2002	43	1	22	6	21
8	Duman et al. 2005	384	14	204	28	180
9	Can et al. 2006	151	1	73	7	78
10	Cindoruk et al. 2007	124	9	62	19	62
11	Bravo et al. 2008	86	3	41	5	45
12	Song et al. 2010	631	11	330	20	331
13	Chu et al. 2012	100	3	50	8	50
14	Pozzoni et al. 2012	185	16	141	13	134
15	Zojaji et al. 2013	160	11	80	24	80
16	Kyriakos et al. 2013	70	1	36	7	34

Table 2.2. Clinical data for probiotic and control group in children

			Probiotic Group		Control Group	
Study_id	**Authors and Year**	**Sample Size**	**Event**	**Total**	**Event**	**Total**
1	Cetina-Sauri 1994	130	19	65	52	65
2	Hafeez et al. 2002	101	32	51	44	50
3	Erdeve, et al. 2004	466	14	244	42	222
4	Kotowska et al. 2004	269	4	132	22	137
5	Kotowska et al.2005	246	9	119	29	127
6	Kurugöl and Koturoğlu 2005	200	20	100	50	100
7	Billoo et al. 2006	100	8	50	10	50
8	Vandenplas et al, 2007	188	13	93	25	95
9	Villarruel et al. 2007	88	22	44	30	44
10	Htwe et al. 2008	100	38	50	12	50
11	Eren et al. 2010	55	15	28	21	27
12	Corrêa et al. 2011	176	29	90	51	86
13	Riaz et al. 2012	90	9	43	22	47
14	Shan et al. 2012	333	6	167	18	166
15	Casem et al. 2013	140	11	69	16	71
16	Zahoo et al. 2014	240	27	120	47	120
17	Bin et al. 2015	205	12	105	26	100

2.5. A Case study of *S.boulardii*

This case study was conducted in Tecnico Lisboa (see Appendix B). After *in vitro*, *in vivo*, clinical literature review and performing a meta-analysis of *S. boulardii* in

children and adults, *S. boulardii* is one of the well-studied probiotic strain was selected to examine on the molecular basis according to Cross-strain promoter analysis.

2.5.1. Cross-strain promoter analysis: *Sc* vs *Sb*

In the beginning of the cross-strain promoter analysis, the data used for the construction of ProBioYeastract considered the following assemblies provided by GenBank for Unique 28 and Biocodex Using scripts, these assemblies were parsed and the data was loaded to the ProBioYeastract database. The information of orthology between *Sc* and *Sb* was provided by the annotation already in the assembly, meaning that the ones submitting the genome, did a functional analysis, and for each gene, they obtained the best hit against *Sc* genes and annotated these as being orthologous genes. (https://www.ncbi.nlm.nih.gov/genome/16045?genome_assembly_id=256035) (https://www.ncbi.nlm.nih.gov/genome/16045?genome_assembly_id=256034)
Afterwards, *S. boulardii* Unique28 and Biocodex gene promoters were retrieved from the ProBioYeastract database and *S. cerevisiae* gene promoters were retrieved from the YEASTRACT database. (http://146.193.39.124/ ProBioYeastract/sboulardii/index.php)
After that, the existence of *S. cerevisiae* putative transcription factor binding sites in the promoters of the three strains were compared by using the YEASTRACT database (http://www.yeastract.com/) query "Find TF binding site".

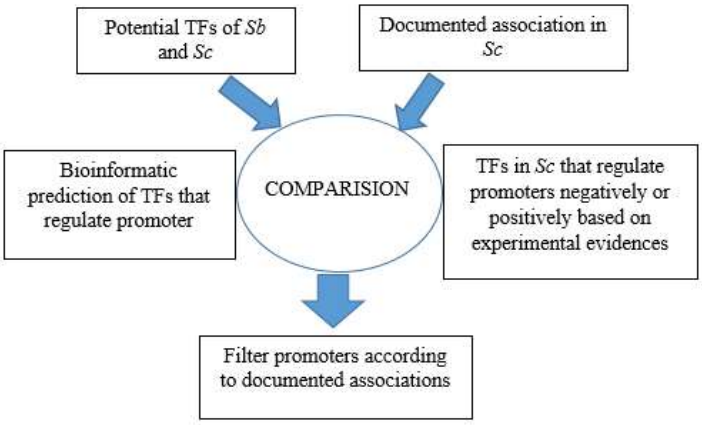


Figure 2.2. The pipeline of cross-strain promoter comparison using YEASTRACT bioinformatics tools

As an example, the analysis of the *FLO5* gene (encoding a lectin-like cell wall protein (flocculin) involved in flocculation) is detailed below. This process was repeated

for each predicted probiotic-related genes, compiled from the literature (Table 1.2). The pipeline design was used as the basis for the development of the "Cross-strain comparison" tool that is now present in the ProBioYeastract database (http://146.193.39.124/probioyeastract/sboulardii/formcrossstrain.php)

The promoters of the *FLO5* genes from Biocodex, Unique28 and S288C strains were extracted (Table 2.3) and compared in terms of presence or absence of *S. cerevisiae* TFBS (Table 2.3) Afterwards, the results were filtered to consider only the TFs that are known to regulate the *S. cerevisiae* S288C *FLO5*, obtained by using the YEASTRACT "Find TF" query. As a result of this *in silico* analysis, 26 out of the 83 probiotic-related genes were found to have TFBS that appeared uniquely in the *S. boulardii* promoters. Among these 6 were chosen for experimental evaluation, representative of the probiotic effects attributed to *S. boulardii* (Table 2.4).

2.5.2. Gene Expression Analysis

To assess the expression of the selected genes in *Sb* and *Sc* strains, three steps were taken, as illustrated in Figure 2.3: cell cultures to retrieve biomass; RNA extraction and RT-PCR, to measure relative gene expression.

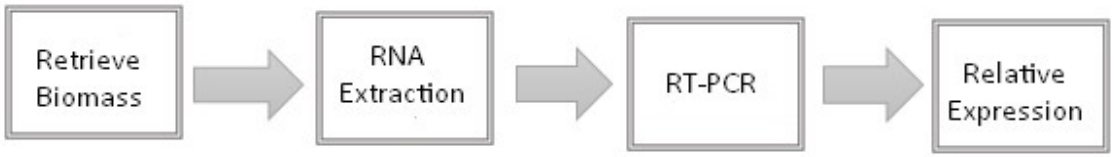


Figure 2.3. Scheme of experimental methodology to determine the expression of the selected genes.

2.5.2.1. Yeast strains and Growth Conditions

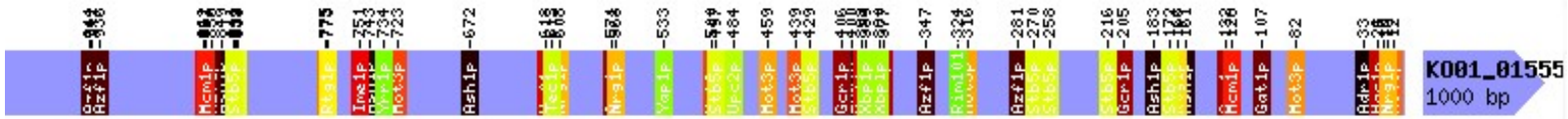
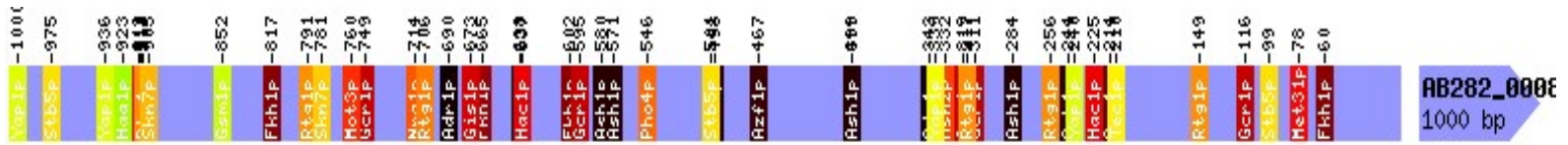
S. boulardii CNCM I-745 was isolated from an ULTRA-LEVURE® (Biocodex, Beauvais, France) sachet in YPD solid agar. *S. cerevisiae* BY4741 strain was obtained from Euroscarf collection.

Table 2.3. An example of a cross-strains promoter analysis, using ProBioYeastract and YEASTRACT database

Promotor Sequence extraction	
For *S. boulardii* Biocodex by using ProBioYeastract database	>KO01_01555upstream sequence, from -1000 to -1, size 1000 AAAAAAATCCATTACATTACGTTCCTTGCTCTGGATCTCTTCACCTGCAATTACTGAAAAGAAAAAAAGAAA TATAATTTTCCTTTGAGCAGCCTTGGTAACTTATCTAAGAACACGCGATGTAATCGAAATTTAATAACCTAA AAAAGAAATAGAGATCGGTGACGTTTGACGCTAGCAAATCTCCACGAGATTTATCTGGAAGGGCATCACCC GTCAAAACACTAAGAAATTTGACACCTTATTTTTTTTTCTCCGAAACGTTCTGAGACGTTTCTTTTTTTTTCAG GACGCCACCCTAAATCCGAAAAACCTTACTTGGCATTAGTGTTGTAAAAATTTCTTTTTTCCATCGTGGTTCT GAAAAGCTTGCAGAAAAAAAGTTCGAGAACGTTTTCGTATGACTTATTAAGCTGCCCTTGTACTTGCCTTCC CTCTTCTTTACTATTTTCAGCCAGGGACAGAAATAAAGCAAAATCGTTTAACTTTTATGACCGTAACAGGTA ACAATATCTGCTCAATTCGGTTAGTAAAAAAAAAAAAGGTTATCGTAGGTTTACTATTTTCACATCGGTGCA TAGAATCTTTACTCTACTTCCTTTGGAGGGAATTTAGTAGTGCTCGATTAGCAGATAACAGCGCAGGGTTCT ATGTAGTACGAAGCACTATCAGGTTTTTTAACGTACGTGAGTTGTTTTTTGTTTCTTATTTTTGGCTTGCCTGG GATAAACGGTGCCCATCAAATTATTAAGACCTGGTGAAAAGAGCGTACCTACATGGGTATCGGGGTTTTTGC CAGAAAGAGCTTTAACTGCAATTGATGCCTACCGGAGATTATTTTGATATCAAAATGCAGGAACGATATCTA CTTCCCTAAAGCAGAAAACAAAACATTTTTTTAAAAGTCTTTCTTATATAAAGGTAGCCTTCATTTCATGATA GCCAGATGATAATCTTAATGTAACAATTGGAGGATACCAGCATCCCTCCACACCTACAA
For *S. boulardii* Unique28 by using ProBioYeastract database	>AB282_00080upstream sequence, from -1000 to -1, size 1000 TGACAATGCCTCATCGCTATATGTTTTTGGCAGTCTTTACACTTCTGGCACTAATTAATGTGGCCTCAGGAGC CACAGAGGCGTGCTTACCAGCAGGCCAGAGGAAAAGTGGGATGAATATAAATTTTTACCAGTATTCATTGA AAGATTCCTCCACGTATTCTAATGCAGCATATATGGCTTACCAATATGCAGACAAAGTCAAATTGGGCTCTG TTAGTGGGCAAACGGATATATCTATCAACTATAATCTTCCTTGTGTTACAACCTCAGGGACATATCAGTGCC CTCAAGAAGATGCATATGGTAATTGGGGATGCAGAGGTAAGGGGAGATGCTCCAACAGTCAAGCAGTTTCA TACTGGAGTACAGATCTGTTTGGCTTTTATACCACTCCAACAAACATCACCCTAGAAATGACAGGTTACTTTT TACCACCACAGACAGGTTCTTACACGTTTTCTTTTGCAACAATAGATGATTCTGCAATTTTATCAGTCGGTGG TAGCATTGCGTTCGAATGTTGTGCACAAGAACAACCTCCCATCACATCGACTAACTTCACCATCAATGGTAT CAAGCCATGGCATGGAAGTCTCCCTGATAATATCGCAGGGACTGTCTACATGTATGCTGGTTTCTATTATCC AATGAAGATTGTTTACTCAAATGCCGTTTCCTGGGGTACACTTCCAATTAGTGTGACACTACCAGATGGCAC TACCGTTAGTGATGACTTTGAAGGGTACGTATATACCTTTGACAACAATCTAAGCCAGCCAAACTGTACCAT

(cont. on next page)

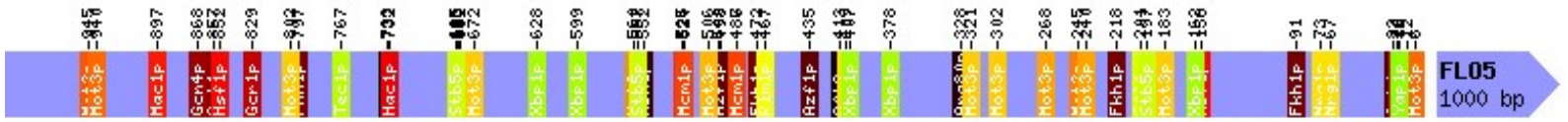
Table 2.3 (cont.)

	TCCAGACCCTTCAAATTATACTGTCAGTACTACCATAACTACAACCGAGCCATGGACCGGTACTTTCACCTCT ACGTCTACTGAGATGACTACTATCACTGGCACCAACGGTGTACCAACTGACGAAACCATCATTGTTGTCAAA ACACCAACAACTGCTAGCACCATCATAACTACGACCGAAGCATGGACTGGCACTTTCACATC
S. cerevisiae S288C by using YEASTRACT Database	>FLO56322005 upstream sequence, from -1000 to -1, size 1000 CCTCTTTCTTTTTTGTAAAAAATTCTGTTTTTAATAGCCAGTTCTTTAGTGATTACAGGTAAGAGGGTTTCATA TTTTAGAAGTGCAGCCATGATGAAGCACTTTTGCTCATTTATTGCGAGAAGTTTAATAAGTAGTATGGTTCCA TTTTCAAGAATCGAGGCACTGTTCCTTCCCAACCTTGGAATCATACTCCGAAAGGATTTCAAGCCGATTTAA ATTCACCTGGTAACTTTCCTACGGTTTGGCCCAAGGTGATTATAATTAACTTGCGGCTTGTTTTCAGCCTGCG ATCGAACCTTTTTTACGCAAAAAAACCTTATTAATTAAGGTTTTGAAAATTTTCTTCTTTCCGGGAGATTTTC ATGTAGCCTCGAGCTTCTGGATTCTCACGGGATTATCTCGCGTTACATTTTTTACTTTCTTCTTTCTTTTTGAC TTAGGATATACAGATGATACGTCATTGTGTCATAAAACCCGCTGTTGTGCAACAAAAGGGAAAAAGAAAAA TACTCCTTTTTAGGTCTTATAAATATTTTTAGCAGCCATCAAGTCCGGCTTTCAAACTTAATTTCACCCTTTTT CACGGCACCCTCGAGAATTACACTTTGGTTGCATGCAGGAGTACGCGAAATGCAGCATAAGCTACACATCTA TGCGTAGATCGCTTAACCTCTAAAGGCCGTAAACTTTTATTTTGTTTTGCGCTCATTAAAACCTAGTGGGAGC TGGTAGGAAATAAGCTAGTAGCTTCTATGGATAGAATGGAAATAAACGTAGGTGTAAACACTATTGGTAGA GAAGTTCCTCTGGTCAAATTTTCATGGGAGATACGTTAAATCTTTCACAGTCTTATCGTTTTGAATCACTGGA CGGTTCTGGTATTCTGCTTCATATTTCGACAAGATAATAAATATAAAAAGAGCACCCTCATGATTTCTTGCTC TGCAGTAAATTCCGCAAATGATTTTCTTTAAATTGATTAGCACCACTAAAAAAA
Determination of TFs Binding Sites Location	
For Biocodex *FLO5*	
For Unique28 *FLO5*	

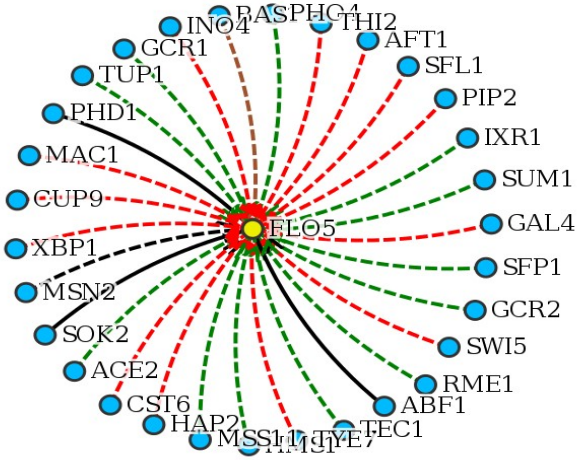
(cont. on next page)

Table 2.3 (cont.)

For *S. cerevisiae* S288C *FLO5*



Determination of documented S. cerevisiae TFs that regulate FLO5



TFs for which there is at least one TFBS in the *FLO5* gene promoters, known to regulate *S. cerevisiae FLO5* expression

Biocodex (*S. boulardii*)	Unique 28 (*S. boulardii*)	*S. cerevisiae*
x	x	Aft1p

TFs for which there are unique TFBS in the *FLO5* gene promoters

Unique to *Sc*	Aft2p, Aft1p, Arg80p, Gcn4p, Mac1p, Rlm1p, Sfl1p
Unique to Biocodex	Cst6p, Ime1p, Sko1p, Upc2p, Cad1p, Yap3p, Cin5p, Yap5p, Yrr1p, Rim101p
Unique to Unique28	Haa1p, Gsm1p, Skn7p, Pho4p, Gis1p, Msn2p, Msn4p, Rph1p, YER130C, Crz1p
Unique to *Sc* and active in *Sc*	Aft1p
Unique in both Biocodex and Unique28	x

Table 2.4. Summary of selected genes for experimental evaluation after cross-strain promotor analysis

Related Disease	**Pathways**	**Selected genes for experimental trials**
AAD related to pathogen infection (Adhesion)	Protein secretory	*FLO5*
Candidiasis (Adhesion)	cAMP-PKA	*EFG1*
Obesity, Type II diabetes	Phosphatidate biosynthesis I (dihydroxyacetone pathway)	*TGL4, YDC1*
Allergic Diseases (Polyamine secretion)	Arginine and proline metabolism	*SPE2*
Gluten Intolerances	Palatinose metabolism	*IMA1*

A small amount of *Sc* and *Sb* strains were collected from solid media and transferred into YPD liquid medium (20g/L glucose (Merck), 10 g/L yeast extract, 20 g/L peptone) (25 ml) in an erlenmeyer flask. The culture was kept under agitation (250 rpm) at 30°C in YPD medium overnight. Cell growth was measured by assessing the optical density (OD) at 600 nm of the cell suspension, to determine the volume of culture to be transferred to a new flask with fresh YPD or YPD+cholate (including 0.5 g/l sodium cholate (Sigma), to mimic human gastrointestinal environmental conditions (Fietto et al. 2004) medium in order to start with an OD600nm = 0.1. the new flasks were kept under agitation (250 rpm) at 30 °C for 5h to ensure 3 cell duplications, when an OD600nm of 0.8 was reached. Afterwards, the cells were harvested by centrifugation at 7 000 rpm at 4°C for 5 min. Prepared samples were stored at -80°C freezer until RNA extraction, as indicated in Fig 2.4.

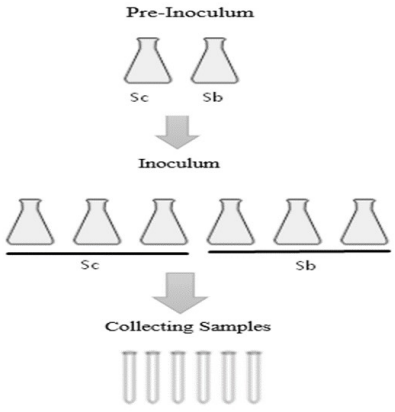


Figure 2.4. Scheme of experiments of the growth of cells carried out to RNA extraction

2.5.2.2. RNA Extraction

The total RNA extraction was carried out for three replicates of *Sb* and *Sc*. Firstly, The pellet of cells was resuspended in 900 µl of AE buffer (50 mM NaAc (Sigma), 10mM EDTA (Aldrich), pH=5.3; 0.1% (v/v) diethylpyrocarbonate (DEPC) treated). Then, 90 µl of SDS 10% were added and mixed by vortexing for 5 seconds. After that, 800 µl of phenol for RNA extraction was added and mixed by vortexing for 5 seconds. After adding phenol, the mix was incubated at 65ºC for 4 minutes. After incubation, the eppendorf tubes were kept on dry ice. Then, each mixture was centrifuged at 15000 rpm at 4ºC for 5 minutes, and the upper liquid phase transferred to a new Eppendorf. 400 µl phenol and 400 µl chloroform were then added and mixed by vortexing for about 5 seconds and centrifuged at 15000 rpm at 4ºC for 5 minutes. The upper liquid phase was transferred to a new Eppendorf and the previous step was subsequently repeated once again. Afterwards, 90 µl sodium acetate (Merck, 3M, pH=5.3, 0.1% DEPC - diethyl pyrocarbonate) and 1 mL 100% ethanol at -20 ºC were added to the collected supernatants, mixed by vortexing for 5s and then stored at -20ºC for 20 minutes, for RNA precipitation. The samples were then centrifuged at 15000 rpm, at 4ºC for 20 minutes, and the supernatant was discarded. Afterwards, 750 µl 70% (v/v) ethanol was added and the samples were centrifuged at 15000 rpm, at 4ºC for 15 minutes. The supernatant was discarded carefully by using a syringe. The pellets were dried in the SpeedVac (V-AL, 20 min, 45ºC) and resuspended in 30 µl distilled H_2O with 0.1% DEPC. NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies) was used to measure RNA concentration and quality. The concentration was then adjusted to 500 ng/µl for the real-time RT-PCR experiments.

2.5.2.3. Real-Time PCR (RT-PCR)

The RT-PCR procedure consisted of two main steps. In the first step, reverse transcription was performed. The reverse transcription (RT) converts RNA into cDNA (complementary DNA), which is then used in the real-time PCR process. PCR reactions were prepared for each sample according to the values indicated in Table 2.5. The retrotranscription program used is described in Table 2.6.

Table 2.5. PCR reaction mixture components and their volumes

Component	Volume in µl (per sample)
10X Buffer (10x)	1.0
$MgCl_2$ (25mM)	2.2
dNTP's (2.5mM)	2.0
Random hexamers (50 µLM)	0.5
RNase Inhibitor (20 U/L)	0.2
MultiScribeTM reverse transcriptas (50 U/L)	0.25
ddH_2O DEPC treated	1.85
RNA sample (500 ng/µL)	2
Total	**10**

Table 2.6. Thermal cycling parameters for the first step of the real-time RT-PCR

Step	Time (min)	Temperature (^{0}C)
Incubation	10	25
Reverse Transcription	30	45
Reverse Transcriptase inactivation	5	95

In the second step, Real-Time PCR reactions were prepared for each sample according to Table 2.7. SYBR® Green reagent was chosen as detection chemistry to perform relative quantification of gene expression. Real-Time PCR was run and analysed using its own software 7500 Systems SDS Software Applied Biosystems Table 2.7.

Table 2.7. RT- PCR reaction components and their volumes for each sample

Component	Volume in µl (per sample)
SYBR®Green PCR Master Mix (2x)	12.5
Forward primer (4 pmol/µL)	2.5
Reverse primer (4 pmol/µL)	2.5
cDNA	2.5
ddH_2O	5.0
Total	**25**

The aim is to find the number of cycles (Ct) necessary to reach a given level of fluorescence above the noise threshold (Wong and Medrano, 2005). Hence, the signal level is registered in an amplification plot, from which Ct is estimated by the intersection

between the exponential phase curve and threshold line. The normalization of the Ct values is performed using an internal control indicated in Equation 1 (Rao et al., 2013).

$$\Delta Ct=(target)-Ct(control) \quad (1)$$

Table 2.8. Primers used in screening and quantitative Real-Time PCR

Gene Name	Primer Sequence (3'-5')
ACT1 *FW*	3'-GGTGTTACTCACGTCGTTCC-5'
ACT1 *RV*	3'-GAAGTCCAAGGCGACGTAAC-5'
FLO5 *FW*	3'-TGGACCGGTACTTTCACCTC-5'
FLO5 *RV*	3'-CACGGTTTCGTCAGTTGGTT-5'
EFG1 *FW*	3'-TCCCAGATAATGGATGCAGGA-5'
EFG1 *RV*	3'-AGCGTTGGCTTTAATCTTATTCT-5'
TGL4 *FW*	3'-ACTCCAACCAAGGGTGACAA-5'
TGL4 *RV*	3'-GCGGACGTAATGGAATACCG-5'
YDC1 *FW*	3'-GTTCTTTCTGGCTGGCTGAC-5'
YDC1 *RV*	3'-TGGCAGGGCCAAATATGTTC-5'
SPE2 *FW*	3'-CAAGCCGCTATCCATCAAA-5'
SPE2 *RV*	3'-TTCGTCGTCATCCTCGATGT-5'
IMA1 *FW*	3'-TGGACCACGTATTCACGAGT-5'
IMA1 *RV*	3'-TAGTTTCGTCGGAGGCATGT-5'

Then, each normalized value correspondent to each gene is compared with the physiological calibrator considered, as shown in Equation 2 (Rao et al. 2013).

$$\Delta\Delta Ct=\Delta C(sample)-\Delta Ct(calibrator) \quad (2)$$

Later, the gene expression level can be estimated using Equation 3 (Rao et al., 2013).

$$2^{-\Delta\Delta Ct} \quad (3)$$

Primers for the amplification of the *FLO5*, *EFG1*, *TGL4*, *YDC1*, *SPE2*, *1MA1* and cDNA for *Sb* and *Sc* were designed using Primer Express Software 444 (Applied Biosystems®) (Table 2.8). The *ACT1* gene for *Sb* and *Sc* was considered as a housekeeping gene to carry out in RT-PCR so as to have internal control. Target genes in *Sb* comparing to *Sc* genes were measured based on Comparative C_T method.

2.5.3. Aggregation Assessment

Sc and *Sb* were cultivated in YPD medium as described in section 2.3.2.1. 7 µl of cell suspension were observed under a bright-field Zeiss Axioplan microscope (Carl Zeiss MicroImaging). 30 images were captured using a CCD camera (Cool SNAPFX, Roper Scientific Photometrics). The number of aggregates and the number of cells per aggregate was calculated for each image using the Metamorph software.

2.5.4. Adhesion to human epithelium cells

The VK2/E6E7 human vaginal epithelial cell line (ATCC CRL-2616) were cultivated in 24-well polystyrene plates (Greiner), in keratinocyte-serum-free medium, containing 0.1ng/ml human recombinant epidermal growth factor (EFG), 0.05 mg/ml bovine pituitary extract and 44.1mg/l calcium chloride, until a density of $2.5x10^5$ cells/ml was reached after 24h of incubation. The culture medium was then removed and substituted by fresh culture medium. *Sb* and *Sc* cells, cultivated in YPD medium as described in section 2.3.2.1, were then added to each well, with a density of $12.5x10^8$ CFU/well. Then, the cells were incubated at 37°C, 5% CO_2, for 30 min. Afterwards, each well was washed 3 times with 500 µL of PBS pH 7.4, following the addition of 500 µL of Triton X-100 0.5% (v/v) and incubation at room temperature for 15 min. The cell suspension in each well was then recovered and spread onto YPD agar plates by using spheres, and incubated at 30ºC for 48h, to determine CFU (Colony Forming Units) count, which represents the proportion of cells adherent to the human epithelium.

2.5.5. Biofilm Quantification

In order to assess the capacity of the biofilm formation of *Sc* and *Sb* cells, the Presto Blue assay was used. Cells were grown in Sabouraud's dextrose broth ((SDB) containing 40 g glucose (Merck) and 10 g peptone (LioChem) per liter, pH 5.6) and collected at mid-exponential phase. A cell suspension was prepared with an OD at 600 nm of 0.1. Cells were then inoculated in 96-well polystyrene titter plates (Greiner), which were previously filled with the appropriated medium, YPD, SDB at pH 5.6 or Roswell Park Memorial Institute (RPMI) 1640 growth medium (containing per 100 mL: 2.08 g

RPMI 1640 (Sigma); 6.91 g 3-(N-morpholino) propanesulfonic acid (MOPS) (Sigma); 3.6 g glucose (Merck)), at pH 4 and 7, so as to have an initial OD600nm = 0.05±0.005. The design of the biofilm formation experiment shown in Figure 2.5.

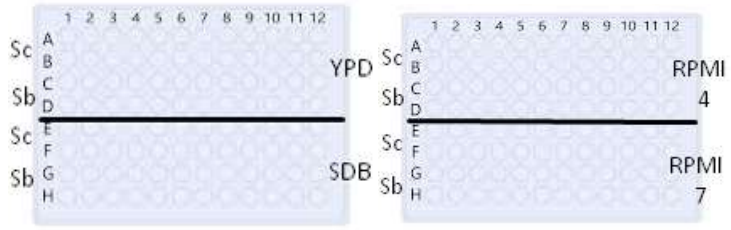


Figure 2.5. Schematic representation of the experimental design of 96-well polystyrene titter plates (Greiner) for biofilm determination.

Afterwards, cell suspensions were sealed with a membrane (Greiner Bio-One) and cultivated at mild orbital shaking (100 rpm), for 24h, at 30ºC. Subsequently, each well was washed two times with 100 µL of sterile PBS pH 7.4 [PBS contained per liter: 8 g NaCl (Panreac), 0.2 g KCl (Panreac), 1.81 g $NaH_2PO_4.H_2O$ (Merck), and 0.24 g KH_2PO_4 (Panreac)] to remove the cells that were not attached to the formed biofilm. Presto Blue reagent was prepared in a 1:10 solution in the medium used for biofilm formation, adding 100 µL of the solution to each well in the dark. Plates were incubated at 37ºC for 30 min. At the end of these processes, absorbance reading was determined in a microplate reader (SPECTROstar Nano, BMG Labtech) at the wavelength of 570 nm and 600 nm for reference.

2.5.6. Statistical Analysis

Statistical analysis of the case study of *Sb* data was performed using Microsoft EXCEL 2016. P-values were calculated performing T test on Microsoft® EXCEL 2016. P-values equal or inferior to 0.05 were considered statistically significant.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. *In vitro*, *In vivo*, Clinical and Meta-Analyses Studies for Probiotics

This study suggests a new approach for probiotics based on their effectiveness in terms of both strain-specificity and disease-specificity. In this regard, important data were collected and some significant points were revealed for different types of probiotic microorganisms.

When the database about clinical trials (https://clinicaltrials.gov/) has been examined, e.g. as seen in the case of a probiotic yeast *S. boulardii*, studies were focused on clinical studies than *in vitro* and *in vivo* studies in the literature. On the other hand, in case of a probiotic bacteria *Bacillus spp*., although clinical trials and meta-analyses are present, these are not published elsewhere; there are more studies available for *in vitro* and *in vivo* studies.

3.1.1. *Lactobacillus spp.* as Probiotics

Recent well-controlled clinical studies have demonstrated that *L. rhamnosus* GG (LGG), *L. reuteri* and *L. plantarum* 299v are able to have effectiveness on shortening, treating or preventing of the duration of diarrhea for adult and children as indicated Table 3.1. LGG is one of the most widely used probiotic strains that are well documented including the prevention and treatment of gastrointestinal infections on human health. Besides, they are resistant to acid and bile, show good growth characteristics with having high adhesion capacity to the intestinal epithelial layer (Segers and Lebeer 2014).

Several studies were examined that LGG was significantly effective in adult and children to prevent AAD and to cure diarrhea caused by CDI. Considering the published results of LGG, when focusing on its effectiveness on the other diarrhea types, it is reasonable to hypothesize that LGG has a positive effect on gastrointestinal health. Thus, the efficacy of LGG on other diseases types should be also concentrated. It has been

reported by Yan and Polk (2012) that LGG exerts impacts on treating and/or preventing several diseases such as ulcerative colitis and atopic dermatitis, as well as diarrhea, in children and adults. Moreover, recent studies have revealed LGG's beneficial effects on human health to prevent and treat more diseases. In addition, there are studies about treating vaginal infections in women health as well, however this hypothesis need to be confirmed. Additionally, the beneficial effects of LGG against rotavirus infection in children have been observed. Supporting this idea, the prophylactic use of *Lactobacillus* GG significantly reduced the risk of diarrhea caused by rotavirus gastroenteritis in infants (Szajewska et al. 2001).

Similarly *L. rhamnosus* probiotic, another well-documented *Lactobacillus* probiotic strain *L. reuteri*, both examined in clinical studies whether they have influences on decreasing symptoms of diarrhea. On the other hand, efficacy studies for *L. plantarum* have been reported in relation to IBS in children and adult.

At last but not least, *Lactobacillus* probiotic has a number of published studies, but some important points are still remains unclear in terms of determining the efficacy. Supporting this, as seen from Table 3.1 there are still not enough investigations for *Lactobacillus* probiotic strains on their dose-effectiveness and duration of usage. The studies shown in Table 3.1, have been mostly displayed at different interval of dosage and duration of use to treat or prevent gastrointestinal diseases.

3.1.2. *Bifidobacterium spp.* as Probiotic

Recently, *Bifidobacterium* species have been reported to treat constipation and travelers' diarrhea, antibiotic-associated diarrhea; preventing remission of disease activity of gut inflammation, moderate ulcerative colitis; treating of necrotizing enterocolitis in newborns; decreasing the development of disease risk for eczema, food allergies and cholesterol-lowering capacities (Fijan, 2014).

In this section as given in Table 3.2, studies on *Bifidobacterium infantis* subsp. has been mostly focused on IBS and IBD.The probiotic strain *Bifidobacterium infantis* 35624 relieves the symptoms of Irritable Bowel Syndrome (IBS) such as abdominal pain/ discomfort and bloating/distension according to *in vitro*, *in vivo* and clinical studies..

Table 3.1. Summary of the major findings on the probiotic efficacy of *Lactobacillus spp.*, according to *in vitro*, *in vivo*, clinical and meta-analysis studies for children and adult patients. *EHEC- Enterohemorrhagic Escherichia coli*, IL-8 Interleukin 8, IL-6 Interleukin 6, IL-1β Interleukin 1 β, TNF-α Tumor Necrosis Factor-alpha, IFN-γ Interferon Gamma, CDI- *C. difficile* infection, AAD Antibiotic-Associated Diarrhea, FAP- Functional Abdominal Pain, PC-Placebo controlled, PG- Parallel-group, R- randomized, MC-Multicentre, RCT-Randomized control trials, Ab-antibiotic, NA-No Available, wk-week, mo-month, yr-year

	Disease	Strain Type	Type of Study	Number of Target group	Dose& Duration	Major findings	Strength of Evidence	References
In vitro	*EHEC* infection	*L. rhamnosus*	Study in Human colon epithelial cell line	x	x	*L. rhamnosus* showed outstanding potential for adhering to the colon epithelial cell line.	Medium Effective ***	(Hirano et al., 2003)
In vivo	Necrotizing enterocolitis	*L.reuteri* DSM 17938	Study in mouse	x	x	The percentage of Treg cells of CD4(+) Foxp3(+) in the intestine reduced during NEC and was restored to normal by L. reuteri DSM 17938.	Medium Effective ***	(Liu et al., 2014)
Clinical studies	**For Adults**							
	CDI	*L.rhamnosus* GG	R, PC	180 inpatient	+Ab	The recurrence rate of CDI reduced, improved earlier disappearance of abdominal cramps and diarrhea, it appears to be a safe and effective therapy for diarrhea caused by *C. difficile*	Effective **	(Pochapin, 2000)

(cont. on next page)

Table 3.1 (cont.)

	CDI	*L.rhamnosus* GG	R, PC	60 outpatient	NA	LGG supplementation showed a positive impact on *H. pylori* therapy-related side-effects and on overall treatment tolerability	Medium Effective ***	(Armuzzi et al., 2001)
	In Children							
	Diarrhea	*L.rhamnosus* GG	x	71 children from 4 to 45 mo	10^{10}cfu/ml twice daily for 5 days	Duration of diarrhea significantly shortened at feeding by *L. rhamnosus* GG group	Significantly Effective ****	(Isolauri et al., 1991)
	Diarrhea caused by Rota-virus	*L. rhamnosus* GG	R	49 children aged 6-35 mo	twice daily for 5 days	The duration of acute rotavirus diarrhea reduced	Effective **	(Majamaa et al., 1995)
	Diarrhea	*L. rhamnosus* GG	R	100 children	x	Duration of diarrhea is reduced for 61 children suffer from rotavirus infection	Effective **	(Guarino et al., 1997)
	Acute onset diarrhea	*L.rhamnosus* GG	PC, DB,ORP	in children aged from 1 mo to 3 yr	10^{10}cfu/ml	Administration of *Lactobacillus* GG to children with acute diarrhea was observed safe and faster discharge from the hospital., duration of diarrhea reduced	Significantly Effective ****	(Guandalini et al., 2000)
	Diarrhea caused by Rota-virus	*L. rhamnosus* GG	R, DB, P	81 children aged 1-6 mo	$6x10^{9}$cfu/ml	*L. rhamnosus* GG significantly reduced the risk of rotavirus gastroenteritis	Significantly Effective ****	(Szajewska et al., 2001)

(cont. on next page)

Table 3.1 (cont.)

	Diarrhea caused by infectious	*L.rhamnosus* GG	R, PC, DB	87 children aged 2 mo to 6 yr	$1.2x10^{10}$ cfu/ml Twice daily &for 5 d	*L. rhamnosus* GG shortened the duration of diarrhea, No observed adverse effect	Effective **	(Syzmanski et al., 2005)
	Prevention of AAD	*L. rhamnosus* GG	R, PC	87 children and adults	x	A fermented multistrain probiotic milk drink may prevent four of five cases of AAD in adult hospitalized patients	Less Effective *	(Wenus et al., 2008)
	Prevention of AAD	*L. rhamnosus* GG	R, PC, DB	240 children aged 3 mo to 14 yr	$2x10^{10}$ cfu/ml+Ab	Reduced the risk of diarrhea	Effective **	(Ruszczyński et al., 2008)
	Functional abdominal pain (FAP)	*L.reuteri* DSM 17938	DB,R, PC	60 children aged 6-16y	$2x10^{8}$ cfu/d for 4 wk	Reduced abdominal pain intensity	Significantly Effective ****	(Romano et al., 2014)
	Functional Chronic constipation	*L.reuteri* DSM 17938	R,DB, PC	44 consecutive infants at least 6 mo	NA&8 wk	Positive effect on bowel frequency, no adverse effect	Effective **	(Coccorullo et al., 2010)
	Acute Diarrhea	*L.reuteri* DSM 17398	MC, R, SB	127 inpatient children	$1x10^{8}$ cfu for 5 days	Effectively reduced the duration of acute diarrhea	Effective **	(Dinleyici and Vandenplas, 2014)
	H.pylori Infection	*L.reuteri* DSM 17398	DB,PC,R	70 treatment	NA	The *L. reuteri* treated group showed significant	Significantly Effective	(Emara et al., 2014)

(cont. on next page)

Table 3.1 (cont.)

				naive patient		improvement in *H. pylori* associated gastritis.	****	
	FAP (IBS)	*L.reuteri* DSM 17938	R,DB,PC	101 children aged 6-15 y	NA&for 4 wk	Significantly reduced the frequency and intensity of FAP in children	Significantly effective ****	(Weizman et al., 2017)
	Diarrhea caused by *Rota virus*	*L. reuteri*	R,P	40 children aged 6 to 36 mo	10^{10}cfu/ml daily &for up to 5 days	After administration of *L. reuteri* effectively colonized GIT and significantly decreased the duration of watery diarrhea associated with rotavirus.	Significantly Effective ****	(Shornikova et al., 1997)
	H.pylori Infection	*L. reuteri* ATTC 55730	DB, PC	40 *H.pylori* positive patient	4wk	*L. reuteri* effectively suppresses *H. pylori* infection in humans and decreases the occurrence of dyspeptic symptoms.	Significantly effective ****	(Francavilla et al., 2008)
	H.pylori Infection	*L. reuteri* ATTC 55570	R,PC	40 *H. pylori* positive children	$1x10^8$ cfu & for 10 days	*L. reuteri* is capable of reducing frequency and intensity of AAD effects	Significantly Effective ****	(Lionetti et al., 2010)
	Acute Diarrhea	*L.reuteri* DSM 17398	R, DP, PC	69 hospitalized children 6 -36 mo	$4x10^8$ cfu/d	*L. reuteri* DSM 17938 as an adjunct to rehydration therapy is efficacious in the treatment of acute diarrhea reducing the frequency, duration and recrudescence rate of the disease, no adverse effect	Significantly Effective ****	(Francavilla et al., 2012)

(cont. on next page)

Table 3.1 (cont.)

	IBS	*L.plantarum* 299v	R, DB, PC, PDS	653 children 1-15 year	NA & for 4 wk	Provided effective symptom relief, especially abdominal pain and bloating IBS patients	Effective **	(Ducrotte et al., 2012)
	IBS	*L.plantarum* 299v	R, PC	60 patient with IBS	For 4 wk	Administration of *L.plantarum* 299v decreased pain and flatulence in patients with IBS	Significantly Effective ****	(Nobaek et al., 2000)
	IBS	*L.plantarum* 299v	PC,DB,R	40 patient	NA&4 wk	All IBS symptoms have an improvement in *L.plantarum* 299v group	Medium effective ***	(Niedzelin et al., 2001)
	CDD	*L.plantarum* 299v		22 ill patient	Twice a wk+Ab	*C. difficile* colonization reduced by treating *L.plantarum* 299v	Effective **	(Klarin et al., 2008)
	AAD	*L.plantarum* 299v	R, PC	NA	10^{10} cfu +Ab	*L. plantarum* could have a preventive effect on milder gastrointestinal symptoms during treatment with antibiotics.	Effective **	(Innermark et al., 2010)
Meta-analysis	Acute Gastroenteretis	*Lactobacillus* GG	15 RCTs 2963 participants	2963 study patients (in children)	$<10^{10}$ cfu daily	*Lactobacillus* GG decreased the duration of diarrhea.	Effective **	(Szajewska et al., 2013)

Moreover, dose range from at about $1x10^6$ to $1x10^8$ cfu/ml has been found as acceptable to show effectiveness for preventing IBS. More researches with *B. infantis* probiotic are needed to be proven with regard to precise beneficial effects on IBS to cure as well.

3.1.3. *Bacillus spp.* as Probiotic

The genus *Bacillus*, with supporting health claimed probiotic features, commonly have included *B. subtilis, B. coagulans, B. clausii, B. cereus*.

Most of the studies have suggested that *Bacillus* spores are effective for preventing and treating of diarrhea types; such as antibiotic-associated diarrhea, caused by a bacterial infection in adult (e.g *C. difficile*), caused by a viral infection (e.g rota-virus) commonly in children, etc.

Table 3.3 presents current research on *in vitro*, *in vivo* and clinical studies on *Bacillus spp.* strains. There are limited *in vitro* studies to conclude the mechanism of action of *Bacillus spp*. One of the *in vitro* study demonstrated that *B. subtilis* spores and cells were able to have the ability to adhere to the gastrointestinal tract having colonisation of the mucosa.

Adhesion of gastrointestinal tract is significant for being good probiotic features. Furthermore, it is concluded that the sporulated phase had a high degree of adhesiveness than the cell in the overt vegetative form (Angioi et al., 1995).

It has been observed that there are effective results on diarrhea types both adult and children. *Bacillus* spores have been found significantly effective with clinical studies for preventing and treating acute diarrhea, antibiotic-associated diarrhea, diarrhea caused by *H. pylori* infection, etc.

Moreover, there is no common adverse effect observed on human health. Therefore, in this section assessment of the effectivity of *Bacillus* probiotics are presented in Table 3.3 with *in vitro*, *in vivo* and clinical studies on human health. In this Table, when a detailed evaluation has been made, a dose of usage of probiotics for the prevention and cure of diseases can be exerted approximately in the range of $1x10^9$ and $2x10^9$, pointed out the dosage for daily use.

Table 3.2. Summary of the major findings on the probiotic efficacy of *Bifidobacterium infantis subsp.*, according to *in vitro*, *in vivo*, clinical and meta-analysis studies for children and adult patients. IECs-Intestinal Epithelial cell, IL-8 Interleukin 8, IL-10 Interleukin 10, T_H1-T helper cells1,T_H17- T helper cells 17, IBS-Irritable Bowel Syndrome, IBD-Inflammatory Bowel Disease, PC-Placebo controlled, PG- Parallel-group, R- randomized, Ab-antibiotic, NA-No Available, wk-week, mo-month

	Disease	Type of Study	Number of Target group	Dose& Duration	Major findings	Strength of Evidence	References
In vitro	IBD	Study in culture media	x	x	*B.infantis* exerts immunomodulatory effects on intestinal epithelial cells (IECs) that mediate host response to flagellin and enteric pathogens	Medium Effective ***	(O'Hara et al., 2006)
	IBD	Study in culture media	x	x	*B. infantis* increased in the secretion of IL-10 and enhanced Fox3p expression in peripheral blood	Medium Effective ***	(Konieczna et al., 2012)
In vivo	IBS	An animal study (in rat)	x	For 14 d, oral gavage	*B. infantis* 35624 is effective in reducing visceral pain, may be decreased in certain IBS disorders	Less Effective *	(McKernan et al., 2010)
	Malabsorption of nutrients & diarrhea	An animal study (in mice)	x	10^6 CFU &for 6 day	Prevented weight loss, protected brush border enzyme activity, decreased small intestinal damage, inhibition the rise in IL10	Medium Effective ***	(Symonds et al., 2012)

(cont. on next page)

Table 3.2 (cont.)

					and IL8, caused by Salmonella infection		
	IBD	An animal study (in mice)	x	NA	Decreased the severity of Dextran sulfate sodium (DSS) induced colitis, T_H1 and T_H17 cells within the lamina propia	Significantly Effective ****	(Konieczna et al., 2013)
Clinical studies	**In Adults**						
	IBS	R, P	362 primary care IBS patients	$1x10^6$,$1x10^8$, $1x10^{10}$ cfu/ml& receiving duration of 4 wk	The dose of $1x10^8$ was found as acceptable, *B. infantis* 35624 relieves many of symptoms of IBS, No observed adverse effect	Medium Effective ***	(Whorwell et al., 2006)
	IBS	R, P	77 Adult Patient	$1x10^{10}$ cfu/ml & for 8 wk	*B. infantis* 35624 alleviates symptoms in IBS	Medium Effective ***	(O'Mahony et al., 2005)
	In Children						
	Diarrhea caused by *rota virus*	DB,PC	4447 of 5-24 mo infant	NA& During 17 months	*B. infantis* can decrease the incidence of acute diarrhea	Less Effective *	(Saavedra et al., 1994)
Meta-analysis	IBS	x	666 study patients	NA	*B. infantis* 35624 may alleviate IBS symptoms such as abdominal pain/discomfort, bloating/distension	Less Effective *	(Yuan et al., 2017)

Table 3.3. Summary of the major findings on the probiotic effectiveness of *Bacillus*, according to *in vitro*, *in vivo*, clinical and meta-analysis studies for children and adult patients. TNF-α Tumor Necrosis Factor-alpha, IFN-γ Interferon Gamma, CDI- *C. difficile* infection, AAD Antibiotic-Associated Diarrhea, PC-Placebo controlled, ORT- oral rehydration therapy, PG- Parallel-group, P-Prospective R- randomized, DB- Double Blind, SB-Single Blind, SC-Single-centre, MC-multicentre Ab-antibiotic, NA-No Available, wk-week, yr-year

	Disease	Strain type	Type of Study	Number of Target group	Dose& Duration	Major findings	Strength of Evidence	References
In vitro	Diarrhea against bacterial Infections (e.g.*C. difficile*)	*B. clausii*	Study in Swiss (peritoneal cells) and C57 BI/6j murine cells (spleen cells)	x	x	NOS II synthetase activity, IFN-production, and CD4+ T-cell proliferation are induced by *B. clausii*	Medium Effective ***	(Urdaci et al., 2004)
	AAD	*B. subtilus*	Study in culture media	x	x	Adhesion of *B. subtilus* to surface of eukaryotic (Caco-2 and HEp-2) cells observed	Effective **	(Angioi et al., 1995)
In vivo	x	*B. subtilus*	An animal study	x	NA	The cellular and humoral immune response induced	Effective **	(Fan et al., 1999)
Clinical studies	**In Adults**							
	H. pylori infection	*B. clausii*	R, PC, DB	60 outpatients	$2x10^9$ cfu for 14 days, during	The mean intensities and frequencies of nausea	Significantly Effective ****	(Nista et al., 2004)

(cont. on next page)

Table 3.3 (cont.)

					and 1 wk thereafter	and diarrhea was significantly observed low in the *B. clausii* group		
	Acute Diarrhea	*B. clausii* UBBC-07	PC	27 outpatient	2×10^9 cfu twice a day &for 10 days.	Significant improvement in the duration of diarrhea, frequency of defecation and stool consistency were observed	Significantly Effective ****	(Sudha et al., 2013)
	AAD	*B. clausii*	R, DB, PC, SC, P	91 patient	1 vial twice a day	*B. clausii* probiotics have been shown a significant effect in the prevention of nausea, bloating, vomiting and abdominal pain, decreased the adverse effect of Ab use	Significantly Effective ****	(Harosheva et al., 2014)
	In Children							
	AAD	*B. subtilus*	NA	35 children 3 and 24 mo	$4x10^9$ & for 5 days	Saccharolytic flora, aerobic increased and anaerobic, no proteolytic flora shown any changes	Effective **	(Benoni et al.,1984)
	Diarrhea	*B. clausii*	ORT	131 children less than 2 yr to 12 yr	NA	*B. clausii* decreased the duration, frequency and hospital stay of diarrhea thereby reducing the treatment and social costs.	Significantly Effective ****	(Lahiri et al., 2015)
	Diarrhea	*B. clausii*	P,MC, SB,R	192 children patients	1×10^9 CFU & for 5 days	*B. clausii* was well tolerated, with no observed adverse events.	Effective **	(Canani et al., 2007)

A meta-analysis conducted in 2018 by Ianiro et al. focused on *Bacillus* probiotics in the treatment of acute diarrhea for children. The authors demonstrated confidence interval of 95% CI: 0.43 to 0.06, p = 0.14 and also concluded that *B. clausii* was able to show an effective therapeutic option in acute diarrhea for children, observed a good safety profile.

One advantage of *Bacillus* spores is to possess not positioning on neuronal systems comparing to other probiotics. Hence, they may safely use when human has neuronal diseases. The missing important idea about probiotics is that the probiotics cannot be used or prescribed for each situation.

All in all, it has been accepted that there are a holistic approach for the efficacy of *Bacillus* spores probiotics on human health with supporting ideas of *in vitro*, *in vivo*, clinical and meta-analysis.

Published data is almost enough to say having co-adjunctive therapy or supporting the immune system and helping digestive system for preventing or treating diseases, however, more research *in vitro*, *in vivo*, clinical studies and also meta-analysis for *Bacillus* subsp. are needed due to the importance of having strain-specific properties of probiotics.

Probiotic preparations containing single probiotic strain might possess several benefits of treatment and prevention since there is not much information on how different strains compete for nutrient, behave and being effective on the human immune system.

In order to retrieve the effectiveness of a probiotic strain, its known good probiotic features are not enough; therefore it would not be possible to make a correct remark on the effectiveness on the mixture of consisted of two or more different species of probiotics such as *Lactobacillus* subsp. and *Bifidobacterium* subsp.

3.1.4. *Saccharomyces spp.* as Probiotic

There is a very common misunderstanding about *Saccharomyces* microorganism. To clarify the confusion between *S.cerevisiae* and *S. boulardii*; the genus *Saccharomyces* includes various yeasts such as *Sc* used for making wine, bread, beer, etc. in industry, therefore, not being accepted as probiotic.

On the other hand, *Sb* is accepted as a marketed probiotic and used as a supplement and/or adjuvant therapy in medicine based on the numerous of clinical and meta-analysis studies. These studies should be given in detail so that the former strain type would confer any confusion not as being a probiotic.

A number of *in vitro*, *in vivo*, clinical and meta-analysis studies have suggested that *S. boulardii* are effective for preventing and treating of different types of diarrhea both in adult and children. Clinical and meta-analysis studies have pointed out the increasing effectiveness of important diseases such as antibiotic-associated diarrhea in adult, caused by a bacterial infection (e.g *C. difficile*, *EHEC,* etc), caused by a viral infection in children (e.g Rota-virus).

As it can be extracted from Table 3.4, the mode of action of *Sb* probiotic is still unclear. This is because *in vitro* and *in vivo* studies are scarce when compared to clinical and meta-analysis. Especially i*n vitro* studies are needed to understand the effective mechanism of action; how *Sb* probiotic can effectively being used for metabolic disorders (e.g. lactose malabsorption, celiac disease, obesity).

When looking at all *Sb* studies together, *Sb* is observed significantly effective on AAD in adult, diarrhea caused by rotavirus in children, diarrhea caused by *H. pylori,* CDI or CDD, and acute diarrhea.

In these studies, information on facts and figures about dosage, duration of *Sb* use are lack. Daily dosage in children has been observed at the interval of 250 and 500 mg approximately. More consistent outcomes can be reached by using oral administration of *Sb*, based on the dosage for diarrhea treatment caused by rotavirus in children studies.

On the other hand, in one a multicenter, phase III, double-masked, randomized, placebo-controlled clinical trial conducted by Ehrhardt et al. (2016), hospitalized patients received systemic antibiotic treatment in 15 hospitals and Perenterol forte 250 mg (includes *S. boulardii subsp*) capsules in Germany. At the end of this treatment, no evidence for an effect of *S. boulardii* in preventing AAD or CDAD in a population of inpatients without particular risk factors apart from systemic antibiotic treatment.

All in all, there are several studies to show efficacy of *S. boulardii* probiotic, as well as a few others without evidences. Therefore, more randomized clinical trials are needed to make definite recommendations about *S. boulardii* probiotics.

3.2. Probiotic use in Turkey and Their Regulations

Recently, probiotic marketing has been rapidly expanded areas in Turkey like all over the world.

In this regard, there are different probiotic types such as dietary supplement, medical preparates, and functional food in Turkey.

In this section, commercially available probiotics and their claims have been examined by contacting related sources. Table 3.5 presents single and mixture of different probiotic strains together with various symbiotic formulations (e.g. probiotic plus prebiotic such as adding vitamins, inulin, fructooligosaccharides, polydextrose). It has been observed that they are being used as medical preparate or dietary supplement as well as functional food ingredient (e.g in yogurt, kefir, beverage, pickle).

However, it should be highlighted that every yogurt product cannot be accepted as naturally probiotic since the type of microorganism may not exert probiotic properties as demonstrated into specific criteria of legislations.

Medical preparates and dietary supplements are mostly preferred at encapsulation forms such as Lyophilised etc. Moreover, this study underlines the importance of scientific evidences with respect to the effectiveness and efficacy in order to make concrete conclusions on health effects.

According to the regulations for probiotic microorganisms used for gastrointestinal and immune system health; any probiotic product should be consisted of at least $1x10^6$ cfu/g live probiotic microorganisms.

Indeed, Regulatory affairs of Probiotics are limited in Turkey, therefore, regulations of them are being examined further to cover complete probiotic characteristics having scientifically evidenced by taking into consideration *in vitro*, *in vivo*, clinical, meta-analyses studies and adverse effects as well.

Another important issue is that all commercially available probiotics should pointed out health claims taking into consideration of live microorganisms, yet it has been observed that they include indicator live microorganisms.

Enterogermina® contains spores of four strains of *Bacillus* subsp. They are resistant toward specially chloramphenicol and tetracyclin antibiotics. Liquid Suspension of these spores includes 2×10^9 cfu/ml. It is used as a medical preparate along with antibiotics against diarrhea in infant (Elshaghabee et al., 2017).

Table 3.4. Summary of the major findings on the probiotic efficacy of *S. boulardii*, according to *in vitro*, *in vivo*, clinical and meta-analysis studies for children and adult patients. EHEC- Enterohemorrhagic Escherichia coli, IL-8 Interleukin 8, IL-6 Interleukin 6, IL-1β Interleukin 1 β, TNF-α Tumor Necrosis Factor-alpha, IFN-γ Interferon Gamma, BBM -Brush Border Membrane, IgA-Immunoglobulin A, CDI- *C. difficile* infection, CDD-*C. difficile* disease, AAD Antibiotic-Associated Diarrhea, TD Traveler's diarrhea PC-Placebo controlled, PG- Parallel-group, R- randomized, DB-Double Blind, DM-Double Mask, MC-Multicentre, Ab-antibiotic, NA-No Available, yr-year, mo-month, wk-week, d-day.

	Disease	**Type of Study**	**Number of Target group**	**Dose& Duration**	**Major findings**	**Strength of Evidence**	**References**
In vitro	Disorders caused by secretion of enzymes	Enzyme activity assay	NA	NA	High sucrose activity and very low alkaline phosphatase activity showed	NA	(Buts et al., 1986)
	C. difficile infection by secretion of enzymes	Study in culture media	NA	150 ml of 100 mg/ml& For 3 days	*S. boulardii* decreased the binding of [^{3}H] toxin A to its BBM receptor	Effective **	(Pothoulakis et al., 1993)
	Diarrhea caused by *Enterohemorrhagic Escherichia coli* (EHEC) infection	Study in culture media	x	x	The protective effect on EHEC infection reduced expression of pro-inflammatory cytokines (IL-8, IL-6, IL-1β, TNF-α, and IFN-γ)	Effective **	(Dahan et al., 2003)
	Diarrhea caused by EHEC infection	Study in culture media	x	x	*S. boulardii* reduced in TNF-α and related apoptosis in EHEC infected T84 intestinal epithelial cells	Effective **	(Dalmasso et al., 2006)

(cont. on next page)

Table 3.4 (cont.)

	Diarrhea	Study in culture media	x	x	Protective effects against diarrheal pathogens by reducing the pro-inflammatory response	Effective **	(Fidan et al., 2009)
In vivo	Disorders caused by BBM enzymes and nutrient transporters	An animal study (in rats)	6 rats/ group	1.5 mg for Wistars rats	No change of jejunum BBM, no intracellular or BBM lesions, BBM sucrose, lactase, and maltase activities increased	Effective **	(Buts et al., 1989)
	Disorders caused by BBM enzymes and nutrient transporters	An animal study (in rats)	8 rats/ group	1.5 mg for Wistars rats &14 to 22 days	Content of the jejunum and ileum significantly increased Secretion of a secretory component of immunoglobulins and secretory IgA enhanced	Significantly Effective ****	(Buts et al., 1990)
	Disorders caused by BBM enzymes and nutrient transporters	An animal study (in rats)	8 and 10 rats/group	1 mg	Spermine/spermidine levels in the jejunal mucosa and sucrose and maltase induction increased by *Sb*	Effective **	(Buts et al., 1994)
	CDI	An animal study (in rat and rabbit ileal loop)	x	NA	Removed toxin receptors with protease activity, decreased brush border glycoproteins	Effective **	(Pothoulakis et al., 1993)
	CDI	An animal study (in rat)	x	x	Reduced the *C. difficile* colitis, enhanced the intestinal mucosal immune response.	Effective **	(Castagliuolo et al., 1998)

(cont. on next page)

Table 3.4 (cont.)

Clinical studies	**For Adults**						
	Effect on BBM, enzymes and nutrient transporters	Pre-post comparison	12 human volunteer	750 mg capsules	The activity of lactase, α-glucosidase (glucoamylase), and alkaline phosphatase increased	Effective **	(Jahn et al.,1990)
	Effect on BBM, enzymes and nutrient transporters	Pre-post comparison	7 human volunteer	NA	No alteration in human BBM morphology, BBM sucrase, lactase and maltase activity are increased	Significantly Effective ****	(Buts et al., 1989)
	CDI	PC	69 Elderly patient	113 mg twice daily during Ab	No evidence that *Sb* prevented the appearance of CDI in the stool	Inactive -	(Lewis et al., 1998)
	AAD	DB, PC, PG	193 Adult patient	1g/d & receiving duration of Ab+2 wk	AAD rate is decreased by *Sb*	Effective **	(McFarland et al., 1994)
	AAD caused by *H.pylori*	MC,P,R	389 Adult Patient	500 mg twice daily for 14d	AAD rate was decreased, suggested an effective and safe treatment by administrating *Sb* for prevention of AAD	Medium Effective ***	(Duman et al., 2005)
	H. pylori Infection	P, R, PC	124 Adult patient	14 d TT+Ab for 10 d+1 g/d & for 28 d	A significant decrease in recurrences	Significantly Effective ****	(Cindoruk et al., 2007)
	AAD	R,PC	89 outpatient	$1x10^{10}$ cfu/ml daily+Ab	*Sb* did not prevent diarrhea related to amoxicillin	Inactive -	(Bravo et al., 2009)

(cont. on next page)

Table 3.4 (cont.)

	CDD	P, R, PC	82 Adult patient	1g /d Ab(for 10 d)+P for 28 d)	Recurrence was decreased, no adverse effect was observed	Significantly Effective ****	(Surawicz et al., 2000)
	AAD or CDAD	MC, DM,R,PC, Phase III	477 inpatient	250 mg +Ab & during 6 wk	No evidence for an effect of *Sb* in preventing AAD or CDAD in a population of inpatients without particular risk factors apart from systemic antibiotic treatment, Nine serious adverse events was observed in *Sb* group	Inactive -	(Ehrhardt et al., 2016)
	For Children						
	Sucrose-isomaltase deficiency	NA	8 children	300 mg	Reduced in breath hydrogen excretion and reduction in clinical symptoms e.g. diarrhea	Effective **	(Harms et al., 1987)
	Prevention of AAD caused by *C.difficile*	P, DB	180 children, over 23 mo	NA	No adverse effect observed, decreased the incidence of AAD in hospitalized patients	Medium Effective ***	(Surawicz et al., 1989)
	Acute Diarrhea	DB, PC	130 children,3 mo-3 yr	200 mg/d &3 times	The cure for diarrhea was observed as 85% in *Sb* group	Significantly Effective ****	(Cetina-Sauri et al., 1994)
	Acute Diarrhea	DB, R, PC	40 children,6-36 mo	NA& for 1 month	The recovery was significantly increased, no adverse effect was observed	Significantly Effective ****	(Guillot et al., 1995)
	AAD	DB, R, PC	children	NA&1 y	Duration of diarrhea decreased, recovery observed and risk of prolonged diarrhea decreased	Medium Effective ***	(Villaruel et al., 2000)
	Acute Diarrhea	RCT	101 children 6-60 mo	500 mg/d &for 6 d	The duration of illness was reduced in *Sb* group	Effective **	(Hafeez et al., 2002)

(cont. on next page)

Table 3.4. (cont.)

	AAD	R, DB, PC	269 children 6 mo to 14 yr	250 mg Twice daily &Duration of Ab treatment	*Sb* has effectively decreased the risk of AAD	Effective **	(Katowska et al., 2004)
	AAD	PC	653 children 1-15 year	NA	It was reduced rate of diarrhea by *Sb*	Significantly Effective ****	(Erdeve et al., 2004)
	ADD	R, DB, PC	269 children 6 mo to 14 yr	250 mg	Decreased risk of AAD caused by C. *difficile* no adverse effect observed	Medium Effective ***	(Kotowska et al., 2005)
	Rota-virus infection	DB, PC	200 inpatient children 3 mo-7 yr	250 mg/d &7 day	It was decreased duration of diarrhea and hospitalization by *Sb*	Significantly effective ****	(Kurugöl and Koturoğlu, 2005)
	Diarrhea	R	55 children patient, from 2 mo to 12 yr	250 mg/d &NA	Decreased the frequency and duration of diarrhea, improved the consistency of stool, the probiotic is well-tolerated	Significantly Effective ****	(Billoo et al., 2006)
	Acute Diarrhea	R,DB,PC	88 children 3-24 mo	NA	Duration of diarrhea is significantly reduced	Significantly Effective ****	(Villarruel et al., 2007)
	Acute Diarrhea	P,SB,PC	600 children 3-36 mo	500 mg/d &NA	There is no difference duration of diarrhea in *Sb* and control groups	Inactive -	(Canani et al., 2007)
	Acute Diarrhea	R, DB, PC	188 children 3-33 mo	500 mg/d &NA	The duration of diarrhea significantly was reduced by *Sb*	Significantly Effective ****	(Vandenplas et al., 2007)

(cont. on next page)

Table 3.4 (cont.)

	Acute Gastroenteretis	R, PC	27 children 6 mo-10 yr	250-500 mg/d	Daily stool frequency is reduced, IgA is significantly increased	Significantly Effective ****	(Ozkan et al., 2007)
	AAD	RCT	100 inpatient children 3 mo-10 yr	500 mg/d &NA	The duration of diarrhea was shortened by *Sb*	Medium Effective ***	(Htwe et al., 2008)
	Acute Diarrhea	OP	90 children 1-15 yr	NA+Ab &for 10 days	There was no side effect, no significant difference in the frequency of diarrhea	Inactive -	(Savas-Erdeve et al., 2009)
	Acute Diarrhea	R, P, OL	55 children 5-16 mo	250-500 mg/d	Duration of diarrhea was significantly decreased in *Sb* group	Significantly Effective ****	(Eren et al., 2010)
	Acute Diarrhea	R, DB	64 children 1-23 mo	500 mg/d &NA	Duration of diarrhea was significantly reduced	Significantly Effective ****	(Grandy et al., 2010)
	Acute Diarrhea	P, DB, R	108 children 3-59 mo	500 mg/d	Duration of diarrhea was significantly decreased	Significantly Effective ****	(Riaz et al., 2011)
	Acute Diarrhea	DB, R, PC	176 children 6-48 mo	NA& for 5 days	Duration of diarrhea was significantly decreased	Significantly Effective	(Corrêa et al., 2011)
	Acute Diarrhea	P, O, R	480 children 1-28 mo	250 mg/d&NA	The duration of diarrhea significantly reduced	Significantly Effective ****	(Dalgic et al., 2011)
	AAD	Two-phase, OLS, R	333 children aged 1-5 yr	500 mg/d	The rate of diarrhea was decreased, higher recurrences was observed	Significantly Effective ****	(Shan et al., 2013)

(cont. on next page)

Table 3.4 (cont.)

	AAD	PC	90 children aged 1 to 5 yr	250 mg/d	The frequency and duration of acute watery diarrhea significantly were reduced	Significantly Effective ****	(Azim et al., 2014)
	H.pylori infection	R, PC	194 *H. pylori* positive patient	NA&during 2 wk	It was observed a beneficial effect on the prevention and treatment of diarrhea during *H. pylori* infection	Effective **	(Bin et al., 2015)
	AAD	RCT	200 children aged 6 mo-5 yr	NA&for 5 d	*Sb* has more efficacy compared to LAB	Effective **	(Asmat et al., 2018)
Meta-analyses	Traveler's Diarrhea (TD)	x	5029 study patients	250-1000 mg/day, During of trip, 3wk	Effective in the prevention of TD	Effective **	(McFarland, 2007)
	AAD	9 R, DB, PC	890 children and adults	NA	Significantly effective for prevention of AAD	Significantly effective ****	(D'Souza, 2002)
	AAD	x	5029 study patients	500-1000 mg/d &During Ab with additional, 3 days to 2 weak after	Effective for the prevention of AAD with a daily dose > 10^9 cells	Effective **	(McFarland, 2010)
	AAD	x	4780 participants	x	Effective in reducing the risk of AAD in children and adults.	Effective **	(Szajewska and Kołodziej, 2015)

Table 3.5. Review of Probiotic market in Turkey

Probiotic Product Name	**Manufacturer**	**Strain Name**	**Preparat Form**	**Content (live cells)**	**Ingredients**	**Prebiotic**	**Beneficiary effects given by the manufacturer**	**Proposed use as**
Enterogermina 	Sanofi Drug ind.trade.co. ltd Italy	*Bacillus spores (4 types of B. clausii strains)*	Flacon	$4x10^9$ cfu	Probiotic	x	Prevention and treatment of kinds of diarrhea in children (Elshaghabee et al., 2017) and adults	Dietary supplement
Reflor 	Biocodex ind.trade.co. ltd France	*Saccharomyces boulardii* CNCM I-745®	Lyophilised Capsule and Sachet Form	$2.5x10^9$ cfu (250 mg)	Probiotic	x	*Prevention and cure of Travelers' diarrhea (Mcfarland, 2007) *Preventing and treating of AAD *Prevention and treatment of CID	Medical preparate
SYMBIOSYS Alflorex (Lyseng-Williamson, 2017)	Biocodex ind.trade.co. ltd Italy	*Bifidobacterium infantis* 35624	Capsule Form	$1x10^9$ cfu	Probiotic without gluten	x	Helping to the regulation of the gastrointestinal system	Dietary supplement

(cont. on next page)

Table 3.5 (cont.)

							(especially symptoms of IBS, diarrhea, constipation, abdominal pain and bloating)	
NBL Probiotic Optima for Adults 	Nobel Drug ind. trade. co.ltd Turkey	*Lactobacillus acidophilus, Enterococcus faecium, Lactobacillus plantarum, Bifidobacterium lactis, Streptococcus thermophilus, Bifidobacterium longum*	Encapsulated chewable tablet	1.5x10 ^{9} cfu	Includes prebiotic, probiotic and vitamin C	Fructooligosaccharide and microcrystalline cellulose	*Regulation of digestive system *Strengthening of the immune system	Dietary supplement
NBL Probiotic GOLD for Adults 	Nobel Drug ind.trade. co.ltd Turkey	*Lactobacillus acidophillus, Lactobacillus rhamnosus, Enterococcus faecium, Bifidobacterium bifidum, Bifidobacterium longum*	Encapsulated sachet	2.5x10 ^{9} cfu	Includes prebiotic,probiotic and vitamin (A,B1,B2, B6,E, C)	Fructooligosaccharide and polydextrose	*Regulation of digestive system *Strengthening of the immune system	Dietary supplement

(cont. on next page)

Table 3.5 (cont.)

NBL Probiotic ATP for infant and children 	Nobel Drug ind.trade. co.ltd Turkey	*Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus plantarum, Bifidobacterium lactis*	Sachet	$2x10^9$ cfu	Includes prebiotic, probiotic and vitamin (B1, B2, B6 ve E)	Fructoolig osaccharid e, galactooli gosacchari de, and polydextro se	*Regulation of digestive system *Strengthening of the immune system	Dietary supplement
NBL Gynobiotic for women 	Nobel Drug ind.trade. co.ltd Turkey	*Lactobacillus acidophilus, Lactobacillus rhamnosus*	Capsule Form	$5x10^9$ cfu	Includes prebiotic, probiotic and anti-bacterial bilberry	Fructoolig osaccharid e	NA	Dietary Supplement
NBL Probiotic Travel 	Nobel Drug ind.trade. co.ltd Turkey	*Streptococcus thermophillus, Bifidobacterium lactis, Lactobacillus acidophilus, Lactobacillus plantarum, Bifidobacterium breve*	Chewabl e tablet	$2.3x10^9$	Includes prebiotic, probiotic and pineapple aroma	Fructoolig osaccharid e	NA	Dietary Supplement

(cont. on next page)

Table 3.5 (cont.)

Kaleidon 	Menarini Group Italy	*Lactobacillus rhamnosus* GG	Sachet Form	$6x10^9$ cfu	Probiotic	x	*Regulation of digestive system *Strengthening of the immune system	Dietary supplement
Bakso Age of 2-10 	Sandoz &Novartis Drug Industry and Trade inc. Switzerland	*Bifidobacterium animalis* subsp *lactis* BB-12	Sachet Form	$1x10^9$ cfu	Probiotic	x	*Regulation of digestive system	Dietary supplement
Bakso Age of 4-10 	Sandoz& Novartis Drug Industry and trade inc. Switzerland	*Lactobacillus acidophilus* *Bifidobacterium animalis* subsp. *lactis* BB-12	Capsule Form	$2x10^9$ cfu	Probiotic	x	*Regulation of digestive system	Dietary Supplement
Bakso Age of 2-10 	Sandoz& Novartis Drug Industry and Trade inc. Switzerland	*Bifidobacterium animalis* subsp. lactis BB-12	Gout	$1x10^9$ cfu	Probiotic	x	*Regulation of digestive System	Dietary Supplement

(cont. on next page)

Table 3.5 (cont.)

Gut feel 	ASSOS Pharmaceuticals Turkey	*Lactobacillus bulgaricus, Lactobacillus acidophilus, Bifidobacterium bifidum, Bifidobacterium longum, Bifidobacterium infantis , Streptococcus thermophilus*	Capsule & powder Form	500 mg	Symbiotic	300 mg inulin	*Regulation of intestinal system	Dietary Supplement
Ntbiotic 	ASSOS Pharmaceuticals Turkey	*Lactobacillus bulgaricus, Lactobacillus acidophilus Bifidobacterium bifidum Bifidobacterium longum Bifidobacterium infantis Streptococcus thermophilus*	Capsule Form	x	Symbiotic	Prebiotic (inulin)	*Regulation of intestinal system	Dietary supplement

(cont. on next page)

Table 3.5 (cont.)

Maflor 	Mamsel Drug Industry and Trade inc. Turkey	*Bifidobacterium animalis ssp lactis B94*	Sachet Form	$5x10^9$ cfu 60 mg for each sachet	Probiotic	Prebiotic Chicory inulin Maltodextrin Ascorbic acid	*Prevention of *C. difficile* infection *Prevention of AAD *Prevention of AAD *Prevention of Traveller's diarrhea	Dietarty supplement
Bioflor 	ORZAX Drug and Chemistry Industry and Trade inc. Turkey	*S. boulardii*	Capsule Form	x	Probiotic	x	*Helping of regulation of the digestive system	Dietary supplement
Bigflor 	ORZAX Drug and Chemistry Industry and Trade inc. Turkey	*S. boulardii*	Sachet Form	$3x10^9$ cfu	Symbiotic without gluten	Prebiotic (Inulin)	*Regulation of digestive system	Dietary Supplement
Probien 	RCFARMA	*Lactobacillus acidophilus Bifidobacterium longum Bifidobacterium bifidum*	Herbal capsule form	800 mg	Symbiotic	Prebiotic (Inulin)	*It cannot be used for preventing and treating diseases	Dietary Supplement

(cont. on next page)

Table 3.5 (cont.)

		Lactobacillus plantarum *Lactobacillus bulgaricus* *Lactobacillus rhamnosus* *Lactobacillus paracasai* *Lactobacillus brevis* *Lactobacillus reuteri* *Bifidobacterium infantis* *Streptococcus Thermophilus*						
Sek Probiotic 	Sek Tat Food and Industry inc. Turkey	*Lactobacillus acidipholus* LA-5® *Bifidobacterium* BB-12®	Beverage	2.5x10^{8} cfu	Probiotic with food additive	x	*Regulation of digestive and immune system	Functional Food
Activia 	Danone HQ France	Probiotic yogurt culture	Yogurt	At least 1x10^{6} cfu	Probiotic	x	*Regulation of gastrointestinal tract	Functional Food

3.3. A meta-analysis of *S. boulardii*

Meta-analysis is a statistical technique for combining the findings from independent studies. A meta-analysis of *S. boulardii* was performed according to adult and children.

3.3.1. In Adults

A meta-analysis of sixteen randomized, controlled trials in adults conducted between probiotic-treated and placebo groups illustrated that *S. boulardii* was significantly protective and curative for diarrhea, with a pooled relative risk of (RR) 0.51 (95% confidence interval [0.40-0.64], P<0.0001). Forest plots demonstrate the effect sizes, confidence intervals (CIs) from included clinical studies, and the computed summary effect size. The width of the confidence interval lines depict the effect estimate of each study.

A random-effect model was used since the random effect approach gives better estimates than fixed effect model in medical decisions about therapeutic effectiveness.

In the forest plot, each study is represented by a horizontal line in the left side of the forest plot, with each end of the line displaying the boundaries of the confidence interval.

The right side of graph shows the outcomes of relative risk (RR) with 95% confidence interval. The x-axis or horizontal line indicates relative risk (RR) with 95% CI for each study. The vertical line (dashed line or line of null effect) shows that there is no effect on overall estimate. The solid black squares represent sample size or weight of each study as indicated in Figure 3.1. In other words, the bigger the black squares, the more subjects in the study. At the end of the forest plot, the width of the solid diamond shows confidence interval for the overall effect estimate based on total sample size for treatment and placebo groups. If the study with 95% CI overlaps the vertical line, there is no statistical significance. Only two studies Lewis et al (1998) and Pozzoni et al. (2012), trended towards placebo, however, these studies were not statistically significant.

Heterogeneity in meta-analysis is defined as the variation in study outcomes between studies. In addition, measuring heterogeneity in meta-analysis studies provides

better interpretation of the results. There was a significant heterogeneity among studies. Therefore, heterogeneity in adults (Q) was found to be 18.75. In addition, The I^2 statistic refers to the percentage of variation across studies that is due to heterogeneity instead of chance, and I^2 was found to be 15.33 in adults.

In a systematic review and meta-analysis, McFarland (2010) reported that *S. boulardii* was significantly protective for antibiotic-associated diarrhea (AAD), with a pooled RR of 0.47, (95% confidence interval [0.35-0.63], P<0.001). Another meta-analysis study in adults indicated that *S. boulardii* reduced the risk of AAD from 17.4% to 8.2% (15 randomised controlled trials, n=3114, RR: 0.49, 95% CI: 0.38-0.63 (Szajewska and Kołodziej, 2015)). This meta-analysis highlighted the importance of *S. boulardii* in reducing the risk of antibiotic-associated diarrhea in adults.

Baujat plot is used to identify studies that excessively contribute to heterogeneity and the overall result (Baujat et al., 2002). Each clinical study has been displayed via the study id number. If a study is located in the top right quadrant, then it has the most contribution to the overall result and to study heterogeneity (Quintana, 2015). Therefore, study 14, located in the top right quadrant, contributes the most on the overall result and heterogeneity (Figure 3.2).

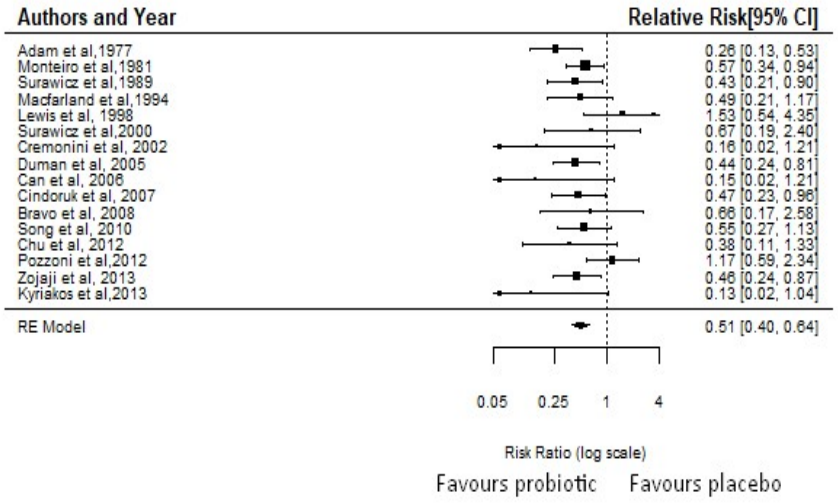


Figure 3.1. Forest plot of meta-analysis of 16 randomized controlled trials based on treatment with *S. boulardii* or placebo for diarrhea in adults. The x-axis depicts effect size, with solid black squares denoting the relative risk and the line indicating 95% CI. The size of the black squares is proportional to study size. (I^2= 15.33%, P<0.0001 ***)

Funnel plots are drawn to visualize potential publication bias in meta-analyses. These plots show the individual effects sizes on the horizontal axis and standard errors on the vertical axis.

Figure 3.2. Baujat plot to identify studies contributing to heterogeneity for *S. boulardii* meta-analysis in adults, The X-axis and Y-axis show the contribution probiotic-treated group and influencing control group of the trial on the overall heterogeneity, respectively.

The standard deviation of sampling distribution (standard error) is proportional to Log Relative Risk (RR). As sample size increases, its precision increases. Each study is demonstrated by a single dot. The vertical line shows the mean effect size on the overall result. The two diagonal lines show 95% confidence interval. Figure 3.3 shows that the study points are equivalently spread on both sides of the vertical summary line. A non-significant Egger's regression test (p = 0.2505) also suggests no publication bias.

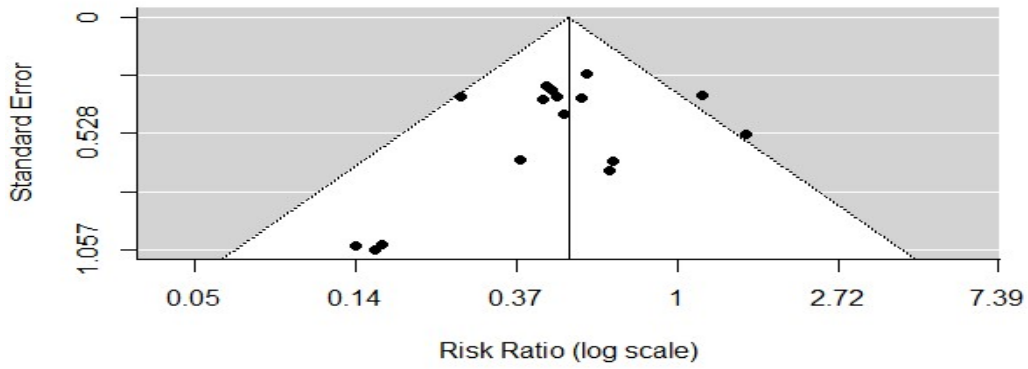


Figure 3.3. Funnel plot to indicate publication bias by using mixed-effects meta-regression model

3.3.2. In children

A meta-analysis of the seventeen randomized, controlled trials in children found that *S. boulardii* was significantly protective and curative for diarrhea, with a pooled relative risk of (RR) 0.55 (95% confidence interval [0.42-0.72], P<0.0001. This result is in agreement with a meta-analysis study of 6 randomized controlled trials with 1653 children patients, which showed that *S. boulardii* decreased the diarrhea risk from 20.9% to 8.8% with RR: 0.43, 95% CI: 0.3-0.6 (Szajewska and Kołodziej, 2015). However, a clinical study by Htwe et al. (2008), demonstrated an increased risk of diarrhea in children with a RR of 3.17, bigger than 1, hence this result was significantly different than other studies.

Notably Billoo et al. (2006) and Casem et al. (2013), did not report a significant effect of *S. boulardii on* diarrhea in children as indicated by the confidence interval lines crossing the null effect vertical line. On the other hand, two ends of horizontal lines of the other studies are located on the left side, meaning that these studies were statistically significant and did clearly favour that treating with *S. boulardii* resulted in lower risk (Figure 3.4).

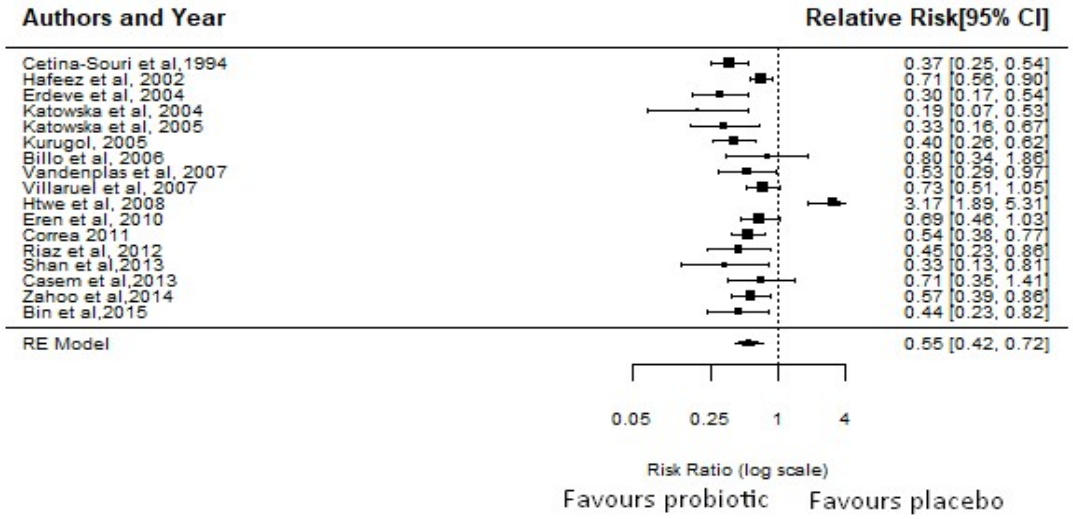


Figure 3.4. Forest plot of meta-analysis of 17 randomized controlled trials based on treatment with *S. boulardii* or placebo for diarrhea in children. The x-axis represents effect size, with solid black squares denoting the relative risk and the line indicating 95% CI. The size of the black squares is proportional to study size (I^2 = 81.31 %, P<0.0001 ***)

Altogether, the black diamond located at the bottom of the forest graph represents heterogeneity on overall study. Heterogeneity (Q) was found to be 70.17 and I^2 was equal to 81.31%. This basically summarizes all of the evidence in probiotic and placebo group. Hence, the overall results favouring use of probiotics is reliable. This is evidenced by the location of solid black diamond that refers to the average value in forest plot and the fact that are both ends are located on the left of the centreline also demonstrate that this meta-analysis in children confirms that there is preventive effects of using *S. boulardii* probiotic in diarrhea.

Study 10 (Htwe et al., 2008) , which favors placebo, was located in the top right quadrant, as demonstrated in Figure 3.5. This study has the most influence on the overall result and heterogeneity.

Figure 3.5. Baujat plot to identify studies contributing to heterogeneity for *S. boulardii* meta-analysis in children, the x-axis and y-axis show the contribution probiotic-treated group and influencing control group of the trial on the overall heterogeneity, respectively.

Figure 3.6 demonstrates that the study points in children are equivalently spread on both sides of the vertical summary line. Similar to meta-analysis outcomes in adults, a non-significant Egger's regression test (p = 0.1841) also suggests no publication bias.

Consequently, results of these meta-analyses confirm the preventive and curative effect of administration of *S. boulardii* on the incidence of different diarrhea in adult and children.

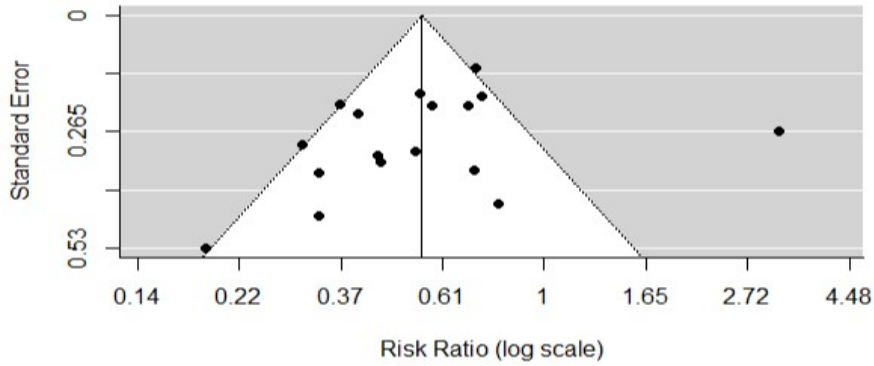


Figure 3.6. Illustration of Funnel plot in children to indicate publication bias by using mixed-effects meta-regression model

3.4. Contribution to the development of the ProBioYeastract Database

As the first stage of this study, ProBioYeastract Database was built, using the recently disclosed genome sequences of *S. boulardii* Biocodex and Unique 28 strains. The contribution of this thesis to the database was the definition of the steps underlying the "Cross-strain Comparision" query.

As the beginning of the study, the Table of Cross-strain comparison of S. *boulardii* Biocodex, Unique 28 and S288C was built manually (Table 3.6), using individual queries available at the YEASTRACT and ProBioYeastract databases, as described section 2.3. in the Methodology chapter. The establishment of the sequencial steps required to reach this final table was done as an iterative process. The "Cross-strain Comparison" query allows the user to search for the Transcription Factors (TFs), predicted to be involved in the regulation of *S. boulardii* Biocodex and Unique 28 genes, but not in the homologous genes in *Sc* S288C, based on the occurrence of *Sc* TFs whose consensus binding site matches a subsequence of the promoter region of the genes. In the ProBioYeastract database, the input required is the names of ORF, so as to reach the cross-species comparison of *S. boulardii* Biocodex, Unique 28 and S288C.

3.5. New clues on the probiotic activity of *Sb*, when compared to *Sc*

Cross-strain promoter comparison of putative probiotic gene regulation and differential gene expression of selected genes were performed when compared to *Sc.*

3.5.1. Cross-strain promotor comparison of putative probiotic gene regulation

The obtained results from Cross-strain promotor comparison as shown in Table 3.6, aiming to find probiotic-related genes, collected from the literature (Table 1.2), whose regulation in *Sb* is different from that in *Sc*. While performing this analysis, it was observed that some genes have two copies in *Sb*, but only one in *Sc*, namely *FLO5*, *CAR1*, and *PRO1* (in blue color in Table 3.6).

Table 3.6 highlights the genes whose promoters share TF binding sites in *Sb* Biocodex and Unique 28 strains that do not exist in *Sc* S288C. Those 26 genes (out of the 83 analysed) are, thus, predicted to be differentially regulated in the *Sb* vs *Sc* strains. If this is the case, their differential expression may contribute to the observed probiotic activity of *Sb* strains, which is not present in *S. cerevisiae.*

3.5.2. Differential gene expression of selected genes: *Sb* vs *Sc*

To evaluate if the observed differences in the *Sb* and *Sc* gene promoter regions result in differences at the level of gene expression, the transcript levels of 6 selected genes, representative of the various mechanisms of probiotic activity exhibited by *S. boulardii*, was measured through RT-PCR.

Gene expression was assessed in exponentially growing cell, cultivated in YPD medium, and YPD with sodium cholate, which mimics, to some extent, human intestinal environmental conditions.

The expression of each selected *Sb* gene was analyzed by RT-PCR, and compared to the corresponding homolog in *S. cerevisiae* (used as a reference), in triplicate.The expression of four genes, *FLO5*, *TGL4*, *YDC1,* and *SPE2*, was found to be down-regulated in *Sb* cells, when compared to *Sc*, while two genes, *EFG1* and *IMA1*, were found to be up-regulated in *Sb* vs *Sc*, in cells cultivated in YPD medium (Figure 3.7).

In YPD supplemented with cholate, the results were similar, with the exception of *IMA1*, whose up-regulation was not observed (Figure 3.8).

Table 3.6. Design of Cross-strain promoter comparison table to help the building of ProBioYeastract Database

Predicted Gene Names	**Unique to *Sc***	**Unique to *Sb* Biocodex**	**Unique to *Sb* Unique28**	**Unique to both *Sc* and active *Sc***	**Unique both Biocodex and Unique28**
Genes Related to Anti-toxin probiotic effect of *S. boulardii*, preventing or treating diarrhea and colitis caused by *C. difficile* infection					
PCR1	x	x	Gcn4p,lys14p,Rgt1p,Rgt3p, Rtg1p,Rtg3pSkn7p,Yap1p, Yap3p,Abf1p,Bas1p,Hsf1p, Mcm1p,Ste12pAce2p,Swi5 p, Ash1p,Rgt1p, Mcm1p	x	x
GLN3	x	x	Azf1p,Ime1p, Mcm1p, Msn2p,Msn4pNrg1p,Rph1p Pho4p,Rlm1pYrr1p, Tda9p Ace2p, Swi5p, Hap2p Hap3p Hap4p, Hap5p	x	x
GAT1	x	Adr1p,Hsf1p, Sfl1p, Ste12p	Pho4p	x	Rlm1p
RRT12	x	x	The gene has no in Unique28	x	There is no comparison due to absence of gene in Unique 28
YSP3	x	x	x	x	x
Predicted genes related to anti-toxin effect of *S. boulardii* , preventing or treating diarrhea caused by *E.coli*					
PHO8	x	x	Aft2p, Aft1p, Bas1p, Gcn4p,Hsf1p,Ime1p,Mcm1 p, Pdr8p,Rtg3p,Yap3p, Ace2p, Swi5p	x	x

(cont. on next page)

Table 3.6 (cont.)

PRP3	x	x	Crz1p, Cup2p, Hap2p, Hap3p, Hap4p, Hap5p, Gis1p, Ime1p, Msn2p	x	x
JIP4	x	x	Aft2p, Aft1p, Hsf1p, Mcm1p, Sfl1p, Ste12p, Yrr1p, Ace2p, Swi5p, Cup2p	x	x
SNF1	Azf1p, Mcm1p Ecm22p, Upc2p, Uga3p, Com2p	Aft2p,Aft1p,Bas1p, Gcn4p,Cup2p,Gln3p,Gcn4p,Skn7p Ndt80p,Sum1p, Stp1p, Stp2p	Cat8p, Sip4p, Cbf1p, Met31p, Met32p,Swi4p, Ace2p, Swi5p	Upc2p	Gat1p, Gln3p, Gzf3p
SNM1	Adr1p, Rim101p	Ace2p,Swi5p,Azf1p,Crz1p,Gat1p, Gln3p, Gzf3p,Mcm1p, Stp2p,Xbp1p, Yrr1p, Haa1p,Gis1p,Msn2p, Msn4p,Rph1p, YER130C,Nrg1p,Rp1p	x	x	x
PEX29	Hcm1p	Crz1p,Ndt80p, Sum1p	Ace2p, Swi5p, Adr1p, Lys14p, Mbp1p, Mcm1p, Rlm1p, Yap1p	x	Cup2p, Hac1p, Tec1p, Xbp1p
CWC2	x	x	Bas1p, Gcn4p, Cbf1p, Crz1p, Fkh1p, Fkh2p, Gcn4p, Hac1p, Hsf1p, Pdr8p, Pho4p, Skn7p, Yap1p, Yap3p, Yrr1p, Tda9p	x	Gat1p, Gln3p, Gzf3p
VPS52	x	Bas1p, Gcn4p, Cup2p, Gat1p, Gln3p, Gzf3p,	x	x	x

(cont. on next page)

Table 3.6 (cont.)

		Gis1p, Msn2p, Msn4p, Rph1p,YER130C, Ste12p, Stp1p, Stp2p, Xbp1p, Yap1p, Com2p			
VPS72	x	x	Adr1p, Bas1p, Gcn4p, Cbf1p, Gat1p, Gln3p, Gzf3p, Hsf1p, Pdr8p, Pho4p, Sfl1p, Yap1p, Hot1p	x	x
RIB3	x	x	Azf1p, Gat1p, Gln3p, Gzf3p,Stp1p, Rim101p	x	x
PAC11	x	x	Ace2p, Swi5p, Adr1p, Mac1p, Yrr1p, Haa1p	x	x
Predicted genes related to Anti-toxin effect of *S. boulardii*, preventing or treating AAD related to pathogen infection					
FLO5	Aft2p, Aft1p, Arg80p, Gcn4p,Mac1p, Rlm1p, Sfl1p	Cst6p, Ime1p, Sko1p, Upc2p, Cad1p, Yap3p, Cin5p, Yap5p, Yrr1p, Rim101p	Haa1p, Gsm1p, Skn7p, Pho4p, Gis1p, Msn2p, Msn4p, Rph1p, YER130C, Crz1p	Aft1p	x
FLO5	Aft2p, Aft1p, Arg80p, Gat1p, Gln3p, Gzf3p, Hsf1p,Mac1p, Rlm1p, Sfl1p	Crz1p,Gis1p,Msn2p, Msn4p,Rph1p, YER130C,Met31p, Met32p,Skn7p, Gsm1p, Haa1p	Yap3p, Rpn4p, Cbf1p, Bas1p	Aft1p	Adr1p Pho4p
FLO8	x	Bas1p, Gcn4p, Crz1p, Gis1p,Msn2p, Msn4p,Rph1p, YER130C,Hsf1p, Mac1p,Msn2p, Msn4p,Nrg1p, Rph1p,Rlm1p,	x	x	x

(cont. on next page)

Table 3.6 (cont.)

		Rpn4p,Skn7p, Tda9p, Com2p			
FLO9	x	Gis1p,Msn2p,Msn4p,Rph1p,YER130C,Gln3p, Hac1p,Pho4p, Skn7p,Gsm1p,Ace2, Swi5p	x	x	x
FLO10	x	x	x	x	x
FLO11	Gsm1p	x	Ace2p,Swi5p,Bas1p, Gcn4p, Cup2p, Hac1p	x	x
FIG2	x	Azf1p, Crz1p, Hap2p, Hap3p,Hap4p,Hap5p,Mcm1p,Ndt80p,Sum1p, Tda9p	x	x	x
EFG1	Ace2p,Swi5p, Azf1p,Gat1p, Gln3p, Gzf3p,Hac1p, Ste12p,Haa1p, Com2p	x	x	Ace2p	Ecm22p,Upc2p, Hsf1p,Lys14p, Tec1p
SAG1	x	x	Cst6p, Met31p, Met32p, Rlm1p, Swi4p, Rim101p, Aca1p, Cst6p, Hac1p, Sko1p, Hap2p, Hap3p, Hap4p, Hap5p, Skn7p	x	x
Predicted genes related to the antimicrobial effect of *S. boulardii*, preventing and treating IBD					
ACC1	x	x	Cbf1p,Hac1p,Mcm1p, Met4p,Pho4p,Sfl1p,Skn7p, Xbp1p,Yap1p,Haa1p	x	x
HFA1	Rlm1p	Msn2p,Msn4p,Nrg1pRph1p,Gsm1p,Hap2,Hap3p,Hap4p,Hap5p,	Adr1p, Bas1p, Gcn4p	Ace2p	Gat1p,Gln3p, Gzf3p

(cont. on next page)

Table 3.6 (cont.)

		Gis1p,Rph1p, YER130C			
Predicted genes related to the trophic effect of *S. boulardii*, preventing of Lactose Intolerance					
MIG1	Azf1p,Cat8p,Sip4p, Hac1p,Gat1p, Gln3p,Gzf3p,Skn7p, Gsm1p	x	x	x	Bas1p,Crz1p, Ime1p,Mbp1p, Nrg1p,Rtg1p, Rtg3p,Stp2p, Tec1p
PGM1	Azf1p, Cst6p, Gat1p, Gln3p,Gzf3p, Hsf1p, Ste12p	x	x	Ste12p	Cup2p,Met4p, Nrg1p,Upc2p, Tda9p
CYC8	Rgt1p	Adr1p, Crz1p, Hsf1p, Mcm1p,Nrg1p, Rme1p, Tec1p	x	x	x
GAL7	x	Cbf1p,Gcn4p, Pho4p, Sfl1p, Skn7p, Ste12p Xbp1p,	x	x	x
PGM2	Pho4p	Ace2p,Swi5p,Cup2p, Gat1p, Gln3p, Gzf3p, Hap2p,Hap3p,Hap4p, Hap5p,Hsf1p, Ime1p, Ndt80p,Sum1p,Nrg1p,S kn7p, Gsm1p Ino2p, Ino4p	x	x	x
TUP1	Rtg1p, Rtg3p	x	Xbp1p, Mbp1p, Hac1p, Ace2p, Swi5p, Yap1p, Mcm1p, Gln3p	x	x
GAL1	x	x	Adr1p, Gcn4p, Mac1p, Ndt80p, Sum1p, Skn7p, Xbp1p, Yrr1p, Gsm1p, Usv1p, Bas1p, Gcn4p,	x	x

(cont. on next page)

Table 3.6 (cont.)

			Hap2p, Hap3p, Hap4p, Hap5p		
GAL10	x	Cup2p,Hac1p, Mcm1p,Ste12p, Ace2p, Swi5p	x	x	x
GAL2	Azf1p,Cbf1p, Gat1p,Gln3p,Gzf3p, Gis1p,Msn2p,Msn4p, Rph1p,YER130C, Gln3p,Hsf1p,Met4p,Msn2p,Msn4p, Nrg1p,Rph1p, Com2p, Usv1p, Rgt1p, Tec1p, Haa1p, Cup2p	x	x	Met4p, Cbf1p Cup2p	Ace2p,Swi5p, Bas1p,Gcn4p, Hac1p,Hap2p, Hap3p,Hap4, Hap5p,Mcm1p, Skn7p,Yap1p, Yap3p,Sko1p, Ste12p, Stp2p
GAL4	x	x	x	x	x
GAL80	x	x	Adr1p, Hsf1p, Pho4p, Rpn4p, Skn7p, Bas1p, Gcn4p	x	x
GAL3	x	Adr1p,Hac1p, Hap2p,Hap3p, Hap4p,Hap5p, Mac1p, Mcm1p	Gcn4p,Ste12p	x	x
Predicted genes promoters related to the trophic effect of *S.boulardii*, preventing and treating Obesity and Type II diabetes					
TGL2	x	x	Adr1p,Cat8p, Sip4p, Nrg1p, Rlm1p	x	x
TGL3	x	Adr1p,Ndt80p, Sum1p,Sfl1p, Ste12p,Tec1p, Uga3p, Bas1p, Gcn4p	Hac1p, Ime1p, Msn2p, Msn4p, Nrg1p, Rph1p, Pho4p	x	x

(cont. on next page)

Table 3.6 (cont.)

TGL4	Adr1p, Azf1p, Cup2p, Ecm22p, Upc2p, Gcn4p, Lys14p, Rgt1p, Rlm1p, Upc2p, Gsm1p, Ste12p	x	x	Ste12p	Ace2p,Swi5p, Gln3p, Sfl1p
TGL5	Gis1p, Msn2p, Msn4p, Rph1p, YER130C, Msn2p, Msn4p, Nrg1p, Rph1p, Pho4p, Mac1p, Mbp1p, Rgt1p, Rlm1p, Ste12p, Stp2p, Swi4p, Gsm1p, Com2p	x	x	Msn2p, Ste12p Swi4p	Adr1p, Azf1p, Ecm22p, Upc2p, Gcn4p, Gln3p, Hsf1p, Met31p, Met32p, Ndt80p, Sum1p, Nrg1p, Stp1p, Stp2p, Tec1p, Upc2p, Yrr1p, Rim101p
AYR1	x	Cbf1p, Hac1p, Ste12p, Rim101p, Haa1p, Pho4p	x	x	x
TGL1	Tec1p	x	x	x	Ime1p,Mcm1p Nrg1p,
YJU3	x	x	x	x	x
YPC1	x	Cbf1p	Adr1p,Azf1p, Crz1p, Gat1p, Gln3p,Gzf3p,Mcm1p, Nrg1p,Rlm1p,Stp2p, Swi4p, Gsm1p, Aft2p, Aft1p, Hsf1p	x	x

(cont. on next page)

Table 3.6 (cont.)

YDC1	Crz1p, Stp2p, Xbp1p, Haa1p, Bas1p	x	x	x	Cbf1p
Predicted genes related to prevention of tight junction distribution of *S. boulardii,* treating diseases of Irritable bowel syndrome (IBS), gluten intolerance, gastroenteritis, and *H. pylori* infections					
STE11	Cup2p, Hac1p, Mcm1p, Met31p, Met32p, Pho4p, Rgt1p, Rlm1p	x	x	Met31p	Adr1p, Hap2p, Hap3p, Hap4p, Hap5p,Hsf1p, Lys14p, Upc2p, Xbp1p, Com2p, Bas1p, Gcn4p
STE7	x	Ace2p, Swi5p, Aft2p, Aft1p, Cbf1p, Crz1p, Hap2p, Hap3p, Hap4p, Hap5p, Met4p, Stp2p, Yrr1p	x	x	x
FUS3	x	x	Aft2p, Aft1p, Crz1p, Gat1p, Gln3p, Gzf3p, Fkh1p, Fkh2p, Hac1p, Swi4p, Yrr1p, Xbp1p	Msn2p	Gcn4p
KSS1	Adr1p, Gcn4p, Gcr1p, Gis1p, Msn2p, Msn4p, Rph1p, YER130C, Ndt80p, Sum1p, Rlm1p	x	x	Msn2p, Msn4p, Rph1p	Yrr1p
SSK2	x	x	Adr1p, Hsf1p, Mac1p, Mbp1p, Rlm1p, Skn7p	x	x
PBS2	Haa1p	x	Adr1p, Aft2p, Aft1p, Crz1p, Gat1p,Gln3p, Gzf3p, Gcr1p,	x	x

(cont. on next page)

Table 3.6 (cont.)

			Hac1p,Mac1p,Stp1p, Stp2p,Tec1p,Upc2p, Ste12p		
HOG1	Ecm22p, Upc2p, Gln3p, Ndt80p, Sum1p, Rlm1p, Upc2p, Zap1p Skn7p, Adr1p	x	x	Skn7p Rlm1p	Crz1p, Lys14p, Mac1p, Mcm1p, Met31p,Me32p Pdr1p,Pdr3p, Pho4p,Ste12p, Rim101p, Hot1p
BCK1	Gis1p, Msn2p, Msn4p, Rph1p,YER130C, Rlm1p,Skn7p, Ste12p, Yrr1p, Tda9p	Crz1p,Cup2p, Hac1p, Hsf1p, Ime1p, Mcm1p, Mot3p, Stp1p, Stp2p, Xbp1p	The gene has no in Unique28	Msn2p	There is no comparison due to absence of gene in Unique 28
SMK1	Azf1p, Gat1p, Gln3p, Gzf3p, Mac1p, Skn7p, Stp1p, Stp2p, Swi4p, Ume6p,Upc2p, Com2p,Ndt80p, Sum1p, Aft2p, Aft1p, Ace2p, Swi5p	x	x	Fkh2p, Ndt80p, Sum1p, Ume6p	Gln3p, Rim101p, Pho4p, Bas1p, Crz1p
Predicted genes promoters related to increasing immune defense in the gut by *S. boulardii*, aiming to treat or prevent allergic diseases					
SPE2	Abf1p, Adr1p, Azf1p, Gln3p, Ime1p, Pdr1p, Pdr3p, Pdr8p, Yrr1p	x	x	x	Cbf1p, Crz1p,Cup2p, Gis1p,Msn2p, Msn4p,Rph1p, YER130CHac1p,Ph o4p, Ste12p, Yap1p, Bas1p, Cst6p
SPE3	x	x	x	x	x

(cont. on next page)

Table 3.6 (cont.)

CAR1	Bas1p,Gcn4p, Ecm22p,Upc2p, Mac1p,Rlm1p, Ste12p,Ume6p, Upc2p,Xbp1p,Yrr1p Gsm1p,Yap3 Gis1p,Rph1p, YER130C, Mcm1p	x	x	Gcn4p, Ume6p	Ace2p,Swi5p, Azf1p,Crz1p, Pho4p,Hac1p
CAR1	x	x	x	x	x
CAR2	Azf1p, Cup2p, Bas1p, Gcn4p, Mcm1p,Gis1p, Msn2p, Msn4p, Rph1p, YER130C, Rph1p	x	x	Msn2p, Mcm1p	Aft2p, Aft1p, Hsf1p,Gcr1p, Skn7p
PUT2	x	Adr1p,Arg80p, Bas1p, Gcn4p,Crz1p, Cst6p,Cup2p, Hac1p, Mcm1p,Sko1p, Uga3pHap2p, Hap3p, Hap4p, Hap5p, Yrr1p	x	x	x
PUT1	Adr1p, Azf1p, Cbf1p, Gat1p, Gzf3p, Gcn4p, Mac1p, Met4p, Pho4p, Rgt1p, Stp2p, Yrr1p, Gsm1p, Haa1p	x	x	Cbf1p, Gat1, Gln3p, Gcn4p, Met4p, Stp2p	Rim101p, Ste12p,Tec1p Met31p, Met32p Hsf1p, Hac1p, Gcr1p,Yap1p, Cad1p,Yap3p, Cin5p, Yap5p
PRO1	x	x	Ecm22p, Upc2p, Ime1p,Yap1p,	x	x

(cont. on next page)

Table 3.6 (cont.)

			Yrr1p		
PRO1	Usv1p,Stp1p, Stp2p, Sfl1p,Mcm1p, Hsf1p,Gat1p, Gzf3p, Adr1p, Ace2p, Swi5p	Crz1p,Mac1p, Mbp1p,Pdr1p, Pdr3p, Rox1p, Gsm1p	Com2p, Upc2p, Swi4p, Pho4p, Met4p, Ecm22p, Cbf1p, Aft2p, Aft1p	Ace2p	Gis1p, Msn2p, Msn4p, Rph1p, YER130C, Nrg1p, Rph1p, Ume6p
PRO2	Hcm1p, Mbp1p, Mcm1p,Ndt80p, Sum1p,Swi4p, Upc2p, Rim101p	x	x	Gcn4p	Gat1p, Gln3p, Gzf3p, Gis1p, Msn2p, Msn4p, Rph1p, YER130C, Nrg1p, Rph1p, Hot1p,Ace2p, Swi5p,Ste12p
PRO3	x	x	Adr1p, Cat8p, Sip4p, Cbf1p, Pho4p, Ste12p, Stp2p, Yrr1p, Rim101p, Haa1p	x	x
Predicted gene promoters related to immunomodulation effect of *S. boulardii*, aiming to treat or prevent gluten intolerances and celiac diseases					
IMA1	Swi4p, Aft2p, Aft1p, Azf1p, Cbf1p,Crz1p Cup2p,Dal81p, Dal82p, Pho4p,Cad1p, Yap3p,Cin5p, Yap5p	x	x	Cin5p	Rim101p, Upc2p, Ste12p, Skn7p, Msn2p, Msn4p, Nrg1p, Rph1p,Hac1p, Gcr1p,Ecm22p, Adr1p,Yrr1p, Gcn4p

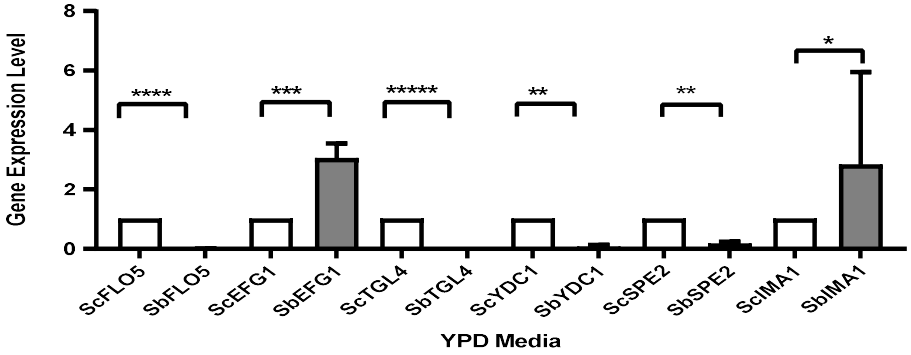


Figure 3.7.Distribution of gene expression level of selected genes by using YPD medium in *Sb* and *Sc* was taken into account as a reference value, identified by RT-PCR analysis to be related to the regulation of genes. The genes found as *EFG1* and IMA1 (up-regulated) and *FLO5*, *TGL4*, *YDC1* and *SPE2* (down-regulated). Error bars represent the corresponding standard deviations. ***** P<0,00001; **** P<0,0001, ***P<0.001; ** P<0,01; * P<0,05.

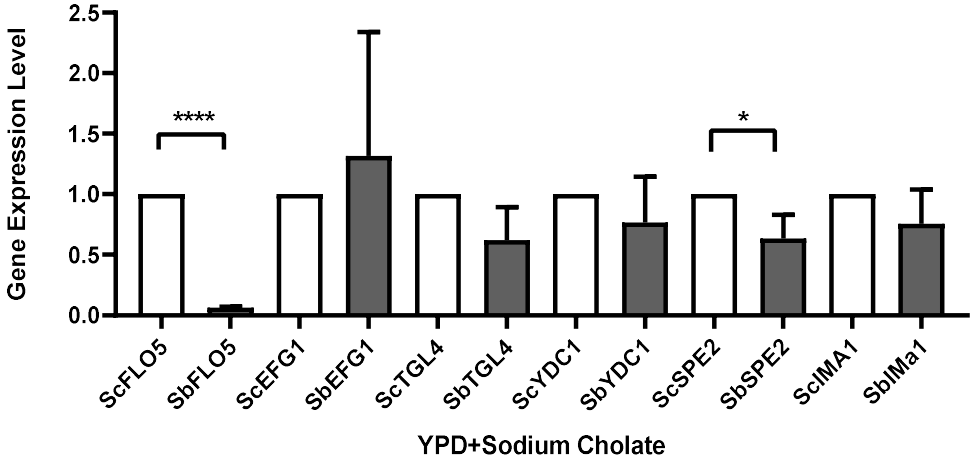


Figure 3.8. Distribution of gene expression level of selected genes by using YPD+Sodium cholate that mimics human gastrointestinal system, in *Sb*, and *Sc* was taken into account as a reference value, identified by RT-PCR analysis to be related to the regulation of genes. The genes found as *EFG1* (up-regulated) and *FLO5*, *TGL4*, *YDC1* and *SPE2* (down-regulated). Error bars represent the corresponding standard deviations. **** P<0,0001;* P<0,05.

Considering the down-regulated genes, it is reasonable to hypothesize that it is not their activity that makes *S. boulardii* a probiotic organism, when compared to *S. cerevisiae*. This appears to be the case for *FLO5*, that contributes to flocculation and adhesion in *S. cerevisiae* (Govender et al., 2008), *TGL4*, that contributes to lipid degradation (Fietto et al., 2004; Rajakumari and Daum, 2010), *YDC1*, that encodes a dehydroceramide hydrolase, involved in sphingolipid degradation (Vandenbosch et al.,

2013) and *SPE2*, involved in the synthesis of polyamines (Balasundaram et al., 1994).

Considering the up-regulated genes, *EFG1* and *IMA1*, their activity may indeed contribute to the probiotic phenotype of *Sb*. Interestingly, Vandenbosch et al. (2013) reported the decreased of biofilm formation upon the deletion of *EFG1* in S288C, suggesting that it plays a role in this process, which is known to be important for the probiotic activity of *Sb*. In *S. cerevisiae*, Efg1 is a protein required for maturation of 18S rRNA, so its link to biofilm formation is likely indirect, through the control of the expression of biofilm related proteins. This hypothesis, of course, requires further confirmation.

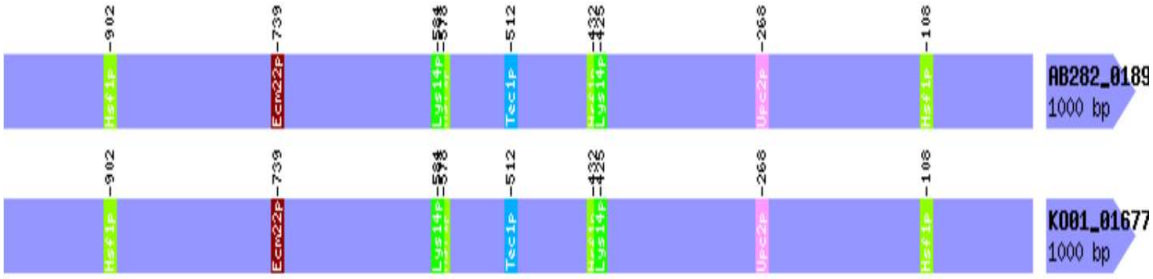


Figure 3.9. Distribution of the putative TF binding sites in the promoter regions of the *EFG1* genes in *S. boulardii* Biocodex (ORF KO01_01677) and Unique28 (ORF AB282_01893) strains, as obtained in the "Search TF" query of the ProBioYeastract database.

Interestingly, when we look at the promoters of the *EFG1* genes in *S. boulardii* Biocodex and Unique28 strains, they share the precise locus for the binding of the TFs that are displayed in Figure 3.9. These transcription factors binding sites exist only in the promoter of the *Sb EFG1* genes, but not in the promoter of the *Sc EFG1* gene, suggesting that at least one of them controls the differential expression of these genes in *Sb* strains, compared to *Sc*. Among these TFs there are two controlling sterol biosynthesis, Ecm22 and Upc2, one regulating lysin biosynthesis, Lys14, one controlling the heat shock response, Hsf1, and one involved in filamentation and biofilm formation, Tec1.

IMA1, on the other hand, encodes a major isomaltase in *Sc* and *Sb*, whose activity may be very important in the fight against gluten intolerance and celiac diseases. However, data presented for *IMA1* gene (with high standard error) in this study does not provide a statistically significant result, and thus, this experiment should be repeated (Figure 3.10).

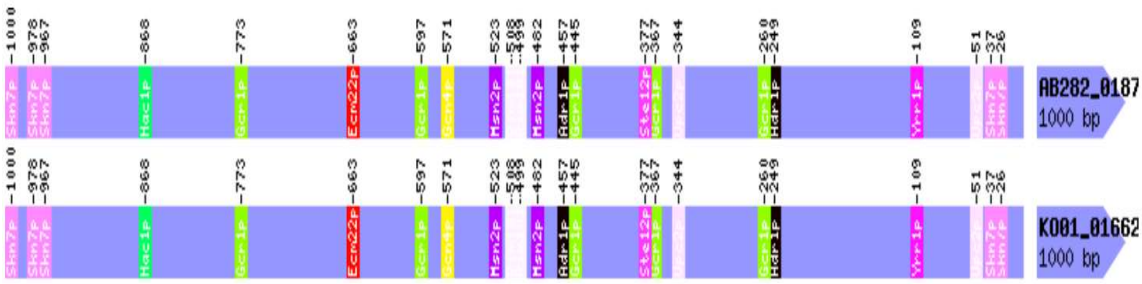


Figure 3.10. Distribution of the putative TF binding sites in the promoter regions of the *IMA1* genes in *S. boulardii* Biocodex (ORF KO01_01662) and Unique28 (ORF AB282_01878) strains, as obtained in the "Search TF" query of the ProBioYeastract database.

Interestingly, when we look at the promoters of the *IMA1* genes in *S. boulardii* Biocodex and Unique28 strains, they share the precise locus for the binding of the TFs that are displayed in Figure 3.10. These transcription factors binding sites exist only in the promoter of the *Sb IMA1* genes, but not in the promoter of the *Sc IMA1* gene, suggesting that at least one of them controls the differential expression of these genes in *Sb* strains, compared to *Sc*. Among these TFs there are eight controlling stress response, Msn2, Msn4, Skn7, Rim101, Yrr1, Hac1, Gcn4 and Rph1, three related to the control of glucose repression/derepression, Nrg1, Adr1 and Gcr1, two controlling sterol biosynthesis, Ecm22 and Upc2, and one involved in filamentation and mating, Ste12. Since *IMA1* encodes an isomaltase the glucose related transcription factors may be particularly relevant.

In general, it is possible to conclude that the expression of selected genes is indeed different in *Sb*, when compared to *Sc*, confirming the promoter analysis outcome. It also shows that the expression of these genes is different depending on the growth media used, which suggest that further experiments should be conducted in media that more faithfully mimics the gastrointestinal tract (Fietto et al., 2004).

3.6. *Sb* exhibits higher aggregation, adhesion to human epithelial cells and biofilm formation than *Sc*

Given the importance of adhesion in the probiotic activity of *S. boulardii*, and the indication that the expression of *EFG1*, related to biofilm formation, is higher in *Sb*, when compared to non-probiotic *Sc* strains, we decide to test if *Sb* Biocodex displays higher

ability than *Sc* to aggregate, adhere to human epithelial cells and form biofilm.

The obtained results show that *S. boulardii* has the ability to aggregate more frequently than *S. cerevisiae* (Figure 3.11). Based on bright-field microscopy, it was possible to assess the percentage of cells that we found as aggregates, versus the total number of cells per image. *S. boulardii* was found to display higher levels of cell-to-cell aggregation (55.6 %) when compared to *S. cerevisiae* (36.5 %).

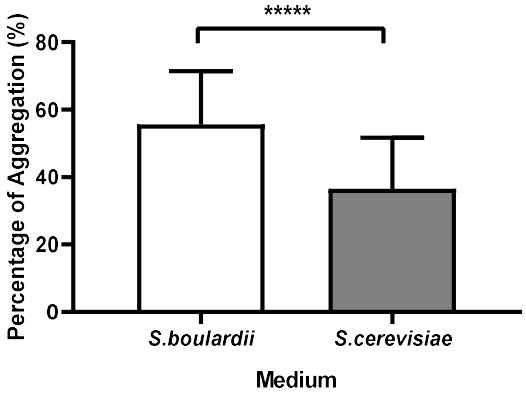


Figure 3.11. The percentage of aggregation in *Sb* and *Sc* cells, *Sb* is formed more aggregation when grown in YPD medium, compared to *Sc* under the same conditions. Standard deviation being represented by the error bars. ***** P<0,00001.

The ability of *S. boulardii* cells to adhere to human epithelial cells was also analyzed, and compared to that of *S. cerevisiae* (Figure 3.12). It was found that indeed the percentage of adhering *S. boulardii* cells (74.3 %) is much bigger that that of *S. cerevisiae* cells (16 %).

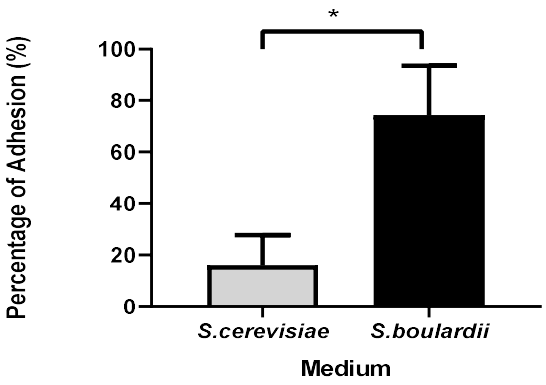


Figure 3.12. Percentage of Adhesion in *Sb* and *Sc* cells, *Sb* is formed more adhesion when grown in YPD medium, compared to *Sc* under the same conditions. Error bar represents the corresponding standard deviation, * P<0,05.

Finally, biofilm formation in polystyrene surfaces by *S. boulardii* and *S. cerevisiae* was evaluated, using the PrestoBlue cell viability assay in four growth media: YPD, SDB and RPMI pH 4 and RPMI pH 7. Except for cells growing in YPD medium, in all cases *S. boulardii* cells were found to form larger biofilms than *S. cerevisiae* cells (Figure 3.13). Interestingly, the difference was found to be particularly strong in RPMI medium, which mimics the composition of human fluids.

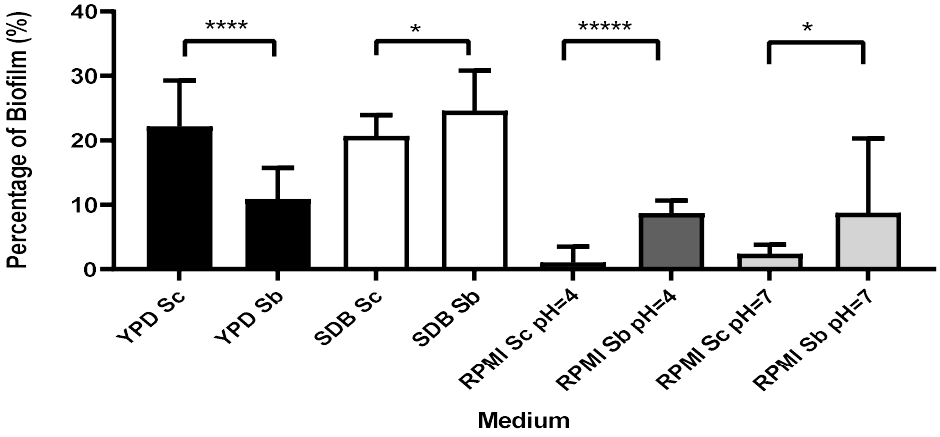


Figure 3.13. Biofilm formation followed by Presto Blue Cell Viability Assay and measurements of absorbance at 570 nm and 600 nm (reference) for the *Sc* and *Sb,* when compared to different medium (YPD, SDB, RPMI both pH=4 and pH=7). Error bars represent the corresponding standard deviations. ***** P<0,00001; **** P<0,0001; * P<0,05.

To the best of our knowledge, this is the first demonstration of the higher adhesion levels of *S. boulardii* cells, when compared to *S. cerevisiae*. The fact that *S. boulardii* displays higher adhesiveness, particularly to human epithelial cells, than *S. cerevisiae* may contribute to its longer period of persistence in the human gut. Besides, it may also contribute to the role of *S. boulardii* in preventing dysbiosis in the gut, providing a healthy balance (homeostasis) between intestinal epithelial cells. Indeed, biofilms of probiotics have been shown to be a protective barrier and provide colonization resistance against pathogenic bacteria (Kechagia et al., 2013). Furthermore, Moré and Vandenplas (2018) reported that *S. boulardii* provides a physical barrier effect and colonization resistance. In support of these, one *in vivo* study on germ-free mice conducted by Tiago and colleagues (2012) has shown that four different strain of *S. boulardii* as a probiotic have ability to exert its antimicrobial effect by adhering to the intestinal mucus membrane and removing pathogens by flow inhibiting their adhesion to the intestine. Altogether, these results provide interesting clues on the molecular basis of the probiotic activity of *S. boulardii*, which is not displayed by *S. cerevisiae.*

CHAPTER 4

CONCLUSION

To date, most studies on probiotics have demonstrated beneficial effect on human health to prevent and treat gastrointestinal tract diseases such as different kinds of diarrhea and IBS in adults and children. Moreover, there are studies on metabolic diseases such as obesity, gluten/lactose intolerances. In this regard, *in vitro*, *in vivo*, clinical and meta-analysis studies were extracted from literature. Effectivity table of *Lactobacillus*, *Bacillus*, *Bifidobacterium* and *Saccharomyces* species has demonstrated that they have a significant effectiveness against gastrointestinal diseases. The sections of bioinformatics database was preliminary designed to develop probiotic guide to be used by consumers, pharmacists, hospitals and pharmaceutical industry. This thesis consists of comprehensive scientific information about probiotics that may provide important data; that covers multidisciplinary approaches including Engineering, Pharmacology, Nutrition, Medicine, Genetic, and Bioinformatics; to carry out *in silico* studies.

Meta-analyses of *S. boulardii* against different types of diarrhea showed consistently protective effects in adults and childen with no evidence of publication bias.

After collecting *in vitro*, *in vivo*, clinical studies data and performing meta-analyis of *S. boulardii*, it has been concluded that the molecular mechanism of *Saccharomyces* probiotic strains is still unclear. In Tecnico Lisboa, molecular studies was conducted to understand the genetic mechanisms of the probiotic characteristics in *S. boulardii*.*S. boulardii* was chosen for the molecular studies because it has 99% genetic homology to non-probiotic *S. cerevisiae* (Douradinha et al., 2014). Within this context, As a result of the *in silico* cross-strain promotor analysis, comparing *Sb* Biocodex and Unique28 strains with *Sc* S288C strain, the expression of 26 probiotic-related genes was manually predicted to be controlled by different transcription factors in probiotic vs non-probiotic strains. Additionally, this work motivated the construction of the ProBioYeastract and the pipeline from this thesis was used as a basis for new functionality in the database and featured the initial development of the contents of ProBioYeastract database that is still under construction. The completion of the ProBioYeastract database may shed light on the better genetic and mechanistic understanding of the gene expression regulation of probiotics which could lead to exert their probiotic features. This new database might

provide an useful mechanism in the future, for grouping a list of probiotic genes depending on their transcription factor binding sites, and compare it with non-probiotics *Sc* strains. The up-regulation of *EFG1* and *IMA1* genes in *Sb* CNCM I-745, when compared to *Sc* BY4741, was observed, leading us to propose that their overexpression in *Sb* strains may underly its probiotic activity. Given the importance of *EFG1* in biofilm formation, the ability of *Sb* CNCM I-745, when compared to *Sc* BY4741, to aggregate, adhere to human epithelial cells and form biofilms was evaluated and shown to be higher in all cases.

Finally, probiotic activity on human health might be highlighted based on *in vitro*, *in vivo*, clinical and meta-analysis study outcomes and mechanism of action how to regulate genes. Further research about probiotic use should be included to clarify the optimal dose of probiotic use and effectiveness of single or combination probiotic formulations or in what form, and for how long based on strain-specific which probiotic product could be specifically used for which illness. Moreover, further studies are needed to elucidate more details in this area and to verify the hypothesis proposed in *S. boulardii* case study.

REFERENCES

Adam J, Barret C, Barret-Bellet A, Benedetti E, Calendini, Haechler M A, Darchen P, Galibert JM, Guerci P, Guiot G, Massonneau A Junqua R, Le Matelot J, Legrand JP, Mallez B, Pidoux A Mestas JJ, Michelet FX, Papel B, Petit P, Pfeiffer G, and Valence P. Portalis H, Prado F, Rameau J. 1977. "Controlled Double- Blind Clinical Trials of Ultra-Levure: Multi Centre Study by 25 Physicians in 388 Cases." *Gazette Medicale de France* 84: 2072–78.

Akazad, Yasuo Ohashia Satoshi Nakaib Taiji Tsukamotoc Naoya Masumoric Hideyuki, Naoto Miyanagad Tadaichi Kitamurae Kazuki Kawabef Toshihiko Kotakeg Masao Kurodag, Seiji Naitoh Hirofumi Kogah Yutaka Saitoi Koichiro Nomataj Motoaki Kitagawak, and Yoshio Aso. 2002. "Habitual Intake of Lactic Acid Bacteria and Risk Reduction of Bladder Cancer." *Urologia Internationalis* 0033: 273–80.

Angioi, A., S. Zanetti, A. Sanna, G. Delogu, and G. Fadda. 1995. "Adhesiveness of *Bacillus subtilis* Strains to Epithelial Cells Cultured *in vitro*." *Microbial Ecology in Health and Disease* 8 (2): 71–77.

Armuzzi, A., F. Cremonini, F. Bartolozzi, F. Canducci, M. Candelli, V. Ojetti, G. Cammarota, et al. 2001. "The Effect of Oral Administration of *Lactobacillus* GG on Antibiotic-Associated Gastrointestinal Side-Effects during *Helicobacter pylori* Eradication Therapy." *Alimentary Pharmacology and Therapeutics* 15 (2): 163–69.

Asmat, Shakila, Fouzia Shaukat, Raheela Asmat, Hafiz Faiq Siddique Gul Bakhat, and Tauseef M. Asmat. 2018. "Clinical Efficacy Comparison Of *Saccharomyces boulardii* And Lactic Acid As Probiotics In Acute Pediatric Diarrhea." *Journal of the College of Physicians and Surgeons Pakistan* 28 (3): 214–17.

Balasundaram, D., J. D. Dinman, C. W. Tabor, and H. Tabor. 1994. "*SPE1* and *SPE2*: Two Essential Genes in the Biosynthesis of Polyamines That Modulate +1 Ribosomal Frameshifting in *Saccharomyces cerevisiae*." *Journal of Bacteriology* 176 (22): 7126–28.

Batista, T M, E T A Marques, G R Franco, and B Douradinha. 2014. "Draft Genome Sequence of the Probiotic Yeast *Saccharomyces*" *Genome Announcements* 2 (6): 578–79.

Baujat, Bertrand, Cédric Mahé, Jean Pierre Pignon, and Catherine Hill. 2002. "A Graphical Method for Exploring Heterogeneity in Meta-Analyses: Application to a Meta-Analysis of 65 Trials." *Statistics in Medicine* 21 (18): 2641–52.

Bernasconi, Jean-paul Buts Paul, Marie-paule V A N Craynest, and Paul Maldague. 1986. "Response of Human and Rat Small Intestinal Mucosa to Oral Administration of *Saccharomyces boulardii*." *Pediatric Research* 20 (2):192-6

Billoo, A. Gaffar, M. A. Memon, S. A. Khaskheli, G. Murtaza, Khalid Iqbal, M. Saeed Shekhani, and Ahson Q. Siddiqi. 2006. "Role of a Probiotic (*Saccharomyces boulardii*) in Management and Prevention of Diarrhoea." *World Journal of Gastroenterology* 12 (28): 4557–60.

Bin, Zhang, Xu Ya-Zheng, Deng Zhao-Hui, Chu Bo, Jiang Li-Rong, and Yvan Vandenplas. 2015. " The Efficacy of *Saccharomyces boulardii* CNCM I-745 in Addition to Standard *Helicobacter pylori* Eradication Treatment in Children." *Pediatric Gastroenterology, Hepatology & Nutrition* 18 (1):17-22

Border, Brush, Membranes Key, Jean-paul Buts, and Nadine De Keyser. 2010. "Interaction of *Saccharomyces boulardii* with Intestinal." *Journal of Pediatric Gastroenterol Nutrition* 1 (4): 532–33.

Bozdogan, Bülent, Sébastien Galopin, Guy Gerbaud, Patrice Courvalin, and Roland Leclercq. 2003. "Chromosomal AadD2 Encodes an Aminoglycoside Nucleotidyltransferase in *Bacillus clausii.*" *Antimicrobial Agents and Chemotherapy* 47 (4): 1343–46.

Bravo, María Verónica, Daniel Bunout, Laura Leiva, María Pía De La Maza, Gladys Barrera, Javier De La Maza, and Sandra Hirsch. 2008. "Efecto Del Probiótico *Saccharomyces boulardii* En La Prevención de La Diarrea Asociada Con Antibióticos En Adultos Ambulatorios En Tratamiento Con Amoxicilina." *Revista Medica de Chile* 136 (8): 981–88.

Bron, Peter A, Michiel Kleerebezem, Robert-jan Brummer, Patrice D Cani, Annick Mercenier, Thomas T Macdonald, Clara L Garcia-ródenas, and Jerry M Wells. 2017. "Can Probiotics Modulate Human Disease by Impacting Intestinal Barrier Function ?" *British Journal of Nutrition* 117 (1): 93–107.

Buts, J. P., P. Bernasconi, J. P. Vaerman, and C. Dive. 1990. "Stimulation of Secretory IgA and Secretory Component of Immunoglobulins in Small Intestine of Rats Treated with *Saccharomyces boulardii*." *Digestive Disease Sci*ence 35: 251–56.

Buts, Jean-paul, Nadine D E Keyser, and Laurence D E Raedemaeker. 1994. "*Saccharomyces boulardii* Enhances Rat Intestinal Enzyme Expression by Endoluminal Release of Polyamines" *Pediatric Research* 36 (4).

Buts, Jean-paul, Nadine D E Keyser, Catherine Stilmant, and Etienne Sokal. 2002. "*Saccharomyces boulardii* Enhances N -Terminal Peptide Hydrolysis in Suckling Rat Small Intestine by Endoluminal Release of a Zinc-Binding Metalloprotease" *Pediatric Research* 51 (4): 528–34.

Buts, Jean Paul, Nadine Dekeyser, Catherine Stilmant, Emilie Delem, Francoise Smets, and Etienne Sokal. 2006. "*Saccharomyces boulardii* Produces in Rat Small Intestine a Novel Protein Phosphatase That Inhibits *Escherichia Coli* Endotoxin by Dephosphorylation." *Pediatric Research* 60 (1): 24–29.

Buts, Jean Paul, and Nadine De Keyser. 2006. "Effects of *Saccharomyces boulardii* on Intestinal Mucosa." *Digestive Diseases and Sciences* 51 (8): 1485–92.

Can, Mehmet, Bulent Ahmet Beşirbellioglu, Ismail Yasar Avci, Can Murat Beker, and Alaaddin Pahsa. 2006. "Prophylactic *Saccharomyces boulardii* in the Prevention of Antibiotic-Associated Diarrhea: A Prospective Study." *Medical Science Monitor* 12 (4):119-22

Canani, R. B., P. Cirillo, G. Terrin, L. Cesarano, M. I. Spagnuolo, A. D. Vincenzo, F. Albano, et al. 2007. "Probiotics for Treatment of Acute Diarrhoea in Children: Randomised Clinical Trial of Five Different Preparations." *British Medical Journal* 335 (7615): 340–340.

Castagliuolo, Ignazio, Andrew C Keates, Chi Chung Wang, Leyla Valenick, Ciaran P Kelly, Sigfus T Nikulasson, Thomas Lamont, and Charalabos Pothoulakis. 2018. "*Clostridium difficile* Toxin A Stimulates Macrophage-Inflammatory Protein-2 Production in Rat Intestinal Epithelial Cells."*Journal of Immunology* 160(12):6039-45

Castagliuolo, Ignazio, J Thomas L A Mont, and Sigfus T Nikulasson. 1996. "Toxin A Effects in the Rat Ileum" *Infection Immunology* 64 (12): 5225–32.

Cerbo, Alessandro Di, Beniamino Palmieri, Maria Aponte, Julio Cesar Morales-Medina, and Tommaso Iannitti. 2016. "Mechanisms and Therapeutic Effectiveness of *Lactobacilli.*"

Cetina-Sauri G., Basto G. S. 1994. "Therapeutic Evaluation of *Saccharomyces boulardii* in Children With Acute Diarrhea." *Annales De Pediatrie* 41 (6): 6–8.

Chu, Yi. 2014. "Intervention Study on *Saccharomyces boulardii* with Proton Pump Inhibitor (PPI)-Based Triple Therapy for *Helicobacter pylori* Related Peptic Ulcer." *African Journal of Pharmacy and Pharmacology* 6 (41): 2900–2904.

Ciccocioppo, R, A Di Sabatino, G R Corazza, and Policlinico San Matteo. 2005. "The Immune Recognition of Gluten in Coeliac Disease" *Clinical &Experimental Immunology* 140 (3) 408–16.

Cindoruk, Mehmet, Gulbanu Erkan, Tarkan Karakan, Ayse Dursun, and Selahattin Unal. 2007. "Efficacy and Safety of *Saccharomyces boulardii* in the 14-Day Triple Anti-*Helicobacter pylori* Therapy: A Prospective Randomized Placebo-Controlled Double-Blind Study." *Helicobacter* 12 (4): 309–16.

Ciorba, Matthew A. 2012. "A Gastroenterologist's Guide to Probiotics." *Clinical Gastroenterology and Hepatology* 10 (9): 960–68.

Coccorullo P, Strisciuglio C, Martinelli M, Miele E, Greco L, Staiano A. 2010. "*Lactobacillus reuteri* (DSM 17938) in Infants with Functional Chronic Constipation: A Double-Blind, Randomized, Placebo-Controlled Study." *Journal of Pediatr*ic 157 (4): 598–602.

Collison, Matthew, Robert P. Hirt, Anil Wipat, Sirintra Nakjang, Philippe Sanseau, and James R. Brown. 2012. "Data Mining the Human Gut Microbiota for Therapeutic Targets." *Briefings in Bioinformatics* 13 (6): 751–68.

Corrêa, Naflesia B.O., Francisco J. Penna, Fátima M.L.S. Lima, Jacques R. Nicoli, and Luciano A.P. Filho. 2011. "Treatment of Acute Diarrhea with *Saccharomyces boulardii* in Infants." *Journal of Pediatric Gastroenterology and Nutrition* 53 (5): 497–501.

Cremonini, F, S Di Caro, EC Nista, F Bartolozzi, G Gasbarrini, and A Gasbarrini. 2002. "Meta-Analysis : The Effect of Probiotic Administration on Antibiotic- Associated Diarrhoea." *Aliment Pharmacology Therapeutics* 16 (8): 1461–67.

Cristofori, Fernanda, Flavia Indrio, Vito Miniello, Maria De Angelis, and Ruggiero Francavilla. 2018. "Probiotics in Celiac Disease." *Nutrients* 10 (12): 1824.

Czerucka, D., T. Piche, and P. Rampal. 2007. "Review Article: Yeast as Probiotics - *Saccharomyces boulardii*." *Alimentary Pharmacology and Therapeutics* 26 (6): 767–78.

Czerucka, Dorota, and Patrick Rampal. 1999. "Effect of *Saccharomyces boulardii* on CAMP- and Ca -Dependent Cl Secretion in T84 Cells" *Digestive Disease and Sciences* 44 (11): 2359–68.

Czerucka. 2002. "Experimental Effects of *Saccharomyces boulardii* on Diarrheal Pathogens" *Microbes Infections* 4 (7): 733–39.

Czerucka, Dorota, Isabelle Roux, and Patrick Rampal. 1994. "*Saccharomyces boulardii* Inhibits Secretagogue-Mediated," Gastroenterology 106 (1): 65–72.

D'Souza, A. L. 2002. "Probiotics in Prevention of Antibiotic Associated Diarrhoea: Meta-Analysis." *British Medical Journal* 324 (7350): 1361.

Dahan, Stephanie, Guillaume Dalmasso, Veronique Imbert, Jean-francois Peyron, Patrick Rampal, and Dorota Czerucka. 2003. "*Saccharomyces boulardii* Interferes with Enterohemorrhagic *Escherichia Coli* -Induced Signaling Pathways in T84 Cells." *Infection and Immunity* 71 (2): 766–73.

Dalgic, Nazan, Mesut Sancar, Banu Bayraktar, Mine Pullu, and Ozlem Hasim. 2011. "Probiotic, Zinc and Lactose-Free Formula in Children with Rotavirus Diarrhea: Are They Effective?" *Pediatrics International* 53 (5): 677–82.

Daliri, Eric Banan-Mwine, and Byong H. Lee. 2015. "New Perspectives on Probiotics in Health and Disease." *Food Science and Human Wellness* 4 (2): 56–65.

Dalmasso, Guillaume, Agnès Loubat, Stéphanie Dahan, Gustavo Calle, and Patrick Rampal. 2006. "*Saccharomyces boulardii* Prevents TNF- α -Induced Apoptosis in EHEC-Infected T84 Cells" *Research Microbiology* 157 (5): 456–65.

Desiere, Frank, Bruce German, Heribert Watzke, Andrea Pfeifer, and Sam Saguy. 2001. "Bioinformatics and Data Knowledge: The New Frontiers for Nutrition and Foods." *Trends in Food Science and Technology* 12 (7): 215–29.

Dinleyici, Ener Cagri, and Yvan Vandenplas. 2014. "*Lactobacillus Reuteri* DSM 17938 Effectively Reduces the Duration of Acute Diarrhoea in Hospitalised Children." *Acta Paediatrica, International Journal of Paediatrics* 103 (7): 300–305.

Distrutti, Eleonora, Lorenzo Monaldi, Patrizia Ricci, and Stefano Fiorucci. 2016. "Gut Microbiota Role in Irritable Bowel Syndrome: New Therapeutic Strategies." *World Journal of Gastroenterology* 22 (7): 2219–41.

Douradinha, Bruno, Viviane C B Reis, Matthew B Rogers, Fernando A G Torres, Jared D Evans, and Ernesto T A Marques. 2014. "Novel Insights in Genetic Transformation of the Probiotic Yeast *Saccharomyces boulardii*." *Bioengineered* Jan-Feb;5(1):21-9.

Duman, Deniz Güney, Serhat Bor, Ömer Özütemiz, Tülin Şahin, Dilek Oğuz, Fahri Işitan, Tümer Vural, et al. 2005. "Efficacy and Safety of *Saccharomyces boulardii* in Prevention of Antibiotic-Associated Diarrhoea Due to *Helicobacter Pylori* Eradication." *European Journal of Gastroenterology and Hepatology* 17 (12): 1357–61.

Edwards-Ingram, Laura, Paul Gitsham, Nicola Burton, Geoff Warhurst, Ian Clarke, David Hoyle, Stephen G. Oliver, and Lubomira Stateva. 2007. "Genotypic and Physiological Characterization of *Saccharomyces boulardii*, the Probiotic Strain of *Saccharomyces cerevisiae*." *Applied and Environmental Microbiology* 73 (8): 2458–67.

Ehrhardt, Stephan, Nan Guo, Rebecca Hinz, Stefanie Schoppen, Jï¿½rgen May, Markus Reiser, Maximilian Philipp Schroeder, et al. 2016. "*Saccharomyces boulardii* to Prevent Antibiotic-Associated Diarrhea: A Randomized, Double-Masked, Placebo-Controlled Trial." *Open Forum Infectious Diseases* 3 (1): 1–7.

Elmer, G. W., L. V. McFarland, S. Lewis, and L. Potts. 1998. "Comment on 'the Lack of Therapeutic Effect of *Saccharomyces boulardii* in the Prevention of Antibiotic-Related Diarrhoea in Elderly Patients' (Multiple Letters) [4]." *Journal of Infection* 37 (3): 307–8.

Elshaghabee, Fouad M.F., Namita Rokana, Rohini D. Gulhane, Chetan Sharma, and Harsh Panwar. 2017. "Bacillus as Potential Probiotics: Status, Concerns, and Future Perspectives." *Frontiers in Microbiology* 8 (AUG): 1–15.

Emara, Mohamed H., Salem Y. Mohamed, and Hesham R. Abdel Aziz. 2014. "*Lactobacillus reuteri* in Management of *Helicobacter pylori* Infection in Dyspeptic Patients: A Double-Blind Placebo-Controlled Randomized Clinical Trial." *Therapeutic Advances in Gastroenterology* 7 (1): 4–13.

Erdeve, Omer, Ulku Tiras, and Yıldız Dallar. 2004. "The Probiotic Effect of *Saccharomyces boulardii* in a Pediatric Age Group." *Journal of Tropical Pediatrics* 50 (4): 234–38.

Eren, Makbule, Ener C. Dinleyici, and Yvan Vandenplas. 2010. “Clinical Efficacy Comparison of *Saccharomyces boulardii* and Yogurt Fluid in Acute Non-Bloody Diarrhea in Children: A Randomized, Controlled, Open Label Study.” *American Journal of Tropical Medicine and Hygiene* 82 (3): 488–91.

Estaki, Mehrbod, Daniella DeCoffe, and Deanna L. Gibson. 2014. “Interplay between Intestinal Alkaline Phosphatase, Diet, Gut Microbes and Immunity.” *World Journal of Gastroenterology* 20 (42): 15650–56.

Everard, Amandine, Sébastien Matamoros, Lucie Geurts, Nathalie M Delzenne, and Patrice D Cani. 2014. “*Saccharomyces boulardii* Administration Changes Gut Microbiota and Reduces Hepatic Steatosis , Low-Grade Inflammation , and Fat Mass in Obese and Type 2 Diabetic Db / Db Mice.” *American Society for Microbiology* 5 (3): 1–9.

Fan, Xiaolong, Felix Stelter, Rene Menzel, Robert Jack, Ingo Spreitzer, Thomas Hartung, and Christine Schütt. 1999. “Structures in *Bacillus subtilis* are recognized by CD14 in a Lipopolysaccharide Binding Protein-Dependent Reaction.” *Infection and Immunity* 67 (6): 2964–68.

Fidan, Isil, Ayse Kalkanci, Emine Yesilyurt, Burce Yalcin, Berna Erdal, Semra Kustimur, and Turgut Imir. 2009. “Effects of *Saccharomyces boulardii* on Cytokine Secretion from Intraepithelial Lymphocytes Infected by *Escherichia Coli* and *Candida Albicans*.” *Mycoses* 52 (1): 29–34.

Fietto, Juliana L.R, Raquel S Araújo, Frederico N Valadão, Luciano G Fietto, Rogelio L Brandão, Maria J Neves, Fátima C.O Gomes, Jacques R Nicoli, and Ieso M Castro. 2004. “Molecular and Physiological Comparisons between *Saccharomyces cerevisiae* and *Saccharomyces boulardii*.” *Canadian Journal of Microbiology* 50 (8): 615–21.

Fijan, Sabina. 2014. “Microorganisms with Claimed Probiotic Properties: An Overview of Recent Literature.” *International Journal of Environmental Research and Public Health* 11 (5): 4745–67.

Fitzpatrick, Leo R. 2013. “Probiotics for the Treatment of *Clostridium difficile* Associated Disease” *World Journal of Gastrointestinal Pathophysiology* 4 (3): 47–52.

Francavilla, R., E. Lionetti, S. Castellaneta, F. Ciruzzi, F. Indrio, A. Masciale, C. Fontana, M. M. La Rosa, L. Cavallo, and A. Francavilla. 2012. “Randomised Clinical Trial: *Lactobacillus reuteri* DSM 17938 vs. Placebo in Children with Acute Diarrhoea - A Double-Blind Study.” *Alimentary Pharmacology and Therapeutics* 36 (4): 363–69.

Francavilla, Ruggiero, Elena Lionetti, Stefania Paola Castellaneta, Anna Maria Magistà, Giovanni Maurogiovanni, Nunzia Bucci, Angela De Canio, et al. 2008. “Inhibition of *Helicobacter pylori* Infection in Humans by *Lactobacillus reuteri* ATCC 55730 and Effect on Eradication Therapy: A Pilot Study.” *Helicobacter* 13 (2): 127–34.

Gao, Xing Wang, Mohamed Mubasher, Chong Yu Fang, Cheryl Reifer, and Larry E. Miller. 2010. "Dose-Response Efficacy of a Proprietary Probiotic Formula of *Lactobacillus acidophilus* CL1285 and *Lactobacillus casei* LBC80R for Antibiotic-Associated Diarrhea and *Clostridium difficile*-Associated Diarrhea Prophylaxis in Adult Patients." *American Journal of Gastroenterology* 105 (7): 1636–41.

Gaudier, E., A. Jarry, H. M. Blottière, P. de Coppet, M. P. Buisine, J. P. Aubert, C. Laboisse, C. Cherbut, and C. Hoebler. 2004. "Butyrate Specifically Modulates *MUC* Gene Expression in Intestinal Epithelial Goblet Cells Deprived of Glucose." *American Journal of Physiology-Gastrointestinal and Liver Physiology* 287 (6): G1168–74.

Ghelardi, Emilia, F. Celandroni, S. Salvetti, S. A. Gueye, A. Lupetti, and S. Senesi. 2015. "Survival and Persistence of *Bacillus clausii* in the Human Gastrointestinal Tract Following Oral Administration as Spore-Based Probiotic Formulation." *Journal of Applied Microbiology* 119 (2): 552–59.

Girard, Philippe, Yannick Pansart, and Jean Marie Gillardin. 2005. "Inducible Nitric Oxide Synthase Involvement in the Mechanism of Action of *Saccharomyces boulardii* in Castor Oil-Induced Diarrhoea in Rats." *Nitric Oxide - Biology and Chemistry* 13 (3): 163–69.

Govender, Patrick, Jody L. Domingo, Michael C. Bester, Isak S. Pretorius, and Florian F. Bauer. 2008. "Controlled Expression of the Dominant Flocculation Genes *FLO1*, *FLO5*, and *FLO11* in *Saccharomyces cerevisiae*." *Applied and Environmental Microbiology* 74 (19): 6041–52.

Graff, Sandrine, Jean-claude Chaumeil, Pierre Boy, René Lai-kuen, and Christine Charrueau. 2008. "Influence of PH Conditions on the Viability of *Saccharomyces boulardii* Yeast." *The Journal of General and Applied Microbiology* 227 (4): 221–27.

Grandy, Giuseppe, Marcos Medina, Richard Soria, Carlos G. Terán, and Magdalena Araya. 2010. "Probiotics in the Treatment of Acute Rotavirus Diarrhoea. A Randomized, Double-Blind, Controlled Trial Using Two Different Probiotic Preparations in Bolivian Children." *BMC Infectious Diseases* 10: 1–7.

Guandalini S, Pensabene L, Zikri MA, Dias JA, Casali LG, Hoekstra H, Kolacek S, Massar K, Micetic-Turk D, Papadopoulou A, de Sousa JS, Sandhu B, Szajewska H, Weizman Z. 2000. "*Lactobacillus* GG Administered in Oral Rehydration Solution to Children with Acute Diarrhea: A Multicenter European Trial." *Journal of Pediatric Gastroenterology Nutrition* 30 (1): 54–60.

Guarino A, Canani RB, Spagnuolo MI, Albano F, Di Benedetto L. 1997. "Oral Bacterial Therapy Reduces the Duration of Symptoms and of Viral Excretion in Children with Mild Diarrhea." *Journal of Pediatric Gastroenterology Nutr.* 25 (5): 516–19.

Gut, Abraham Majak, Todor Vasiljevic, Thomas Yeager, and Osaana N Donkor. 2018. "*Salmonella* Infection – Prevention and Treatment by Antibiotics and Probiotic Yeasts : A Review." *Microbiological Research* 164 (11): 1327–44.

Hafeez A, Tariq P, Ali S. 2002. "The Efficacy of *Saccharomyces boulardii* in the Treatment of Acute Watery Diarrhea in Children: A Multicentre Randomized Controlled Trial." *Journal of College of Physicians and Surgeons Pakistan* 12 (7): 432–34.

Hirano, Jyunko, Tomoaki Yoshida, Tsuyoshi Sugiyama, Naoki Koide, Isamu Mori, and Takashi Yokochi. 2003. "The Effect of *Lactobacillus rhamnosus* on Enterohemorrhagic *Escherichia coli* Infection of Human Intestinal Cells *in vitro.*" *Microbiology and Immunology* 47 (6): 405–9.

Högenauer, Christoph, Heinz F Hammer, Guenter J Krejs, and Emil C Reisinger. 1998. "Mechanisms and Management of Antibiotic-Associated Diarrhea." *Clinical Infection Diseases* 27 (4): 702–10.

Holzapfel, Wilhelm H, Petra Haberer, Rolf Geisen, and Ulrich Schillinger. 2001. "Taxonomy and Important Features of Probiotic Microorganisms in Food and Nutrition." *American Journal of Clinical Nutrition* 73 (2): 365S.

Homan, Matjaž, and Rok Orel. 2015. "Are Probiotics Useful in *Helicobacter pylori* Eradication?" *World Journal of Gastroenterology* 21 (37): 10644–53.

Hong, Huynh A., Hong Duc Le, and Simon M. Cutting. 2005. "The Use of Bacterial Spore Formers as Probiotics." *FEMS Microbiology Reviews* 29 (4): 813–35.

Huang, Yuan, and Zhonge Chen. 2016. "Inflammatory Bowel Disease Related Innate Immunity and Adaptive Immunity." *American Journal of Translational Research* 8 (6): 2490–97.

Hunt, Richard, David Armstrong, Peter Katelaris, Mary Afihene, Abate Bane, Shobna Bhatia, Min Hu Chen, et al. 2017. "World Gastroenterology Organisation Global Guidelines." *Journal of Clinical Gastroenterology* 51 (6): 467–78.

Ianiro, Gianluca, Gianenrico Rizzatti, Manuel Plomer, Loris Lopetuso, Franco Scaldaferri, Francesco Franceschi, Giovanni Cammarota, and Antonio Gasbarrini. 2018. "*Bacillus clausii* for the Treatment of Acute Diarrhea in Children: A Systematic Review and Meta-Analysis of Randomized Controlled Trials." *Nutrients* 10 (8): 1074.

Isolauri E., Juntenen M., Rautanen T., Sillanaukee P., Koivula T. 1991. "A Human *Lactobacillus* Strain (*Lactobacillus casei* Sp Strain GG) Promotes Recovery from Acute Diarrhea in Children." *Pediatrics* 7: 88–90.

Kamboj, Amrit K., Thomas G. Cotter, and Amy S. Oxentenko. 2017. "*Helicobacter pylori*: The Past, Present, and Future in Management." *Mayo Clinic Proceedings* 92 (4): 599–604.

Kareem, Alaa, Naimah Alaa, Jabbar Abd, Al Manhel, Manar Jabbar, and Al Shawi. 2018. “Isolation , Purification and Characterization of Antimicrobial Peptides Produced from *Saccharomyces boulardii*.” *International Journal of Peptide Research and Therapeutics* 24 (3): 455–61.

Kechagia, Maria, Dimitrios Basoulis, Stavroula Konstantopoulou, Dimitra Dimitriadi, Konstantina Gyftopoulou, Nikoletta Skarmoutsou, and Eleni Maria Fakiri. 2013. “Health Benefits of Probiotics: A Review.” *ISRN Nutrition* 2013: 1–7.

Kelesidis, Theodoros, and Charalabos Pothoulakis. 2012. “Efficacy and Safety of the Probiotic *Saccharomyces boulardii* for the Prevention and Therapy of Gastrointestinal Disorders.” *Therapeutic Advances in Gastroenterology* 5 (2): 111–25.

Khan, Fahmi Yousef, and Abdul Naser Elzouki. 2014. “*Clostridium difficile* Infection: A Review of the Literature.” *Asian Pacific Journal of Tropical Medicine* 7 (S1): S6–13.

Khatri, Indu, Akil Akhtar, Kamaldeep Kaur, Rajul Tomar, and Gandham Satyanarayana Prasad. 2013. “Gleaning Evolutionary Insights from the Genome Sequence of a Probiotic Yeast *Saccharomyces boulardii*.” *Gut Pathogens* 5 (1):30

Khatri, Indu, Rajul Tomar, K. Ganesan, G. S. Prasad, and Srikrishna Subramanian. 2017. “Immunological and Trophical Effects of *Saccharomyces boulardii* on the Small Intestine in Healthy Human Volunteers.” *Scientific Reports* 7 (1): 1–13.

Khin Htwe, Khin Saw Yee, Marlar Tin, and Yvan Vandenplas. 2008. “Effect of *Saccharomyces boulardii* in the Treatment of Acute Watery Diarrhea in Myanmar Children: A Randomized Controlled Study.” *American Journel of Tropical Medicine and Hygiene* 78 (2): 214–16.

Kim, Min Jun Song Dong Il Park Jung Ho Park Hong Joo Kim Yong Kyun Cho Chong Il Sohn Woo Kyu Jeon Byung Ik. 2010. “The Effect of Probiotics and Mucoprotective Agents on PPI-Based Triple Therapy for Eradication of *Helicobacter pylori.*” *Helicobacter.* 15 (3): 206–13.

Kindenplas, Yvan. 1999. “Bacteria and Yeasts in the Treatment of Acute and Chronic Infectious.” *European Society of Clinical Infectious Diseases* 5 (7): 389–95.

Klug, Lisa. 2014. “Yeast Lipid Metabolism at a Glance.” *FEMS Yeast Research* 14 (3): 369–88.

Kobyliak, Nazarii, Caterina Conte, Giovanni Cammarota, Andreana P. Haley, Igor Styriak, Ludovit Gaspar, Jozef Fusek, Luis Rodrigo, and Peter Kruzliak. 2016. “Probiotics in Prevention and Treatment of Obesity: A Critical View.” *Nutrition & Metabolism* 13 (1): 14.

Konieczna, Patrycja, Ruth Ferstl, Mario Ziegler, Remo Frei, Dirk Nehrbass, Roger P. Lauener, Cezmi A. Akdis, and Liam O'Mahony. 2013. "Immunomodulation by *Bifidobacterium infantis* 35624 in the Murine Lamina Propria Requires Retinoic Acid-Dependent and Independent Mechanisms." *PLoS ONE* 8 (5):62617

Konieczna, Patrycja, David Groeger, Mario Ziegler, Remo Frei, Ruth Ferstl, Fergus Shanahan, Eamonn M.M. Quigley, Barry Kiely, Cezmi A. Akdis, and Liam O'Mahony. 2012. "*Bifidobacterium infantis* 35624 Administration Induces Foxp3 T Regulatory Cells in Human Peripheral Blood: Potential Role for Myeloid and Plasmacytoid Dendritic Cells." *Gut* 61 (3): 354–66.

Kotowska, M, P Albrecht, and H A Szajewsk. 2005. "*Saccharomyces boulardii* in the Prevention of Antibiotic-Associated Diarrhoea in Children : A Randomized Double-Blind Placebo-Controlled Trial," *Alimentary Pharmacology & Therapeutics* 21(5):583–90.

Kurugöl, Z., and G. Koturoğlu. 2005. "Effects of *Saccharomyces boulardii* in Children with Acute Diarrhoea." *Acta Paediatrica* 94 (1): 44–47.

Kyriakos, Nikolaos, Konstantinos Papamichael, Anastassios Roussos, Ioannis Theodoropoulos, Christos Karakoidas, Alexandros Smyrnidis, Emmanuel Archavlis, Konstantina Lariou, and Gerassimos J Mantzaris. 2013. "A Lyophilized Form of *Saccharomyces boulardii* Enhances the *Helicobacter pylori* Eradication Rates of Omeprazole-Triple Therapy in Patients With Peptic Ulcer Disease or Functional Dyspepsia." *Hospital Chronicles* 8 (3): 127.

Laulund, Svend, Anette Wind, Patrick Derkx, and Véronique Zuliani. 2017. "Regulatory and Safety Requirements for Food Cultures." *Microorganisms* 5 (2): 28.

Lewis SJ, Potts LF, Barry RE. 1998. "The Lack of Therapeutic Effect of *Saccharomyces boulardii* in the Prevention of Antibioticrelated Diarrhoea in Elderly Patients." *Journal of Infections* 36 (2): 171–74.

Liu J-J, Zhang G-c, Kong II, Yun E J, Zheng J-Q, Kweon D-H, Jin Y-S. 2018. "A Mutation in *PGM2* Causing Inefficient Galactose Metabolism in the Probiotic Yeast *S. boulardii*." *Applied and Environmental Microbiology* 84 (10).

Liu, Jing-jing, Iok Kong, Guo-chang Zhang, Lahiru N Jayakody, Heejin Kim, Peng-fei Xia, and Suryang Kwak. 2016. "Metabolic Engineering of Probiotic *Saccharomyces Boulardii*." *Applied and Environmental Microbiology* 82 (8): 2280–87.

Liu, Yuying, Dat Q. Tran, Nicole Y. Fatheree, and J. Marc Rhoads. 2014. "*Lactobacillus reuteri* DSM 17938 Differentially Modulates Effector Memory T Cells and Foxp3 + Regulatory T Cells in a Mouse Model of Necrotizing Enterocolitis ." *American Journal of Physiology-Gastrointestinal and Liver Physiology* 307 (2): G177–86.

Łukaszewicz, Marcin. 2012. “Probiotic Yeast Publications with " *boulardii* ".” In *Probiotics*, 385–97.

Lyseng-Williamson, Katherine A. 2017. “*Bifidobacterium infantis* 35624 as a Probiotic Dietary Supplement: A Profile of Its Use.” *Drugs & Therapy Perspectives* 33 (8): 368–74.

Mackenzie, Donald A, Marianne Defernez, Warwick B Dunn, Marie Brown, Linda J Fuller, Steve A James, John Eagles, Mark Philo, Santiago R M Seco De Herrera, and G Andreas. 2008. “Relatedness of Medically Important Strains of *Saccharomyces cerevisiae* as Revealed by Phylogenetics and Metabolomics.” *Yeast* 25 (7): 501–12.

Majamaa H, Isolauri E, Saxelin M, and Vesikari T. 1995. “Lactic Acid Bacteria in the Treatment of Acute Rotavirus Gastroenteritis.” *Journal of Pediatric Gastroenterology Nutrition* 20(3): 333–38.

Mantis, N J, Rol N, Corthesy B. 2011. “Secretory IgA’s Complex Roles in Immunity and Mucosal Homeostasis in the Gut.” *Mucosal Immunology* 4 (6):603-11.

Maroof, Hamidreza, and Zuhir Mohammad Hassan. 2012. “*Lactobacillus acidophilus* Could Modulate the Immune Response Against Breast Cancer in Murine Model,” 1353–59.

Martins, Flaviano S, P Sousa, Helena R C Arau, Paulo F Pimenta, Fabiana C P Tiago, A Bonjardim, Jacques R Nicoli, and Mauro M Teixeira. 2013. “Inhibition of Tissue Inflammation and Bacterial Translocation as One of the Protective Mechanisms of *Saccharomyces boulardii* against Salmonella Infection in Mice” 15 (4): 270–79.

McFarland, L.V. 2017. “Common Organisms and Probiotics: *Saccharomyces boulardii*.” In *The Microbiota in Gastrointestinal Pathophysiology*, 145–64.

McFarland, L V, C M Surawicz, R N Greenberg, R Fekety, G W Elmer, K A Moyer, S A Melcher, et al. 1994. “A Randomized Placebo-Controlled Trial of *Saccharomyces boulardii* in Combination with Standard Antibiotics for *Clostridium difficile Disease* .” *Jama* 271 (24): 1913–18.

McFarland, Lynne V. 2006. “Meta-Analysis of Probiotics for the Prevention of Antibiotic Associated Diarrhea and the Treatment of *Clostridium difficile* Disease.” *American Journal of Gastroenterology* 101 (4): 812–22.

Mcfarland. 2007. “Meta-Analysis of Probiotics for the Prevention of Traveler’s Diarrhea.” *Travel Medicine and Infectious Disease* 5 (2 SPEC. ISS.): 97–105.

Mcfarland 2010 “Systematic Review and Meta-Analysis of *Saccharomyces boulardii* in Adult Patients.” *World Journal of Gastroenterology* 16 (18): 2202–22.

McKernan, D. P., P. Fitzgerald, T. G. Dinan, and J. F. Cryan. 2010. “The Probiotic *Bifidobacterium infantis* 35624 Displays Visceral Antinociceptive Effects in the Rat.” *Neurogastroenterology and Motility* 22 (9).

Moal, Vanessa Liévin-le, and L Servin. 2013. “Pathogenesis of Human Enterovirulent Bacteria : Lessons from Cultured , Fully Differentiated Human Colon Cancer Cell Lines.” *MMBR* 77 (3): 380–439.

Monteiro E, Fernandes JP, Vieira MR, Correia JP, Caetano, Reis L JM, Ribeiro T, Antunes AB, Noronha R, Batista F, Pecegueiro A Mendes MP, Cabrita A, Roseta A, Abecassis L, and Fernandes D. Rosa S, Ferreira SB. 1981. “Double Blind Clinical Trial on the Use of Ultra-Levure in the Prophylaxis of Antibiotic Induced Gastro-Intestinal and Mucocutaneous Disorders.” *Acta Med Port* 3: 141–45.

Monteiro, Pedro Tiago, Pedro Pais, Catarina Costa, Sauvagya Manna, Isabel Sa-Correia, and Miguel Cacho Teixeira. 2017. “The PathoYeastract Database: An Information System for the Analysis of Gene and Genomic Transcription Regulation in Pathogenic Yeasts.” *Nucleic Acids Research* 45 (D1): D597–603.

Moré, Margret I, and Yvan Vandenplas. 2018. “*Saccharomyces boulardii* CNCM I-745 Improves Intestinal Enzyme Function : A Trophic Effects Review.” *Clinical Medicine Insights Gastroenterology* 11: 1–14.

Muñoz-quezada, Miriam Bermudez-brito Julio Plaza-díaz Sergio, and Carolina Gómez-llorente Angel Gil. 2012. “Probiotic Mechanisms of Action.” *Nutrition & Metabolism* 61: 160–74.

N, Jayanthi, and Ratna Sudha M. 2015. “*Bacillus clausii* - The Probiotic of Choice in the Treatment of Diarrhoea.” *Journal of Yoga & Physical Therapy* 05 (04): 1–4.

Nagpal, Ravinder, Ashwani Kumar, Manoj Kumar, Pradip V. Behare, Shalini Jain, and Hariom Yadav. 2012. “Probiotics, Their Health Benefits and Applications for Developing Healthier Foods: A Review.” *FEMS Microbiology Letters* 334 (1): 1–15.

Nazir, Yusuf, Syed Ammar Hussain, Aidil Abdul Hamid, and Yuanda Song. 2018. “Probiotics and Their Potential Preventive and Therapeutic Role for Cancer , High Serum Cholesterol , and Allergic and HIV Diseases” *BioMed Research International* 2018: 1-17

Nista, E. C., M. Candelli, F. Cremonini, I. A. Cazzato, M. A. Zocco, F. Franceschi, G. Cammarota, G. Gasbarrini, and A. Gasbarrini. 2004. “*Bacillus clausii* Therapy to Reduce Side-Effects of Anti-*Helicobacter pylori* Treatment: Randomized, Double-Blind, Placebo Controlled Trial.” *Alimentary Pharmacology and Therapeutics* 20 (10): 1181–88.

O’Hara, Ann M., Padraig O’Regan, Áine Fanning, Caitlin O’Mahony, John MacSharry, Anne Lyons, John Bienenstock, Liam O’Mahony, and Fergus Shanahan. 2006. “Functional Modulation of Human Intestinal Epithelial Cell Responses by *Bifidobacterium infantis* and *Lactobacillus salivarius.*” *Immunology* 118 (2): 202–15.

O'Mahony, Liam, Jane Mccarthy, Peter Kelly, George Hurley, Fangyi Luo, Kersang Chen, Gerald C. O'Sullivan, et al. 2005. "*Lactobacillus* and *Bifidobacterium* in Irritable Bowel Syndrome: Symptom Responses and Relationship to Cytokine Profiles." *Gastroenterology* 128 (3): 541–51.

Oak, Sophia J., and Rajesh Jha. 2018. "The Effects of Probiotics in Lactose Intolerance: A Systematic Review." *Critical Reviews in Food Science and Nutrition* 9: 1–9.

Olveira, Gabriel, and Inmaculada González-Molero. 2016. "An Update on Probiotics, Prebiotics and Symbiotics in Clinical Nutrition." *Endocrinología y Nutrición (English Edition)* 63 (9): 482–94.

Ozen, M., and E. C. Dinleyici. 2015. "The History of Probiotics: The Untold Story." *Beneficial Microbes* 6 (2): 159–65.

Ozkan, T. B., E. Sahin, G. Erdemir, and F. Budak. 2007. "Effect of *Saccharomyces boulardii* in Children with Acute Gastroenteritis and Its Relationship to the Immune Response." *Journal of International Medical Research* 35 (2): 201–12.

Pandey, Kavita R., Suresh R. Naik, and Babu V. Vakil. 2015. "Probiotics, Prebiotics and Synbiotics- a Review." *Journal of Food Science and Technology* 52 (12): 7577–87.

Papadimitriou, Konstantinos, Georgia Zoumpopoulou, Benoit Foligné, Voula Alexandraki, Maria Kazou, Bruno Pot, and Effie Tsakalidou. 2015. "Discovering Probiotic Microorganisms: *In vitro, in vivo*, Genetic and Omics Approaches." *Frontiers in Microbiology* 6 (FEB): 1–28.

Patel, Rachna, and Herbert L. Dupont. 2015. "New Approaches for Bacteriotherapy: Prebiotics, New-Generation Probiotics, and Synbiotics." *Clinical Infectious Diseases* 60 (Suppl 2): S108–21.

Peniche, Alex G., Tor C. Savidge, and Sara M. Dann. 2013. "Recent Insights into *Clostridium difficile* Pathogenesis." *Current Opinion in Infectious Diseases* 26 (5): 447–53.

Pochapin, Mark. 2000. "The Effect of Probiotics on *Clostridium difficile* Diarrhea." *American Journal of Gastroenterology* 95 (1 SUPPL.): 2–4.

Pontier-bres, Rodolphe, Patrick Munro, Laurent Boyer, Rodolphe Anty, Patrick Rampal, and Emmanuel Lemichez. 2014. "*Saccharomyces boulardii* Modifies *Salmonella typhimurium* Traffic and Host Immune Responses along the Intestinal Tract." *PLoS ONE* 9 (8): 1–11.

Pontier-bres, Rodolphe, Patrick Rampal, Jean-françois Peyron, Patrick Munro, Emmanuel Lemichez, and Dorota Czerucka. 2015. "The *Saccharomyces boulardii* CNCM I-745 Strain Shows Protective Effects against the B. Anthracis LT Toxin," 7 (11): 4455–67.

Pothoulakis, C. 2009. “Review Article: Anti-Inflammatory Mechanisms of Action of *Saccharomyces boulardii*.” *Alimentary Pharmacology and Therapeutics* 30 (8): 826–33.

Pothoulakis, Charalabos. 2000. “Effects of *Clostridium difficile* Toxins on Epithelial Cell Barrier.” *Annals of the New York Academy of Sciences* 915: 347–56.

Pozzoni P, Riva A, Bellatorre AG, Amigoni M, Redaelli E, Ronchetti A, Stefani M, Tironi R, Molteni EE, Conte D, Casazza G, Colli A. 2012. “*Saccharomyces boulardii* for the Prevention of Antibiotic-Associated Diarrhea in Adult Hospitalized Patients: A Single-Center, Randomized, Double-Blind, Placebo-Controlled Trial.” *American Journel of Gastroenterology* 107 (6): 922–31.

Predrag, Stojanović. 2016. “Analysis of Risk Factors and Clinical Manifestations Associated with *Clostridium difficile* Disease in Serbian Hospitalized Patients.” *Brazilian Journal of Microbiology* 47 (4): 902–10.

Probiotic chart in Canada,http://www.probioticchart.ca (accessed April 15, 2019)

Quintana, Daniel S. 2015. “From Pre-Registration to Publication: A Non-Technical Primer for Conducting a Meta-Analysis to Synthesize Correlational Data.” *Frontiers in Psychology* 6 : 1549 (OCT).

Rajakumari, Sona, and Günther Daum. 2010. “Multiple Functions as Lipase, Steryl Ester Hydrolase, Phospholipase, and Acyltransferase of Tgl4p from the Yeast *Saccharomyces cerevisiae*.” *Journal of Biological Chemistry* 285 (21): 15769–76.

Rao, Xiayu, Xuelin Huang, Zhicheng Zhou, and Xin Lin. 2013. “An Improvement of The2–Delta Delta CT) Method for Quantitative Real-Time Polymerase Chain Reaction Data Analysis.” *Biostat Bioinforma Biomath.* 3 (3): 71–85.

Ratna Sudha, M., S. Bhonagiri, and M. Asin Kumar. 2013. “Efficacy of *Bacillus clausii* Strain UBBC-07 in the Treatment of Patients Suffering from Acute Diarrhoea.” *Beneficial Microbes* 4 (2): 211–16.

Riaz, Musheer, Seema Alam, Abida Malik, and S. Manazir Ali. 2012. “Efficacy and Safety of *Saccharomyces boulardii* in Acute Childhood Diarrhea: A Double Blind Randomised Controlled Trial.” *Indian Journal of Pediatrics* 79 (4): 478–82.

Ricci, Antonia, Ana Allende, Declan Bolton, Marianne Chemaly, Robert Davies, Rosina Girones, Kostas Koutsoumanis, et al. 2017. “Update of the List of QPS recommended Biological Agents Intentionally Added to Food or Feed as Notified to EFSA 6: Suitability of Taxonomic Units Notified to EFSA until March 2017.” *EFSA Journal* 15 (7).

Ríos-covián, David, Patricia Ruas-madiedo, Abelardo Margolles, Miguel Gueimonde, Clara G De Los Reyes-gavilán, and Nuria Salazar. 2016. “Intestinal Short Chain Fatty Acids and Their Link with Diet and Human Health.” *Frontiers in Microbiology* 7 (February): 1–9.

Romano, Claudio, Valeria Ferrau', Francesca Cavataio, Giuseppe Iacono, Massimo Spina, Elena Lionetti, Fabrizio Comisi, Annalisa Famiani, and Donatella Comito. 2014. "*Lactobacillus reuteri* in Children with Functional Abdominal Pain (FAP)." *Journal of Paediatrics and Child Health* 50 (10): E68–71.

Ruszczyński, M., A. Radzikowski, and H. Szajewska. 2008. "Clinical Trial: Effectiveness of *Lactobacillus rhamnosus* (Strains E/N, Oxy and Pen) in the Prevention of Antibiotic-Associated Diarrhoea in Children." *Alimentary Pharmacology and Therapeutics* 28 (1): 154–61.

Saavedra, J. M., N. A. Bauman, J. A. Perman, R. H. Yolken, J. M. Saavedra, N. A. Bauman, and I. Oung. 1994. "Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to Infants in Hospital for Prevention of Diarrhoea and Shedding of Rotavirus." *The Lancet* 344 (8929): 1046–49.

Sanders, Mary Ellen, Louis M.A. Akkermans, Dirk Haller, Cathy Hammerman, James Heimbach, Gabriele Hörmannsperger, Geert Huys, et al. 2010. "Safety Assessment of Probiotics for Human Use." *Gut Microbes* 1 (3): 164–85.

Savas-Erdeve S, Gokay S, and Dallar Y. 2009. "Efficacy and Safety of *Saccharomyces boulardii* in Amebiasis-Associated Diarrhea." *Turkish Journal of Pediatrics* 51 (3): 220–24.

Schaeffer, Hans J, and Michael J Weber. 1999. "MINIREVIEW Mitogen-Activated Protein Kinases : Specific Messages from Ubiquitous Messengers" 19 (4): 2435–44.

Schneider, Stéphane M, Fernand Girard-pipau, Jérôme Filippi, Xavier Hébuterne, Dominique Moyse, Gustavo Calle, Anne Pompei, et al. 2005. "Effects of *Saccharomyces boulardii* on Fecal Short-Chain Fatty Acids and Microflora in Patients on Long-Term Total Enteral Nutrition" 11 (39): 6165–69.

Seekatz, Anna M., and Vincent B. Young. 2014. "*Clostridium difficile* and the Microbiota." *Journal of Clinical Investigation* 124 (10): 4182–89.

Segers, Marijke E., and Sarah Lebeer. 2014. "Towards a Better Understanding of *Lactobacillus rhamnosus* GG - Host Interactions." *Microbial Cell Factories* 13 (Suppl 1): S7.

Shan, L. S., P. Hou, Z. J. Wang, F. R. Liu, N. Chen, L. H. Shu, H. Zhang, et al. 2013. "Prevention and Treatment of Diarrhoea with *Saccharomyces boulardii* in Children with Acute Lower Respiratory Tract Infections." *Beneficial Microbes* 4 (4): 329–34.

Shornikova, a V, I a Casas, E Isolauri, H Mykkanen, and T Vesikari. 1997. "*Lactobacillus reuteri* as a Therapeutic Agent in Acute Diarrhea in Young Children." *Journal of Pediatic Gastroenterology Nutrition* 24 (4): 399–404.

Siciliano, Rosa Anna, and Maria Fiorella Mazzeo. 2012. “Molecular Mechanisms of Probiotic Action: A Proteomic Perspective.” *Current Opinion in Microbiology* 15 (3): 390–96.

Sivananthan, Kavitha, and Andreas Munk Petersen. 2018. “Review of *Saccharomyces boulardii* as a Treatment Option in IBD.” *Immunopharmacology and Immunotoxicology* 17 (0): 1–11.

So¨ren Nobaek, M.D., Marie-Louise Johansson, Ph.D., Go¨ran Molin, Ph.D., Siv Ahrne´, Ph.D., And, and F.R.C.S. Bengt Jeppsson, M.D., Ph.D. 2000. “Alteration of Intestinal Microflora Is Associated With Reduction in Abdominal Bloating and Pain in Patients With Irritable Bowel Syndrome.” *American College of Gastroenterology* 95 (5): 1231–38.

Spiller, R., Q. Aziz, F. Creed, A. Emmanuel, L. Houghton, P. Hungin, R. Jones, et al. 2007. “Guidelines on the Irritable Bowel Syndrome: Mechanisms and Practical Management.” *Gut* 56 (12): 1770–98.

Stier H., Bischoff S.C. 2016. “Influence of *Saccharomyces boulardii* CNCM I-745 on the Gut-Associated Immune System.” *Clinical Experimental of Gastroenterology* 13 (9): 269–79.

Surawicz, C M, L V McFarland, R N Greenberg, M Rubin, R Fekety, M E Mulligan, R J Garcia, et al. 2000. “The Search for a Better Treatment for Recurrent *Clostridium difficile Disease: Use of High-Dose Vancomycin Combined with* Saccharomyces Boulardii.” *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America* 31 (4): 1012–17.

Surawicz, Christina M., Gary W. Elmer, Pieter Speelman, Lynne V. McFarland, Janet Chinn, and Gerald Van Belle. 1989. “Prevention of Antibiotic-Associated Diarrhea by *Saccharomyces boulardii*: A Prospective Study.” *Gastroenterology* 96 (4): 981–88.

Symonds, Erin L., Caitlin O’Mahony, Susan Lapthorne, David O’Mahony, John Mac Sharry, Liam O’Mahony, and Fergus Shanahan. 2012. “*Bifidobacterium infantis* 35624 Protects against *Salmonella*-Induced Reductions in Digestive Enzyme Activity in Mice by Attenuation of the Host Inflammatory Response.” *Clinical and Translational Gastroenterology* 3 (November 2011): 1–10.

Szajewska, H., A. Horvath, and M. Kołodziej. 2015. “Systematic Review with Meta-Analysis: *Saccharomyces boulardii* Supplementation and Eradication of *Helicobacter Pylori* Infection.” *Alimentary Pharmacology and Therapeutics* 41 (12): 1237–45.

Szajewska, H., and M. Kołodziej. 2015. “Systematic Review with Meta-Analysis: *Saccharomyces boulardii* in the Prevention of Antibiotic-Associated Diarrhoea.” *Alimentary Pharmacology and Therapeutics* 42 (7): 793–801.

Szajewska, H., A. Skórka, M. Ruszczyński, and D. Gieruszczak-Białek. 2013. "Meta-Analysis: *Lactobacillus* GG for Treating Acute Gastroenteritis in Children - Updated Analysis of Randomised Controlled Trials." *Alimentary Pharmacology and Therapeutics* 38 (5): 467–76.

Szajewska, Hanna, Maria Kotowska, Jacek Z. Mrukowicz, Malgorzata Armánska, and Wieslaw Mikolajczyk. 2001. "Efficacy of *Lactobacillus* GG in Prevention of Nosocomial Diarrhea in Infants." *Journal of Pediatrics* 138 (3): 361–65.

Tao, Lin, Bohua Wang, Yafen Zhong, Siok Hoon Pow, Xian Zeng, Chu Qin, Peng Zhang, et al. 2017. "Database and Bioinformatics Studies of Probiotics." *Journal of Agricultural and Food Chemistry* 65 (35): 7599–7606.

Teixeira, M. C. 2006. "The YEASTRACT Database: A Tool for the Analysis of Transcription Regulatory Associations in *Saccharomyces cerevisiae*." *Nucleic Acids Research* 34 (90001): D446–51.

Teixeira, Miguel C., Pedro T. Monteiro, Margarida Palma, Catarina Costa, Cláudia P. Godinho, Pedro Pais, Mafalda Cavalheiro, et al. 2018. "YEASTRACT: An Upgraded Database for the Analysis of Transcription Regulatory Networks in *Saccharomyces cerevisiae*." *Nucleic Acids Research* 46 (D1): D348–53.

Tewari, Vishal Vishnu, Sachin Kumar Dubey, and Girish Gupta. 2015. "*Bacillus clausii* for Prevention of Late-Onset Sepsis in Preterm Infants: A Randomized Controlled Trial." *Journal of Tropical Pediatrics* 61 (5): 377–84.

Thomas, S, I Przesdzing, D Metzke, J Schmitz, and A Radbruch. 2009. "*Saccharomyces boulardii* Inhibits Lipopolysaccharide-Induced Activation of Human Dendritic Cells and T Cell Proliferation." *Clinical Experimental of Immunol*ogy156 (1): 78–87.

Tiago, F. C.P., F. S. Martins, E. L.S. Souza, P. F.P. Pimenta, H. R.C. Araujo, I. M. Castro, R. L. Brandão, and Jacques R. Nicoli. 2012. "Adhesion to the Yeast Cell Surface as a Mechanism for Trapping Pathogenic Bacteria by *Saccharomyces* Probiotics." *Journal of Medical Microbiology* 61 (9): 1194–1207.

Tuo, Yanfeng, Xing Song, Yinglong Song, Wenwen Liu, Yu Tang, Yuan Gao, Shujuan Jiang, Fang Qian, and Guangqing Mu. 2018. "Screening Probiotics from *Lactobacillus* Strains According to Their Abilities to Inhibit Pathogen Adhesion and Induction of Pro-Inflammatory Cytokine IL-8." *Journal of Dairy Science* 101 (6): 4822–29.

Urdaci, C. 2008. "Exported Proteins in Probiotic Bacteria : Adhesion to Intestinal Surfaces, Host Immunomodulation and Molecular Cross-Talking with the Host." *FEMS Immunology &Medical Biology* 54 (1): 1–17.

Urdaci, Maria, Philippe Bressollier, and Irina Pinchuk. 2004. "*Bacillus clausii* Probiotic Strains." *Journal of Clinical Gastroenterology* 38 (Supp. 2): 86–90.

Vandenbosch, Davy, Evelien De Canck, Inne Dhondt, Petra Rigole, Hans J. Nelis, and Tom Coenye. 2013. "Genomewide Screening for Genes Involved in Biofilm Formation and Miconazole Susceptibility in *Saccharomyces cerevisiae.*" *FEMS Yeast Research* 13 (8):

Vandenplas, Yvan, Oscar Brunser, and Hania Szajewska. 2009. "*Saccharomyces boulardii* in Childhood." *European Journal of Pediatrics* 168 (3): 253–65.

Vandenplas, Yvan, Geert Huys, and Georges Daube. 2015. "Probiotics: An Update." *Jornal de Pediatria* 91 (1): 6–21.

Varankovich, Natallia VVarankovich, N. V, Nickerson, M. T. and Korber, D. R. 2015. "Probiotic-Based Strategies for Therapeutic and Prophylactic Use against Multiple Gastrointestinal Diseases." *Frontiers in Microbiology* 6 (July): 1–14.

Villarruel, Gladys, Daniel Martinez Rubio, Fani Lopez, Julio Cintioni, Rubén Gurevech, Gladis Romero, and Yvan Vandenplas. 2007. "*Saccharomyces boulardii* in Acute Childhood Diarrhoea: A Randomized, Placebo-Controlled Study." *Acta Paediatrica, International Journal of Paediatrics* 96 (4): 538–41.

Wang, Yu huan, and Ying Huang. 2014. "Effect of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* Supplementation to Standard Triple Therapy on *Helicobacter pylori* Eradication and Dynamic Changes in Intestinal Flora." *World Journal of Microbiology and Biotechnology* 30 (3): 847–53.

Wang, Yuan, Jing Zhang, Xian Jin Yi, and Fu Shin X. Yu. 2004. "Activation of ERK1/2 MAP Kinase Pathway Induces Tight Junction Disruption in Human Corneal Epithelial Cells." *Experimental Eye Research* 78 (1): 125–36.

Weizman, Zvi, Ghaleb Asli, and Ahmed Alsheikh. 2017. "Effect of a Probiotic Infant Formula on Infections in Child Care Centers: Comparison of Two Probiotic Agents." *Pediatrics* 115 (1): 5–9.

Wenus, C., R. Goll, E. B. Loken, A. S. Biong, D. S. Halvorsen, and J. Florholmen. 2008. "Prevention of Antibiotic-Associated Diarrhoea by a Fermented Probiotic Milk Drink." *European Journal of Clinical Nutrition* 62 (2): 299–301.

Wong, Marisa L., and Juan F. Medrano. 2005. "Real-Time PCR for mRNA Quantitation." *BioTechniques* 39 (1): 75–85.

Yan, Fang, and D Brent Polk. 2012. "*Lactobacillus rhamnosus* GG: An Updated Strategy to Use Microbial Products to Promote Health." *Functional Food Reviews (Print)* 4 (2): 77–84.

Yuan, Fuqiang, Huijuan Ni, Carl V. Asche, Minchul Kim, Saqib Walayat, and Jinma Ren. 2017. "Efficacy of *Bifidobacterium infantis* 35624 in Patients with Irritable Bowel Syndrome: A Meta-Analysis." *Current Medical Research and Opinion* 33 (7): 1191–97.

Zanello, Galliano, François Meurens, Mustapha Berri, and Henri Salmon. 2009. "*Saccharomyces boulardii* Effects on Gastrointestinal Diseases." *Current Issues in Molecular Biology* 11 (33): 47–58.

Zaouche, A, C Loukil, P De Lagausie, M Peuchmaur, J Macry, F Fitoussi, P Bernasconi, et al. 2000. "Effects of Oral *Saccharomyces boulardii* on Bacterial Overgrowth, Translocation, and Intestinal Adaptation after Small-Bowel Resection in Rats." *Scandinavian Journal of Gastroenterology* 35 (2): 160–65.

Zhu X.Y., Du J., Wu J., Zhao L. W., Meng X., and Liu G. F. 2017. "Influence of *Saccharomyces boulardii* Sachets Combined with Bismuth Quadruple Therapy for Initial *Helicobacter pylori* Eradication." *Zhonghua Yi Xue Za Zhi* 97 (30): 2353–56.

Zojaji H., Ghobakhlou M., Rajabalinia H., Ataei E., Sherafat S.J., and Moghimi-Dehkordi B. 2013. "The Efficacy and Safety of Adding the Probiotic *Saccharomyces boulardii* to Standard Triple Therapy for Eradication of *H. pylori*: A Randomized Controlled Trial." *Gastroenterology and Hepatology from Bed to Bench* 6 (SUPPL.): S99–104.

APPENDIX A

R CODES

A.1. R codes for Meta-analysis *S. boulardii* in adult

```
test2 <- read_excel("C:/Users/melike yılmaz/Desktop/test2.xlsx")
View(test2)
View(test2)
library(metafor)
dat <- escalc(measure="RR", ai=ai, n1i=n1i, ci=ci, n2i=n2i, data=test2, append =
TRUE)
View(dat)
metanal2 <- rma(yi, vi, data=dat)
View(metanal2)
metanal2
confint(metanal2)
predict(metanal2, digits=3, transf=exp)
predict(metanal2, digits=2, transf=exp)
confint(metanal2)
test2[1:16,"Author+year"]
forest(metanal2, transf=exp)
forest(metanal2, slab=paste(dat$Author, sep=","), xlim=c(-16,6), at=log(c(0.05, 0.25, 1,
4)), atransf=exp, cex=0.6)
op <-par(cex=0.8, font=2)
text(-16,18, "Authors and Year", pos=4)
text(6,18, "Relative Risk[95% CI]", pos=2)
par(op)
baujat(metanal2)
funnel(metanal2, atransf=exp)
summary(metanal2)
regtest(metanal2, model="rma")
```

Rstudio Data Results for adults

```
Random-Effects Model (k = 16; tau^2 estimator: REML)

tau^2 (estimated amount of total heterogeneity): 0.0330 (SE = 0.0714)
tau (square root of estimated tau^2 value):      0.1815
I^2 (total heterogeneity / total variability):   15.33%
H^2 (total variability / sampling variability):  1.18

Test for Heterogeneity:
Q(df = 15) = 18.7461, p-val = 0.2255

Model Results:

estimate      se     zval    pval    ci.lb    ci.ub
 -0.6760  0.1180  -5.7274  <.0001  -0.9074  -0.4447  ***

---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> confint(metanal2)

       estimate  ci.lb   ci.ub
tau^2    0.0330 0.0000  0.5461
tau      0.1815 0.0000  0.7390
I^2(%)  15.3341 0.0000 75.0062
H^2      1.1811 1.0000  4.0010

> predict(metanal2, digits=3, transf=exp)
  pred ci.lb ci.ub cr.lb cr.ub
 0.509 0.404 0.641 0.333 0.778
> predict(metanal2, digits=2, transf=exp)
 pred ci.lb ci.ub cr.lb cr.ub
 0.51  0.40  0.64  0.33  0.78
> confint(metanal2)

       estimate  ci.lb   ci.ub
tau^2    0.0330 0.0000  0.5461
tau      0.1815 0.0000  0.7390
I^2(%)  15.3341 0.0000 75.0062
H^2      1.1811 1.0000  4.0010

> test2[1:16,"Author+year"]
# A tibble: 16 x 1
   `Author+year`
   <chr>
 1 Adam et al,1977
 2 Monteiro et al,1981
 3 Surawicz et al,1989
 4 Macfarland et al,1994
 5 Lewis et al, 1998
 6 Surawicz et al,2000
 7 Cremonini et al, 2002
 8 Duman et al, 2005
 9 Can et al, 2006
10 Cindoruk et al, 2007
11 Bravo et al, 2008
12 Song et al, 2010
13 Chu et al, 2012
14 Pozzoni et al,2012
```

```
15 Zojaji et al, 2013
16 Kyriakos et al,2013

Random-Effects Model (k = 16; tau^2 estimator: REML)

  logLik  deviance       AIC       BIC      AICc
-12.6676   25.3351   29.3351   30.7512   30.3351

tau^2 (estimated amount of total heterogeneity): 0.0330 (SE = 0.0714)
tau (square root of estimated tau^2 value):      0.1815
I^2 (total heterogeneity / total variability):   15.33%
H^2 (total variability / sampling variability):  1.18

Test for Heterogeneity:
Q(df = 15) = 18.7461, p-val = 0.2255

Model Results:

estimate      se     zval    pval    ci.lb    ci.ub
 -0.6760  0.1180  -5.7274  <.0001  -0.9074  -0.4447  ***

---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> regtest(metanal2, model="rma")

Regression Test for Funnel Plot Asymmetry

model:     mixed-effects meta-regression model
predictor: standard error

test for funnel plot asymmetry: z = -1.1491, p = 0.2505
```

A.2. R codes for Meta-analysis *S. boulardii* in children

```
test3 <- read_excel("C:/Users/melike yılmaz/Desktop/test3.xlsx")
View(test3)
View(test3)
library(metafor)
dat <- escalc(measure="RR", ai=ai, n1i=n1i, ci=ci, n2i=n2i, data=test3, append =
TRUE)
View(dat)
metanal2 <- rma(yi, vi, data=dat)
View(metanal2)
metanal2
confint(metanal2)
predict(metanal2, digits=3, transf=exp)
```

```
predict(metanal2, digits=2, transf=exp)
confint(metanal2)
test3[1:17,"Author+year"]
forest(metanal2, transf=exp)
forest(metanal2, slab=paste(dat$Author, sep=","), xlim=c(-16,6), at=log(c(0.05, 0.25, 1,
4)), atransf=exp)
op <-par(cex=0.8, font=2)
text(-16,20, "Authors and Year", pos=4)
text(6,20, "Relative Risk[95% CI]", pos=2)
par(op)
par(op)
baujat(metanal2)
funnel(metanal2, atransf=exp)
summary(metanal2)
regtest(metanal2, model="rma")
```

Rstudio Data Results for children

```
Random-Effects Model (k = 17; tau^2 estimator: REML)

tau^2 (estimated amount of total heterogeneity): 0.2513 (SE = 0.1175)
tau (square root of estimated tau^2 value):      0.5013
I^2 (total heterogeneity / total variability):   81.31%
H^2 (total variability / sampling variability):  5.35

Test for Heterogeneity:
Q(df = 16) = 70.1689, p-val < .0001

Model Results:

estimate      se     zval    pval    ci.lb    ci.ub
 -0.6002  0.1407  -4.2670  <.0001  -0.8759  -0.3245  ***

---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> confint(metanal2)

       estimate   ci.lb   ci.ub
tau^2    0.2513  0.1045  0.7309
tau      0.5013  0.3233  0.8549
I^2(%)  81.3073 64.4006 92.6739
H^2      5.3497  2.8090 13.6498

> predict(metanal2, digits=3, transf=exp)
  pred ci.lb ci.ub cr.lb cr.ub
 0.549 0.416 0.723 0.198 1.522
```

```
> predict(metanal2, digits=2, transf=exp)
 pred ci.lb ci.ub cr.lb cr.ub
 0.55  0.42  0.72  0.20  1.52
> confint(metanal2)

       estimate   ci.lb   ci.ub
tau^2    0.2513  0.1045  0.7309
tau      0.5013  0.3233  0.8549
I^2(%)  81.3073 64.4006 92.6739
H^2      5.3497  2.8090 13.6498

> test3[1:17,"Author+year"]
 [1] "Cetina-Souri et al,1994" "Hafeez et al, 2002"      "Erdeve et al
, 2004"
 [4] "Katowska et al, 2004"    "Katowska et al, 2005"    "Kurugol, 200
5"
 [7] "Billo et al, 2006"       "Vandenplas et al, 2007"  "Villaruel et
al, 2007"
[10] "Htwe et al, 2008"        "Eren et al, 2010"        "Correa 2011"
[13] "Riaz et al, 2012"        "Shan et al,2013"         "Casem et al,
2013"
[16] "Zahoo et al,2014"        "Bin et al,2015"



Random-Effects Model (k = 17; tau^2 estimator: REML)

  logLik  deviance       AIC       BIC      AICc
-14.3107   28.6214   32.6214   34.1665   33.5444

tau^2 (estimated amount of total heterogeneity): 0.2513 (SE = 0.1175)
tau (square root of estimated tau^2 value):      0.5013
I^2 (total heterogeneity / total variability):   81.31%
H^2 (total variability / sampling variability):  5.35

Test for Heterogeneity:
Q(df = 16) = 70.1689, p-val < .0001

Model Results:

estimate      se     zval    pval    ci.lb    ci.ub
 -0.6002  0.1407  -4.2670  <.0001  -0.8759  -0.3245  ***

---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

> regtest(metanal2, model="rma")

Regression Test for Funnel Plot Asymmetry

model:     mixed-effects meta-regression model
predictor: standard error

test for funnel plot asymmetry: z = -1.3283, p = 0.1841
```

APPENDIX B

PROJECT COVER

"A case of *S. boulardii* study was performed and written at Tecnico Lisboa Biological Engineering Department under the supervisors of Assoc. Prof. Dr. Miguel Teixeira and Asst. Prof. Dr. Pedro Monteiro."

TÉCNICO LISBOA

Predicting the mechanisms of probiotic activity in *Saccharomyces boulardii*: a contribution to the development of the ProBioYeastract database

Melike Yılmaz

Thesis to obtain the Master of Science Degree in

Biological Engineering

Supervisor: Professor Miguel Nobre Parreira Cacho Teixeira

Co-supervisor: Professor Pedro Tiago Gonçalves Monteiro

Examination Committee

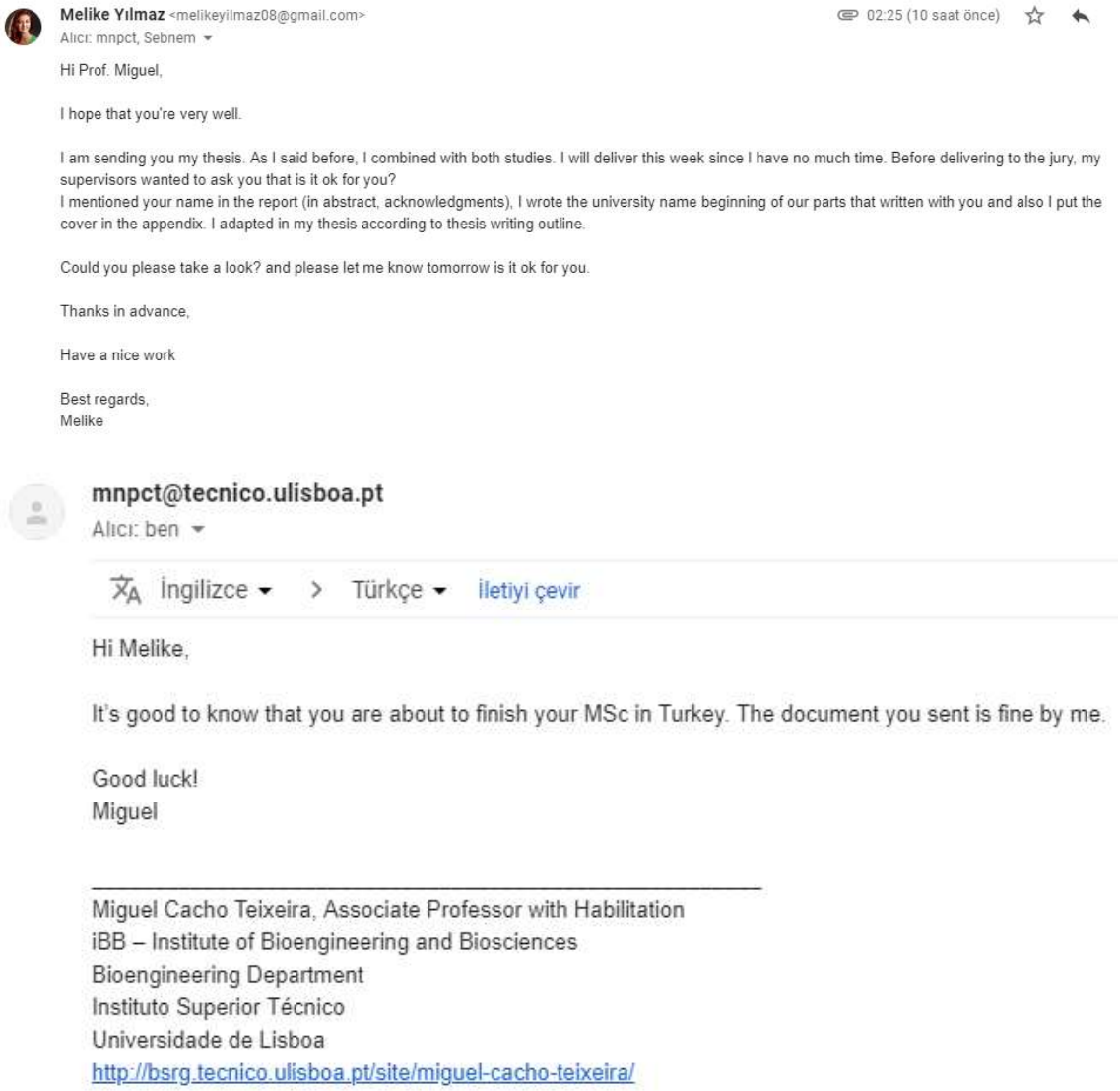
Chairperson: Professor Gabriel António Amaro Monteiro

Supervisor: Professor Miguel Nobre Parreira Cacho Teixeira

Member of the committee: Professor Jorge Humberto Gomes Leitão

Janeiro 2019

Figure B.1. Cover of Project in Portugal



Melike Yılmaz <melikeyilmaz08@gmail.com> 02:25 (10 saat önce)
Alıcı: mnpct, Sebnem

Hi Prof. Miguel,

I hope that you're very well.

I am sending you my thesis. As I said before, I combined with both studies. I will deliver this week since I have no much time. Before delivering to the jury, my supervisors wanted to ask you that is it ok for you?
I mentioned your name in the report (in abstract, acknowledgments), I wrote the university name beginning of our parts that written with you and also I put the cover in the appendix. I adapted in my thesis according to thesis writing outline.

Could you please take a look? and please let me know tomorrow is it ok for you.

Thanks in advance,

Have a nice work

Best regards,
Melike

mnpct@tecnico.ulisboa.pt
Alıcı: ben

İngilizce > Türkçe İletiyi çevir

Hi Melike,

It's good to know that you are about to finish your MSc in Turkey. The document you sent is fine by me.

Good luck!
Miguel

Miguel Cacho Teixeira, Associate Professor with Habilitation
iBB – Institute of Bioengineering and Biosciences
Bioengineering Department
Instituto Superior Técnico
Universidade de Lisboa
http://bsrg.tecnico.ulisboa.pt/site/miguel-cacho-teixeira/

Figure B.2. Screenshot of mail from Assoc. Prof. Dr. Miguel Teixeira