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Escola Superior de Biotecnologia

DEVELOPMENT OF A FUNCTIONAL ORANGE JUICE POWDER BY SPRAY DRYING

Thesis submitted to the Universidade Católica Portuguesa to attain
the degree of PhD in Biotechnology - with specialization in Microbiology

By

Joana Inês Bastos Barbosa

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Under the supervision of Professor Paula Cristina Maia Teixeira

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To my family

Abstract

The concept of functional food products has acquired a huge dimension with the enormous number of people concerned about consuming foods with health benefits. An innovative non-dairy functional food would be very attractive, not only to these consumers but also to specific niches in the market (e.g. lactose intolerants and vegetarians).

The aim of this work was to develop a powdered functional orange juice. For this, cultures with probiotic characteristics were used and the necessary conditions to obtain the dried product by spray drying were defined. Two cultures were selected, a commercial probiotic, *Lactobacillus plantarum* 299v and an isolate from a food matrix, *Pediococcus acidilactici* HA-6111-2. Safety and the presence of some functional characteristics of *P. acidilactici* were investigated and compared with those of the commercial probiotic. None of the bacteria produced any of the virulence factors investigated; they did not exhibit significant resistance to antibiotics and reductions lower than one logarithmic cycle were observed after exposure to simulated gastro-intestinal tract conditions. It was also verified that both bacteria survived during the spray drying process and subsequent storage for 60 days.

Powdered orange juice was initially obtained in a Büchi Mini Spray Dryer at constant feed temperature (ºC), flow rates of feed (mL/min), drying air (%) and compressed air (L/h) and varying inlet and outlet air temperatures (ºC) as well as the ratio of total soluble solids (orange juice): drying aid (10DE maltodextrin or gum Arabic). At selected inlet and outlet air temperatures of 120 ºC and 65 ºC, respectively, and ratio of soluble solids: drying aid of 0.5: 2, high drying yields and powders with low water activities (a_w) were obtained. No cell inactivation was observed for each culture after incorporated into orange juice and spray dried. Their survival during storage at 4 ºC was higher than at

room temperature under uncontrolled a_w. *Pediococcus acidilactici* was more resistant than *L. plantarum*, especially during storage at 4 ºC, with logarithmic reductions lower than 1 log-unit during a period of at least seven months.

To enhance survival of spray dried bacteria during storage, different pre-spray drying conditions were tested: i) different sugars were added to the culture media used (cell growth in the presence of lactose, followed by glucose resulted in the highest survival) and ii) cells were exposed to different sub-lethal stresses (only survival of *L. plantarum* cells during storage at room temperature was increased). For both cases, powders obtained were stored under different conditions of temperature, light exposure and a_w. Generally, survival was enhanced at 4 °C, a_w of 0.03 and absence of light. Cell viability during passage through simulated gastro-intestinal tract conditions was also investigated at the end of the storage at 4 ºC; the viability of both cultures was not enhanced by prior exposure to sub-lethal stresses.

Survival of cultures in orange fruit powders obtained by spray-, freeze- and convective hot air drying was investigated during drying and subsequent storage. Cell inactivation was only observed during convective hot air drying (about 2 logarithmic cycle reduction). Minimal reductions were observed for *P. acidilactici* for most conditions of storage while for *L. plantarum* only during storage at 4 ºC. Taking into account the initial number of cells obtained after each drying process, the techniques which allowed survival of an increased number of cells after storage, were spray- and freeze drying. Better dissolution, color and vitamin C retention were also obtained when these techniques were used.

Despite the scale-up and validation at industrial scale being still necessary, spray drying at pilot scale allowed producing a new functional orange juice powder with probiotic characteristics. The bacteria used demonstrated to be good candidates for the development of this product.

Resumo

O conceito de alimento funcional tem adquirido uma enorme dimensão graças ao grande número de pessoas preocupadas em consumir alimentos com benefícios para a saúde. Um alimento funcional inovador, não lácteo, poderá ser de grande interesse não só para este grupo de consumidores, mas também para nichos de mercado específicos (ex. intolerantes à lactose e vegetarianos).

Este trabalho teve como objetivo o desenvolvimento de um sumo de laranja funcional desidratado. Foram usadas culturas com características probióticas e definiram-se as condições necessárias para obtenção do produto desidratado através de secagem por atomização. Selecionaram-se duas culturas, um probiótico comercial, *Lactobacillus plantarum* 299v, e um isolado de uma matriz alimentar, *Pediococcus acidilactici* HA-6111-2. Testou-se a segurança e a presença de algumas características funcionais de *P. acidilactici* e comparou-se com o probiótico comercial. Nenhuma das bactérias apresentou quaisquer dos factores de virulência; não exibiram resistências significativas a antibióticos e observou-se uma redução inferior a um ciclo logarítmico após exposição às condições do trato gastro-intestinal simulado. Verificou-se ainda a capacidade de ambas as bactérias sobreviverem ao processo de secagem por atomização e posterior armazenamento durante 60 dias.

O sumo de laranja em pó foi obtido, numa primeira fase, por desidratação num *Büchi Mini Spray Dryer* mantendo constantes a temperatura de alimentação (ºC), as taxas de fluxo de alimentação (ºC), do ar de secagem (%) e do ar comprimido (L/h), variando as temperaturas do ar de entrada e de saída (ºC) e a proporção de sólidos solúveis totais (do sumo): auxiliar de secagem (10DE maltodextrina ou goma arábica). Às temperaturas de entrada e de saída selecionadas de 120 e 65 ºC, respetivamente, e proporção de sólidos solúveis: auxiliar de secagem de 0.5: 2, elevados rendimentos e pós com baixas actividades de água (a_w) foram obtidos. Não foi observada inativação celular das culturas após incorporação em sumo de laranja e desidratação por atomização. Observou-se uma

sobrevivência superior durante o armazenamento a 4 ºC do que à temperatura ambiente, sob valores de a_w não controlados. *Pediococcus acidilactici* apresentou maior resistência que *L. plantarum*, especialmente a 4 ºC, com perdas de viabilidade inferiores a um ciclo logarítmico durante um período de, pelo menos, sete meses.

Para aumentar a sobrevivência das bactérias desidratadas, diferentes condições pré-secagem foram testadas: i) diferentes açúcares foram adicionados ao meio de cultura (maior sobrevivência após crescimento na presença de lactose e glucose) e ii) exposição das células a diferentes stresses sub-letais (aumento da sobrevivência de *L. plantarum* durante o armazenamento à temperatura ambiente). Em ambos os casos, os pós obtidos foram armazenados em diferentes condições de temperatura, exposição de luz e a_w. Globalmente, a sobrevivência foi superior a 4 ºC, com a_w controlada a 0.03 e na ausência de luz. A viabilidade celular das duas culturas durante a passagem pelo trato gastro-intestinal simulado foi também investigada no fim do período de armazenamento a 4 ºC e não aumentou com a exposição aos stresses sub-letais.

Avaliou-se a sobrevivência das culturas durante a secagem por diferentes técnicas (atomização, liofilização e convecção por ar quente) e posterior armazenamento nos pós de laranja obtidos. Apenas a secagem por convecção causou inativação celular (redução de 2 ciclos logarítmicos). Reduções mínimas foram observadas para *P. acidilactici* na maioria das condições de armazenamento, enquanto que para *L. plantarum* apenas a 4 ºC. Tendo em conta o número inicial de células obtidas após cada processo de secagem, as técnicas que permitiram a sobrevivência de um maior número de células após armazenamento foram as de secagem por atomização e liofilização. Melhor dissolução, cor e retenção de vitamina C foram também obtidas por estas técnicas.

Apesar de ser ainda necessário o *scale-up* e validação à escala industrial, a secagem por atomização à escala piloto permitiu produzir um novo sumo de laranja funcional com características próbioticas. Os microrganismos estudados demonstraram ser bons candidatos para o desenvolvimento desse produto.

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Keywords

Acidic stress

Antibiotic susceptibility

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Color

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Dried powder

Gastro-intestinal tract

Gum Arabic

Heat shock

Lactic acid bacteria

Lactobacillus plantarum 299v

Maltodextrin

Oxidative stress

Pediococcus acidilactici HA-6111-2

Probiotic juice

Spray drying

Storage

Sugars

Survival

Viability

Virulence factors

Vitamin C.

LIST OF CONTENTS

Abstract vii

Resumo ix

Acknowledgments xi

Keywords xiii

List of Contents xiv

List of symbols and abbreviations xxi

List of tables xxiii

List of figures xxv

Scope and Outline xxviii

CHAPTER 1 Introduction 1

1.1. Spray drying 2

1.1.1. Principles of the technique 2

1.1.1.1. Atomization 3

1.1.1.2. Contact of sprayed particles and hot air 4

1.1.1.3. Evaporation of droplets water 5

1.1.1.4. Separation of dried product and humid air 6

1.2. Drying of fruit juices by spray drying 6

1.2.1. Stickiness and glass transition temperature 7

1.2.2. Drying aids 8

1.2.3. Influence of drying parameters and conditions on the fruit juice powders properties 9

1.2.3.1. Feed temperature 10

1.2.3.2. Feed rate 10

1.2.3.3. Type and concentration of drying aids 11

1.2.3.4. Type and speed of atomizers 12

1.2.3.5. Drying air flow rate 12

1.2.3.6. Inlet air temperature 13

1.3. Functional fruit juices powders 14

1.3.1. Probiotics and probiotic foods 14

1.3.2. Spray drying of probiotics and factors influencing their survival ... 15

1.3.2.1. Factors influencing survival of probiotics before spray drying 17

1.3.2.1.1. Strains characteristics 17

1.3.2.1.2. Growth phase 17

1.3.2.1.3. Sub-lethal stress exposure 18

1.3.2.1.4. Growth medium 18

1.3.2.2. Factors influencing survival of probiotics during spray drying 19

1.3.2.2.1. Drying medium 19

1.3.2.2.2. Drying parameters 20

1.3.2.3. Factors influencing survival of dried probiotics after spray drying 22

1.3.2.3.1. Packaging 23

1.3.2.3.2. Storage conditions 23

1.3.2.3.3. Rehydration 24

1.3.3. Retention of functional properties by the probiotics after spray dried 25

1.3.4. Spray drying of probiotic fruit juices 27

1.4. Conclusion 28

CHAPTER 2 *Pediococcus acidilactici* HA-6111-2 as a potential probiotic to be used in food industry 29

Abstract..30

2.1. Introduction 31

2.2. Materials and Methods 32

2.2.1. Origin, growth and storage conditions of LAB isolates 32

2.2.2. Virulence factors: production of gelatinase, lipase and DNase and hemolytic activity 33

2.2.3. Virulence genes 34

2.2.4. Antibiotic susceptibility..34

2.2.5. Gastrointestinal tract simulation 35

2.2.5.1. Inoculum 35

2.2.5.2. Simulated gastrointestinal conditions 35

2.2.6. Spray drying of *P. acidilactici* HA-6111-2 36

2.2.6.1. Preparation of cultures 36

2.2.6.2. Spray drying...36

2.2.6.3. Analysis of powders...36

2.2.6.4. Storage conditions..37

2.2.6.5. Enumeration...37

2.2.7. Statistical analysis..37

2.3. Results and Discussion 38

2.4. Conclusions 44

CHAPTER 3 Drying conditions for orange juice incorporated with lactic acid bacteria by spray drying 45

Abstract 46

3.1. Introduction 47

3.2. Materials and methods 48

3.2.1. Origin, growth and storage conditions of LAB isolates 48

3.2.2. Conditions of the drying process of orange juice 49

3.2.2.1. Materials 49

3.2.2.2. Orange juice preparation 49

3.2.2.3. Spray drying 49

3.2.2.4. Analysis of powders 50

3.2.2.4.1. Drying yield 50

3.2.2.4.2. Water activity 50

3.2.3. Spray drying of orange juice with LAB 51

3.2.3.1. Orange juice preparation 51

3.2.3.2. Preparation of LAB cultures 51

3.2.3.3. Spray drying and powder analysis 51

3.2.3.4 Storage conditions 52

3.2.3.5 Enumeration of LAB cultures 52

3.2.4. Statistical analysis 52

3.3. Results and discussion 53

3.4. Conclusions 60

CHAPTER 4 Effect of different conditions of growth and storage of two lactic acid bacteria after spray drying in orange juice 63

Abstract 64

4.1. Introduction 65

4.2. Materials and methods 66

4.2.1. Origin, growth and storage conditions of LAB cultures 66

4.2.2. Spray drying of orange juice incorporated with LAB 67

4.2.2.1. Orange juice preparation 67

4.2.2.2. Preparation of LAB cultures 67

4.2.2.3. Spray drying 68

4.2.2.4. Analysis of powders 68

4.2.2.4.1. Drying yield 68

4.2.2.4.2. Water activity 68

4.2.2.5. Storage conditions 68

4.2.2.6. Enumeration of LAB cultures 69

4.2.3 Gastro-intestinal tract simulation 69

4.2.3.1. Inoculum 69

4.2.3.2. Simulated gastro-intestinal conditions 70

4.2.4. Statistical analysis 70

4.3. Results and discussion 71

4.4. Conclusion 82

CHAPTER 5 Influence of sub-lethal stresses on the survival of lactic acid bacteria after spray drying in orange juice 83

Abstract 84

5.1. Introduction 85

5.2. Materials and Methods 86

5.2.1. Origin, growth and storage conditions of LAB cultures 86

5.2.2. Inoculum 86

5.2.3. Determination of the sub-lethal conditions 87
5.2.4. Spray drying of orange juice incorporated with LAB cultures 87
5.2.4.1. Materials and orange juice preparation 87
5.2.4.2. Exposure of LAB cultures to sub-lethal stresses 88
5.2.4.3. Spray drying 88
5.2.4.4. Analysis of powders 89
5.2.4.4.1. Drying yield 89
5.2.4.4.2. Water activity 89
5.2.4.5. Storage conditions 89
5.2.4.6. Enumeration of LAB cultures 90
5.2.5. Gastro-intestinal tract simulation 90
5.2.5.1. Inoculum 90
5.2.5.2. Simulated gastro-intestinal conditions 90
5.2.6. Statistical analysis 91
5.3. Results and Discussion 91
5.4. Conclusion 103

CHAPTER 6 Comparison of spray drying, freeze drying and convective hot air drying for the production on an orange juice powder 105

Abstract 106
6.1. Introduction 107
6.2. Materials and Methods 109
6.2.1. Lactic acid bacteria: origin, growth and storage conditions 109
6.2.2. Spray drying of orange juice with incorporated LAB 109
6.2.2.1. Orange juice preparation 109

6.2.2.2. Preparation of LAB cultures 110

6.2.2.3. Spray drying 110

6.2.3. Freeze drying of orange juice incorporated with LAB 111

6.2.3.1. Orange juice and LAB cultures preparation 111

6.2.3.2. Freeze drying 111

6.2.4. Convective hot air drying of orange incorporated with LAB 111

6.2.4.1. Orange and LAB cultures preparation 111

6.2.4.2. Convective hot air drying 112

6.2.5. Analysis of powders 112

6.2.5.1. Drying yield for spray dried powders 112

6.2.5.2. Water activity 113

6.2.5.3. Dissolution test 113

6.2.5.4. Color measurement 113

6.2.5.5. Vitamin C content 114

6.2.6. Storage conditions 115

6.2.7. Enumeration of LAB cultures 115

6.2.8. Statistical analysis 116

6.3. Results and Discussion 116

6.4. Conclusions 130

CHAPTER 7 Main conclusions 133

CHAPTER 8 Future work proposals 137

REFERENCES 141

List of symbols and abbreviations

AA………….. Ascorbic acid

agg………….. Aggregation protein gene

ANOVA…….. Analysis of variance

ATCC……….. American Type Culture Collection

a_w……………. Water activity

BPW………… Buffered peptone water

C*…………… Chroma

cfu………….... Colony forming unit

CIE………….. Commission Internationale de L'Eclairage

CLSI………… Clinical and Laboratory Standards Institute

CNCM………. Collection nationale de cultures de micro-organismes

Cyl………….. Cytolysin gene

DE………….. Dextrose equivalents

DHAA………. Dehydroascorbic acid

DNA………… Deoxyribonucleic Acid

Dnase……….. Deoxyribonuclease

esp………….. Enterococcal Surface Protein gene

efaAfm………. Cell wall adhesins of *Enterococcus faecium* gene

efaAfs……….. Cell wall adhesins of *Enterococcus faecalis* gene

EFSA……….. European Food Safety Authority

FAO………… Food and agriculture organization

FEEDAP……. Panel on additives and products or substances in animal feed

gelE………… Extracellular metallo-endopeptidase gene

GIT………….. Gastro-instestinal tract

GRAS……….. Generally regarded as safe

Hº…………… Hue angle

HCl………….. Hydrochloric acid

HPLC……….. High-performance liquid chromatography

H_2O_2.................. Hydrogen peroxide

IAA…………. Isoascorbic acid

INSA………... Instituto Nacional de Saúde Doutor Ricardo Jorge

LAB…………. Lactic acid bacteria

LiCl…………. Lithium chloride

MHA………… Muller-Hinton agar

MIC…………. Minimum inhibitory concentration

MRS…………. de Man, Rogosa and Sharpe

NaOH………... Sodium hydroxide

NFSM……….. Nonfat skimmed milk

OMAIAA……. Observatório dos mercados agrícolas e das importações agro-alimentares

OPDA……….. 1,2-phenylenediamine dihydrochloride

RSM…………. Reconstituted skim milk

SXT………….. Trimethoprim/sulphamethoxazole

tet……………. Tetracycline genes

USDA/FAS…. United States Department of Agriculture/Foreign Agricultural Service

UV…………… Ultraviolet

WHO………… World Health Organization

List of tables

Table 1.1. Literature on different probiotic fruit juice powders by spray drying………..27

Table 2.1. Minimum Inhibitory Concentration (MIC; µg/mL) of sixteen antibiotics for LAB isolates…………………………………………………………………………...39

Table 2.2. Survival of LAB isolates through simulated quick and slow digestions……..41

Table 3.1. Yield and a_w of orange juice powder at selected drying conditions………….53

Table 3.2. Survival of *L. plantarum* 299v and *P. acidilactici* HA-6111-2 before and after spray drying (SD) in RSM and orange juice supplemented with 10DE maltodextrin or gum Arabic……………………………………………………………………………….55

Table 4.1. Survival of LAB isolates before and after spray drying (SD) in RSM or in orange juice with 10DE maltodextrin (OJM) after grown in standard or modified MRS and the a_w values and yield of the respective powders…………………………………..71

Table 4.2. Survival of LAB isolates after 12 months of storage after spray-drying (SD) in RSM or in orange juice with 10 DE maltodextrin, through quick and slow digestions simulations………………………………………………………………………………..80

Table 5.1. Selected sub-lethal conditions for each LAB culture………………………...92

Table 5.2. Survival of LAB before and after spray drying (SD) in orange juice with 10DE maltodextrin after being exposed to different sub-lethal conditions……………………..92

Table 6.1. Results of the analyses of the obtained orange powders incorporated with both LAB after each drying method…………………………………………………………116

Table 6.2. Number of viable cells of both LAB in orange juice (or orange for convective drying) before each drying method and in the respective rehydrated powder after each processing……………………………………………………………………………….118

Table 6.3. Colorimetric results of the orange dried powders for each drying method and during 180 days of storage…………………………………………………………...126

Table 6.4. Vitamin C content (mg/100 mL) of diluted orange juice and orange powders obtained after each drying method and during storage…………………………………128

List of figures

Figure 1.1. Schematic representation of the spray drying process……………………….3

Figure 1.2. Drying parameters and conditions that influence the fruit juice powders properties……………………………………………………………………………10

Figure 1.3. Important factors that influence the probiotics survival before, during and after being spray dried…………………………………………………………………16

Figure 2.1. Logarithmic reduction of *P. acidilactici* HA-6111-2 (black) and *L. plantarum* 299v (grey) incorporated in 10% of RSM after spray drying and during 60 days of storage at 4 ºC (dotted lines) and room temperature (solid lines)………………………………...43

Figure 3.1. Logarithmic reduction of *L. plantarum* 299v (**A**) and *P. acidilactici* HA-6111-2 (**B**) incorporated in orange juice or RSM after spray drying and during 210 days of storage at room temperature: (—◆—) control (inoculum in 10% (w/v) of RSM); (—■—) orange juice with 2% of 10 DE maltodextrin and (—▲—) orange juice with 2% of gum Arabic. The dotted lines mean that the isolate was reduced to values below the detection limited of the enumeration technique…………………………………………………….56

Figure 3.2. Logarithmic reduction of *L. plantarum* 299v (**A**) and *P. acidilactici* HA-6111-2 (**B**) incorporated in orange juice or RSM after spray drying and during 210 days of storage at temperature of 4 ºC: (—◆—) control (inoculum in 10% (w/v) of RSM);(—■—) orange juice with 2% of 10 DE maltodextrin and (—▲—) orange juice with 2% of gum Arabic……………………………………………………………………………….57

Figure 4.1. Logarithmic reduction of *L. plantarum* 299v incorporated in RSM or orange juice with 2% of 10DE maltodextrin after spray drying and during 12 months of storage at different conditions (A, B, C or D): control (inoculum in 10% (w/v) of RSM); inoculum in orange juice with 2% of 10DE maltodextrin after grown in MRS supplemented with 20 g/L of different sugars (2, 3, 4, 5 and 6). The dotted lines mean that survival was lower than the detection limit of the enumeration technique…………………………………...74

Figure 4.2. Logarithmic reduction of *P. acidilactici* HA-6111-2 incorporated in RSM or orange juice with 2% of 10DE maltodextrin after spray drying and during 12 months of storage at different storage conditions (A, B, C or D): control (inoculum in 10% (w/v) of RSM); inoculum in orange juice with 2% of 10DE maltodextrin after grown in MRS supplemented with 20 g/L of different sugars (2, 3, 4, 5 and 6). The dotted lines mean that survival was lower than the detection limit of the enumeration technique………………76

Figure 5.1. Logarithmic reductions of *L. plantarum* 299v (●) without sub-lethal treatment; (□) acidic stress (HCl); (△) acidic stress (Lactic acid); (○) temperature stress and (◇) oxidative stress and incorporated in orange juice with 2% of 10DE maltodextrin after spray drying and during 180 days of storage in the presence of light with a_w of 0.03 at room temperature (**A**) and at 4 ºC (**B**), in the absence of light with a_w of 0.03 at room temperature (**C**) and in the presence of light with a_w of 0.11 at room temperature (**D**)….94

Figure 5.2. Logarithmic reductions of *P. acidilactici* HA-6111-2 (●) without sub-lethal treatment; (□) acidic stress (HCl); (△) acidic stress (Lactic acid); (○) temperature stress and (◇) oxidative stress and incorporated in orange juice with 2% of 10DE maltodextrin after spray drying and during 180 days of storage in the presence of light with a_w of 0.03 at room temperature (**A**) and at 4 ºC (**B**), in the absence of light with a_w of 0.03 at room temperature (**C**) and in the presence of light with a_w of 0.11 at room temperature (**D**).....98

Figure 5.3. Survival of *L. plantarum* 299v cells spray-dried in orange juice and after 180 days of storage through quick (**A**) and slow (**B**) simulated digestions after exposure to (□) acidic stress (HCl); (△) acidic stress (Lactic acid); (○) thermal stress; (◇) oxidative stress; and control without prior sub-lethal treatment (●)…………………………………...100

Figure 5.4. Survival of *P. acidilactici* HA-6111-2 spray-dried in orange juice and after 180 days of storage, through quick (**A**) and slow (**B**) simulated digestions after exposure to (□) acidic stress (HCl); (△) acidic stress (Lactic acid); (○) thermal stress; (◇) oxidative stress and control without sub-lethal treatment (●)……………………………………..101

Figure 6.1. Survival of *L. plantarum* 299v incorporated in RSM (open circle), in orange powder with 10 DE maltodextrin (closed square) or in orange powder without 10 DE maltodextrin (open square) after spray drying (**SD**), freeze drying (**FD**) or convective drying (**CD**) and during 180 days of storage in different conditions: room temperature in the presence of light with a_w of 0.03 (**1**); room temperature in the absence of light with a_w of 0.03 (**2**); room temperature in the presence of light with a_w of 0.11 (**3**) and at 4 ºC in the presence of light with a_w of 0.03 (**4**)……………………………………………...…120

Figure 6.2. Survival of *P. acidilactici* HA-6111-2 incorporated in RSM (open circle), in orange powder with 10 DE maltodextrin (closed square) or in orange powder without 10 DE maltodextrin (open square) after spray drying (**SD**), freeze drying (**FD**) or convective drying (**CD**) and during 180 days of storage at different conditions: room temperature in the presence of light with a_w of 0.03 (**1**); room temperature in the absence of light with a_w of 0.03 (**2**); room temperature in the presence of light with a_w of 0.11 (**3**) and at 4 ºC in the presence of light with a_w of 0.03 (**4**)……………………………………………..121

Scope and Outline

The use of spray drying for the production of different powdered fruit juices has been a common practice in the last years since this is an advantageous technique. Studies on probiotic fruit juice powders obtained by spray drying have been performed only very recently, and so far there is no study with probiotic orange juice powders. Therefore, this study aimed the development of a spray dried orange juice powder containing a probiotic culture.

To achieve this main goal, specific objectives were defined:

- To select the probiotic cultures to be incorporated into the orange juice;
- To select the spray drying conditions resulting in maximum yield of orange juice powder and maximum survival of probiotics;
- To evaluate the effect of the drying process in the degradation of vitamin C and color of the orange juice;
- To evaluate the effect of the drying process on the retention of probiotics functional proprieties;
- To compare spray drying with freeze drying and convective drying as methods for the preparation of dried orange juice powder containing probiotic cultures.

This thesis is structured in five parts which comprises 8 chapters. The different chapters are presented by the order in which the practical work was developed.

Part I contains chapter 1, in which a literature revision is presented. Each step of the spray drying process is discussed in detail. The influence of different spray drying parameters on the properties of the powdered fruit juices is described. The selection process for probiotics to be incorporated into fruit juices and how the probiotics cause health benefits to consumer are briefly approached. As the viability of probiotics is extremely important

in the development of a functional product, the factors that influence this viability, in all pre- and post-spray drying steps, and how to improve it are also detailed. Finally, few studies on probiotic fruit juices obtained by spray drying are cited.

Part II comprises chapters 2 and 3, which are the results of early experiments where microorganisms to be incorporated and the spray drying conditions were selected to obtain a probiotic orange juice powder. In Chapter 2, results of the study of some probiotic characteristics of an isolate from a food matrix, *P. acidilactici* HA-6111-2 (previously characterized by our research team) are presented and compared with a commercial probiotic culture, *L. plantarum* 299v. Some technological proprieties of both bacteria were also evaluated. Based on these results these two lactic acid bacteria were selected for subsequent studies. In Chapter 3 the chosen conditions of the spray drying conditions to develop the probiotic orange juice powder were established. The highest survival of bacteria during drying and storage as well as the highest yield and lowest water activity of orange juice powder were the main goals.

Part III encompasses chapters 4 and 5, in which pre- and post-spray drying attempts to enhance the survival of both lactic acid during spray drying and subsequent storage and during passage through the simulated GI tract were investigated. Pre-spray drying conditions were tested in chapter 4, including the addition of different sugars to the culture media used for the growth of both bacteria, and in chapter 5, including the exposure of cells to different sub-lethal stresses. Post-spray drying conditions were investigated in both chapters by subsequent storage of dried orange juice with incorporated bacteria under different conditions of temperature, light exposure and water activity.

Part IV includes chapter 6 where the comparison of spray drying with other two drying techniques (freeze- and convective hot air drying) were performed. This comparison was

based on the highest bacterial survival in orange powders during and after drying, and also on the characteristics of the final powders (dissolution, water activity, color and retention of vitamin C).

Part V comprises Chapter 7, in which the main conclusions of this study are presented, and Chapter 8, with the proposals of future work.

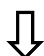
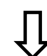
PART I: Literature Review

CHAPTER 1
Introduction

PART II: Selection of microorganisms and drying conditions

CHAPTER 2
Pediococcus acidilactici as a potential probiotic to be used in food industry

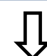
CHAPTER 3
Drying conditions for orange juice incorporated with lactic acid bacteria by spray drying

PART III: Improvement of pre- and post-spray drying conditions

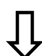
CHAPTER 4
Effect of different conditions of growth and storage on the survival of two lactic acid bacteria after spray drying in orange juice

CHAPTER 5
Influence of sub-lethal stresses on the survival of lactic acid bacteria after spray-drying in orange juice



PART IV: Spray drying *versus* other drying techniques

CHAPTER 6
Comparison of spray drying, freeze drying and convective hot air drying for the production of a probiotic orange powder



PART V: Conclusion and future work

CHAPTER 7
Main conclusions

CHAPTER 8
Future Work

The work presented in this thesis comprises six articles, five submitted and one published in a peer-reviewed scientific journal:

Chapter 1

Barbosa, J., Teixeira, P. 2015. Development of functional fruit juices powders by spray drying: a review. *Food Reviews International* (submitted)

Chapter 2

Barbosa, J., Borges, S., Teixeira, P. 2015. *Pediococcus acidilactici* as a potential probiotic to be used in food industry. *International Journal of Food Science and Technology*. DOI:10.1111/ijfs.12768

Chapter 3

Barbosa, J., Borges, S., Teixeira, P. 2015. Drying conditions for orange juice incorporated with lactic acid bacteria by spray drying. *Food and Bioproducts Processing* (submitted).

Chapter 4

Barbosa, J., Borges, S., Teixeira, P. 2015. Effect of different conditions of growth and storage on the survival of two lactic acid bacteria after spray drying in orange juice. *International Journal of Food Microbiology* (submitted).

Chapter 5

Barbosa, J., Borges, S., Teixeira, P. 2015. Influence of sub-lethal stresses on the survival of lactic acid bacteria after spray-drying in orange juice. *Food Microbiology* (submitted).

Chapter 6

Barbosa, J., Borges, S., Amorim, M., Pereira, M.J., Oliveira, A., Pintado, M.E., Teixeira, P. 2015. Comparison of spray drying, freeze drying and convective hot air drying for the production of a probiotic orange powder. *Journal of Functional Foods* (submitted).

CHAPTER 1

Introduction

1.1. Spray drying

Drying is one of the oldest techniques used to preserve foodstuffs. The first patent on the use of spray drying technique dates back to 1872 (Percy, 1872), but it was the urgent need to preserve foods, as well as to reduce its weight and volume, which led to the height of the spray drying technique that occurred during the Second World War (Cal and Sollohub, 2010).

Spray drying is a common process to obtain dry powders with low moisture content from liquid products as solutions, emulsions or suspensions (Peighambardoust *et al.*, 2011). Several advantages are recognized to this technique such as its low cost, its high reproducibility and its applicability to thermo-labile materials due to the short drying time. Additionally it is easy to operate and to scale-up, and it is very fast, since the particles are produced while they are dried (Burgain *et al.*, 2011; Silva *et al.*, 2011).

Although several factors may influence the quality of spray-dried products (Chegini *et al.*, 2008), using this technique, it is possible to obtain a final product with low water activity, ensuring its microbiological stability, reduced weight and volume, facilitating its storage, transportation and commercialization.

1.1.1. Principles of the technique

The process comprises four important steps, as schematized in Figure 1.1. First, the liquid sample is converted into a spray by an atomizer device. These small droplets are subjected to the contact with heated air in a drying chamber, resulting in moisture evaporation and formation of dry solid particles. Finally, the solid particles are separated from the air flow and collected in a collecting device (Cal and Sollohub, 2010; Gharsallaoui *et al.*, 2007; Peighambardoust *et al.*, 2011). Each of the process steps will be discussed in detail in subsequent sections.

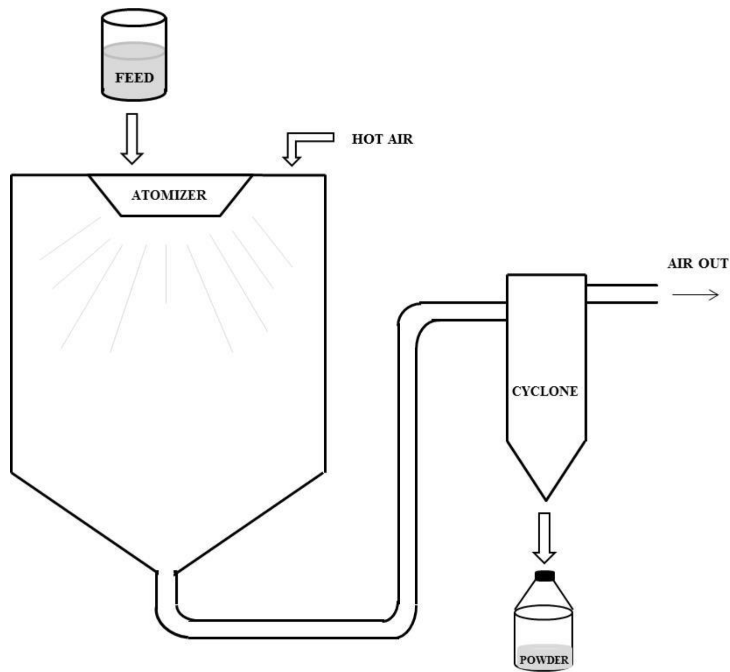


Figure 1.1. Schematic representation of the spray drying process.

1.1.1.1. Atomization

This is the most important process in the spray dryer, where the liquid, usually pumped by a peristaltic pump, is disintegrated into a large number of spray droplets. For this process an atomizer is required which is located inside the drying chamber. The higher the energy supplied the smaller is the size of the droplets obtained. Maintaining constant the energy supply and increasing the feed rate result in increased droplet size, as well as higher viscosity and surface tension of the initial sample (Gharsallaoui *et al.*, 2007). Depending on the sample and the intended powder characteristics, different atomizers can be chosen: centrifugal or rotary atomizer, hydraulic or pressure nozzle, two-fluid or pneumatic nozzle and ultrasonic nozzle (Cal and Sollohub, 2010; Silva *et al.*, 2011). Centrifugal atomizers, more expensive comparing with other atomizers, utilize the energy of a wheel, disc or bowl with high rotation speed to disintegrate the liquid into uniform and very fine droplets, under the effect of centrifugal force and under low feed pressure (Brennan, 2006; Cal and Sollohub, 2010). Pressure nozzles are inexpensive and appropriate to low

viscosity liquids, since through their small orifice, the liquid is pumped under elevated pressure and is disintegrated into a spray. If pumping pressure remains stable, the droplets will have a uniform size. In two-fluid nozzle atomizer, which is costly, a gas, generally compressed air, exits through an orifice at high speed. The liquid feed exits through a separated orifice and the impact with high speed air determines its disintegration into small droplets. These require lower drying chambers and are frequently used in laboratory spray driers. The size of droplets produced by this type of atomizer is not so uniform, especially when the liquids are very sticky (Brennan, 2006; Peighambardoust *et al.*, 2011). The ultrasonic atomizer disintegrates the liquid into very small droplets (below 50 microns) after its passage by the head of the atomizer, which has an integrated ultrasonic generator (Silva *et al.*, 2011; Walzel, 2011).

Despite their differences, the various atomizers are intended to form small droplets with a high surface area, thereby resulting in a short drying time during exposure to the hot air (Peighambardoust *et al.*, 2011).

1.1.1.2. Contact of sprayed particles and hot air

In the drying chamber, still during the atomization, the sprayed particles that are being formed come in contact with hot air and the drying process starts.

The shape of the vertical drying chambers most commonly used is cylindrical with an inverted cone in the lower part and can be tall-form or short-form designed, taking into account their height to diameter ratio of 5:1 and 2:1, respectively (Cal and Sollohub, 2010). The most often used are the short-form design, especially because they enable the use of rotary atomizers, while in tall-form design pressure or two-fluid nozzles atomizers are required (Langrish and Fletcher, 2001). Their selection should be based on the desired

particle size, the temperature at which the particles can be subjected and the route of dispersion of the particles (Cal and Sollohub, 2010).

Three systems of contact of sprayed particles and hot air can be achieved: i) concurrent design, where both liquid and hot air are introduced at the top of the drying chamber and sprayed particles fall in the same direction as the hot air flow down to its bottom; ii) counter-current design, in which the sprayed particles and hot air flow in different directions and particles contact with dry air at high temperatures; and iii) mixed flow design, that is a combination of concurrent and counter-current flows (Brennan, 2006, Cal and Sollohub, 2010; Paudel *et al.*, 2013). Concurrent design of drying chamber is the most frequently used. In these dryers, different flow paths are obtained taking into account their design and atomizers used. In tall-form chambers, designed to nozzle atomizers, a laminar flow path occurs, whereas in short-form chambers, with rotary atomizers, occurs a more complex spiral flow (Brennan, 2006). The concurrent with laminar flow path reduces drying efficiency and therefore it is compensated by the increase of the height of the chamber (Bork, 2001). The concurrent dryers also provide a low thermal degradation, since evaporation of sprayed particles occurs immediately at high inlet temperatures and dried particles rapidly are exposed to mild temperatures (Gharsallaoui *et al.*, 2007).

1.1.1.3. Evaporation of droplets water

In the contact between the sprayed particles and hot air, the heat transfer is carried out by convection from air to the droplets and the moisture transfer is carried out in the reverse direction due to vapor pressure difference (Masters, 1985).

Evaporation occurs through migration of moisture from inside of the droplet at a constant temperature and constant partial vapor pressure at the surface of the droplet. Moisture

diffusion rate from the inner to the surface of the droplet is constant to be able to maintain the saturated conditions at the surface. When the moisture content inside the particle reaches a critical value a dry skin is formed at its surface, so that evaporation become dependent on the diffusion rate of moisture through the dry skin. Its thickness increases and the evaporation rate is reduced. The drying is completed when the temperature of the particle becomes equal to the temperature of the air (Gharsallaoui *et al.*, 2007; Masters, 1985).

The heat and mass transfer rate depends on the particle size and the relative rate of air and particles (Murugesan and Orsat, 2012).

1.1.1.4. Separation of dried product and humid air

The final step of the spray drying process consists in the separation of dried product from the humid drying air. This separation often occurs outside the drying chamber in suitable devices, such as cyclone separators. It may also occur inside the drying chamber, but scraping tools are required for the collection of the dried product, such as mechanical brushes or vibratory devices (Cal and Sollohub, 2010). The separation by cyclone is based on the difference of density between dry product and humid air. It is produced a rotating vortex by a high speed flow field, which throws the dry particles towards the walls and downwards of the cyclone (Paudel *et al.*, 2013). Along with the cyclones, dryers can also be fitted with bag filters which allow collecting finest particles (Gharsallaoui *et al.*, 2007). Nevertheless, powders with very fine particles, with diameter less than 50 µm, may exhibit weak rehydration characteristics (Brennan, 2006).

1.2. Drying of fruit juices by spray drying

The use of spray drying to dry fruit juices is not a new practice. In 1978, Ashis S. Gupta patented a series of combinations of spray drying parameters to obtain a high quality

orange juice powder. Since then, this technique has been studied by many authors to obtain powders from juices of numerous fruits, such as açai (Tonon *et al*., 2011), acerola (Righetto and Netto, 2005), bayberry (Fang and Bhandari, 2012), black mulberry (Fazaeli *et al*., 2012), lime (Roustapour *et al*., 2006), mango (Cano-Chauca *et al*., 2005), pineapple (Abadio *et al*., 2004), pomegranate (Yousefi *et al*., 2011), watermelon (Quek *et al*., 2007), among others.

Economically this technique has great potential, since converting these products into powders, can increase significantly their shelf life, as well as reduce their volume and weight (Adhikari *et al*., 2003).

Fruit juices are constituted by large amounts of low molecular weight sugars, such as glucose, fructose and sucrose, which make them very sticky during spray drying and turn impossible their drying in their pure state. Besides drying problems, also caking can occur during powder storage. This is related to their low glass transition temperature which can be increased by the addition of drying aids with high molecular weights (Bhandari *et al*., 1997).

1.2.1. Stickiness and glass transition temperature

Stickiness of fruit juices during spray drying is assigned by thermal plasticization of low molecular weight sugars that are part of its constitution (Truong *et al*., 2005). In an attempt to minimize stickiness problems it is common to obtain a sticky point temperature curve subjecting the powder to a controlled temperature and moisture, as developed by Lazar *et al*. (1956). This sticky point temperature is 10 to 20 °C above the glass transition temperature, being both closely related (Roos and Karel, 1991). During spray drying, the moisture of particles is removed very quickly, which results in amorphous products (Aguilera *et al*., 1995). Glass transition temperature is defined as the temperature at which

occurs a glass-rubber transition of an amorphous product, which becomes sticky when the temperature of the particles surface is higher than their sticky point temperature (Bhandari *et al.*, 1997). This rubbery state causes a deposit of particles on the surfaces of dryer (Wang and Langrish, 2009). According to Bhandari and collaborators (1997), stickiness is apparently affected by a combination of physical properties besides glass transition temperature, such as solubility (ability of powder to dissolve), melting point (temperature at which the solid and liquid forms of a substance can be in equilibrium) and hygroscopicity (equilibrium moisture content after exposure to air humidity under given conditions).

1.2.2. Drying aids

The stickiness problem is easily solved with the addition of drying aids, which reduces the thermal plasticity of fruit juices. They can generate an external layer on the particle and change the stickiness of their surface through its conversion into a glassy state (Adhikari *et al.*, 2004). This is achieved once these drying aids have high molecular weights and hence have high glass transition temperatures, raising the glass transition temperature of the solution (Bhandari *et al.*, 1997).

Various drying aids are used in the food industry, such as different carbohydrates and proteins, but maltodextrins and gum Arabic are the most often used for fruit juices (Fazaeli *et al.*, 2012; Patil *et al.*, 2014; Tonon *et al.*, 2010). Maltodextrins, cheaper than gum Arabic, have different dextrose equivalent (DE), which are closely related to its mean molecular weight. This means that the lower the dextrose equivalent, the higher will be the glass transition temperature, which ranges from 100 to 243 ºC (Roos and Karel, 1991). Gum Arabic has a glass transition temperature of 126 ºC (reviewed by Schutyser *et al.*, 2012).

Furthermore, gum Arabic and maltodextrins are also used as encapsulating agents, being able to preserve antioxidant properties, nutritional value and color, and to retain aroma and flavors of different spray dried products (Boiero *et al.*, 2014; Chin *et al.*, 2010; Desobry *et al.*, 1999; Kha *et al.*, 2010; Osorio *et al.*, 2011; Pitalua *et al.*, 2010). Unlike gum Arabic, maltodextrins are usually associated with other encapsulating agents to enhance encapsulation efficiency, such as proteins (Castro-Muñoz *et al.*, 2014), modified starches (Soottitantawat *et al.*, 2003) and even gum Arabic (Bule *et al.*, 2010).

They are also recognized as prebiotics, i.e., benefit host health since they are non-digestible food ingredients that reaching the intestine of the host will stimulate the growth and/or activity of certain bacteria, as *Bifidobacterium* spp. and lactobacilli (Gibson and Roberfroid, 1995). Calame and collaborators (2008) found significantly higher numbers of *Bifidobacterium* spp. and lactobacilli after 4 weeks of gum Arabic consumption, attesting its prebiotic efficacy. Anekella and Orsat (2013) confirmed that maltodextrin acted as a moderate prebiotic, after enabling a high survival of probiotics *Lactobacillus acidophilus* NRRL B-4495 and *Lactobacillus rhamnosus* NRRL B-442 after spray drying.

1.2.3. Influence of drying parameters and conditions on the fruit juice powders properties

Several drying factors affect the spray dried fruit juices (Figure 1.2). Hygroscopicity, particle morphology, moisture content, bulk density (ratio of powder weight and its volume), solubility and drying yield (ratio of the weight of the powder obtained with the initial solid content) are some of the powder properties that are influenced by these spray drying factors (Fazaeli *et al.*, 2012; Tonon *et al.*, 2011).

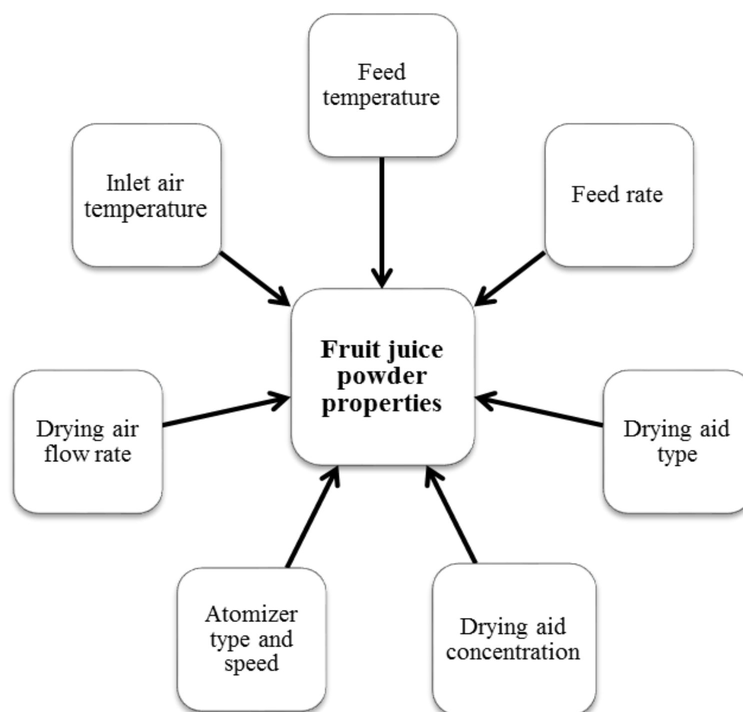


Figure 1.2. Drying parameters and conditions that influence the fruit juice powders properties.

1.2.3.1. Feed temperature

Feed temperature of the sample is an important factor, since it alters its viscosity contributing to an uniform atomization of the particles. This temperature must not be too high to avoid degradation of thermolabile compounds, such as the vitamins found in fruit juices (Gharsallaoui *et al.*, 2007). On a study using the spray drying to encapsulate *d*-limoneno, Paramita and collaborators (2010) found that high feed temperatures resulted in the formation of a thicker layer of wall material, which acted as a barrier of the flavor release.

1.2.3.2. Feed rate

Feed rate is related to the moisture content of the powders, since, if the atomizer is fed too quickly, the sprayed droplets will have a shorter contact time with hot air, so the

evaporation of water droplets will be lower, as well as the drying yield, due to the deposit on the dryer walls (Chegini and Ghobadian, 2007). Tonon and collaborators (2008) reported that an increase of feed flow rate from 5 to 25 g/min resulted in high moisture content, lower drying yield, and lower hygroscopicity of spray dried açai powders.

1.2.3.3. Type and concentration of drying aids

Drying aids play an important role in the drying of fruit juices, as already mentioned. Besides reducing stickiness by raising the glass transition temperature of the solution, they also influence the powder properties. Different drying aids affect the moisture content and hygroscopicity of the powders obtained (Tonon *et al*., 2011). Differences in moisture adsorption are due to differences in their chemical structure; drying aids composed by a higher number of ramifications with hydrophilic groups absorb air humidity more readily (Phisut, 2012). Also the size of the particles varies with the type of drying aid used, which is related to its molecular size (Tonon *et al*., 2011).

Moreover, the concentration of drying aid used influences the properties of the powders. Fazaeli and collaborators (2012) found a significant increase in the drying yield of black mulberry juice after increasing the concentration of maltodextrin. Several researchers found an inverse relationship between maltodextrin concentration and hygroscopicity (Moreira *et al*., 2009), bulk density (Fazaeli *et al*., 2012) and moisture content (Abadio *et al*., 2004; Kha *et al*. 2010) of spray dried powders. Bhusari and collaborators (2014) reported different sizes and shapes of tamarind pulp powder particles as a result of different concentrations of drying aids and Zareifard *et al*. (2012) found bigger particles of lime juice powders after increasing maltodextrin concentration. High concentrations of maltodextrin led to an increase of mango powders solubility (Cano-Chauca *et al*., 2005) and tamarind pulp powders porosity (Bhusari *et al*., 2014). The concentration of

maltodextrin also seems to have an effect on the color of the powders. In two studies with different products, the authors reported that the use of maltodextrin in concentrations equal to or greater than 10% resulted in loss of the attractive color of the powders due to a whitish appearance (Caliskan and Dirim, 2013; Quek *et al*., 2007).

1.2.3.4. Type and speed of atomizers

Different types of atomizers can be used to form sprayed particles, which will influence their size. Finney and collaborators (2002) found that the use of centrifugal wheel atomizer led to formation of larger orange oil particles in comparison with spray nozzle atomizer. Using the same type of nozzle atomizer, increasing the atomization pressure results in smaller droplet sizes (reviewed by Ishwarya *et al*. 2015). Several authors have studied the effect of the atomizer speed. Chegini and Ghobadian (2005) reported that maintaining feed flow rate and raising the atomizer speed resulted in the decrease of moisture content and particle size and the increase of bulk density of orange juice powders. For Abadio and collaborators (2004), the atomizer speed was inversely related to good solubility of the pineapple juice powder.

1.2.3.5. Drying air flow rate

Drying air flow rate should be the maximum considering that the energy available to evaporate the sprayed particles varies with the amount of drying air. However, Goula and Adamopoulos (2005a) found that the increase of the drying air flow rate resulted in the increase of the moisture content of tomato pulp powders. Nevertheless, the drying yield increased after the increase of drying air flow rate, but in combination with the increase of other parameters, such as compressed air flow rate and inlet air temperature (Goula and Adamopoulos, 2005b).

1.2.3.6. Inlet air temperature

Inlet air temperature is the temperature of the drying air, which causes the evaporation of sprayed particles (Cal and Sollohub, 2010). Different inlet air temperatures have been studied in the spray drying of various fruit juices over the years and this drying parameter affected all properties of the obtained powders. At higher inlet air temperatures an excessive evaporation occurs, which results in lower moisture content of powders. Innumerous authors reported this fact (Fazaeli *et al.*, 2012; Quek *et al.*, 2007; Tonon *et al.*, 2011). An increase of the inlet air temperature can also cause a quick formation of an impermeable dried layer in the droplet surface, which lead to the formation of porous particles, resulting in low bulk densities (Kha *et al*, 2010), larger and morphological different particles (Tonon *et al.*, 2011) and high hygroscopicity (Santhalakshmy *et al.*, 2015). While Fazaeli *et al.* (2012) reported an increase in the solubility of the powders of black mulberry juice dried at an inlet air of 150 ºC, in the study of Quek *et al.* (2007), watermelon powders showed less solubility at 175 ºC. This difference could be explained by the higher inlet air temperature used by Quek *et al* (2007), which might had resulted in the formation of a rigid layer on the powder particle surface increasing its insoluble solids percentage. Due to the efficient transfer of heat and mass at higher inlet air temperatures, the drying yield is elevated (Fazaeli *et al.*, 2012; Tonon *et al.*, 2008). Nonetheless, at very high temperatures the yield decreases, since melting of the powder occurs and this results in adherence to the dryer walls; at inlet air temperature of 150 ºC, Santhalakshmy and collaborators (2015) found the highest drying yield of jamun fruit juice powder comparing with lower (140 and 145 ºC) and higher temperatures (155 and 160 ºC). High temperatures also influenced the colors of dried powders by the significant degradation of carotenoids content (Kha *et al.*, 2010; Quek *et al.*, 2007) and also resulted in great losses of antioxidant properties (Caliskan and Dirim, 2013).

1.3. Functional fruit juices powders

There are few studies on fruit juices with incorporated probiotics and dried by spray drying. Survival of probiotics is influenced by several factors, but can be improved by some changes, such as the exposure to sub-lethal stresses before spray drying or different storage conditions of the powders.

1.3.1. Probiotics and probiotic foods

The concept of "probiotic" was introduced in 1907 by Eli Metchnikoff, who observed that consumption of fermented milk in high quantities resulted in longer lives of nomads in Bulgaria and Russian Steppes (Metchnikoff, 1907). According to Hamilton-Miller and collaborators (2003), the word "probiotic" was firstly used by Werner Georg Kollath in 1953 to describe active substances required to restore health of patients suffering from a malnutrition form. The definition of "probiotic" was improved throughout the years and, currently, probiotics are defined as "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host" by the Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO, 2002). The most used microorganisms belong to genera *Lactobacillus* (e.g. *Lactobacillus plantarum*, *L. acidophilus, L. rhamnosus)* and *Bifidobacterium* (e.g. *Bifidobacterium bifidum, Bifidobacterium longum*), but other bacteria (e.g. *Enterococcus faecium*, *Lactococcus lactis*) as well as yeasts (e.g. *Saccharomyces boulardii*) may also demonstrate probiotic properties (Champagne *et al*., 2011). Both *Lactobacillus* and *Bifidobacterium* genera belong to the group of lactic acid bacteria (LAB) and are the most used commercially in probiotic foods. LAB have a generally recognized as safe (GRAS) status (Tripathi and Giri, 2014). Selection requirements for probiotic strains to develop probiotic foods include: i) safety: they should be of human origin and do not possess

virulence factors nor be pathogenic; ii) functional properties: they should be able to survive through gastrointestinal tract (GIT) and adhere to mucosal surface of gut; iii) technological properties: they should be genetically stable, be produced in large scale and should survive during processing and storage; and iv) physiological properties: it is desirable that a probiotic could be able to inhibit the growth of pathogens in the GIT, reduce serum cholesterol, improve lactose tolerance and possess antimutagenic and anticarcinogenic properties (Iannitti and Palmieri, 2010; Tripathi and Giri, 2014). Several reviews on the health benefits provided by probiotics have been published recently (e.g. Butel, 2014, Tripathi and Giri, 2014); Iannitti and Palmieri (2010) published a detailed review on significant clinical trials using probiotics to treat several pathologies. In order to cause health benefits to the consumers, probiotic foods should contain viable probiotic microorganisms in amounts of about 10^6 to 10^7 cfu (colony forming units)/g or ml at the time of consumption. Moreover, probiotic cultures should maintain their properties during processing and storage, as well as to survive passage through the GIT of the consumers (FAO/WHO, 2002; Sanz, 2007; Talwalkar *et al*., 2004).

1.3.2. Spray drying of probiotics and factors influencing their survival

The addition of probiotic cultures to food products requires previous encapsulation by methods such as spray drying, to guarantee their easy storage, handling and longer shelf life (Santivarangkna *et al*., 2006). Microencapsulated probiotics by spray drying are more resistant to the severe conditions imposed by the food matrices (e.g. low pH, low water activity, high salt or sugars concentrations, natural antimicrobial compounds), to storage and to passage through GIT (Champagne and Fustier, 2007; Ying *et al*., 2012). Although spray drying is a moderate technique, due to the short residence time and the quite low temperatures reached by the particles (Mazza *et al*., 2003), decreases on the survival of

probiotics during drying and subsequent storage have been often reported (Ananta *et al*., 2005; Barbosa *et al*., 2015). Ways to improve survival of probiotics during spray drying have been widely investigated (Golowczyc *et al*., 2011a; Perdana *et al*., 2014; Reddy *et al*., 2009) since this technique has numerous advantages (e.g. lower costs and less time consuming) over freeze drying, the most used technique to produce probiotic cultures (Santivarangkna *et al*., 2007). Since production of dried probiotics begins with cell growth in culture medium and ends with their storage in a powder form, several parameters can be improved before, during and after spray drying to enhance the probiotic survival (Figure 1.3); these will be discussed in subsequent sections. Nevertheless, some more detailed reviews about this subject have been published recently (Peighambardoust *et al*., 2011; Silva *et al*., 2011; Tripathi and Giri, 2014).

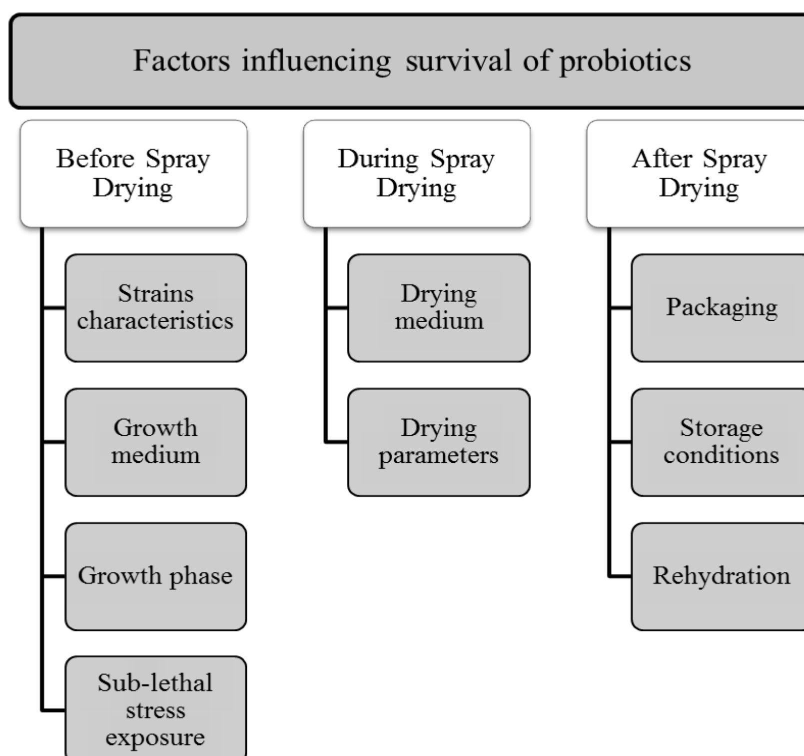


Figure 1.3. Important factors that influence the probiotics survival before, during and after being spray dried.

1.3.2.1. Factors influencing survival of probiotics before spray drying

Cell viability during spray drying is influenced by some conditions prior to drying as the intrinsic resistance of the strains, growth medium, growth phase and the exposure of cells to stress conditions (Fu and Chen, 2011).

1.3.2.1.1. Strains characteristics

Intrinsic resistance of the strains results in different behaviors during spray drying. Corcoran and collaborators (2004) reported survivals of 25% and 50% for *L. rhamnosus* E800 and *L. rhamnosus* GG, respectively, after being spray dried in reconstituted skim milk (20% w/v). Also Golowczyc *et al*. (2011a) studied two strains of *Lactobacillus kefir* spray dried in reconstituted skim milk (11% w/v) and found that *L. kefir* 8348 was more sensitive to the process than *L. kefir* 8321. Moreover, the authors reported that this more sensitive strain loss the ability to adhere to intestinal cells after spray drying.

Therefore, the selection of resistant strains should be done, to guarantee the best performance during spray drying and subsequent storage.

1.3.2.1.2. Growth phase

It is widely accepted that cells at stationary phase are more resistant to spray drying and subsequent storage than cells at lag or exponential phases (Corcoran *et al*., 2004; Lavari *et al*., 2015; Teixeira *et al*., 1995a). This is partially explained by the fact the stresses encountered during growth (e.g. starvation of nutrients and low pH) induce cell resistance to other stresses (Teixeira *et al*., 1994; van de Guchte *et al*., 2002).

1.3.2.1.3. Sub-lethal stress exposure

Previous exposure of cells to sub-lethal stress conditions could induce resistance to spray drying. Paéz and collaborators (2012) studied the effect of a mild heat treatment on the survival of five lactobacilli to spray drying. For *Lactobacillus casei* Nad and *L. plantarum* 8329, the authors observed a significantly higher loss of viability when cells were not exposed to the thermal stress. Silva and collaborators (2005a) studied the effect of acidic stresses and found that cells of *Lactobacillus bulgaricus* ESB285 were more resistant to heat stress and spray drying when grown under non-controlled pH (final pH of 4.5) instead of controlled pH (adjusted to 6.5). It has been reported that cell exposure to stresses led to the synthesis of heat shock proteins (Lim *et al.*, 2000; Silva *et al.*, 2005b), which, according to Teixeira *et al.* (1994) occurs at exponential phase. This makes sense, since Teixeira *et al.* (1995a) observed that the same cells, not exposed to any stress condition, were more resistant at the stationary phase.

1.3.2.1.4. Growth medium

The composition of the growth medium greatly affects the survival of probiotics during spray drying and storage. During spray drying, cells are exposed to low water activities and high temperatures, i.e. cells are subjected to both osmotic and thermal stresses. Cellular accumulation of compatible solutes, such as amino acids, quaternary amines and sugars (Kets *et al.*, 1996), enhances resistance to these stresses (Carvalho *et al.*, 2004a; Welsh, 2000). Ferreira and collaborators (2005) found that during storage after spray drying, *Lactobacillus sakei* CTC 494 survived 60% longer when grown in medium containing 20 g/L of sucrose or 12.5 g/L of monosodium glutamate. Compatible solutes can be synthetized by some bacteria (other than LAB) and be accumulated when they are adapting to harsh conditions, but during the drying process, this is not possible due to the

short drying time (Morgan *et al.*, 2006). Thus, these compatible solutes should be accumulated before spray drying, such as in the growth medium used, to allow the adaptation of cells (Fu and Chen, 2011).

1.3.2.2. Factors influencing survival of probiotics during spray drying

Survival of probiotics during spray drying is influenced by the drying medium used but also by some parameters associated with the drying process itself, such as drying temperatures.

1.3.2.2.1. Drying medium

Protective carriers are added to the drying medium, individually or in combination with others, to increase cell viability during spray drying and storage. Skim milk is probably the most common carrier and its protective effect has been demonstrated for different species by several authors (Ananta *et al.*, 2005; Barbosa *et al.*, 2015; Paéz *et al.*, 2012; Teixeira *et al.*, 1995a,b). Still in an attempt to improve this effect, other protective carriers have been added to skim milk. Ananta and collaborators (2005) included oligofructose-based or polydextrose-based prebiotics besides skim milk into drying medium. Despite high survival rates obtained, the authors found that the storage stability of *L. rhamnosus* GG was higher when only skim milk was added to the drying medium. On the contrary, Golowczyc *et al.* (2011a) observed that the addition of different protective carriers plus skim milk allowed a better survival of two *L. kefir* strains during spray drying than skim milk alone. For the production of non-dairy probiotic foods, it is necessary to use other protective carriers as an alternative to skim milk. Various sugars have been added to the drying medium to assess their protective effect. Semyonov and collaborators (2011)

tested the effect of trehalose together with other protective carriers added to the drying medium on the survival of the probiotic *L. casei* subsp. *paracasei* LMG P-21380 during drying in an ultrasonic vacuum spray dryer. The authors found that the addition of maltodextrin 5DE in combination with trehalose resulted in a significant increase of probiotic survival. Ying and coworkers (2012) incorporated glucose into the drying medium and observed that despite it did not had a marked influence on the survival of *L. rhamnosus* GG during spray drying, survival was increased during storage. Besides skim milk and sugars, other carriers such as gum Arabic (Desmond *et al*., 2002a; Salar-Behzadi *et al*., 2013) and maltodextrin (Anekella and Orsat, 2013; Reddy *et al*., 2009) have demonstrated to be effective protectants, contributing to high survival rates during spray drying. Semyonov and collaborators (2011) reported significantly higher survival of *L. casei* subsp. *paracasei* LMG P-21380 after dried in encapsulation matrices based on low DE maltodextrins in comparison with high DE. As mentioned above, the drying medium conferring the highest protection during spray drying is not necessarily the same which confers the best protection during storage of dried probiotics.

Moreover, the solid content of drying medium has influence on probiotic survival. High solids content results in bigger particles which will take more time to dry, so that the probiotics will be more time in contact with high temperatures, decreasing their survival (Santivarangkna *et al*., 2007).

1.3.2.2.2. Drying parameters

Decrease of probiotics survival during spray drying and subsequent storage is also correlated with the inlet and outlet air temperatures used. Various authors have studied the effect of inlet air temperatures (Anekella and Orsat, 2013; Mestry *et al*., 2011) and the outlet air temperatures (Kingwatee *et al*., 2014; Golowczyc *et al*., 2010) on the survival of

different species. After spray drying, Teixeira and collaborators (1995b) reported that the loss of viability of spray dried *Lactobacillus delbrueckii* ssp. *bulgaricus* NCFB 1489 was associated to cell membrane, cell wall and DNA damages. It is considered that the outlet air temperature is the main parameter affecting the survival of probiotics and it depends of several other parameters, such as inlet air temperature, drying air and feed flow rates and the size of atomized droplets (Santivarangkna *et al*., 2007). Ananta and collaborators (2005) studied the effect of a variety of outlet air temperatures from 70 to 100 ºC and found that the higher the outlet air temperature, the higher was the degree of cell membrane damage, decreasing the survival of *L. rhamnosus* GG; higher outlet air temperatures also led to lower moisture content of the powders. Kim and Bhowmik (2006) observed that higher inlet and outlet air temperatures resulted in a decreased viability of various LAB strains and that this viability was more affected by the outlet air temperature. Cell inactivation is indirectly influenced by inlet air temperature, which apparently has a small effect. After studying the effect of both inlet air temperature (120 to 160 ºC) and feed flow rate (0.12 to 0.3 L/h) on the survival of a *L. acidophilus* isolate, Mestry and collaborators (2011) found that survival was lower when high inlet air temperatures and low feed flow rate were used. This was related with the exposure time of cells to high temperatures, since at higher feed flow rates this time is reduced. Teixeira and collaborators (1995a,b) had already reported that combinations of temperature-time during spray drying affected the survival of bacteria. It is important to highlight that, as mentioned before in this review, other parameters of spray dried powders are also influenced by drying air temperatures. Lower drying air temperatures used in the spray drying of a probiotic fruit juice result in powders with high probiotic survival but also with high moisture contents. Thus, it is necessary to choose the suitable air drying temperatures to guarantee the quality of the final product.

As already mentioned in the topic of spray drying technique, atomization is the most important step, since liquid sample is atomized into small droplets. During atomization, probiotic cells are exposed to shear forces, which according to several authors do not cause significant cell inactivation (Riveros *et al.*, 2009; Santivarangkna *et al.*, 2008a). Nonetheless, in a fairly recent study conducted by Ghandi and collaborators (2012) to test the effect of different shear rates on the survival of *Lactococcus lactis* subsp. *cremoris* ASCC930119, the authors observed that the survival decreased significantly with an increased shear rate, regardless of the gas used during atomization. Survival of probiotics could also be influenced by atomization pressure. Riveros and coworkers (2009) observed higher *L. acidophilus* cell numbers at lower nozzle pressures. Low shear forces are produced at lower atomization pressures, which enhanced the survival of bacteria (Riveros *et al.*, 2009). According to Ghandi *et al.* (2012), the survival of probiotics is also affected indirectly by the initial droplet size and distribution by modifying the temperature of droplet/particle, the trajectories of moisture and the exposure to oxygen. The authors demonstrated that besides shear stress, damages caused by exposure to oxygen resulted in higher cell inactivation in comparison with thermal and dehydration stresses during the drying process.

1.3.2.3. Factors influencing survival of dried probiotics after spray drying

Besides the efforts to guarantee the maximum survival of probiotics until their dried form, the subsequent processes to spray drying are also extremely important in this sense. The viability of probiotic cultures should be maintained during probiotic food production, but also during their storage. In the case of a dried probiotic fruit juice, product rehydration should also not affect their viability.

1.3.2.3.1. Packaging

The choice of suitable packaging is an important issue, since type of packaging materials, their light and gas permeability, and packaging technique (as vacuum) could affect probiotics survival (reviewed by Tripathi and Giri, 2014).

Different types of packaging materials have been used to store different spray dried probiotics (Hsiao *et al*., 2004; Maciel *et al*., 2014). Wang and collaborators (2004) reported better survivals of *L. acidophilus* CCRC 14079 and *Streptococcus thermophilus* CCRC 14085 dried in fermented soymilk after storage in laminated pouches followed by glass and polyester bottles. Maciel and coworkers (2014) reported good survival rates of spray dried *L. acidophilus* La-5 after storage in metallized polyester-polyethylene packaging. The authors attributed the high survivals through the barrier characteristics of the packaging due to the water vapor barrier of polyethylene, gas barrier of polyester and, finally, light barrier of metallization (Maciel *et al*., 2014). A package that is impermeable to gas and light might reduce the oxidation of the cell membrane lipids, preventing the formation of free radicals, which once diffusing into cell can cause cell death by DNA damage (Santivarangkna *et al*., 2008b).

Regarding storage gaseous atmosphere, Golowczyc *et al*. (2010) did not found significant differences among the survival of *L. kefir* CIDCA 8348, *L. plantarum* CIDCA 83114 and *Saccharomyces lipolytica* CIDCA 812 stored under vacuum or air. However, Chávez and Ledeboer (2007) reported high survival rates of *Bifidobacterium lactis* BB12 stored under vacuum in comparison with air.

1.3.2.3.2. Storage conditions

Temperature and relative humidity of storage are crucial and greatly affect the viability of dried probiotics.

In several studies on spray dried bacteria authors found that their survival during storage was favored at lower temperature conditions (Barbosa *et al.*, 2015; Golowczyc *et al.*, 2010; Perdana *et al.*, 2014; Teixeira *et al.*, 1995b). At higher temperatures, lipid oxidation and protein denaturation might occur, leading to loss of cell viability (Fu and Chen, 2011).

The water portion available after drying affects the viability of the dried microorganisms, not only after drying but also during storage (Zayed and Roos, 2004). Both water activity and moisture content of dried probiotics should be low and remained constant during storage to improve their survival (Chávez and Ledeboer, 2007). Teixeira and collaborators (1995b) studied the effect of storage under different water activities (0.03, 0.11, 0.23, 0.43 and 0.75) on the survival of *L. delbrueckii* ssp. *bulgaricus* NCFB 1489 and observed higher survival at 0.11 and 0.23. Other authors reported higher survival rates at low water activities (Golowczyc *et al.*, 2011a; Ying *et al.*, 2012). Relative humidity of storage also influences the glass transition temperature of the dried powders. High relative humidity during storage induces caking in dried powders which lead to viability loss. The addition of protective carriers allow dried powders to keep in a glassy state, improving the probiotics survival during storage by controlling free radical formation (Fu and Chen, 2011; Meng *et al.*, 2008).

1.3.2.3.3. Rehydration

Another critical step is the rehydration of dried probiotics, since it consists in another osmotic stress (Muller *et al.*, 2010). Several authors reported that the solution used, the time and temperature of rehydration affects the recovery of spray dried probiotics (Muller *et al.*, 2010; Teixeira *et al.*, 1995a; Wang *et al.*, 2004).

Although Teixeira and collaborators (1995a) had reported that rehydration should be done slowly (30 minutes of soaking instead of 2 min of vigorous shaking), Muller *et al*. (2010) observed a better recovery of spray dried *B. longum* NCC3001 after 30 minutes of reconstitution than after 60 minutes. Based on both studies, 30 minutes appears to be an adequate time for rehydration and the same authors reported that at 30 minutes reconstitution, no significant differences were obtained between the different rehydration media used. Desmond and collaborators (2001) observed maximum differences of 0.5 log cfu/mL in the recovery of *L. paracasei* ssp. *paracasei* NFBC338 after using various rehydration media.

Increasing the rehydration temperature until 50 ºC was reported to enhance the viability of spray dried probiotics (Teixeira *et al*., 1995a; Wang *et al*., 2004). Nevertheless, at rehydration temperatures above the temperatures of membrane phase transition, an increased proportion of saturated fatty acids can occur, leading to cell membrane rupture, which may result in loss of viability (Santivarangkna *et al*., 2008a).

Once behavior during rehydration is species/strain dependent and a probiotic fruit juice powder has to be rehydrated before consumption, the best rehydration conditions must be achieved and described on product packaging to guarantee the best survival of a specific probiotic.

1.3.3. Retention of functional properties by the probiotics after spray dried

Extremely important, besides maintaining their viability during all processes of drying and storage, is the ability of a dried probiotic culture to retain its functional properties.

Some authors studied different spray dried probiotic species and evaluated the retention of their specific properties. Reddy and collaborators (2009) studied the functional

properties as acid and bile salt tolerance and cholesterol assimilation of three probiotics: *L. plantarum* CRF 2191, *Lactobacillus salivarius* CFR 2158 and *P. acidilactici* CFR 2193, spray dried in nonfat skimmed milk (NFSM) or maltodextrin. The authors found that despite that NFSM being a good carrier during storage due to the high survival rate of probiotics, it did not act as a good protector of their functional characteristics. The retention of functional properties was carrier- and cell concentration-dependent, but also genera- and species-dependent. Of the three probiotics investigated, *L. plantarum* CRF 2191 was the one that had better retention of tolerance to acid and bile, but *L. salivarius* CFR 2158 and *P. acidilactici* CFR 2193 were the ones that demonstrated higher retention of cholesterol assimilation (just for cells spray dried in maltodextrin in the case of *L. salivarius*). In another study, Silva and collaborators (2002) evaluated the effect of spray drying on the retention of bacteriocinogenic activity of three LAB: *L. sakei* CTC 494, *L. salivarius* CTC 2197 and *Carnobacterium divergens* against *Listeria monocytogenes*, *Listeria innocua* and *Staphylococcus aureus*. Spray drying and subsequent storage did not affect bacteriocin production by *L. sakei* and *L. salivarius* and their inhibitory activity against all of the tested pathogens, with the exception of *C. divergens*, that despite inhibiting both *Listeria* spp., lost the ability to inhibit *S. aureus*. Golowczyc and collaborators (2011b) studied the adhesion ability of three lactobacilli after spray drying. The authors found that spray dried *L. plantarum* 83114 and *L. kefir* 8321 maintained the ability to adhere to intestinal cells, but spray dried *L. kefir* 8348 lost significant adhesion ability. This strain-dependent retention of functional properties reinforces the need to evaluate each probiotic case by case. Such evaluation has not been made for probiotics after being spray dried in fruit juices. Nevertheless, further studies on the development of this type of products must take into account the capacity of the probiotic chosen to maintain its properties, so that they can be considered as a true functional product.

1.3.4. Spray drying of probiotic fruit juices

Despite the development of innumerous fruit juice powders, studies regarding spray drying of probiotics incorporated into fruit juices are scarce. All the research articles found so far, comprising the type of fruit, the probiotic culture used and the drying conditions are listed in Table 1.1. To our knowledge, no spray dried probiotic fruit juices are available in the market.

Table 1.1. Literature on different probiotic fruit juice powders by spray drying

Probiotic	Fruit Juice	Drying aid	In/Outlet air temperatures (°C)	Reference
L. acidophilus	Watermelon (plus carrot)	10-15% maltodextrin	120-160/—	Mestry *et al.* (2011)
L. acidophilus MTCC 447	Pomegranate	15% GA 5% MD	130, 140, 150/70	Simha *et al.* (2012)
L. acidophilus NRRL B-4495 *L. rhamnosus* NRRL B-442	Raspberry	MD ratios 1:1; 1:1.5 or 1:2	100, 115, 130/67-97	Anekella and Orsat (2013)
L. casei NRRL B-442	Cashew apple	20% MD 10%GA plus 10% MD	120/75	Pereira *et al.* (2014)
L. casei 01	Lychee	20% MD 20% GA 15% MD plus 5% Inulin 15% GA plus 5% Inulin	—/60-90	Kingwatee *et al.* (2014)

1.4. Conclusion

The increase in demand for healthy products has caused a rapid development of new functional products. Consumers are always looking for different products, which mean that industries have the challenge to innovate, either in product type, in the feature that makes it functional or in product processing technique, as would be a probiotic fruit juice powder. The biggest challenge in the production of probiotic fruit juice powders by spray drying is to obtain a powder that when rehydrated is, for instance, faithful to the organoleptic characteristics of the juice, but also ensuring maximum survival of the incorporated probiotic. Despite this technique having so many variables that may impair survival of probiotics, not only during processing but also during storage, it is an attractive technique for industrial use due to its low cost and simplicity and to allow obtaining a product with reduced weight and volume, which also contributes to reducing costs. Selecting a robust probiotic culture and adjusting some parameters and conditions, such as the use of protective carriers or suitable packaging during storage, will guarantee the maintenance of probiotic viability until the time of consumption of the product. It should be noted that besides the viability, the probiotic properties should also be maintained to ensure beneficial health effects to those who consume them. Given the few studies carried out and the numerous fruits available, the industry should invest in this type of product. Development of a probiotic fruit juice powder has all the characteristics to be a successful functional product with great acceptance by consumers.

CHAPTER 2

Pediococcus acidilactici HA-6111-2 as a potential probiotic to be used in food industry

Abstract

The objective of this study was to compare the results obtained between a commercial probiotic bacterium and a strain of *P. acidilactici* HA-6111-2 isolated from a food matrix. Besides the characterization of the isolates, the main focus of this work was to evaluate their ability to survive through simulated GIT passage as well as the effect of the spray drying process in order to be used as concentrated cultures in the food industry.

Both lactic acid bacteria produced none of the virulence determinants investigated; they exhibited no significant antibiotic resistances and less than 1 log-unit reduction in viable counts was obtained after exposure to simulated GIT conditions. Concentrated cultures of *P. acidilactici* in 10 % (w/v) of reconstituted skim milk were obtained by spray drying and, after 60 days of storage at 4 ºC, less than 1 log unit-reduction in viable count was recorded.

In conclusion, from these preliminary characterization tests and its extended viability in the dried form, this *P. acidilactici* strain should be considered as a potentially useful probiotic.

2.1. Introduction

Nowadays, there is a large and increasing demand for functional foods and therefore various products with incorporated probiotics have emerged on the market. Most are dairy products, such as yoghurts, milk and cheeses (Abadía-García *et al*., 2013; Senaka Ranadheera *et al*., 2012; Wang *et al*., 2012), but also have expanded to other non-dairy products, such as fruit juices (Ying *et al*., 2013) and cereals (Charalampopoulos and Pandiella, 2010), among others. Strains of LAB belonging to the genera *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and *Enterococcus* have been widely used in the manufacture of probiotic food products (Champagne *et al*., 2011; Modzelewska-Kapituła *et al*., 2008). Lactic acid bacteria are generally recognized as safe (GRAS) and, additionally, some strains are commensal microbes of intestinal microbiota and therefore well-adapted to this environment. According to Food and Agriculture Organization/World Health Organization (2002), probiotic microorganisms are able to confer a health benefit to consumers if ingested in adequate amounts (FAO/WHO, 2002). The beneficial effects of probiotics are strain dependent and include the reduction in the production of toxic substances, stimulation of the immune system, reduction of serum cholesterol, prevention and treatment of diarrhea and reduction of pathogen colonization of the GIT (Ezendam and van Loveren, 2006; Lourens-Hattingh and Viljoen, 2001; Ramos-Cormenzana *et al*., 2005; Saad, 2006; Sanz, 2007). A successful probiotic must have characteristics such as resistance to the acidic environment of the stomach and to bile salts of the small intestine, antimicrobial activity against important pathogens and also the capacity to adhere and colonize the intestine (FAO/WHO, 2002; Mishra and Prasad, 2005).

Therefore, a probiotic food is a processed product containing viable probiotic microorganisms in amounts of about 10^6-10^7 cfu/g, which are able to maintain their characteristics during the production and commercialization of the product, as well as to

survive during the gastrointestinal tract of the consumer (FAO/WHO, 2002; Sanz, 2007; Talwalkar *et al*., 2004). Although their use is more common in food products, probiotics are also available as dietary supplements (in the form of powder, capsules or tablets) or as dried concentrates which can be subsequently added to foods (Klayraung *et al*., 2009; Teixeira *et al*., 1995a).

Although most of the probiotics belong to the genus *Lactobacillus*, some strains of *P. acidilactici* have also been proposed (Balgir *et al*., 2013). The additive Bactocell PA (preparation with *P. acidilactici* (CNCM) MA 18/5 M) has already been authorized by the European Food Safety Authority (EFSA, 2009) to be used as a feed additive for shrimp, as well as pigs and chickens.

The objective of this study was to compare the results obtained between a commercial probiotic bacterium *L. plantarum* 299v and the strain *P. acidilactici* HA-6111-2 concerning their absence of virulence factors and antibiotic resistances. Also their ability to survive through simulated GIT passage was investigated since it is an important characteristic of a probiotic culture. Finally, in order to be used as concentrated cultures in the food industry we tested the ability of both strains to survive during spray drying and subsequent storage at room temperature and 4 ºC.

2.2. Materials and Methods

2.2.1. Origin, growth and storage conditions of LAB isolates

Two LAB strains were used: *P. acidilactici* HA-6111-2 (Albano *et al*., 2009), from *Escola Superior de Biotecnologia* (ESB) culture collection and *L. plantarum* 299v (Probis Probiotika, Lund, Sweden).

The isolates were grown aerobically on de Man, Rogosa and Sharpe (MRS) agar (Lab M, Bury, United Kingdom) at 37 ºC for 24 h and stored at -80 ºC in MRS broth (Lab M) containing 30% (v/v) of glycerol (Sigma, Steinheim, Germany), and sub-cultured twice before use in assays.

2.2.2. Virulence factors: production of gelatinase, lipase and DNase and hemolytic activity

Gelatinase activity was assessed according to Tiago *et al.* (2004) by using the modified Luria-Bertani (MLB) broth supplemented with 50.0 g/L of gelatin. The presence of sufficient gelatinase turned the medium liquid even when placed in the refrigerator.

Lipase activity was tested according to Tiago *et al.* (2004) and a positive reaction was indicated by a clear halo around the colonies.

DNase activity was studied as described by Ben Omar *et al.* (2004) by using the medium DNase agar (Pronadisa, Madrid, Spain) with 0.05 g/L of methyl green (Sigma). A clear halo around the colonies was indicative of a positive result.

Production of hemolysin was determined by streaking isolates on a plate of Columbia agar with 5% v/v of sheep blood (BioMérieux, Marcy l'Etoile, France). The presence or absence of zones of clearing around the colonies was interpreted as beta-hemolysis (positive hemolytic activity) or gamma hemolysis (negative hemolytic activity), respectively. When observed, greenish zones around the colonies were interpreted as alpha-hemolysis and taken as negative for the assessment of hemolytic activity (Semedo *et al.*, 2003).

All experiments were performed in duplicate and *S. aureus* ATCC 25213 was used as a positive control.

2.2.3. Virulence genes

The amplification of the surface adhesin genes (*esp*, *efaAfs* and *efaAfm)*, the aggregation protein gene (*agg*), the extracellular metallo-endopeptidase (*gelE*) and the cytolysin genes (*cylA*, *cylB* and *cylM*, $cylL_L$ and $cylL_S$) were done according to Barbosa *et al.* (2010).

2.2.4. Antibiotic susceptibility

The minimum inhibitory concentration – MIC (µg/mL) was determined by Ɛ-test for three antibiotics - trimethoprim/sulphamethoxazole (SXT), meropenem and imipenem (all from AB Biodisk, Solna, Sweden) and by the agar microdilution method for thirteen antibiotics, according to the Clinical and Laboratory Standards Institute (CLSI, 2007) – ampicillin, vancomycin and chloramphenicol (Fluka), penicillin G, oxacillin, nitrofurantoin, kanamycin and streptomycin (Sigma), erythromycin, tetracycline, gentamicin, ciprofloxacin and rifampicin (kindly supplied by the company Labesfal, Tondela, Portugal). Each test was carried on Muller-Hinton agar (MHA) (BioMérieux) with cations adjusted for penicillin G (Sigma) and ampicillin (Fluka, Steinheim, Germany) and on MHA for the other antibiotics. Antibiotic concentrations ranged from 0.03 to 512 µg/mL.

In order to classify the isolates according to their susceptibility (sensitive, intermediate or resistant), Table 1 (Microbiological cut-off values (mg/L)) of the Panel on Additives and Products or Substances in Animal Feed (FEEDAP) of the European Food Safety Authority (EFSA, 2012) was consulted.

Each experiment was performed in duplicate and all the isolates were grown on plates of MHA and MHA with cations adjusted with no added antibiotic as controls. The quality control strains *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 25922 were used to monitor the accuracy of MICs (CLSI, 2007).

2.2.5. Gastrointestinal tract simulation

2.2.5.1. Inoculum

From MRS agar incubated at 37 ºC for 24 h, one colony of each LAB isolate was transferred to 10 mL of MRS broth and incubated in the same conditions. For the final inoculum, 0.1 mL of the last culture was transferred to 10 mL of MRS broth (1:100) and incubated at 37 ºC for 24 h to reach stationary phase. Each isolate was harvested by centrifugation (8877 x g, 10 min, 37 ºC; Rotina 35R, Hettich, Germany), re-suspended in 10 mL of sterile quarter strength Ringer's solution (Lab M) and mixed to obtain an inoculum of approximately 10^7 cfu/mL.

2.2.5.2. Simulated gastrointestinal conditions

The simulation was achieved according to Barbosa *et al.* (2012), with some modifications. To obtain the conditions of the stomach, Buffered Peptone Water (BPW, Lab M) was adjusted to pH 3.0 with Hydrochloric Acid (1 M HCl, Pronalab, Lisbon, Portugal) with 1000 units/mL of a filter sterilized solution of pepsin (Sigma). For the simulated conditions of the small intestine, the pH of each sample was increased from 3.0 to 7.0 with a sterile solution of Sodium Hydroxide (1 M NaOH, Pronalab) and a sterile solution of bile salts (Pronadisa) was also added to achieve a final concentration of 0.3% (w/v). All assays were done in triplicate.

Serial decimal dilutions of each sample were made in sterile quarter strength Ringer's solution and enumerated by the drop count technique (Miles and Misra 1938). Each dilution was plated on MRS agar in duplicate. After incubation at 37 ºC for 48 h, the colonies were counted and the cfu/mL calculated. Microbial counts were transformed to

logarithmic reduction using the equation: log (N/N_0), where N is the microbial cell density at a particular sampling time and N_0 is the initial cell density.

2.2.6. Spray drying of *P. acidilactici* HA-6111-2

2.2.6.1. Preparation of cultures

The inocula were obtained as described above, and after reaching stationary phase, cells were harvested by centrifugation (8877 x g, 10 min, 37 ºC; Rotina 35R), washed twice in sterile quarter strength Ringer's solution (Lab M) and re-suspended in the same volume of 10 % (w/v) reconstituted skim milk (RSM) powder (Oxoid, Basingstoke, UK).

2.2.6.2. Spray drying

The drying of the prepared solution was achieved in a laboratory scale Büchi Mini-Spray Dryer Model B-191 (Büchi Laboratoriums-Technik, Flawil, Switzerland) with a two-fluid nozzle atomizer with a 1 mm inside diameter and a concurrent drying chamber of 10.5 cm. The drying air flow rate was kept at 86%, the compressed air flow rate at 550 L/h and the solution (stirring at 40 ºC) was fed into the chamber at a constant flow rate of 5 mL/min. The inlet and outlet air temperatures used were 120 ºC and 65 ºC, respectively. It was used a single cyclone air separator system and the dried powders were collected from the base of the cyclone. Two replicates were conducted for each experiment.

2.2.6.3. Analysis of powders

Immediately after the spray drying, drying yield was calculated and the a_w of the powder determined. The drying yield was determined as the percentage ratio between the weight of total mass of product collected by the total mass fed to the spray drier. The a_w of the powders was determined using a water activity meter (Aqualab, Series 3, Decagon

Devices Inc., Pullman, WA) at a constant temperature of 23 ± 1 ºC. Two readings were made.

2.2.6.4. Storage conditions

Dried samples were stored in plastic containers, in normal atmosphere (air) at 4 ºC and at room temperature.

2.2.6.5. Enumeration

The survival of *P. acidilactici* HA-6111-2 was assessed immediately after spray drying and at regular intervals throughout storage by rehydration of each dried sample to their initial solids content in sterile quarter strength Ringer's solution (Lab M), followed by serial decimal dilutions and plated in duplicate for enumeration by the drop count technique (Miles and Misra, 1938) on MRS agar. The colonies were counted after incubation at 37 ºC for 48 h and the cfu/mL calculated. Microbial counts were transformed to logarithmic reduction using the equation: $\log (N/N_0)$, where N is the microbial cell density at a particular sampling time and N_0 is the microbial cell density after spray drying.

2.2.7. Statistical analysis

An analysis of variance (one-way ANOVA) was performed to evaluate any significant effects of slow and quick gastric transit simulations and digestions on the survival of isolates in simulated GIT. Also significant differences between the temperatures of storage of the dried powders were tested. Multiple comparisons were evaluated by Tukey's post-hoc test. All calculations were carried out using the software Kaleidagraph (version 4.4, Synergy Software, Reading, USA).

2.3. Results and Discussion

Isolates neither produced any virulence factors nor harbored the virulence genes, assessed in this study (data not shown). Although the American Food and Drug Administration had given the "generally regarded as safe" status to LAB, their safety must be evaluated before use as probiotics (FAO/WHO, 2002). The absence of virulence factors and virulence genes is a prerequisite to consider a bacterium as probiotic. The virulence factors and genes investigated were selected since they are associated with other LAB, such as *Enterococcus* spp. (Barbosa *et al.*, 2010; Johansson and Rasmussen, 2013). *Lactobacillus plantarum* 299v did not produce any of the virulence factors and genes tested, as well as for *P. acidilactici* HA-6111-2 as previously demonstrated by Albano *et al.* (2009). The absence of virulence factors of *Lactobacillus* and *Pediococcus* species has been reported by several authors (Borges *et al.*, 2013; Muñoz-Atienza *et al.*, 2013; Vesterlund *et al.*, 2007).

Together with the absence of virulence factors, the absence of antibiotic resistances is a very important aspect for LAB to be considered as safe. The MIC values obtained of the sixteen antibiotics tested for both isolates are presented in Table 2.1.

Table 2.1. Minimum Inhibitory Concentration (MIC; μg/mL) of sixteen antibiotics for LAB isolates

Class	Antibiotic	MIC (μg/mL)	
		***L. plantarum* 299v**	***P. acidilactici* HA-6111-2**
β-lactam	Ampicillin	2	1*
	Penicillin G	32	1*
	Oxacilin	64	0.25*
Fluoroquinolone	Ciprofloxacin	8	2*
Chloramphenicol	Chloramphenicol	8	4*
Macrolide	Erithromycin	0.125	4*
Nitrofuran	Nitrofurantoin	256	16*
Ansamycins	Rifampicin	8	0,25*
Tetracycline	Tetracycline	64	64*
Glycopeptide	Vancomycin	512	512*
	Kanamycin	8	4*
Aminoglycoside	Gentamycin	0.25	0.25*
	Streptomycin	8	8*
Sulfonamide and Pyrimidine	SXT	0.094	>32
Carbapenem	Meropenem	0.38	1.5
	Imipenem	0.032	0.094

*MIC's previously described by Albano *et al.* (2009)

The Panel on Additives and Products or Substances in Animal Feed (FEEDAP) of European Food Safety Authority defined the microbiological cut-off values for some antimicrobials (EFSA, 2012). According to their microbiological breakpoints, *L. plantarum* 299v and *P. acidilactici* HA-6111-2 were sensitive to ampicillin, chloramphenicol, erythromycin, kanamycin, gentamicin, and streptomycin. Both are considered intrinsically resistant to vancomycin and pediococci to SXT (Ammor *et al.*, 2007; Klein *et al.*, 2000), which means that these resistances are not horizontally

transferable (Ammor *et al.*, 2007). Due to the high MIC values obtained to tetracycline for both LAB, they were classified as resistant to this antibiotic. However, many researchers tried to propose cut-off values in order to recognize if some antimicrobial resistances are intrinsic or acquired (Danielsen and Wind, 2003; Klare *et al.*, 2007). Danielsen and Wind (2003) proposed a cut-off value of 64 µg/mL for tetracycline for *L. plantarum* species. In the study of Tankovic *et al.* (1993), MIC values higher than 128 µg/mL were reported for *P. acidilactici* species, and none of the genes *tetK*, *tetL*, *tetM* and *tetO* were detected in any of the strains. Also Klare and others (2007) did not find any acquired antibiotic resistance to tetracycline for all isolates of *Pediococcus* spp. Thus, tetracycline resistance of the strain *P. acidilactici* HA-6111-2 is likely to be intrinsic. Due to the lack of microbiological breakpoints for the other antibiotics tested in this work by FEEDAP, we analyzed our results comparing with those of other studies, which, in general, are in agreement to those we found (Abbasiliasi *et al.*, 2012; Belletti *et al.*, 2009; Danielsen *et al.*, 2007; Danielsen and Wind, 2003; Flórez *et al.*, 2006; Tankovic *et al.*, 1993; Zarazaga *et al.*, 1999).

As probiotic microorganisms must be able to survive in the GIT and confer a health benefit to the host (FAO/WHO, 2002), the ability of LAB cultures to survive in both short and long digestions in simulated conditions of the GIT is presented by their logarithmic reductions in Table 2.2. No significant differences ($p>0.05$) between the types of digestion for both stomach and small intestine conditions were found.

Table 2.2. Survival of LAB isolates through simulated quick and slow digestions

	log (N/N_0)[a]					
	Quick digestion simulation			**Slow digestion simulation**		
LAB	0 min	60 min[b]	120 min[c]	0 min	120 min[b]	240 min[c]
L. plantarum	0.00±0.00	0.11±0.08	-0.06±0.04	0.00±0.00	-0.04±0.09	-0.18±0.16
P. acidilactici	0.00±0.00	0.01±0.13	-0.11±0.09	0.00±0.00	-0.02±0.10	-0.17±0.07

[a]Survival is represented as the mean of the logarithmic reduction: log (N/N_0) ± the standard error of the mean

N is the CFU/mL at each sampling time

N_0 is the CFU/mL at time zero

[b]Survival after exposure to pH 3.0 in the presence of pepsin

[c]Survival after exposure to pH 3.0 in the presence of pepsin and subsequent exposure to bile salts at pH 7.0

All the values are not significantly different (p>0.05)

When the gastric transit was simulated at pH 3.0 with pepsin, no significant differences (p=0.32) between the types of digestion were obtained for both isolates. In quick digestion, the exposure to the stomach conditions did not cause any reduction for *L. plantarum* 299v and *P. acidilactici* HA-6111-2. In slow digestion, since the cells are exposed to conditions of the stomach for a longer period, there was a slight reduction of less than 0.05 log-unit for both microorganisms. Other authors have studied the behavior of LAB at those conditions. For *P. acidilactici*, the strain Kp10 from the study of Abbasiliasi *et al.* (2012) showed a survival of more than 97% at pH 3.0 after 3 hours of incubation and the strain L169 from the study of Kaboré *et al.* (2012) showed a survival of 96.2% at pH 2.5 after 4 hours of incubation. In both studies, only the pH effect was tested, since pepsin was not added. Kumar *et al.* (2011) studied the strain *L. plantarum* VR1, which had an increase of 0.3 log unit after 3 hours at pH 2.0, also without pepsin.

When bile salts were added (small intestine digestion), no significant differences were found (*p*=0.60) between short and long digestions, since slight decreases (about 0.1 log-unit reduction for short digestion and about 0.2 log-unit reduction for the long digestion) were found for both microorganisms. All the strains from the studies mentioned above showed high survival rates after the addition of 0.3% (w/v) of bile salts (Abbasiliasi *et al.*, 2012; Kaboré *et al.*, 2012; Kumar *et al.*, 2011).

As expected, the probiotic *L. plantarum* 299v was not affected by the conditions of the gastrointestinal tract, even in conditions of long digestion. A previous study had already revealed that *L. plantarum* 299v was able to survive and colonize the GIT (Johansson *et al.*, 1993). *Pediococcus acidilactici* HA-6111-2 proved to be a good candidate to be considered as a probiotic culture, by its tolerance to GIT conditions. Furthermore, this strain shows antimicrobial activity against important foodborne pathogens such as *L. monocytogenes,* by the production of a bacteriocin PA-1, as previously demonstrated by Albano *et al.* (2007). This is an important advantage of *P. acidilactici* HA-6111-2, which can also be used as a food preservative.

The survival of LAB cultures was not affected during the drying process, since no reduction in the number of cells was obtained. Immediately after the spray drying, the number of viable cells detected was 6.5×10^{10} cfu/mL for *P. acidilactici* HA-6111-2 and 2.3×10^{11} cfu/mL for *L. plantarum* 299v.

After spray drying, the powder recovery, represented as the mean of percentages of powder collected ± the standard error of the mean, and the a_w values, represented as the mean of the values obtained ± the standard error of the mean, were 50.5% ± 4.0 and 0.370 ± 0.060, respectively.

The survival of both strains during storage at 4 ºC and room temperature is presented as their logarithmic reductions in Figure 2.1. Significant differences were obtained between the storage temperatures tested ($p<0.05$).

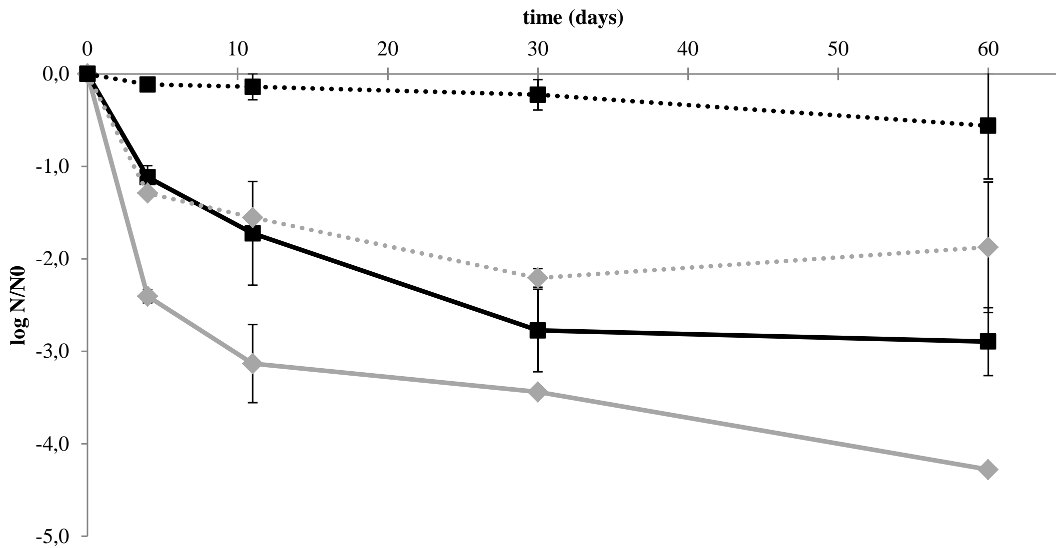


Figure 2.1. Logarithmic reduction of *P. acidilactici* HA-6111-2 (black) and *L. plantarum* 299v (grey) incorporated in 10% of RSM after spray drying and during 60 days of storage at 4 ºC (dotted lines) and room temperature (solid lines).

Drying bacterial cultures by methods such as spray drying is a common procedure (Ananta *et al.*, 2005; Reddy *et al.*, 2009; Teixeira *et al.*, 1995b). With this inexpensive and rapid method it is possible to obtain concentrated cultures to be further added to food products. Both LAB survived during drying process, resulting in no reduction in the number of cells. The storage at 4 ºC resulted in a higher survival than at room temperature. As previously reported, the survival of spray dried bacteria is higher at low temperatures (Teixeira *et al*., 1995b). After 60 days of storage at room temperature, viable counts detected were 1.8×10^8 cfu/mL of *P. acidilactici* HA-6111-2 and 1.2×10^7 cfu/mL of

L. plantarum 299v (reduced 2.9 and 4.3 log–units, respectively), whilst at 4 °C, the reduction was lesser: 0.6 log-unit reduction for *P. acidilactici* HA-6111-2 (viable counts of 3.9x10^{10} cfu/mL) and 1.9 log-unit reduction for *L. plantarum* 299v (viable counts of 4.1x10^{9} cfu/mL). So, *P. acidilactici* HA-6111-2 demonstrated to be more resistant to storage after spray drying than *L. plantarum* 299v.

These preliminary results indicate that it is possible to obtain a concentrate of *P. acidilactici* HA-6111-2 by spray drying, which is viable for at least 60 days at 4 °C, for further use in the food industry.

2.4. Conclusions

In conclusion, *P. acidilactici* HA-6111-2 passed most of the *in vitro* properties recommended by FAO/WHO (2002); did not produce any of the virulence factors or virulence genes tested; has no significant antibiotic resistances, and was capable of surviving during the simulated gastrointestinal tract, during both short and long digestions, at least as well as the commercial probiotic *L. plantarum* 299v, tested simultaneously. This *Pediococcus* strain was able to survive a drying process and during storage at 4 °C for 60 days, the reductions were minimal. Overall, although additional *in vivo* tests are still required, our results indicate that *P. acidilactici* HA-6111-2 is a potential probiotic candidate and, in the future, could be successfully used in functional foods.

CHAPTER 3

Drying conditions for orange juice incorporated with lactic acid bacteria by spray drying

Abstract

A well-known tactic to increase shelf life of a fruit juice is to make it into powder. If to this fruit juice were added probiotic cultures it is possible to develop a product with health benefits. This work aimed to develop an orange juice powder by spray drying and incorporate viable LAB (*L. plantarum* 299v and *P. acidilactici* HA-6111-2), ensuring their survival both during drying and storage (at room temperature and at 4 ºC).

An inlet air temperature of the spray drying of 120 °C and a 0.5: 2 ratio of soluble solids of the orange juice and drying agent added (prebiotics: 10DE maltodextrin or gum Arabic) were selected. The drying process did not affect both LAB and their survival was higher at 4 ºC. The additive 10DE maltodextrin conferred a slightly higher protection than gum Arabic, in the case of *L. plantarum* and at 4 ºC. *Pediococcus acidilactici* was more resistant than *L. plantarum* during storage at 4 ºC, with logarithmic reductions lower than 1 log-unit.

It was demonstrated that it is possible to produce a functional non-dairy product, orange juice powder supplemented with prebiotic compounds, containing viable LAB for at least 7 months, when stored at 4 ºC.

3.1. Introduction

The production of oranges in Portugal is more than 200,000 tons per year (OMAIAA, 2011) and the possibility of producing natural orange juice powder would be an advantage at economic level, not only by the reduction in volume and weight of the packages, easier transportation and storage, but especially by increasing the shelf life of the product.

Spray drying is the most common method used for converting liquid food products into dry powder, because it is inexpensive and easy to operate. Briefly, the process involves the pumping of liquid sample into the atomizer that transforms the liquid into small droplets, which rapidly lose their moisture on contact with the hot and dry air (Silva *et al.*, 2011). It could be a good method to get a natural orange juice powder, if the characteristics of the natural juice allowed the powder production. Fruit juices are extremely sticky, which is related to their low glass transition temperatures (presence of low molecular weight sugars and organic acids in their composition), their high hygroscopicity, water solubility and low melting point (Bhandari *et al.*, 1997). The glass transition temperature is the temperature at which the transition from a glassy to a rubbery state of an amorphous system occurs (Bhandari and Howes, 1999). The usual strategy is the addition of some materials with high molecular weight that can lead to an increase of the glass transition temperature of the fruit juices. Several authors have been using drying aids to a variety of fruit juices, being maltodextrins and gum Arabic the more common agents used (Cano-Chauca *et al.*, 2005; Martinelli *et al.*, 2007; Roustapour *et al.*, 2006; Tonon *et al.*, 2010). Maltodextrins are low cost oligosaccharides, made from starch, that have dextrose equivalents (DE) and gum Arabic is a complex heteropolysaccharide and a natural exudate of Acacia tree (Bemiller and Whistler, 1996). Together with their ability as drying agents, several studies have provided evidence that both maltodextrin and gum

Arabic also have prebiotic effects (Anekella and Orsat, 2013; Calame *et al*., 2008; Slavin, 2013). Prebiotics are defined as "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health" (Gibson and Roberfroid, 1995). Some of their health benefits are the reduction of the inflammation and symptoms associated with inflammatory bowel disease, the decreasing of the risk of cardiovascular disease, the prevention of colon cancer, among others (Slavin, 2013). Studies on spray drying of fruit juices with probiotic bacteria incorporated are rare (Anekella and Orsat, 2013; Mestry *et al*., 2011; Pereira *et al*., 2014) and to our knowledge, there is no study with spray dried orange juice incorporating probiotics. The use of probiotics as food supplements is increasing, because of their health benefits, as well as the increased diversity in food choices they provide. Producing an orange juice with prebiotics and probiotic bacteria can be an innovative way to increase this diversity, especially among consumers who preferred functional non-dairy based foods. Whilst dairy products are the priority of the development of novel probiotic foods, an increase of vegetarianism, milk cholesterol content, and lactose intolerance justify the need for non-dairy probiotic products (Granato *et al*., 2010).

This work aimed to develop an orange juice powder dried by spray drying and incorporate viable LAB ensuring their survival both during drying and storage.

3.2. Materials and methods

3.2.1. Origin, growth and storage conditions of LAB isolates

Two LAB were used: *Lactobacillus plantarum* 299v (Probis Probiotika, Lund, Sweden) and *Pediococcus acidilactici* HA-6111-2 from *Escola Superior de Biotecnologia* (ESB) culture collection.

The isolates were grown on de MRS agar (Lab M) at 37 ºC for 24 h and stored at -80 ºC in MRS broth (Lab M) containing 30% (v/v) of glycerol (Sigma), and sub-cultured twice before use in assays.

3.2.2. Conditions of the drying process of orange juice

3.2.2.1. Materials

Mature oranges exclusively originated in Portugal were randomly purchased from local commercial establishments (Porto, Portugal) and stored at room temperature until used (for no more than 24 h before experiments).

The drying agents used were 10DE maltodextrin (Sigma) and gum Arabic (Merck, Darmstadt, Germany).

3.2.2.2. Orange juice preparation

Oranges were squeezed using a domestic juicer and the juice was filtered in order to eliminate the solids in suspension, preventing the obstruction of the atomizer of the spray dryer. The content of the total soluble solids of the juice was measured using a digital refractometer (model PR-32α (alpha), Brix 0–32%, Atago U.S.A., Inc., WA, U.S.A.) and adjusted to 0.5 or 1% (w/v). The drying agents 10DE maltodextrin and gum Arabic were added, both at the concentrations of 1 or 2% (w/v), under magnetic stirring at 40 ºC, until complete dissolution.

3.2.2.3. Spray drying

The drying of orange juice was performed in a laboratory scale Büchi Mini Spray Dryer Model B-191 (Büchi Laboratoriums-Technik) with a two-fluid nozzle atomizer with a 1 mm inside diameter and a concurrent drying chamber of 10.5 cm. The solution previously

prepared (stirring at 40 ºC) was fed into the chamber through a peristaltic pump at a constant flow rate (5 mL/min). The flow rate of the drying air was kept at 86% and the compressed air flow rate at 550 L/h. The inlet air temperatures tested were 120 and 130 ºC. The outlet air temperature cannot be regulated, resulting from a combination of the inlet air temperature, the feed rate, the drying gas flow rate and the solids content of the feed. A single cyclone air separator system was used and the dried powders were collected from the base of the cyclone. Two replicates were conducted for each experiment.

3.2.2.4. Analysis of powders

The analysis of the dried powders was performed immediately after the spray drying.

3.2.2.4.1. Drying yield

The drying yield was determined as the percentual ratio between the weight of total mass of product collected with the initial amount of solids present in the solution fed to the spray drier.

3.2.2.4.2. Water activity

The water activity of the powders was determined using a water activity meter (Aqualab, Series 3, Decagon Devices Inc.) at a constant temperature of 23 ± 1 ºC. Two readings were done for each sample.

3.2.3. Spray drying of orange juice with LAB

3.2.3.1. Orange juice preparation

The orange juice was prepared as described in section 3.2.2.2. The total soluble solids content of the juice was adjusted to 0.5% (w/v) and at this solution was added 2% (w/v) of the drying agent (10DE maltodextrin or gum Arabic), under magnetic stirring at 40 ºC, until complete dissolution.

3.2.3.2. Preparation of LAB cultures

From MRS agar incubated at 37 ºC for 24 h, one colony of each LAB isolate was transferred to MRS broth and incubated at the same conditions. For the final inoculum, the last culture was transferred to a new MRS broth (1:100) and incubated at 37 ºC for 24 h to reach stationary phase. Each isolate was harvested by centrifugation (8877 x g, 10 min, 37 ºC; Rotina 35R), washed twice in sterile quarter strength Ringer's solution (Lab M) and re-suspended in the same volume of the final solution prepared before (in section 3.2.2.2.).

As control, 10 % (w/v) of reconstituted skim milk (RSM) powder (Oxoid, Basingstoke, UK) was used to re-suspend the LAB cultures.

3.2.3.3. Spray drying and powder analysis

The drying of LAB cultures incorporated either in orange juice or in the RSM was achieved as described in section 3.2.2.3. The drying conditions chosen for both LAB cultures were: feed temperature of 40 ºC, feed flow rate of 5 mL/min; 86% of drying air flow rate; compressed air flow rate of 550 L/h, inlet air temperature of 120 ºC and outlet air temperature of about 65 ºC.

Each experiment was done in duplicate for both LAB cultures and dried products.

The analysis of dried powders obtained was done as described in section 3.2.2.4.

3.2.3.4. Storage conditions

Dried samples were stored in plastic containers, hermetically sealed in glass flasks, in normal atmosphere (air), in the presence of daylight, at 4 ºC and room temperature.

3.2.3.5. Enumeration of LAB cultures

The survival of each microorganism was assessed immediately after spray drying and at regular intervals throughout storage by rehydration of each dried sample to their initial solids concentration in sterile quarter strength Ringer's solution (Lab M). Each rehydrated sample was homogenized for 1 minute and kept at room temperature for 30 minutes followed by serial decimal dilutions and plated in duplicate for enumeration by the drop count technique (Miles and Misra, 1938) on MRS agar. The enumeration of each microorganism re-suspended in both orange juice or RSM before spray drying was also performed.

The colonies were counted after incubation at 37 ºC for 48 h and the cfu/mL calculated. Microbial counts were transformed to logarithmic reduction using the equation: log (N/N_0), where N is the microbial cell count at a particular sampling time and N_0 is the microbial cell count after spray drying.

3.2.4. Statistical analysis

Any significant difference between drying agents used and temperatures of storage was tested by an analysis of variance (one-way ANOVA). Multiple comparisons were evaluated by Tukey's post-hoc test. All calculations were carried out using the software Kaleidagraph (version 4.4, Synergy Software, Reading, USA).

3.3. Results and discussion

To develop a new product such as an orange juice powder with functional properties, two LAB were selected to be incorporated: *L. plantarum* 299v - a commercial probiotic and *P. acidilactici* HA-6111-2 an isolated strain from a food matrix and with probiotic characteristics found after preliminary characterization (Barbosa *et al*., 2015).

Optimization of a drying process is the initial step to gather the best conditions for obtaining a powdered product of good quality. The different parameters evaluated to optimize the drying process of orange juice by spray drying were i) the content of soluble solids in orange juice, as well as the optimal ratio of drying agents tested, allowing the juice drying with the lowest loss of powder, and ii) the inlet air temperature of the spray drying. With the different tests carried out, conditions leading to the highest drying yield and product with low a_w were selected.

The best ratio of soluble solids content of the orange juice and drying agents was 0.5: 2 and the selected inlet air drying temperature was 120 °C. Drying yield and a_w values obtained for the selected conditions are presented in Table 3.1.

Table 3.1. Yield and a_w of orange juice powder at selected drying conditions

	Orange juice (0.5% of total soluble solids)	
Drying agent	**Yield[a]**	**a_w[b]**
2% 10DE Maltodextrin	40.90 ± 1.13	0.409 ± 0.004
2% gum Arabic	53.05 ± 1.48	0.321 ± 0.021

[a]The powder yield is represented as the media of percentages of powder obtained ± the standard error of the mean

[b]The a_w is represented as the media of a_w values obtained ± the standard error of the mean

Another important parameter in spray drying is the outlet air temperature. In the Büchi Mini Spray Dryer used this is not an adjustable parameter, resulting from the combination of the various parameters such as inlet air temperature, pump and aspirator settings and feed concentration. Several researchers reported that outlet air temperatures above 85 ºC were lethal for probiotic cultures (Corcoran *et al.*, 2004; Gardiner *et al.*, 2000). Since we intended to incorporate probiotics into orange juice and the outlet air temperatures are such an important parameter in the survival of bacteria during drying, all the conditions selected (Table 3.1) resulted in low outlet air temperatures (close to 65 ºC) and, simultaneously, high drying yields and low a_w values.

The drying yield obtained with both drying agents, was close to 50%. According to Bhandari *et al.* (1997) a total powder recovery of 50% in a laboratory scale spray dryer is considered to be the reference point for a marginally successful drying. From both concentrations of the drying agents tested, the addition of 2% (w/v) to the orange juice allowed a better drying yield than 1% (w/v). This is in agreement with some studies which stated that the increasing of drying agents concentration in fruit juices, also increased the powder yield (Fazaeli *et al.*, 2012; Quek *et al.*, 2007). Values of a_w between 0.3 and 0.4 were obtained for the orange juice powders. With the subsequent addition of probiotic cultures to orange juice, it was important that the selected conditions allowed to obtain low a_w values, as water remaining after drying affects the viability of cultures, after the drying process and also during storage (Zayed and Roos, 2004). Moreover, dried products with a_w values below 0.6 are considered microbiologically stable (Quek *et al.*, 2007).

After the optimization of the process of orange juice powder production, we proceeded to the incorporation of each lactic acid bacteria to the juice with each drying agent - 10DE maltodextrin and gum Arabic - and subsequent drying.

Drying of the selected LAB in RSM was used as a control since it has been demonstrated that it is as an efficient protector, both during drying and also during subsequent storage (Ananta *et al*., 2005; Gardiner *et al*., 2000; Teixeira *et al*., 1995a).

The survival of LAB was not affected during the drying process, as shown in the Table 3.2.

Table 3.2. Survival of *L. plantarum* 299v and *P. acidilactici* HA-6111-2 before and after spray drying (SD) in RSM and orange juice supplemented with 10DE maltodextrin or gum Arabic

	log cfu/mL					
	L. plantarum 299v			*P. acidilactici* HA-6111-2		
	RSM	**MD**	**GA**	**RSM**	**MD**	**GA**
Before SD	10,0±0,09	10,0±0,53	9,4±0,44	10,7±0,29	9,0±0,07	9,1±0,04
After SD	11,3±0,10	11,2±0,05	9,9±0,70	11,0±0,11	9,1±0,23	9,9±0,15

MD – 10DE maltodextrin

GA – gum Arabi

The survival of each microorganism during storage at room temperature and at 4 °C is presented in Figures 3.1 and 3.2, respectively.

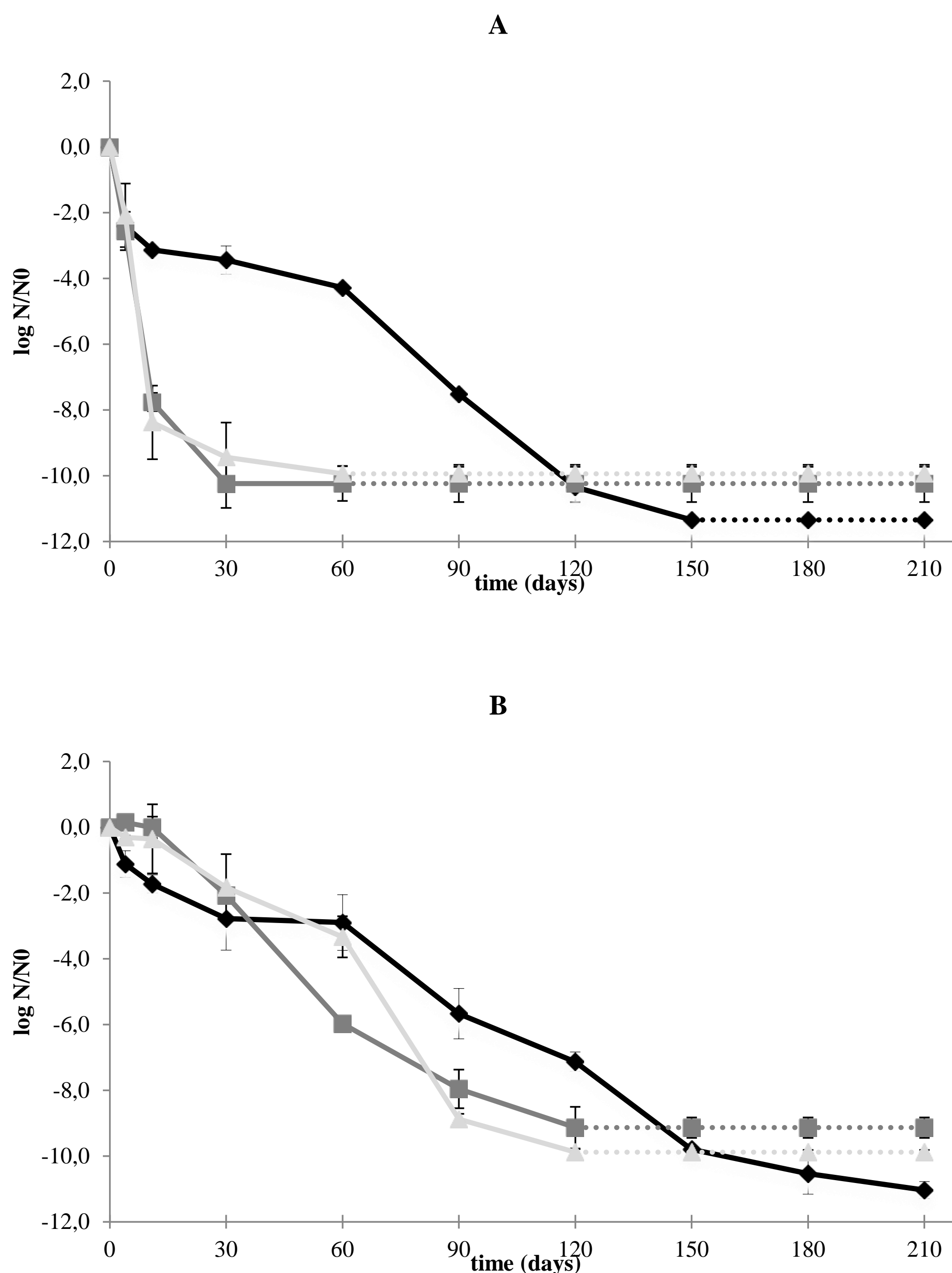


Figure 3.1. Logarithmic reduction of *L. plantarum* 299v (**A**) and *P. acidilactici* HA-6111-2 (**B**) incorporated in orange juice or RSM after spray drying and during 210 days of storage at room temperature: (—◆—) control (inoculum in 10% (w/v) of RSM); (—■—) orange juice with 2% of 10DE maltodextrin and (—▲—) orange juice with 2% of gum Arabic. The dotted lines mean that the isolate was reduced to values below the detection limited of the enumeration technique.

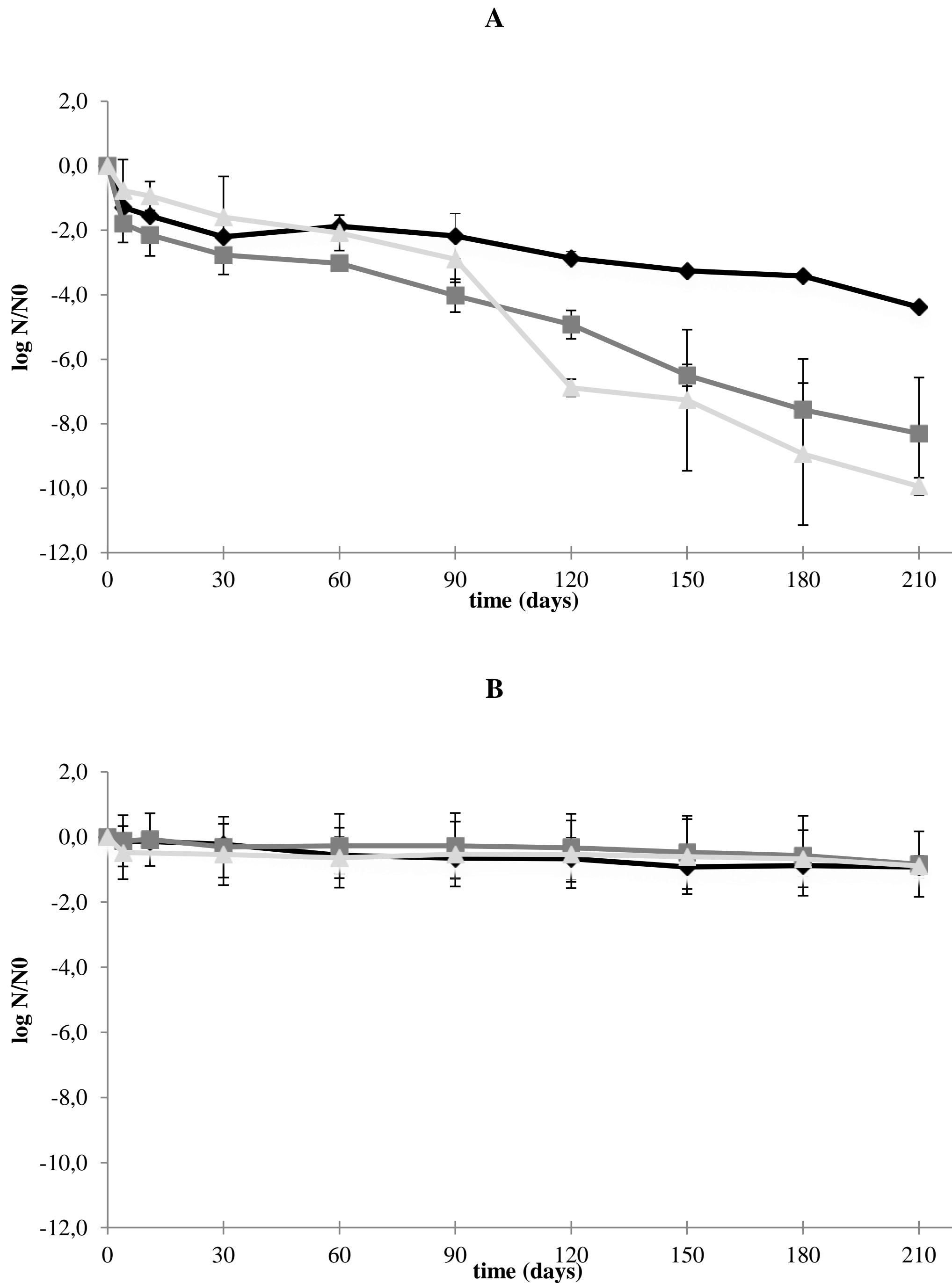


Figure 3.2. Logarithmic reduction of *L. plantarum* 299v (**A**) and *P. acidilactici* HA-6111-2 (**B**) incorporated in orange juice or RSM after spray drying and during 210 days of storage at temperature of 4 ºC: (—◆—) control (inoculum in 10% (w/v) of RSM);(—■—) orange juice with 2% of 10DE maltodextrin and (—▲—) orange juice with 2% of gum Arabic.

For both LAB, survival during storage at room temperature was higher when cells were spray dried in RSM. During storage at room temperature, the reduction of both microorganisms was lower for RSM, during 60 days of storage. Thereafter, both were reduced to values below the level of the detection limit of the enumeration technique after 150 (*L. plantarum* 299v) or 210 days of storage (*P. acidilactici* HA-6111-2). In orange juice dried with drying agents, no significant differences were obtained between the drying agents regarding to the survival of LAB during storage ($p>0.05$). The survival of *L. plantarum* was similar in orange juice dried with maltodextrin or gum Arabic, i.e. after 11 days of storage, an accentuated reduction in the number of cells occurred, and after 60 days the logarithmic reduction was to values below the level of the detection limit of the enumeration technique. For *P. acidilactici* the behavior was also the same for both drying agents used in orange juice, i.e., the reduction became more pronounced after 30 days and values below the level of the detection limit were attained after 120 days of storage.
At room temperature the behavior of both bacteria was very similar since none were able to survive in orange juice powder.

A higher survival was observed during storage at 4 ºC than at room temperature, for both LAB.
In the case of *L. plantarum*, the drying with RSM also conferred a protective effect during storage at 4 °C, comparing with the other drying agents. Significant differences were found among the additives used ($p<0.001$). In the orange juice dried with 10DE maltodextrin, there was a 4 log-units reduction in the survival of *L. plantarum* 299v up to 90 days of storage and of 8 log-units until the end of the storage period (210 days). In the presence of gum Arabic, despite the reduction had been lower up to 90 days, after this

period this reduction was higher than in the presence of 10DE maltodextrin, reaching values below the detection limit after 210 days (> 9.9 log reduction).

For all the spray drying media investigated, RSM and orange juice supplemented with 10DE maltodextrin or gum Arabic, no significant differences were observed in the survival of *P. acidilactici* HA-6111-2 during storage at 4 ºC ($p>0.05$). At this storage temperature, *P. acidilactici* demonstrated a higher survival than *L. plantarum* ($p<0.001$), showing less than 1 log-unit reduction after 210 days.

Orange juice dried with 10DE maltodextrin conferred a slightly higher protection on the survival of *L. plantarum* 299v during storage at 4 ºC.

As expected, significant differences were obtained between the temperatures of storage used ($p<0.001$). Many authors reported the higher survival of spray dried bacteria during storage at low temperatures (Gardiner *et al*., 2000; Silva *et al*., 2002; Teixeira *et al*., 1995b).

Of the drying agents used in this study, 10DE maltodextrin allowed better results on the survival of the bacteria in comparison with gum Arabic. Other studies have demonstrated the importance of maltodextrin as a drying agent in fruit juices, as well as its protective ability of probiotic cultures during drying and subsequent storage (Anekella and Orsat, 2013; Pereira *et al*., 2014).

Despite the scarce literature regarding the drying of *L. plantarum* and *P. acidilactici* strains incorporated in fruit juices by spray drying, many authors have studied the behavior of different strains after spray drying using maltodextrin as carrier. Lapsiri and collaborators (2012) found high survival rate of *L. plantarum* TISTR 2075 after spray drying. During storage at different temperatures, survival of spray dried cells was affected by elevated temperatures; while at 25 °C no cells have survived up to 90 days of storage,

at 4 °C this strain had a decrease of only 1.62 log cfu/g after 12 months of storage in the absence of light. In the study of Reddy and collaborators (2009), using maltodextrin and nonfat skimmed milk as carriers, during 60 days of storage, the high temperatures also affected the survival rate of *L. plantarum* and *P. acidilactici* strains tested. At 4 ºC a survival rate of 60% was obtained for both strains and carriers. At 30 ºC, using maltodextrin as carrier, the survival rate decreased to 50% for both strains and using nonfat skimmed milk, the survival rate decreased to 67% for *L. plantarum* and to 53% for *P. acidilactici*. The authors concluded that maltodextrin is a good substitute of nonfat skimmed milk.

Although maltodextrin could be a good encapsulating agent during spray drying, it also acts as a prebiotic, allowing the survival of the cultures (Anekella and Orsat, 2013; Lapsiri *et al*., 2012; Pereira *et al*., 2014; Reddy *et al*., 2009).

Extra experiments need to be performed, especially in terms of storage conditions improvement and validation of results in a larger industrial drier. Nonetheless, the present data is promising and allowed to prove that it is possible to produce a functional non-dairy based food, such as an orange juice powder that incorporates both prebiotic and probiotic ingredients.

3.4. Conclusions

As conclusions of this study, the conditions to obtain orange juice powder by spray drying were pooled. Another challenge was the incorporation of bacteria with probiotic characteristics and that they were able to survive during drying process and storage. In this study it was demonstrated that it is possible to produce a healthy product, not only for

the advantage of using a powder made from natural fruit juice, as well as the beneficial characteristics conferred by a prebiotic and probiotic incorporated.

Within various additives which can be used in food industry, the prebiotic maltodextrin turned possible not only to obtain a powder of good quality, but also a higher survival of the bacteria incorporated into orange juice, along its storage at 4 °C. If this product is stored and sold at refrigerated conditions it can have a long shelf life, depending on the probiotic used. The potential probiotic *P. acidilactici* HA-6111-2 studied was more resistant than the probiotic *L. plantarum* 299v. The challenge continues to be the preservation of these products at room temperature, so it is necessary to improve the storage conditions in order to increase their shelf life at the lowest possible cost.

CHAPTER 4

Effect of different conditions of growth and storage of two lactic acid bacteria after spray drying in orange juice

Abstract

Consumers increasingly require food products with health benefits. So, a dried orange juice incorporating probiotics could be a novel challenge.

In this context, we investigated whether different sugars added to the culture media used for two LAB growth contributed to their protection during spray drying in orange juice and subsequent storage under different conditions of temperature, light exposure and water activity. Cell viability during passage through simulated GIT conditions was also investigated.

Cells grown in MRS containing fructose resulted in the worst survival rates during storage. High survival was observed for cells grown in the presence of lactose, followed by glucose. The survival of dried bacteria was enhanced at 4 °C, water activity of 0.03 and absence of daylight. For cells grown in standard MRS and after 12 months of storage at 4 ºC in orange juice (about 10^9 cfu/mL), there was a reduction of approximately 2 log-units for both LAB after GIT passage simulation.

Using the conditions of growth and storage investigated, it is possible to improve the survival rate of LAB and produce an orange juice powder with pre- and probiotic characteristics with shelf life of at least 12 months.

4.1. Introduction

Development of foods containing probiotic organisms is still an emerging field, due to the continuous demand of consumers for different products with health promoting characteristics (Ying *et al*., 2013). In addition to traditional dairy products, non-dairy probiotic products like fruit juices are a good alternative for consumers who prefer foods low in cholesterol, which are vegetarians or even lactose intolerant (Granato *et al*., 2010). Producing a dried fruit juice incorporating probiotic bacteria through spray drying is a good way to preserve the fruit juice and simultaneously to present an innovative product.

Spray drying has been extensively used to produce powdered foods since it is an economic technique and easy to control and to operate (Silva *et al*., 2011). Dried products are convenient to ship and to store, and have an increased shelf life. A major problem with the spray drying of fruit juices is their viscosity since they are extremely sticky due to the presence of low molecular weight sugars and organic acids in their composition. Moreover, dried juices are highly hygroscopic and present low solubility and low melting point (Bhandari *et al*., 1993). However, these problems may be overcome through the addition of high molecular weight drying agents, such as maltodextrins. These compounds decrease viscosity and prevent operational problems during spray drying; structural changes such as crystallization during storage are also minimized (Anekella and Orsat, 2013; Tonon *et al*., 2010). The prebiotic characteristics of some drying agents, also promotes the beneficial effects of the final products (Slavin, 2013).

The viability of probiotic cultures incorporated into fruit juices may be compromised during spray drying; this technique triggers a series of stresses due to high temperatures and dehydration (Tripathi and Giri, 2014). To be considered probiotic, foods must contain viable probiotic microorganisms in amounts of about 10^6-10^7 cfu/g at the time of consumption. This level of viable cells must be maintained during passage through the

GIT (FAO/WHO, 2002). It is therefore crucial to guarantee the best performance of probiotic cultures, and for this, some factors like the composition of the growth media and the storage conditions should be considered (Tripathi and Giri, 2014). Many authors have stated that the presence of some carbohydrates in the culture media influence survival of microorganisms during drying and subsequent storage (Carvalho *et al*., 2004b; Ferreira *et al*., 2005; Strasser *et al*., 2009). The viability of dried probiotics is also affected by the storage conditions, i.e. temperature, presence of oxygen and moisture (Strasser *et al*., 2009; Teixeira *et al*., 1995b).

This study aimed i) to investigate the survival of two LAB (a commercial probiotic and a potential probiotic) during spray drying in orange juice and subsequent storage as affected by the incorporation of different sugars in the culture medium; ii) to investigate the effects of different conditions of temperature, light exposure and water activity on the survival of the two LAB during storage of the dried powders and iii) to test the ability of both dried LAB to resist during passage through simulated GIT conditions at the end of storage time.

4.2. Materials and methods

4.2.1. Origin, growth and storage conditions of LAB cultures

Two strains of LAB were used: *L. plantarum* 299v (probiotic culture supplied by Probis Probiotika, Lund, Sweden) and *P. acidilactici* HA-6111-2, a potential probiotic (Barbosa *et al*., 2015), deposited in the culture collection of *Escola Superior de Biotecnologia*.

Cells were grown in de MRS agar (Lab M) at 37 ºC for 24 h and stored at -80 ºC in MRS broth (Lab M) containing 30% (v/v) of glycerol (Sigma), and sub-cultured twice in MRS broth before use in assays.

4.2.2. Spray drying of orange juice incorporated with LAB

4.2.2.1. Orange juice preparation

Ripe oranges grown in Portugal were randomly purchased from local commercial establishments (Porto, Portugal), and stored at room temperature until used (for no more than 24 h before experiments).

Oranges were squeezed using a domestic juicer and the juice was filtered to prevent obstruction of the atomizer of the spray dryer. The total soluble solids content of the juice was adjusted to 1.5% (w/v) using a digital refractometer (model PR-32α (alpha), Brix 0–32%, Atago U.S.A., Inc.) and 2% (w/v) of the drying agent 10DE maltodextrin (Sigma) was added. This mixture was dissolved with magnetic stirring at 40 °C.

4.2.2.2. Preparation of LAB cultures

To test the effect that different sugars exerted on the growth and survival of both LAB during spray drying and subsequent storage, cells were grown into standard MRS broth or MRS broth modified by replacing 20 g/L of glucose by 20 g/L of each sugar: fructose, sorbitol, lactose or sucrose (Carvalho *et al.*, 2004b).

One colony of each LAB, grown in MRS agar at 37 ºC during 24 h, was transferred to standard or modified MRS broth and incubated in the same conditions. These cultures were then transferred (1:100) to new standard or modified MRS broth and incubated at 37 ºC for 24 h to reach stationary phase. Cells were harvested by centrifugation (8877 x g, 10 min, 37 ºC; Rotina 35R), washed twice in sterile quarter strength Ringer's solution (Lab M) and re-suspended to the same volume of the final solution prepared in section 4.2.2.1.

Cultures grown in standard MRS and re-suspended in 3.5% (w/v) of RSM powder (Oxoid) were used as a control.

4.2.2.3. Spray drying

The drying of orange juice with each LAB culture was performed in a pilot-scale Spray Dryer (Niro Atomizer, Soborg, Denmark) with a vaned wheel rotating at high speed and a concurrent drying chamber (0.8 m diameter and 0.6 m height). The solution previously prepared (stirring at 40 ºC) was fed into the chamber through a peristaltic pump at a constant flow rate (25 mL/min). The inlet and outlet air temperatures were adjusted to 150 ºC and 70 ºC, respectively. The dried powders were collected in a single cyclone air separator system. Each experiment was repeated three times.

4.2.2.4. Analysis of powders

The dried powders were analyzed immediately after spray drying.

4.2.2.4.1. Drying yield

The drying yield (%) was determined as the % weight fraction of the total solids contained in the liquid feed that could be recovered from the collecting vessel attached to the bottom of the cyclone.

4.2.2.4.2. Water activity

The water activity of the powders was measured using a water activity meter (Aqualab, Series 3, Decagon Devices Inc.) at a constant temperature of 23 ± 1 ºC. Two readings were made for each sample.

4.2.2.5. Storage conditions

Dried samples were stored in plastic containers, hermetically sealed in glass flasks, in normal atmosphere (air), at two different temperatures (4 ºC and room temperature), in

the presence or absence of daylight (hereafter referred to as light) and under controlled a_w. The a_w was controlled by equilibrium with saturated aqueous solution of LiCl (Sigma) and dried silica gel (Pronalab) to give a_w values of 0.11 and 0.03, respectively (Teixeira *et al.*, 1995b).

4.2.2.6. Enumeration of LAB cultures

Enumeration of each LAB, in orange juice or RSM, was performed before and immediately after spray drying and at regular intervals throughout storage. Dried samples were rehydrated to their initial solids concentration in sterile quarter strength Ringer's solution. Each rehydrated sample was homogenized for 1 minute and kept at room temperature for 30 minutes and suitable dilutions were plated in duplicate by the drop count technique (Miles and Misra, 1938) on MRS agar. Colonies were counted after incubation at 37 ºC for 48 h and the cfu/mL calculated. Microbial counts were transformed to logarithmic reduction using the equation: log (N/N_0), where N is the microbial cell count at a particular sampling time and N_0 is the microbial cell count after spray drying.

4.2.3 Gastro-intestinal tract simulation

4.2.3.1. Inoculum

At the end of the storage period at 4 ºC, powders (a_w of 0.03) containing LAB grown in standard MRS were rehydrated to their initial solids concentration in sterile quarter strength Ringer's solution. Cells dried in RSM were used as a control.

4.2.3.2. Simulated gastro-intestinal conditions

As described by Barbosa *et al*. (2012), aliquots of 0.5 mL of each inoculum prepared as described above were placed into glass flasks with 49.5 mL of BPW (Lab M) adjusted to pH 3.0 with HCl (1M, Pronalab) and with 1000 units/mL of a filter sterilized solution of pepsin (Sigma) to simulate the stomach conditions. Samples were taken at time 0 (time of inoculation) and after 30 min and 60 min (quick gastric transit simulation) or for a total of 120 min (slow gastric transit simulation). To simulate the conditions of the small intestine, a sterile solution of bile salts was added (final concentration of 0.3% (w/v), Pronadisa), after increasing the pH from 3.0 to 7.0 with a sterile solution of Sodium Hydroxide (1M NaOH, Pronalab). Again, samples were taken at time 0 (time of bile salts addition) and every 30 min for a total of 60 min (quick digestion simulation) or for a total of 120 min (slow digestion simulation). Enumeration of survivors was done as described in section 4.2.2.6. All assays were done in triplicate.

4.2.4. Statistical analysis

Significant differences in the survival of LAB cultures previously grown into standard or modified MRS broth and stored under different conditions of temperature, a_w and light, were tested by an analysis of variance (one-way ANOVA). Significant differences between slow and quick gastric transit simulations and digestions on the survival of microorganisms in simulated GIT conditions were also investigated. Multiple comparisons were evaluated by Tukey's post-hoc test. All calculations were carried out using the software KaleidaGraph (version 4.4, Synergy Software, Reading, USA).

4.3. Results and discussion

The drying conditions for orange juice incorporated with the cultures used in this study - commercial probiotic *L. plantarum* 299v and the potential probiotic *P. acidilactici* HA-6111-2 - had been previously selected (chapter 3).

Table 4.1 shows cell counts observed for the two LAB before and after spray drying for all the growth conditions investigated as well as the a_w values of the dried powders and the drying yield.

Table 4.1. Survival of LAB isolates before and after spray drying (SD) in RSM or in orange juice with 10DE maltodextrin (OJM) after grown in standard or modified MRS and the a_w values and yield of the respective powders

		[a] log cfu/mL		Powder	
LAB	Condition	Before SD	After SD	a_w [b]	Yield (%) [c]
L. plantarum 299v	MRS-Glucose + RSM	9.9 ± 0.12	10.4 ± 0.12	0.383 ± 0.125	62.8 ± 5.4
	MRS-Glucose + OJM	9.4 ± 0.03	9.5 ± 0.30	0.421 ± 0.076	53.7 ± 5.5
	MRS-Fructose + OJM	9.2 ± 0.04	9.6 ± 0.12	0.398 ± 0.032	43.8 ± 5.7
	MRS-Sorbitol + OJM	9.4 ± 0.11	9.1 ± 0.39	0.386 ± 0.015	38.9 ± 6.0
	MRS-Lactose + OJM	8.4 ± 0.12	7.9 ± 0.57	0.379 ± 0.116	48.4 ± 6.6
	MRS-Sucrose + OJM	8.2 ± 0.33	8.4 ± 0.37	0.374 ± 0.054	56.0 ± 8.9
P. acidilactici HA-6111-2	MRS-Glucose + RSM	9.2 ± 0.02	10.3 ± 0.03	0.358 ± 0.122	61.5 ± 1.2
	MRS-Glucose + OJM	9.0 ± 0.06	9.4 ± 0.23	0.357 ± 0.038	53.8 ± 8.6
	MRS-Fructose + OJM	9.4 ± 0.12	9.4 ± 0.50	0.488 ± 0.095	58.4 ± 7.7
	MRS-Sorbitol + OJM	8.6 ± 0.24	8.3 ± 0.43	0.372 ± 0.052	41.1 ± 5.1
	MRS-Lactose + OJM	8.7 ± 0.05	8.7 ± 0.12	0.392 ± 0.045	59.4 ± 7.6
	MRS-Sucrose + OJM	8.8 ± 0.06	8.9 ± 0.19	0.359 ± 0.022	65.8 ± 6.4

[a]Survival is represented as the mean of the log cfu/mL ± the standard error of the mean

[b]The a_w is represented as the mean of a_w values ± the standard error of the mean

[c]The powder yield is represented as the mean of percentages of powder obtained ± the standard error of the mean

The drying process did not affect the number of *L. plantarum* 299v and *P. acidilactici* HA-6111-2 cells in all experiments. No significant differences were observed (p>0.05) between the number of cells obtained before and after spray drying.

In most samples, the drying yield was high and the a_w of the powders was low. These two parameters are important and reflect the drying efficiency.

It has been previously demonstrated that when *L. plantarum* 299v and *P. acidilactici* HA-6111-2 were spray dried in orange juice with 10 DE maltodextrin and stored at room temperature without controlled a_w survivors decreased to values below the detection limit of the enumeration technique after 60 days (*L. plantarum* 299v) or 120 days (*P. acidilactici* HA-6111-2). At 4 °C, while *P. acidilactici* HA-6111-2 cells have been reduced by less than1 log unit, *L. plantarum* 299v decreased by 8 log units after 210 days of storage (chapter 3). Thus, in order to improve survival of these strains, in this study we investigated different storage temperatures (room and 4 ºC) and, at room temperature, we varied other conditions (the presence/absence of light and a_w controlled to 0.03 or to 0.11).

Figures 4.1 and 4.2 show the survival of both LAB evaluated over 12 months at room temperature and exposed to light (graphs A1 to A6), at 4 °C and light exposure (graphs B1 to B6), at room temperature and in the absence of light (graphs C1 to C6), all with a_w values controlled to 0.03 and, finally, at room temperature with light exposure and a_w value controlled to 0.11 (graphs D1 to D6).

For all the storage conditions investigated, no significant (p>0.001) reductions in cell viability were observed for both LAB after spray drying in RSM.

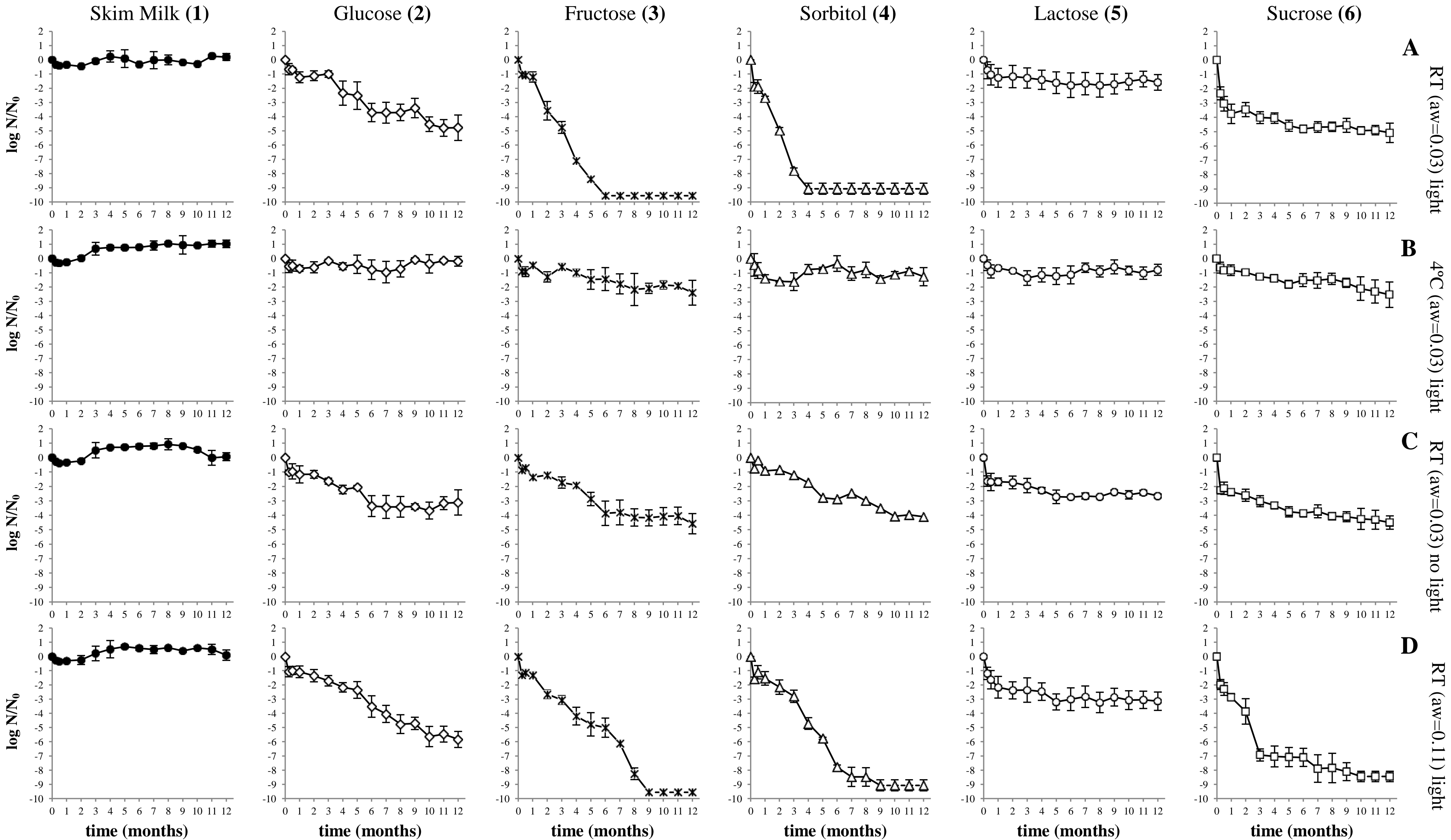
After spray drying in orange juice and subsequent storage at room temperature in the presence of light and a_w controlled to 0.03 (Figure 4.1, graphs A2 to A6), the highest survival of *L. plantarum* 299v was observed for cells grown in MRS with lactose (1.6 log unit reduction), MRS with glucose (4.8 log unit reduction) and MRS with sucrose (5.1 log unit reduction). *Pediococcus acidilactici* HA-6111-2 showed logarithmic reductions of less than 1 log-unit for all conditions (Figure 4.2, graphs A2 to A6), except for cells grown in MRS containing fructose (2.1 log-unit reduction).

For both LAB, stored at 4 °C under controlled a_w (0.03; graphs B2 to B6) logarithmic reductions were minimal. The maximum reduction in cell viability of *P. acidilactici* (Figure 4.2) was observed for cells grown in MRS with fructose. For *L. plantarum* (Figure 4.1), significant reductions in cell viability were only observed for cells grown in MRS with fructose or sucrose.

In the absence of light (graphs C2 to C6), with the exception of cells grown in the presence of lactose, for all the other sugars investigated, survival of *L. plantaru*m (Figure 4.1) increased. This effect was not demonstrated for *P. acidilactici* (Figure 4.2) where no significant decrease in survival was observed when cells were stored at an a_w controlled to 0.03 and in absence of light.

During storage at an a_w controlled to 0.11 (graphs D2 to D6), for *L. plantarum*, only cells grown in MRS with lactose (3.1 log unit reduction) or glucose (5.8 log unit reduction) were not reduced to values below the detection limit of the technique. For *P. acidilactici*, with the exception of fructose, there were no significant differences in the viability of cells grown in the presence of lactose, glucose, sorbitol or sucrose (p>0.05).

Survival during storage at a_w controlled to 0.03 (graphs A2 to A6) was significantly (p<0.0001) higher than when cells were stored at an a_w of 0.11.



Skim Milk (1)
Glucose (2)
Fructose (3)
Sorbitol (4)
Lactose (5)
Sucrose (6)
A
RT (aw=0.03) light
B
4°C (aw=0.03) light
C
RT (aw=0.03) no light
D
RT (aw=0.11) light
log N/N0
time (months)

Figure 4.1. Logarithmic reduction of *L. plantarum* 299v incorporated in RSM or orange juice with 2% of 10DE maltodextrin after spray drying and during 12 months of storage at different conditions (A, B, C or D): control (inoculum in 10% (w/v) of RSM); inoculum in orange juice with 2% of 10DE maltodextrin after grown in MRS supplemented with 20 g/L of different sugars (2, 3, 4, 5 and 6). The dotted lines mean that survival was lower than the detection limit of the enumeration technique.

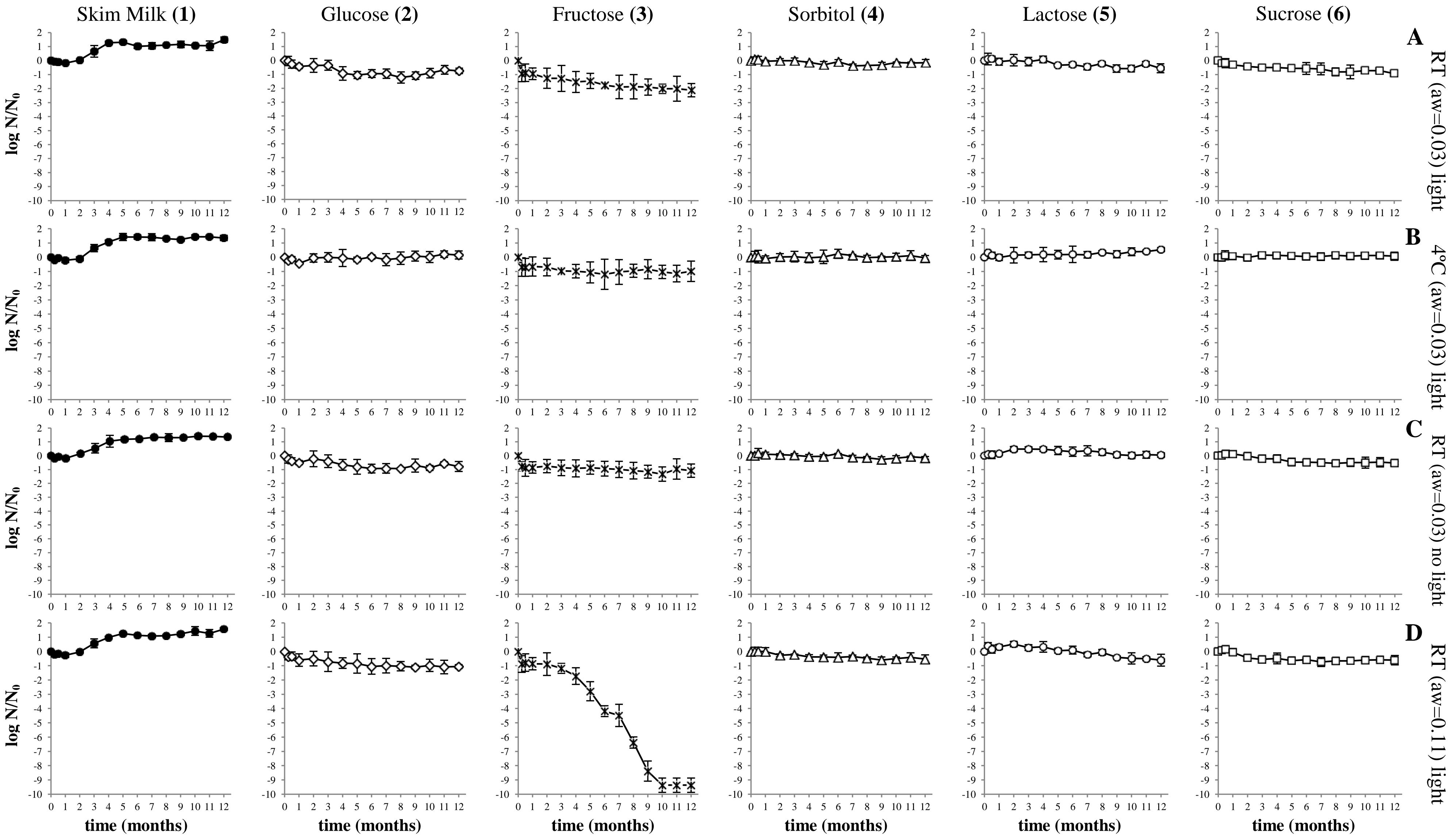

Skim Milk (1)
Glucose (2)
Fructose (3)
Sorbitol (4)
Lactose (5)
Sucrose (6)
A
RT (aw=0.03) light
B
4°C (aw=0.03) light
C
RT (aw=0.03) no light
D
RT (aw=0.11) light
log N/N0
time (months)

Figure 4.2. Logarithmic reduction of *P. acidilactici* HA-6111-2 incorporated in RSM or orange juice with 2% of 10DE maltodextrin after spray drying and during 12 months of storage at different storage conditions (A, B, C or D): control (inoculum in 10% (w/v) of RSM); inoculum in orange juice with 2% of 10DE maltodextrin after grown in MRS supplemented with 20 g/L of different sugars (2, 3, 4, 5 and 6). The dotted lines mean that survival was lower than the detection limit of the enumeration technique.

For all the conditions investigated, the potential probiotic *P. acidilactici* HA-6111-2 proved to be more resistant than the commercial probiotic *L. plantarum* 299v. Other investigators had previously reported that resistance to spray drying is species/strain-dependent (Corcoran *et al*., 2004; Páez *et al*., 2012).

It was also evident that the RSM, used in this study as a control, conferred a high protective effect, as observed in other studies (Ananta *et al*., 2005; Fritzen-Freire *et al*., 2012).

The addition of sugars to the drying medium to improve survival of LAB during drying by different drying techniques has been reported by several authors (Carvalho *et al*., 2004b; Golowczyc *et al*., 2011a). Although the survival of LAB during storage at room temperature after spray drying in orange juice was demonstrated to be very low in previous studies (chapter 3), the addition of sugars to the juice was excluded as a potential alternative to improve survival as this would result in an increase in the viscosity of the juice with detrimental effects during the drying process. In addition to the use of maltodextrin, recognized as a good protectant during drying (Anekella and Orsat, 2013; Reddy *et al*., 2009), other factors were combined to increase the survival of LAB. It has

been previously demonstrated that the addition of certain sugars to the media used for the growth of various LAB cultures influenced their survival during spray drying (Ferreira *et al.*, 2005), lyophilization (Carvalho *et al.*, 2004b), and other drying methods (Tymczyszyn *et al.*, 2007). This effect was also observed in the present study.

The logarithmic reductions of both LAB were minimal during storage at 4 ºC. It has been demonstrated that the highest survival of dried bacteria occurs at low storage temperatures (Teixeira *et al.*, 1995b), probably resulting from a lower rate of membrane fatty acid oxidation (Castro *et al.*, 1995).

The absence of light during storage resulted in an increasing survival of *L. plantarum* 299v, comparing with the storage in the presence of light. Several authors have already mentioned the negative effects of light exposure on the survival of dried microorganisms (Strasser *et al.*, 2009; Tripathi and Giri, 2014).

Survival of both LAB during storage at a_w controlled to 0.03 was higher than when cells were stored at an a_w of 0.11. Abe *et al.* (2009) also recorded better survival rates at lower a_w values. However, Golowczyc *et al.* (2011a) reported higher survival rates at both a_w values of 0.03 and 0.11.

Growth in the presence of various sugars results in cells with different morphological and physiological characteristics, which leads to different behaviors to different stresses (Carvalho *et al.*, 2004a). One possible explanation for this increase in resistance is related to the accumulation of compatible solutes by cells which makes them more resistant to hyperosmotic stress. But LAB cannot synthesize these compatible solutes (such as sugars) that favor the increase of viability during the drying processes (Morgan *et al.*, 2006). Adding these solutes to the drying medium may not be sufficient, since the drying time is

short. Thus, by growing the cells in contact with sugars, they have time to accumulate these compatible solutes (Ferreira *et al*., 2005). Sugars may preserve the structures of the membrane as well as delay protein denaturation (Chávez and Ledeboer, 2007).

Some differences in the results obtained by other authors when the same sugars were incorporated into the growth media, could be explained by the different species/strains, different drying techniques or the different drying media used. For example, Carvalho *et al*. (2004b) reported good survival of freeze dried cells of *L. delbrueckii* ssp. *bulgaricus* during storage, after their growth in MRS containing fructose or lactose. In our study, cells grown in the presence of fructose demonstrated the lowest survival during storage. Similarly, Panoff *et al*. (2000) found that growth of a *L. delbrueckii* ssp. *bulgaricus* strain in the presence of lactose and sucrose increased their survival during freezing and thawing. Also the growth of a *L. sakei* strain in the presence of sucrose enhanced its survival after spray drying (Ferreira *et al*., 2005). Sorbitol also protected lyophilized cells of *L. plantarum* during storage, but only when this was incorporated in the drying medium (Carvalho *et al*. 2002). After the incorporation of glucose into the drying medium, the survival of spray dried cells of *L. rhamnosus* during storage was improved (Ying *et al*., 2012). Unlike the study by Carvalho *et al*. (2004b), the high survival rate during storage of the cells grown in MRS medium with glucose in our study, may be due not only to the fact that drying techniques were different, but also the storage conditions of the dried powders.

Since the viability of probiotics should be maintained not only in the food product, but also during the storage period and throughout passage of the GIT of the consumer, the conditions of the GIT were simulated after 12 months of storage to verify that both LAB could survive a quick or a slow digestion. As there were many variables, cells from

cultures grown in MRS medium with glucose (high survival, after lactose, and sugar naturally embedded in MRS) and powders stored at 4 °C (longer survival of both microorganisms) and derived from orange juice with maltodextrin, were chosen. Powders stored at 4 °C and derived from the cultures dried in RSM, were used as a control. The survival of both LAB are presented in Table 4.2.

Table 4.2. Survival of LAB isolates after 12 months of storage after spray-drying (SD) in RSM or in orange juice with 10 DE maltodextrin, through quick and slow digestions simulations

		log (N/N_0)[a]		
		Quick digestion simulation		
LAB	**Condition**	**0 minutes**	**60 minutes[b]**	**120 minutes[c]**
L. plantarum 299v	SD in RSM	0.00±0.00	-0.24±0.18	-1.38±0.15
	SD in orange juice	0.00±0.00	-0.51±0.29	-2.02±0.45
P. acidilactici HA-6111-2	SD in RSM	0.00±0.00	-0.09±0.01	-0.66±0.16
	SD in orange juice	0.00±0.00	-0.83±0.18	-1.96±0.43
		Slow digestion simulation		
		0 minutes	**120 minutes[b]**	**240 minutes[c]**
L. plantarum 299v	SD in RSM	0.00±0.00	-0.15±0.10	-1.35±0.45
	SD in orange juice	0.00±0.00	-0.76±0.28	-2.31±0.27
P. acidilactici HA-6111-2	SD in RSM	0.00±0.00	-0.10±0.07	-1.16±0.29
	SD in orange juice	0.00±0.00	-1.03±0.35	-2.08±0.37

[a]Survival is represented as the mean of the logarithmic reduction: log (N/N_0) ± the standard error of the mean

[b]Survival after exposure to pH 3.0 in the presence of pepsin

[c]Survival after exposure to pH 3.0 in the presence of pepsin and subsequent exposure to bile salts at pH 7.0

N: cfu/mL at each sampling time

N_0: cfu/mL at time zero

For *L. plantarum* 299v, there were no significant differences ($p>0.05$) between cells dried in RSM and orange juice with maltodextrin, during both quick and long simulated digestions. However, the reduction was greater when the cells were subsequently exposed to bile salts than to pH of 3.0 with pepsin. For *P. acidilactici* HA-6111-2, the decrease was lower for cells dried in RSM. Again, for orange juice with maltodextrin and RSM, the reduction was higher when the cells were exposed to the conditions of the small intestine digestion than to the acidic conditions of the stomach.

The ability to survive better in low pH rather than in the presence of bile salts was also reported for *P. acidilactici* (Abbasiliasi *et al*., 2012) and *L. plantarum* (Mirlohi *et al*., 2009) strains.

Several authors have shown that the survival of cells from different species, through a simulated GIT, was enhanced, after being spray dried (Maciel *et al*., 2014; Páez *et al*., 2012).

In summary, after 12 months of storage in orange juice, there was a reduction of approximately 2 log-unit for *L. plantarum* 299v and *P. acidilactici* HA-6111-2 during both the quick and the long simulated digestions. This result is important since after the storage period studied, the dried powders which have the minimum amount required of viable cultures in order to be considered as a probiotic product (~10^9 cfu/mL), suffer a reduction of only 2 log-units after gastro-intestinal passage, thereby presenting viable counts of these cultures of ~10^7 cfu/mL, thus potentially exerting a beneficial effect on the health of the consumer.

4.4. Conclusion

From the present study we conclude that: i) the use of carbohydrates such as lactose (or glucose) for the growth of LAB before spray drying in orange juice, could improve their ability to survive during the drying processes and during subsequent storage;
ii) in addition to the high survival of both LAB studied, they were able to resist simulated conditions of the GIT, even after 12 months of storage; and iii) storage conditions are very important and, although 4 °C was shown to be the best temperature, room temperature without light exposure also allowed a high survival when the a_w was controlled to 0.03. Costs of storage could be reduced by eliminating refrigerated storage and survival enhanced by a light-proof package.

Thus, using the conditions of growth and storage mentioned above, it should be possible to produce an orange juice powder with pre- and probiotic characteristics with an shelf life date of at least 12 months in a package protected from daylight exposure.

CHAPTER 5

Influence of sub-lethal stresses on the survival of lactic acid bacteria after spray drying in orange juice

Abstract

The demand for new functional non-dairy based products makes the production of an orange juice powder with pre- and probiotic characteristics, an encouraging challenge. However, in the drying process and during storage in the dried state, loss of viability of the probiotic cultures can occur, since the cells are exposed to various stresses. The influence of sub-lethal conditions of temperature, acidic pH and hydrogen peroxide on the viability of *P. acidilactici* HA-6111-2 and *L. plantarum* 299v during spray drying in orange juice and subsequent storage under different conditions was investigated. At the end of storage, the survival of both microorganisms through simulated GIT conditions was also determined. The viability of cells previously exposed to each stress was not affected by the drying process. However, during 180 days of storage at room temperature, survival of *L. plantarum* 299v was enhanced by prior exposure to sub-lethal conditions. Exposure of *P. acidilactici* HA-6111-2 to sub-lethal stresses prior to spray drying did not result in the enhancement of its viability during storage. Previous exposure to sub-lethal stresses of each microorganism did not improve their viability after passage through simulated GIT. However, as cellular inactivation during 180 days of storage was low, both microorganisms were present in numbers of ca. 10^7 cfu/mL at the end of GIT. This is an indication that both *P. acidilactici* HA-6111-2 and *L. plantarum* 299v are good candidates for use in the development of an orange juice powder with functional characteristics.

5.1. Introduction

Spray drying is a powerful tool to transform liquid food products into dry powder of high quality and at low cost. The process consists of pumping the liquid sample which is atomized into small droplets, which then lose their moisture on contact with hot and dry air (Silva *et al*., 2011). The occurrence of stickiness of sugar-rich food products during this process, and also during storage, requires the addition of some drying agents with high molecular weights such as maltodextrins, to prevent this problem (Caliskan and Dirim, 2013; Fazaeli *et al*., 2012). In addition to decreasing stickiness, maltodextrins are also recognized prebiotics, i.e., improve the host's health by stimulating the growth and/or activity of specific bacteria in the colon (Gibson and Roberfroid, 1995).

Since the 1970's spray drying has been used to dry orange juice (Gupta, 1978). More recently, some researchers have added probiotic cultures to dehydrated fruit juices because of their beneficial health effects (Anekella and Orsat, 2013; Pereira *et al*., 2014). It would be highly advantageous to combine the benefits of probiotics and prebiotics with those of natural orange juice.

However, in the spray drying process loss of viability of the probiotic cultures can occur, since the cells are exposed to stresses such as high temperatures and rapid dehydration (Tripathi and Giri, 2014). Nevertheless, it is possible to increase the resistance of microorganisms to the drying process and storage, by prior exposure to sub-lethal stresses, inducing the production of protective cellular components (Broadbent and Lin, 1999; Hurst, 1977).

The maximum viability of probiotics during spray drying and storage is necessary since probiotic microorganisms should be present in foods in numbers of about 10^6-10^7 cfu/g or cfu/mL until the time of consumption and must remain viable during passage through the GIT of the consumer (FAO/WHO, 2002; Sanz, 2007).

The purpose of this study was to investigate the influence of sub-lethal conditions such as high temperature, acidic pH and presence of hydrogen peroxide (H_2O_2) on the viability of two probiotic LAB during spray drying in orange juice and subsequent storage under different conditions of temperature, light and water activity. The ability of both LAB to survive during passage through simulated GIT conditions, was also evaluated at the end of storage.

5.2. Materials and Methods

5.2.1. Origin, growth and storage conditions of LAB cultures

Lactobacillus plantarum 299v (commercial probiotic; Probis Probiotika, Lund, Sweden) and *P. acidilactici* HA-6111-2 (Escola Superior de Biotecnologia culture collection) were used in this study. *Pediococcus acidilactici* HA-6111-2 is recognized as a potential probiotic strain (Barbosa *et al*., 2015). Cultures were grown on de MRS agar (Lab M) at 37 ºC for 24 h and preserved at -80 ºC in MRS broth (Lab M) containing 30% (v/v) of glycerol (Sigma).

5.2.2. Inoculum

One colony of each LAB grown on MRS agar (24 h at 37 ºC) was transferred to MRS broth and incubated in the same conditions. This last culture was transferred to fresh MRS broth (1:100 v/v) and incubated at 37 ºC for 24 h to reach stationary phase. Each isolate was harvested by centrifugation (8877 x g, 10 min, 37 ºC; Rotina 35R), washed twice and re-suspended in the same volume of sterile quarter strength Ringer's solution (Lab M).

5.2.3. Determination of the sub-lethal conditions

Both LAB were exposed to different pH values, temperatures and concentrations of H_2O_2. Aliquots of 0.5 ml of each inoculum (obtained as described in section 5.2.2) were placed into glass flasks with 49.5 ml of BPW (Lab M) adjusted to pH values from 2.0 to 2.8 with HCl(1 M, Pronalab) or to pH values from 2.0 to 3.5 with 1 M lactic acid (José M. Vaz Pereira, Lda, Lisbon, Portugal) for acidic stresses; in BPW containing various concentrations of H_2O_2 (aga – álcool e géneros alimentícios, S.A., Prior Velho, Portugal) from 0.25 to 60 mM for oxidative stresses and, finally, for stress temperatures, the glass flasks were kept at temperatures from 48 ºC to 60 ºC. As control, an aliquot of 0.5 ml of inoculum was placed into glass flasks with 49.5 ml of BPW at pH 7.0 and held at 37 ºC. All experiments were done in duplicate. For each sample, taken at time 0 (time of inoculation) and after 30 and 60 minutes, serial decimal dilutions were made in sterile quarter strength Ringer's solution (Lab M) and plated in duplicate for enumeration by the drop count technique (Miles and Misra, 1938) on MRS agar. The colonies were counted after incubation at 37 ºC for 48 h and the cfu/mL calculated. Microbial counts were transformed to logarithmic reduction using the equation: $\log (N/N_0)$, where N is the microbial cell density at a particular sampling time and N_0 is the initial microbial cell count. Those conditions resulting in a loss of viability lower than 1 log unit after 60 minutes of exposure were selected for subsequent experiments, described in section 5.2.4.2.

5.2.4. Spray drying of orange juice incorporated with LAB cultures

5.2.4.1. Materials and orange juice preparation

Mature oranges originating in Portugal and randomly purchased from local commercial establishments (Porto, Portugal) were squeezed using a domestic juicer and the juice was

filtered in order to eliminate the solids in suspension, preventing the obstruction of the atomizer of the spray dryer The content of the total soluble solids of the juice was adjusted to 1.5% (w/v) with a digital refractometer (model PR-32α (alpha), Brix 0–32%, Atago U.S.A., Inc.) and 2% (w/v) of the drying agent 10DE maltodextrin (Sigma) was added. This mixture was dissolved under magnetic stirring at 40 °C.

5.2.4.2. Exposure of LAB cultures to sub-lethal stresses

The inoculum of LAB was prepared as described above in section 5.2.3. Instead of sterile quarter strength Ringer's solution (Lab M), cells were re-suspended in the same volume of BPW adjusted to each sub-lethal condition to be investigated. Cells were exposed to these conditions during 1 h and the samples were taken at time 0 (time of inoculation) and after 60 minutes. After the exposure to each sub-lethal condition, each suspension was harvested by centrifugation and the cells re-suspended in the same volume of the final solution prepared in 5.2.4.1.

Cells obtained from the inoculum prepared as described in 5.2.2 were directly re-suspended in the same volume of the final solution prepared in 5.2.4.1 and used as a control.

Each experiment was repeated three times.

5.2.4.3. Spray drying

The drying of orange juice containing sub-lethal stressed LAB cultures and the respective control, was performed in a pilot-scale Spray Dryer (Niro Atomizer, Soborg, Denmark) with a vaned wheel rotating at high speed and a co-current drying chamber (0.8 m diameter and 0.6 m height). Each solution, stirred at 40 ºC, was fed into the chamber through a peristaltic pump at a constant flow rate of 25 mL/min and at inlet and outlet air

temperatures adjusted to 150 and 70 ºC, respectively. The dried powders were collected in a single cyclone air separator system.

5.2.4.4. Analysis of powders

The dried powders were analyzed immediately after spray drying.

5.2.4.4.1. Drying yield

The drying yield (%) was determined as the % weight fraction of the total solids contained in the liquid feed that could be recovered from the collecting vessel attached to the bottom of the cyclone.

5.2.4.4.2. Water activity

The water activity of the powders was determined using a water activity meter (Aqualab, Series 3, Decagon Devices Inc.) at a constant temperature of 23 ± 1 ºC. Two readings were made for each sample.

5.2.4.5. Storage conditions

Dried samples were stored during 180 days in hermetically sealed glass containers, under the following conditions:

A) room temperature, in the presence of daylight; water activity (a_w)=0.03

B) 4 ºC, in the presence of daylight; a_w=0.03

C) room temperature, in the absence of daylight; a_w=0.03

D) room temperature, in the presence of daylight; a_w=0.11

Water activity was controlled by equilibrium with saturated aqueous solution of LiCl (Sigma) and dry silica gel (Pronalab) to give a_w values of 0.11 and 0.03, respectively (Teixeira *et al.*, 1995b).

5.2.4.6. Enumeration of LAB cultures

The enumeration of each LAB culture was determined before spray drying. Immediately after spray drying and at regular intervals throughout storage survival was evaluated by rehydration of each dried sample to their initial solids concentration in sterile quarter strength Ringer's solution (Lab M). After homogenizing for 1 minute and kept at room temperature for 30 minutes, each rehydrated sample was enumerated as described above in section 5.2.3.

Microbial counts were transformed to logarithmic reduction using the equation: log (N/N_0), where N is the microbial cell count at a particular sampling time and N_0 is the microbial cell count after spray drying.

5.2.5. Gastro-intestinal tract simulation

5.2.5.1. Inoculum

At the end of the storage period, powders that were stored at 4 ºC were rehydrated to their initial solids concentration in sterile quarter strength Ringer's solution.

5.2.5.2. Simulated gastro-intestinal conditions

These assays were done according to Barbosa *et al*. (2012) with some modifications. Aliquots of 0.5 mL of each inoculum were placed into glass flasks with 49.5 mL of BPW adjusted to pH 3.0 with 1 M HCl and with 1000 units/mL of a filter sterilized solution of pepsin (Sigma) to achieve the conditions of the stomach. Samples were taken at time 0

(time of inoculation) and each 30 min interval until a total of 60 min (quick gastric transit simulation) or for a total of 120 min (slow gastric transit simulation). To simulate the conditions of the small intestine, a sterile solution of bile salts was added (final concentration of 0.3% (w/v), Pronadisa), after increasing the pH from 3.0 to 7.0 with a sterile solution of Sodium Hydroxide (1 M NaOH, Pronalab). Again, samples were taken at time 0 (time of bile salts addition) and every 30 min for a total of 60 min (quick digestion simulation) or for a total of 120 min (slow digestion simulation). Survivors were enumerated as described above in section 5.2.3. Survival was represented as the mean of the logarithmic reduction: log (N/N_0) ± the standard error of the mean. Three independent assays were carried out.

5.2.6. Statistical analysis

An analysis of variance (one-way ANOVA) was performed to evaluate any significant differences in the survival of LAB cultures obtained with the different sub-lethal stresses applied and the different storage conditions taking into account temperatures, a_w and light. Also any significant effects between slow and quick gastric transit simulations and digestions, both with and without sub-lethal stresses, on the survival of LAB in simulated GI tract were tested. Multiple comparisons were evaluated by Tukey's post-hoc test. All calculations were carried out using the software KaleidaGraph (version 4.4, Synergy Software, Reading, USA).

5.3. Results and Discussion

The microorganisms used in this study have been previously dried into orange juice with 10DE maltodextrin and stored at room temperature without controlled a_w. At these conditions a high loss of viability was observed (data not shown).

In an attempt to increase the survival of these microorganisms during spray drying and during subsequent storage, cells were exposed to different sub-lethal conditions (Table 5.1) before drying.

Table 5.1. Selected sub-lethal conditions for each LAB culture

	*Sub-lethal value	
Stress	*L. plantarum* 299v	*P. acidilactici* HA-6111-2
Thermal	49.5 ºC	50.0 ºC
Acidic (adjusted with HCl)	pH 2.8	pH 2.7
Acidic (adjusted with Lactic acid)	pH 3.0	pH 3.1
Oxidative (H_2O_2)	45 mM	25 mM

*Loss of viability was lower than 1 log-unit after 60 minutes of exposure to each condition imposed (data not shown).

As shown in Table 5.2, for all the sub-lethal conditions investigated, there were no significant (p>0.05) reductions in cell viability during the drying process.

Table 5.2. Survival of LAB before and after spray drying (SD) in orange juice with 10DE maltodextrin after being exposed to different sub-lethal conditions

	log cfu/mL[a]			
	L. plantarum 299v		*P. acidilactici* HA-6111-2	
Sub-lethal stress	Before SD	After SD	Before SD	After SD
Without	9.4 ± 0.03	9.5 ± 0.30	9.0 ± 0.06	9.4 ± 0.23
Acidic pH (HCl)	9.1 ± 0.47	9.3 ± 0.68	10.1 ± 0.03	10.3 ± 0.40
Acidic pH (Lactic acid)	10.1 ± 0.40	10.2 ± 0.45	9.5 ± 0.12	10.2 ± 0.08
Thermal	10.0 ± 0.16	9.2 ± 0.08	8.3 ± 0.14	8.0 ± 0.12
Oxidative (H_2O_2)	10.3 ± 0.13	9.5 ± 0.17	8.7 ± 0.15	7.9 ± 0.64

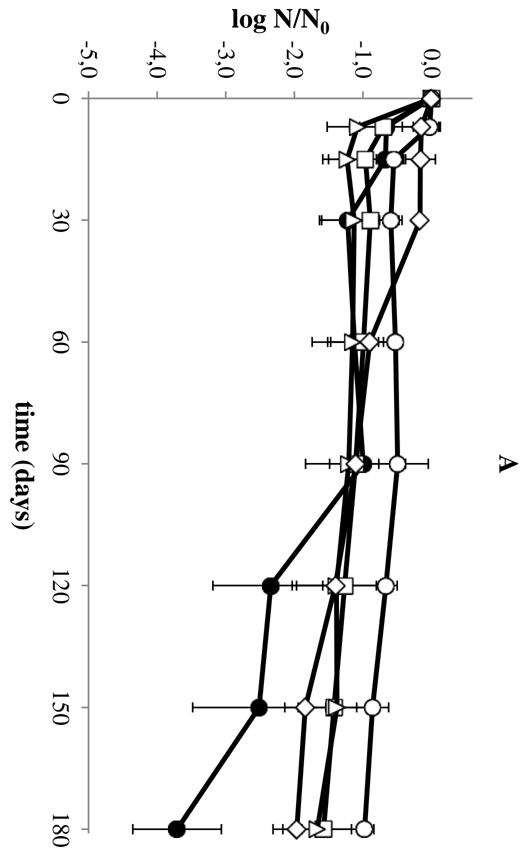
[a]Survival is represented as the mean of the log cfu/mL ± the standard error of the mean

The drying yield obtained for orange juice powders with *L. plantarum* 299v, ranged between 40 and 54% and with *P. acidilactici* HA-6111-2 between 45 and 64%. A powder recovery of 50% in a laboratory scale spray dryer is considered an efficient drying (Bhandari *et al.*, 1997). All the powders obtained had values of a_w between 0.3 and 0.4. The values of a_w should be as low as possible in order to increase survival of dried cells during storage (Chávez and Ledeboer, 2007); values around 0.2 are commonly observed in industrial spray dryers (Adhikari *et al.*, 2009).

Survival of *L. plantarum* 299v and *P. acidilactici* HA-6111-2 during storage at different conditions of temperature, a_w values and light exposure are presented in Figures 5.1 and 5.2, respectively.

Survival of *L. plantarum* was not significantly different (p>0.05) in the presence or absence of light (Figure 5.1, graphs A and C, respectively). Prior exposure of *L. plantarum* to any of the sub-lethal stresses applied, increased their survival during 180 days of storage at room temperature; this effect was not observed (p>0.05) for cells stored at 4 ºC (Figure 5.1C). At this temperature, cell inactivation was very low, as previously demonstrated by other researchers (Gardiner *et al.*, 2000; Teixeira *et al.*, 1995b).

For storage at a_w controlled to 0.03 and at room temperature (in the presence and absence of light, in figure 5.1 graphs A and C, respectively), initially, reductions in viable cell numbers were greater for cells exposed to lactic acid (about -2 log units), the number of viable *L. plantarum* cells remained constant until the end of storage. However, from 90 days until the end of the storage, the reduction in viable cells not exposed to sub-lethal stress was greater (more than 3 log units). Temperature conferred the highest protection, followed by treatment with HCl, H_2O_2, and finally by lactic acid.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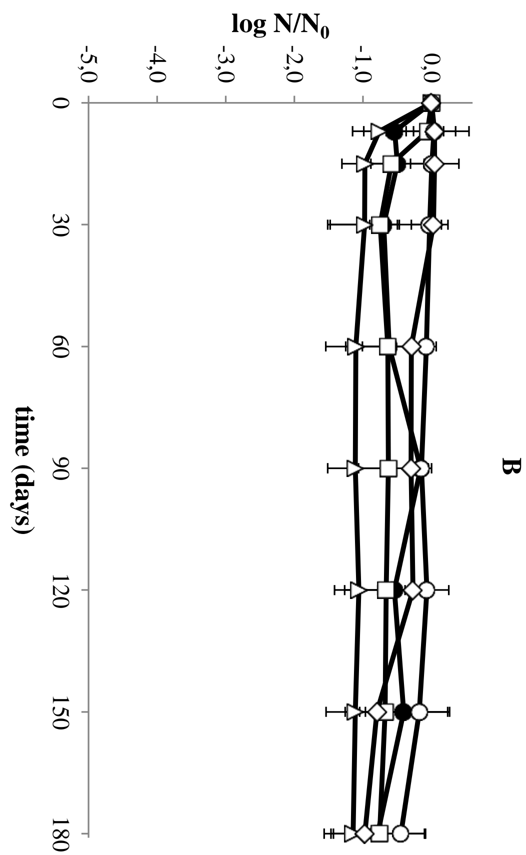
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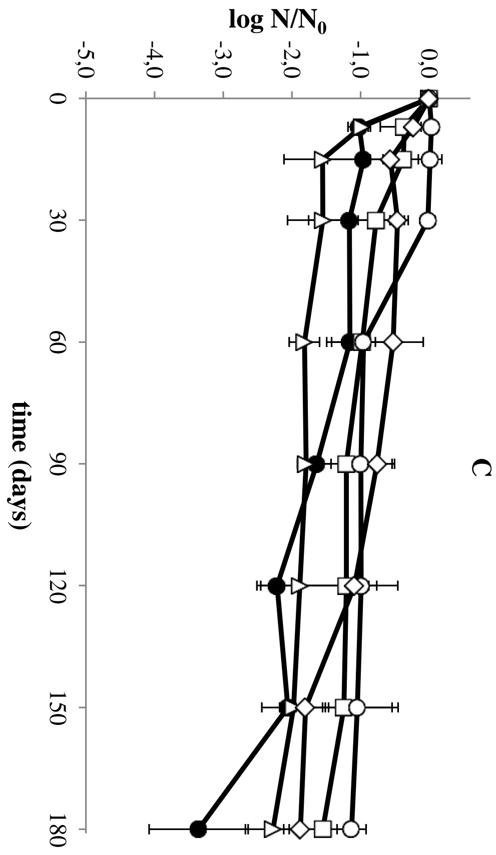
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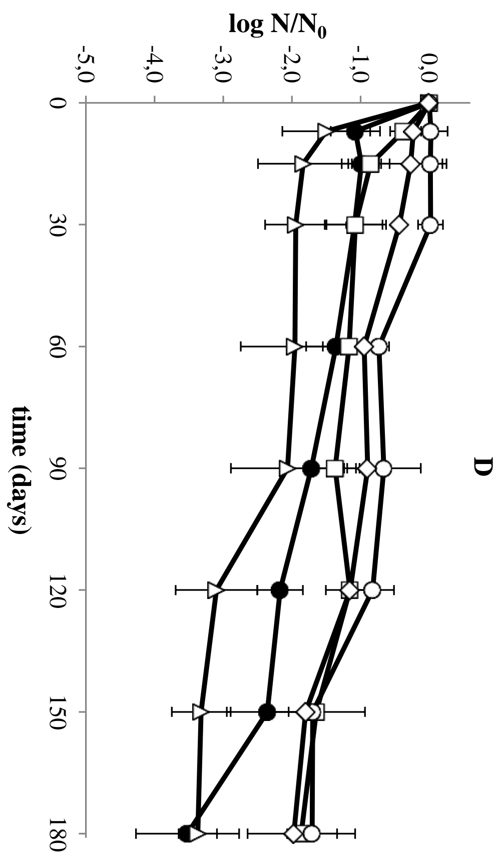
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Figure 5.1. Logarithmic reductions of *L. plantarum* 299v (●) without sub-lethal treatment; (□) acidic stress (HCl); (∆) acidic stress (Lactic acid); (○) temperature stress and (◇) oxidative stress and incorporated in orange juice with 2% of 10DE maltodextrin after spray drying and during 180 days of storage in the presence of light with a_w of 0.03 at room temperature (**A**) and at 4 ºC (**B**), in the absence of light with a_w of 0.03 at room temperature (**C**) and in the presence of light with a_w of 0.11 at room temperature (**D**).

At room temperature with a_w controlled to 0.11 (fig. 1, graph D), greatest reductions in viability were recorded for *L. plantarum* 299v cells not exposed to sub-lethal stresses or exposed to lactic acid, followed by cells exposed to H_2O_2, HCl and temperature. For these last three conditions, no significant differences in viability were obtained (p>0.05).

Increased survival of sub-lethal stressed lactobacilli following spray drying has also been observed by other researchers (Paéz *et al*., 2012).

Acid is an important stress, since it occurs during fermentation of foods, where the growth of LAB generates acidic end products creating a hostile environment for other organisms. Even for *L. plantarum* which is resistant to lactic acid, its growth can be strongly inhibited by the presence of high concentrations of this organic acid (Russell and Diez-Gonzalez, 1998). Also when a probiotic culture reaches the stomach it is exposed to extremely acidic conditions. Many species of lactic acid bacteria exhibit an acid tolerance response generated by a short exposure to a sub-lethal pH value, increasing their survival during subsequent exposure to a lethal pH value. In this study, cells of *L. plantarum* 299v exposed to lactic acid and HCl prior to spray drying become more resistant during storage. Other authors reported an increased resistance of *Lactobacillus* to different

stresses following exposure to acidic conditions. Broadbent and collaborators (2010) studied the acid tolerance response in *L. casei* ATCC 334. The authors treated the cells with a broad range of pH values using HCl for acid adaptation and then, the cells were acid challenged at pH 2.0. They concluded that the acid adaptation improved acid tolerance of cells. Also Silva and coworkers (2005a) showed that the growth of *L. bulgaricus* ESB285 under non-controlled pH (final pH of 4.5) resulted in cells more resistant to heat stress and spray drying than cells grown under controlled pH (adjusted to 6.5).

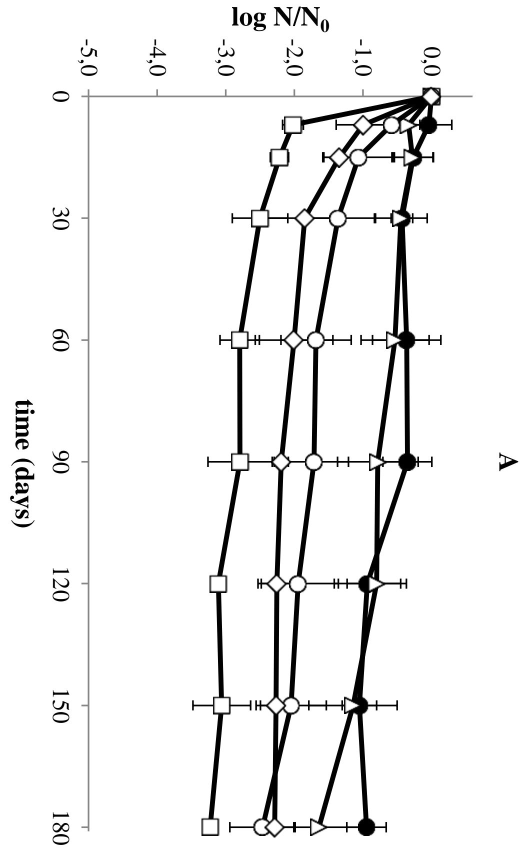
Oxygen alone does not cause damage to the bacterial cell, but its partial reduction to water and the formation of reactive oxygen species such as H_2O_2, superoxide radical anion O_2^- or hydroxyl radical HO^-, lead to oxidative stress. When it occurs at high levels, oxidative stress can lethally damage all cellular components (Miyoshi *et al*. 2003; Silva *et al*., 2005b). Exposure of *Lactobacillus* to mild concentrations of H_2O_2 has resulted in an increase in the resistance of these bacteria to a subsequent heat treatment. Desmond and collaborators (2002b) found that exposure of *L. paracasei* NFBC 338 to 3 mM H_2O_2 slightly increased its viability following heat treatment, but exposure to H_2O_2 was less effective when compared to exposure to a sub-lethal thermal stress.

Cells of *L. plantarum* 299v exposed to sub-lethal temperatures prior to spray drying were the most resistant over the 180 days of storage. Anekella and Orsat (2013) studied two species of *Lactobacillus* and found that a pretreatment with a sub-lethal temperature (50 ºC for *L. acidophilus* NRRL B-4495 and 52.5 ºC for *L. rhamnosus* NRRL B-442) allowed them to survive during spray drying in raspberry juice using outlet temperatures which normally would be lethal for LAB. In the study of Paéz and collaborators (2012) a previous heat treatment (at 52 °C) applied to two *Lactobacillus* spp. (*L. casei* Nad and *L. plantarum* 8329), enhanced their survival after spray drying in skim milk and for *L.*

plantarum its survival was improved during storage. Similar results were obtained even using different microorganisms and techniques. Nguyen and collaborators (2014) stated that the exposure of *Bifidobacterium bifidum* THT 0101 to a sub-lethal temperature of 42 ºC increased significantly the cells' resistance to freeze-drying. This protection conferred by thermal stress is probably due to the synthesis of specific stress proteins, known as heat shock proteins (Silva *et al*., 2005b). Some of these proteins are also induced by other stresses, such as acidic conditions (Lim *et al*., 2000), which indicates that this protein synthesis is a more general stress response instead of just a thermal stress response. Some authors stated that protein synthesis only occurred for cells in the exponential phase (Teixeira *et al*., 1994), but other studies have shown that the stress response was also induced in stationary phase cells (Guzzo *et al*. 1997).

In Figure 5.2, it is notable that exposure to any of the sub-lethal treatments prior to spray drying, did not result in the enhancement of survival of *P. acidilactici* HA-6111-2 during storage. On the contrary, survival during storage was negatively influenced by previous exposure to all the sub-lethal conditions investigated.

To our knowledge, there are no publications reporting the effects of sub-lethal treatments in the survival of *P. acidilactici* during spray drying and subsequent storage.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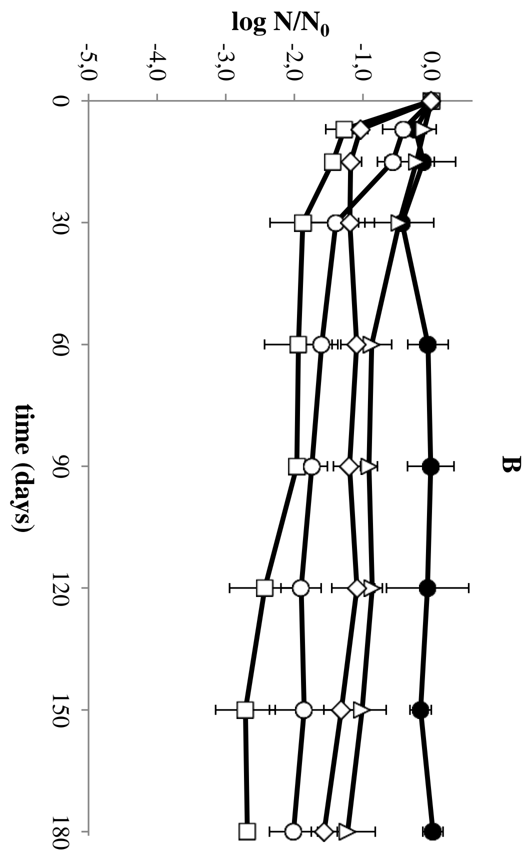
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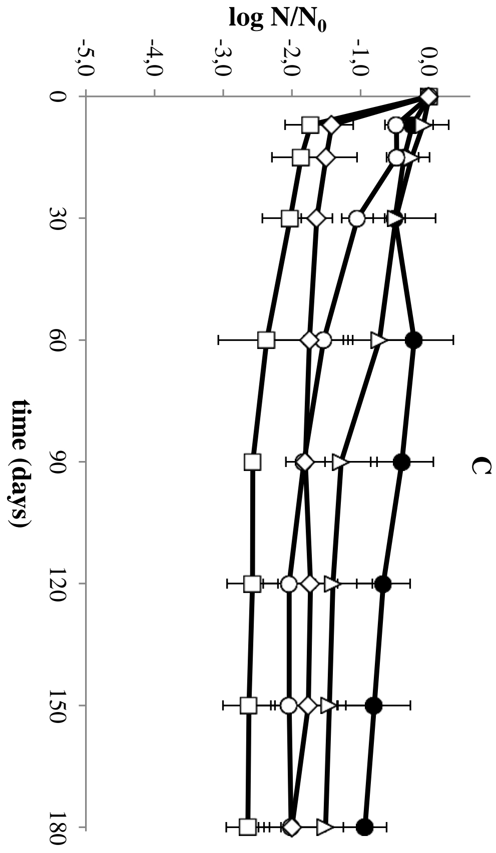
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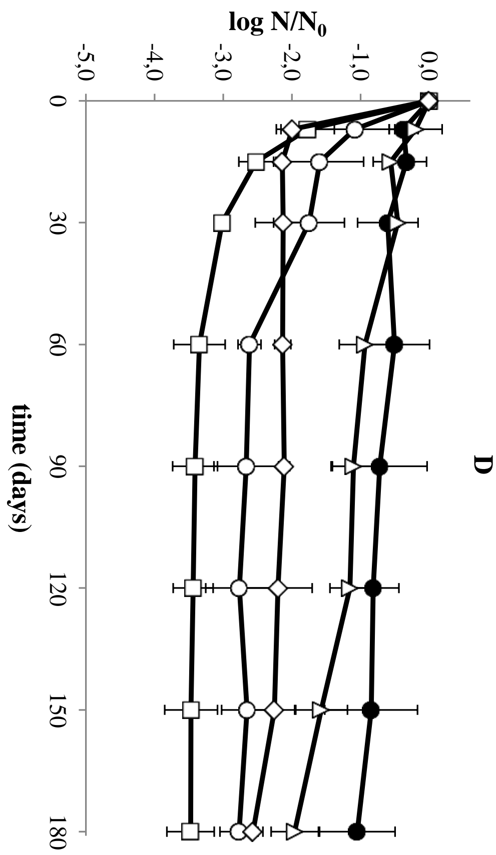
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time (days)

Figure 5.2. Logarithmic reductions of *P. acidilactici* HA-6111-2 (●) without sub-lethal treatment; (□) acidic stress (HCl); (△) acidic stress (Lactic acid); (○) temperature stress and (◇) oxidative stress and incorporated in orange juice with 2% of 10DE maltodextrin after spray drying and during 180 days of storage in the presence of light with a_w of 0.03 at room temperature (**A**) and at 4 ºC (**B**), in the absence of light with a_w of 0.03 at room temperature (**C**) and in the presence of light with a_w of 0.11 at room temperature (**D**).

It is very important to minimize cell death of putative probiotics, not only during the spray drying process and subsequent storage, but also to ensure minimal loss of viability during exposure to the conditions of the simulated GIT, since the viability of probiotic cultures should be maintained during the GIT passage of the consumer to guarantee their beneficial effects in the lower intestine (colon). Therefore, survival of *L. plantarum* 299v (Figure 5.3) and *P. acidilactici* HA-6111-2 (Figure 5.4) cells, exposed or not to sub-lethal stresses, was evaluated after spray drying in orange juice and after 180 days of storage at 4 °C (longest expected survival) during both quick and slow digestions in the simulated GIT conditions.

For both LAB and for both types of digestion, when gastric transit was simulated (pH 3.0 with pepsin) no significant differences (p>0.05) were observed between conditions (exposure to sub-lethal stresses). During both digestion simulations cell inactivation was lower than 1 log cycle.

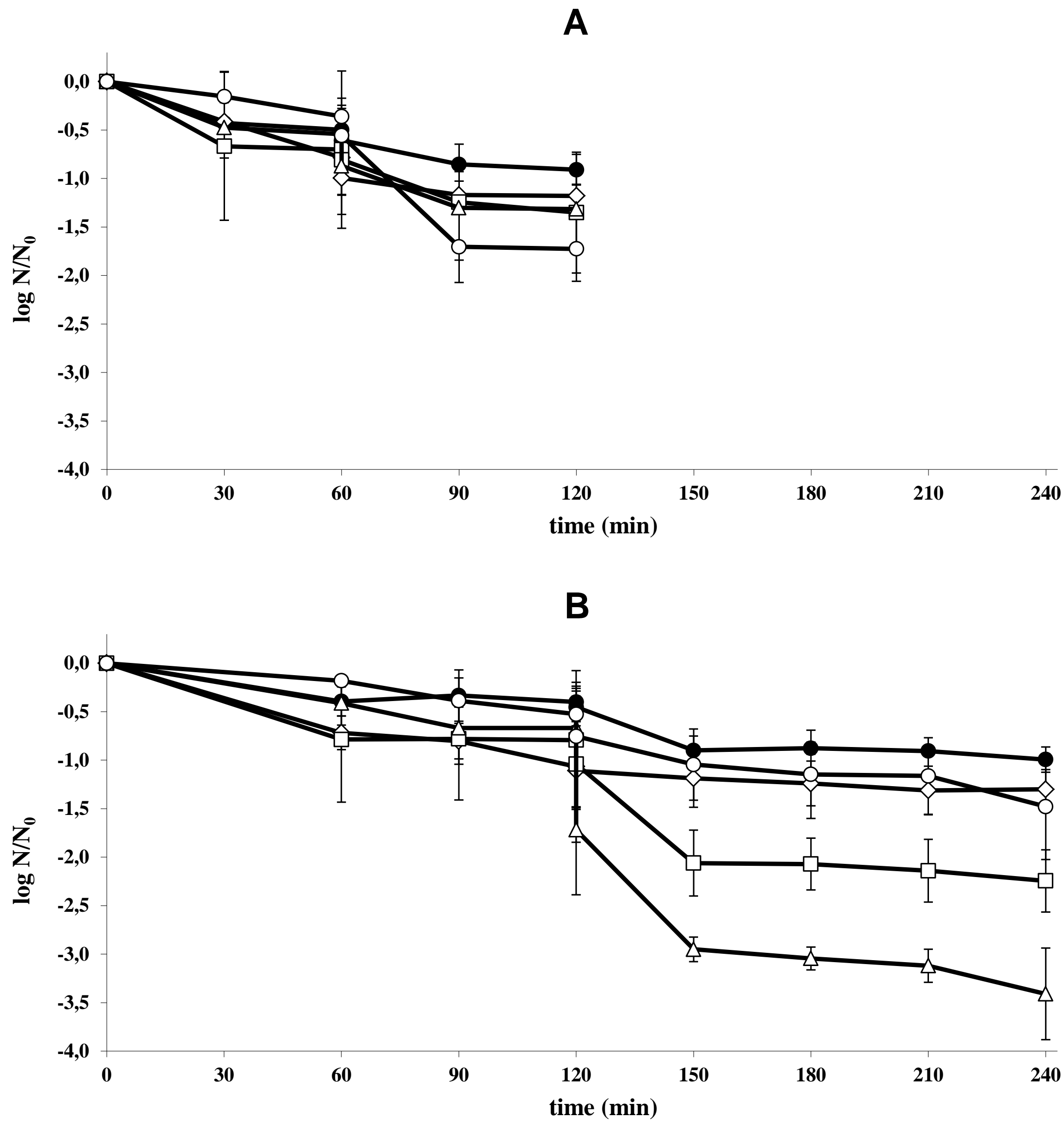

Figure 5.3. Survival of *L. plantarum* 299v cells spray-dried in orange juice and after 180 days of storage through quick (**A**) and slow (**B**) simulated digestions after exposure to (□) acidic stress (HCl); (△) acidic stress (Lactic acid); (○) thermal stress; (◇) oxidative stress; and control without prior sub-lethal treatment (●).

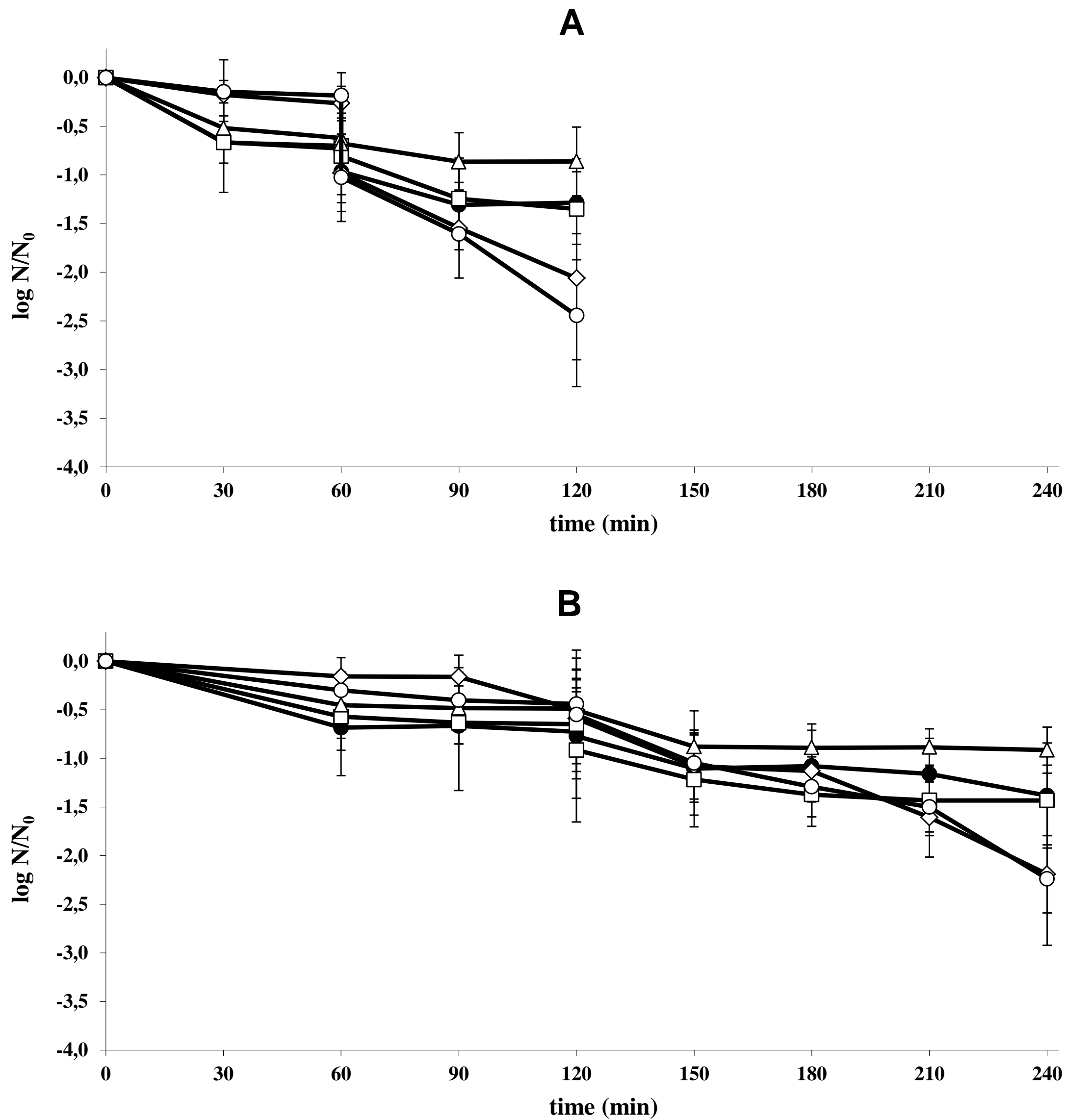

Figure 5.4. Survival of *P. acidilactici* HA-6111-2 spray-dried in orange juice and after 180 days of storage, through quick (**A**) and slow (**B**) simulated digestions after exposure to (□) acidic stress (HCl); (△) acidic stress (Lactic acid); (○) thermal stress; (◇) oxidative stress and control without sub-lethal treatment (●).

When *L. plantarum* 299v was exposed to the simulated small intestine conditions (with bile salts), higher inactivation was observed than when cells were exposed to pH 3.0 with pepsin. Significant differences were obtained between digestions (p<0.05). For quick digestion (Figure 5.3A) no significant differences (p=0.98512) were obtained between conditions (exposure to sub-lethal stresses). However, significant differences (p<0.05) were observed for long digestion (Figure 5.3B), with lower survival of cells previously exposed to sub-lethal stresses.

In the case of *P. acidilactici* HA-6111-2, after the addition of bile salts, no significant differences (p>0.05) were obtained between conditions (exposure to sub-lethal stresses) and types of digestion (quick or long).

According to Begley and collaborators (2005), bile affects the phospholipids and proteins in bacterial cell membranes, altering cellular homeostasis. For all the conditions, digestions and LAB, the cell reduction was greater when the cells were exposed to bile salts than to pH 3.0 with pepsin. Other researchers (Abbasiliasi *et al.*, 2012; Zheng *et al.*, 2013) have already reported similar results. However, to perform their beneficial properties, probiotics have to resist the low pH in the stomach and then exposure to alkaline pH together with bile to reach the lower intestine whilst maintaining their viability. Prior exposure to the sub-lethal stresses investigated did not enhance viability of both LAB after passage through simulated GIT. Nevertheless, since low inactivation occurred, during storage at 4 ºC, after passage through simulated GIT viable cell numbers of *L. plantarum* 299v and *P. acidilactici* HA-6111-2 cells were approximately of 10^7 cfu/mL and 10^8 cfu/mL, respectively.

5.4. Conclusion

Survival of *L. plantarum* 299v and *P. acidilactici* HA-6111-2 during storage, after exposure to sub-lethal stresses and spray drying in orange juice with maltodextrin, was influenced in a positive and negative manner, respectively. Their viability during passage through the simulated GIT was not improved by sub-lethal stresses exposure.

Selection of storage conditions of the dried powders was crucial to ensure a high survival of LAB. These conditions were found to be strain dependent. Considering that to have a beneficial effect on health, a probiotic must be present in minimum quantities of 10^7 cfu/mL in the product and remain viable during passage through the GIT, both *L. plantarum* 299v and *P. adicilactici* HA-6111-2 were demonstrated to be good candidates for incorporation and to be spray dried in an orange juice with maltodextrin, in order to produce a new functional orange juice powder.

CHAPTER 6

Comparison of spray drying, freeze drying and convective hot air drying for the production on an orange juice powder

Abstract

Survival of *L. plantarum* 299v and *P. acidilactici* HA-6111-2 in orange powders obtained by three drying techniques (spray drying, freeze drying and convective hot air drying) was investigated during drying and subsequent storage. Color and vitamin C content of the powders were also evaluated. There was no decrease in the number of cells during spray- and freeze drying, but a reduction of about 2 log cycles was obtained in convective hot air drying, for both LAB. During storage at 4 ºC no significant differences ($p>0.05$) in the survival of *L. plantarum* were observed for the orange powder obtained by the different techniques. However, during storage at room temperature, survival of *L. plantarum* was similar or higher in orange powders prepared by convective drying. Survival during storage at water activity values of 0.11 or 0.03 was significantly different only for freeze dried powders. For *P. acidilactici*, reductions during storage were minimal for most of the conditions investigated. Taking into account the initial number of cells obtained after each drying process, the techniques which allowed survival of an increased number of cells after 180 days of storage, were spray- and freeze drying. Another disadvantage besides the low number of viable cells surviving during convective drying was the fact that the powders did not dissolve in water. The most yellowish color was obtained by freeze drying, when no maltodextrin was added, and the higher retention of total vitamin C was achieved in spray- and freeze dried powders. By the high production costs and long drying times of freeze drying unlike the spray drying, it can be concluded that spray drying could be a good method to produce a new functional non-dairy product, such as a probiotic orange juice powder.

6.1. Introduction

Orange (*Citrus sinensis* L. Osbeck) is the fresh fruit, following apple, with higher production in Portugal: 208,000 metric tons in the marketing year 2013/2014 (OMAIAA, 2011; USDA/FAS, 2014). Orange is a fruit with a high water content, protein, sugars, fiber, minerals and vitamins such as vitamin C (57 mg per 100 ml) and carotene (120 mg per 100 ml), this last responsible for the typical color (INSA, 2014). It can be consumed in several ways and plays an important role in national and international gastronomy and nutrition. Orange juice is also highly appreciated, and if probiotics are incorporated, the nutritional content of juice can improve survival of the added organisms during storage (Ding and Shah, 2008). This is an advantage since a probiotic food should contain viable probiotic microorganisms in amounts of about 10^6-10^7 cfu/g or mL until the expiry date of products, and additionally should survive during the passage of the gastrointestinal tract of the consumer (FAO/WHO, 2002; Sanz, 2007). Orange juice with probiotics would be an ideal product for consumers who, besides orange juice, also like products which have health benefits and which are not milk-based, such as yoghurts and cheeses (Abadía-García *et al.*, 2013; Senaka Ranadheera *et al.*, 2012). Nonetheless, several disadvantages are found in the production and sale of an orange juice with probiotics, such as their short shelf-life, the possible need for refrigeration and high volume and weight of packaging. Conversion of the liquid orange juice into a powdered orange juice with probiotic characteristics will potentially enhance the stability of the product, resulting in a novel and healthy product. For the production of a probiotic orange juice powder, various drying methods could be used, such as spray-, freeze- and convective hot air drying, each having both advantages and disadvantages.

Spray drying allows the transformation of a solution into a dried powder in a single operation. The feed solution is sprayed into a chamber where hot dry air rapidly

evaporates the small droplets leaving the spray dried particles (Silva *et al*., 2011). Beyond being a rapid drying process, this technique is also inexpensive and its operation is simple and continuous (Duffie and Marshall, 1953).

Nevertheless, the high temperatures in this process may lead to the decrease or loss of vitamin C and carotene, as well as the flavor and aroma of orange (Dziezak, 1988). Also the presence of low molecular weight sugars such as fructose, glucose and sucrose and organic acids in the composition of orange juice affect its drying by this method, since they cause problems of stickiness, resulting in low process yield and operating difficulties (Bhandari *et al*., 1997). Drying agents, such as maltodextrins have been extensively used to reduce the stickiness of sugar-rich fruit juices (Tonon *et al*., 2010). Besides being effective during drying, maltodextrins minimize crystallization during storage, are inexpensive, have a mild flavor, and can be used as encapsulating agents; it has been also reported that maltodextrins are effective in preserving carotenoids (Desobry, 1997). Evidence of their prebiotic properties, conferring beneficial characteristics to the final product has been provided (Anekella and Orsat, 2013; Slavin, 2013).

Freeze drying is a process in which the water is removed from a frozen solution by sublimation under reduced pressure (Castro *et al*., 1997) giving rise to high quality dried products (Ratti, 2001). During the process no significant losses of vitamin C occur (Lin *et al*., 1998) and there is high retention of nutrients and flavorings; freeze dried products can be easily rehydrated before use (Tsami *et al*., 1999). However, freeze drying is expensive (about six times more expensive per kg of water removed in comparison with spray drying) and is time-consuming (Castro *et al*., 1997; Knorr, 1998).

The convective drying technique allows exposure of a solid to a continuous flow of hot air evaporating the moisture (Ratti, 2001). Whilst this fast hot air drying process can cost from 4 to 8 times less than freeze drying (Flink, 1977), and the obtained dried products

have a long shelf-life, their quality can be much lower than the original, with a drastic reduction in the volume, with deformation and color change. In contrast to spray- and freeze drying, rehydration of dried products by hot air drying is low (Ratti, 2001). Studies with different fruits demonstrated an increase in retention of ascorbic acid and color or an increase in retention of β-carotene and better rehydration when the fruit was exposed to an intermittent change in temperature or air flux, respectively (Lewicki, 2006).

The present study was carried out to investigate the effect of three different drying methods on the survival of two lactic acid bacteria (LAB) in orange powders during drying and storage, as well as on the color and vitamin C content of orange powder.

6.2. Materials and Methods

6.2.1. Lactic acid bacteria: origin, growth and storage conditions

Two LAB were selected due to their probiotic characteristics: *L. plantarum* 299v, a commercial probiotic (Probis Probiotika, Lund, Sweden) and *P. acidilactici* HA-6111-2, a potential probiotic previously characterized (Barbosa *et al.*, 2015) from *Escola Superior de Biotecnologia* culture collection. The isolates were grown on MRS agar (Lab M) at 37 ºC for 24 h and stored at -80 ºC in MRS broth (Lab M) containing 30% (v/v) of glycerol (Sigma), and sub-cultured twice before use in assays.

6.2.2. Spray drying of orange juice with incorporated LAB

6.2.2.1. Orange juice preparation

Ripe oranges grown in Portugal and randomly purchased from local commercial establishments (Porto, Portugal) were squeezed using a domestic juicer and the juice was filtered to prevent obstruction of the atomizer of the spray dryer. The total soluble solids content of the juice was adjusted to 1.5% (w/v) using a digital refractometer (model PR-

32α (alpha), Brix 0–32%, Atago U.S.A., Inc.). The drying agent 10DE maltodextrin (Sigma) was added to the juice at 2% (w/v) and the mixture was dissolved under magnetic stirring at 40 °C.

6.2.2.2. Preparation of LAB cultures

After each LAB had grown on MRS agar at 37 ºC for 24 h, one colony was transferred to MRS broth and incubated in the same conditions. Then these last cultures were transferred (1:100) to MRS broth and incubated at 37 ºC for 24 h in order to reach stationary phase. Cells were harvested by centrifugation (8877 x g, 10 min, 37 ºC; Rotina 35R), washed twice by centrifugation in sterile quarter strength Ringer's solution (Lab M) and re-suspended to the same volume of the final solution prepared as described in section 6.2.2.1.

As control, cultures grown in MRS were re-suspended in 3.5% (w/v) of RSM powder (Oxoid).

6.2.2.3. Spray drying

The drying of orange juice with each LAB culture and their control in RSM was carried out in a pilot-scale Spray Dryer (Niro Atomizer, Soborg, Denmark) with a vaned wheel rotating at high speed and a concurrent drying chamber (0.8 m diameter and 0.6 m height). The solutions, stirred continuously at 40 ºC, were fed into the chamber through a peristaltic pump at a constant flow rate (25 mL/min) and at inlet and outlet air temperatures adjusted to 150 ºC and 70 ºC, respectively. The dried powders were collected in a single cyclone air separator system. Three replicates were conducted for each experiment.

6.2.3. Freeze drying of orange juice incorporated with LAB

6.2.3.1. Orange juice and LAB cultures preparation

Orange juice was prepared with and without 10DE maltodextrin as described in section 6.2.2.1. The preparation of LAB cultures to be incorporated into the mixtures was done as described in section 6.2.2.2. As control, each LAB culture was also re-suspended in 3.5% (w/v) of RSM.

Each cellular suspension was maintained for 1 h at room temperature for equilibration and then distributed in plastic flasks before being freeze dried.

6.2.3.2. Freeze drying

Each sample of 50 mL was initially frozen at -80 ºC and then desiccated under vacuum (50 mTorr) for 7 days in a freeze-drier (Martin Christ, Osterad am Harz, Germany) at room temperature and the condenser was cooled at -55 ºC. Two replicates were conducted for each experiment.

6.2.4. Convective hot air drying of orange incorporated with LAB

6.2.4.1. Orange and LAB cultures preparation

Each orange was peeled, cut into very small and thin pieces, which were spread over 4 trays. With this technique it is possible to use the entire fruit, without the need to extract its juice. This possibility is an advantage in so far as this does not lose orange fiber that may be incorporated into the final powder.

The cells of each LAB were obtained as mentioned in section 6.2.2.2, but after washing twice in sterile quarter strength Ringer's solution, the cells were re-suspended in the same volume of sterile quarter strength Ringer's solution, or to use as a control, the same volume of 3.5% (w/v) RSM.

The orange pieces of each tray were placed in separate sterile containers and immersed (for 1 h to allow cell adherence) in suspensions prepared in Ringer's or RSM with each LAB (4 trays in total). Then the orange pieces with incorporated LAB were filtered with sterile gauze and were again distributed in their respective trays.

6.2.4.2. Convective hot air drying

Each tray was placed in a pilot-scale tray drier (Armfield, Ringwood, United Kingdom), which dehydrates wet solid products by a flow of hot air through the trays. During 48 h, the temperature and speed of air flow were maintained at 40 ºC and 1.5 m/s, respectively (Rêgo *et al*., 2013). The dried powders were obtained after grinding the dried pieces of orange. Each experiment was done twice.

6.2.5. Analysis of powders

Each of the dried powders was analyzed immediately after the end of each drying technique for various parameters described below. The color measurement and quantification of vitamin C parameters were done for orange juice dried by each drying method but without incorporated LAB and stored in the absence of daylight (as described below in section 6.2.6).

6.2.5.1. Drying yield for spray dried powders

After each spray drying the drying yield (%) was determined as the % of the powder weight collected from the collecting vessel attached to the bottom of the cyclone to the initial amount of solids contained in the liquid feed.

6.2.5.2. Water activity

The water activity of the powders was measured using a water activity meter (Aqualab, Series 3, Decagon Devices Inc.) at a constant temperature of 23 ± 1 ºC. Two readings were made for each sample.

6.2.5.3. Dissolution test

In a test tube, 50 mg of each sample were mixed with 1 mL of distilled water using a vortex at half speed. Dissolution was measured as the time (in seconds) taken to dissolve the powders completely (Quek *et al.*, 2007). The measurements were done in triplicate and at room temperature.

6.2.5.4. Color measurement

The measurements were performed in the CIE (Commission Internationale de L'Eclairage) Lab color scale, using a Konica Minolta CR-400 portable colorimeter (Konica Minolta, Tokyo, Japan) with the D65 illuminant. This system uses three values to describe the exact site of a color within a three-dimensional visible color space: L^*, a^* and b^*. The L^* value measures the lightness of the sample and ranges from 0 to 100, ranging from black to white, respectively; a^* varies between red ($+a^*$) to green ($-a^*$) and b^* varies between yellow ($+b^*$) to blue ($-b^*$) color space. The instrument was calibrated with a white ceramic plate ($L^* = 91.0$, $a^* = +0.3165$, $b^* = +0.3326$) before each sample analysis (Quek *et al.*, 2007). Three color measurements were performed at three different locations. For each powder sample, immediately after the end of each drying technique and at regular intervals during storage, was calculated the chroma [$C^*=(a^{*2}+b^{*2})^{1/2}$], which is the intensity or saturation of the color and the hue angle [$H^o=\tan^{-1}(b^*/a^*)^{1/2}$], which characterizes the perception of the color. Values of hue angle of 0°, 90°, 180° and

270° indicate pure color of red, yellow, green and blue, respectively (Quek *et al*., 2007; Santhalakshmy *et al*., 2015).

6.2.5.5. Vitamin C content

The total vitamin C content (ascorbic acid – AA - plus dehydroascorbic acid - DHAA) was based on HPLC UV detection, using isoascorbic acid (IAA) as internal standard, as described by Zapata and Dufour (1992).

Each dried sample was rehydrated to their initial solids concentration in water, immediately after the end of each drying technique and at regular intervals during storage. To a volume of 5 mL of each rehydrated sample was added 1 mL of IAA (0.03 g/50 mL; Fluka), the pH adjusted to 2.45 with HCl (1 M, Pronalab) and, finally, added methanol-ultra pure water (5:95 v/v) to reach a volume of 20 mL in a volumetric flask. The solution was centrifuged (8877 x g, 5 min, 4 ºC; Rotina 35R), 3 mL of supernatant was transferred to a new test tube and 1 mL of 1,2-phenylenediamine dihydrochloride (OPDA; 0.03 g/50 mL; Fluka) was added. The mixture was maintained in the dark at room temperature and after 37 min, 20 μL were injected into the HPLC system, which consisted of a controller (Hercule Lite – Chromatography interface, Tokyo, Japan), a solvent pump (Jasco PU – 1580, Tokyo, Japan), an injector with a 20 μL sample loop (Jasco AS – 1555, Tokyo, Japan), a reversed phase column (Macherey–Nagel, Chromcart 100–10 Nucleosil, 250 x 4.6 mm) and a UV detector (Jasco UV – 1575, Tokyo, Japan). Detector wavelength was first scheduled to run at 348 nm, to detect DHAA, and then at 261 nm, to detect AA and IAA. Standard solutions were prepared in methanol-ultra pure water (5:95 v/v) and the mobile phase with 13.61 g of potassium dihydrogen phosphate (Fluka), 3.64 g of cetrimide (Fluka) and 2 L of methanol-ultrapure water (5:95 v/v), filtered under vacuum by a 0.45 μm membrane and degassed for 15 min in an ultrasonic bath (Alexandre *et al*.,

2012; Zapata and Dufour, 1992). As a control, the total vitamin C content was measured for 5 mL of orange juice with total soluble solids adjusted to 1.5% (w/v), as described in section 6.2.2.1. Three replicate analyses were performed.

6.2.6. Storage conditions

During 180 days of storage, each dried sample was stored in plastic containers, hermetically sealed in glass flasks, in normal atmosphere (air), at two different temperatures (4 ºC and room temperature), in the presence or absence of daylight and under controlled water activity (a_w) values of 0.03 and 0.11, by equilibrium with dried silica gel (Pronalab) and saturated aqueous solution of LiCl (Sigma), respectively (Teixeira *et al*., 1995b).

6.2.7. Enumeration of LAB cultures

Before and immediately after each drying technique and at regular intervals throughout storage, each LAB in orange or RSM powders was enumerated. For convective hot air drying, enumeration of LAB was also done in several stages of the process, namely after immersion, drying and grinding.

After rehydration of each dried sample to their initial solids concentration in sterile quarter strength Ringer's solution, homogenized for 1 minute and kept at room temperature for 30 minutes, suitable dilutions were plated in duplicate by the drop count technique (Miles and Misra, 1938) on MRS agar. After incubation at 37 ºC for 48 h, colonies were counted and the cfu/mL calculated. Microbial counts were transformed to logarithmic reduction using the equation: log (N/N_0), where N is the microbial cell count at a particular sampling time and N_0 is the microbial cell count immediately after drying.

6.2.8. Statistical analysis

An analysis of variance (one-way ANOVA) was performed to evaluate any significant differences between the three drying techniques used and the different storage conditions on the survival of *L. plantarum* 299v and *P. acidilactici* HA-6111-2. Significant differences between the color of powders and the concentration of vitamin C after each drying method and during storage were also investigated. Multiple comparisons were evaluated by Tukey's post-hoc test. All calculations were carried out using the software KaleidaGraph (version 4.4, Synergy Software, Reading, USA).

6.3. Results and Discussion

Orange powders incorporated with each microorganism and obtained with the three drying techniques were analyzed and the results are shown in Table 6.1.

Table 6.1. Results of the analyses of the obtained orange powders incorporated with both LAB after each drying method

	Spray drying^{MD}	Freeze drying^{MD}	Freeze drying	Convective drying
L. plantarum				
Water activity	0.42 ± 0.08	0.38 ±0.01	0.35 ± 0.01	0.39 ± 0.01
Dissolution (s)	19.5 ± 0.6	3.5 ± 0.2	3.4 ± 0.2	—
P. acidilactici				
Water activity	0.36 ± 0.04	0.38 ± 0.00	0.34 ± 0.02	0.37 ± 0.02
Dissolution (s)	19.9 ± 0.3	3.2 ± 0.3	3.9 ± 0.0	—

^{MD}Orange juice powder with 10 DE Maltodextrin

— not dissolvable

All the results are represented by the mean ± the standard error of the mean

There were no significant differences (p>0.05) between *L. plantarum* 299v and *P. acidilactici* HA-6111-2 for each of the tested parameters. Values of a_w of all the orange powders incorporated with each LAB was about 0.40 (p>0.05). Despite the low a_w values obtained, they are not as low as those obtained in industrial spray dryers, with values around 0.2 (Adhikari *et al*., 2009). However, storage under controlled a_w is a form, already described (Teixeira *et al*., 1995b), to improve the survival of microorganisms. The dissolution test is expressed as the time required for the powder obtained to be completely reconstituted in water. Significant differences (p<0.0001) were obtained between each drying method. The spray dried powders were completely reconstituted in water in a very short time (about 19 s). Quek and collaborators (2007) had already described short dissolution times for spray dried watermelon powders, especially for those that had been obtained at lower inlet temperatures; these powders had a higher moisture content which translated into a greater tendency to agglomerate, increasing the reconstitution of powders in water. The freeze-dried powders, with or without 10 DE maltodextrin, dissolved almost instantaneously (about 3 s) but none of the convectively dried powders were completely dissolved in water. The porous structure of freeze dried powders and the low porosity achieved with the convective drying technique appears to be related to the rehydration capacity (Ratti, 2001).

For spray dried samples, the average of the process yields obtained for each of LAB was higher than 50%. Similar values after spray drying of fruit juices have been described by other authors (Fang and Bhandari, 2012).

The enumeration of LAB was performed before and after each drying method (Table 6.2).

Table 6.2. Number of viable cells of both LAB in orange juice (or orange for convective drying) before each drying method and in the respective rehydrated powder after each processing

		log CFU/g or ml [a]	
Drying method	**Process**	***L. plantarum* 299v**	***P. acidilactici* HA-6111-2**
Spray drying[MD]	Before	9.4 ± 0.0	9.0 ± 0.1
	After	9.5 ± 0.3	9.4 ± 0.2
Freeze drying[MD]	Before	10.1 ± 0.0	10.3 ± 0.0
	After	10.4 ± 0.5	11.6 ± 0.2
Freeze drying	Before	10.3 ± 0.2	10.5 ± 0.2
	After	11.3 ± 0.1	11.2 ± 0.3
Convective drying	Immersion	8.1 ± 0.5	8.6 ± 0.1
	During (5h)	8.4 ± 0.2	8.5 ± 0.3
	During (24h)	7.4 ± 0.3	6.6 ± 0.3
	During (48h)	6.4 ± 0.0	6.3 ± 0.1
	Grinding	6.5 ± 0.0	6.2 ± 0.1

[MD]Orange juice powder with 10 DE Maltodextrin

[a]Survival is represented as the mean of the log cfu/mL ± the standard error of the mean

No significant differences ($p>0.05$) were obtained between *L. plantarum* 299v and *P. acidilactici* HA-6111-2 for each method. For spray- and freeze drying techniques, there was no decrease in the number of viable cells ($p>0.05$). The same was not observed for convective drying, since after 48 hours of drying, the number of cells of *L. plantarum* 299v and *P. acidilactici* HA-6111-2 had reduced almost 2 log cycles. Other authors have already reported good survival rates of probiotics after spray drying in fruit juices (Anekella and Orsat, 2013), but to our knowledge, no studies have been performed on freeze drying probiotics directly in fruit juices. Some authors just studied the influence of different protectants on the survival of freeze dried probiotics in other matrices (Zayed

and Roos, 2004); others have studied the survival of freeze dried probiotics added to freeze dried fruit powders (Nualkaekul *et al.*, 2012). Studies of dried probiotics by convective drying are also scarce. Betoret and collaborators (2003) impregnated apple cylinders samples with probiotics and observed a decrease in microbial content of 3 log cycles after being air dried at 40 ºC. After incorporating probiotics into apple cubes, Rêgo *et al*. (2013) also observed a decrease of about 2 log cycles in probiotic cell numbers after convective drying.

Survival during 180 days under different storage conditions (numbers 1 to 4 in each graph) after each drying technique (different letters in each graph) for *L. plantarum* 299v and *P. acidilactici* HA-6111-2 are shown in Figures 6.1 and 6.2, respectively.

Comparing both LAB for each drying method, protection by RSM was most pronounced during storage of spray dried powders. For any of the storage conditions, survival was always higher when compared to the spray dried cells in orange juice (p<0.0001). For freeze drying, the protective effect of RSM was absent for cells of *L. plantarum* (Figure 6.1) at room temperature with (graph FD1) and without light (graph FD2), and for *P. acidilactici* cells (Figure 6.2), under all conditions of storage at room temperature (graphs FD1, FD2 and FD3). There was also no protection for the dried *L. plantarum* cells by convective drying and stored at room temperature without light (Figure 1, graph CD2; p>0.05) and in the case of *P. acidilactici*, there was no protection by any of the storage conditions, as there were no significant differences (p>0.05) in survival of cells dried with RSM or orange.

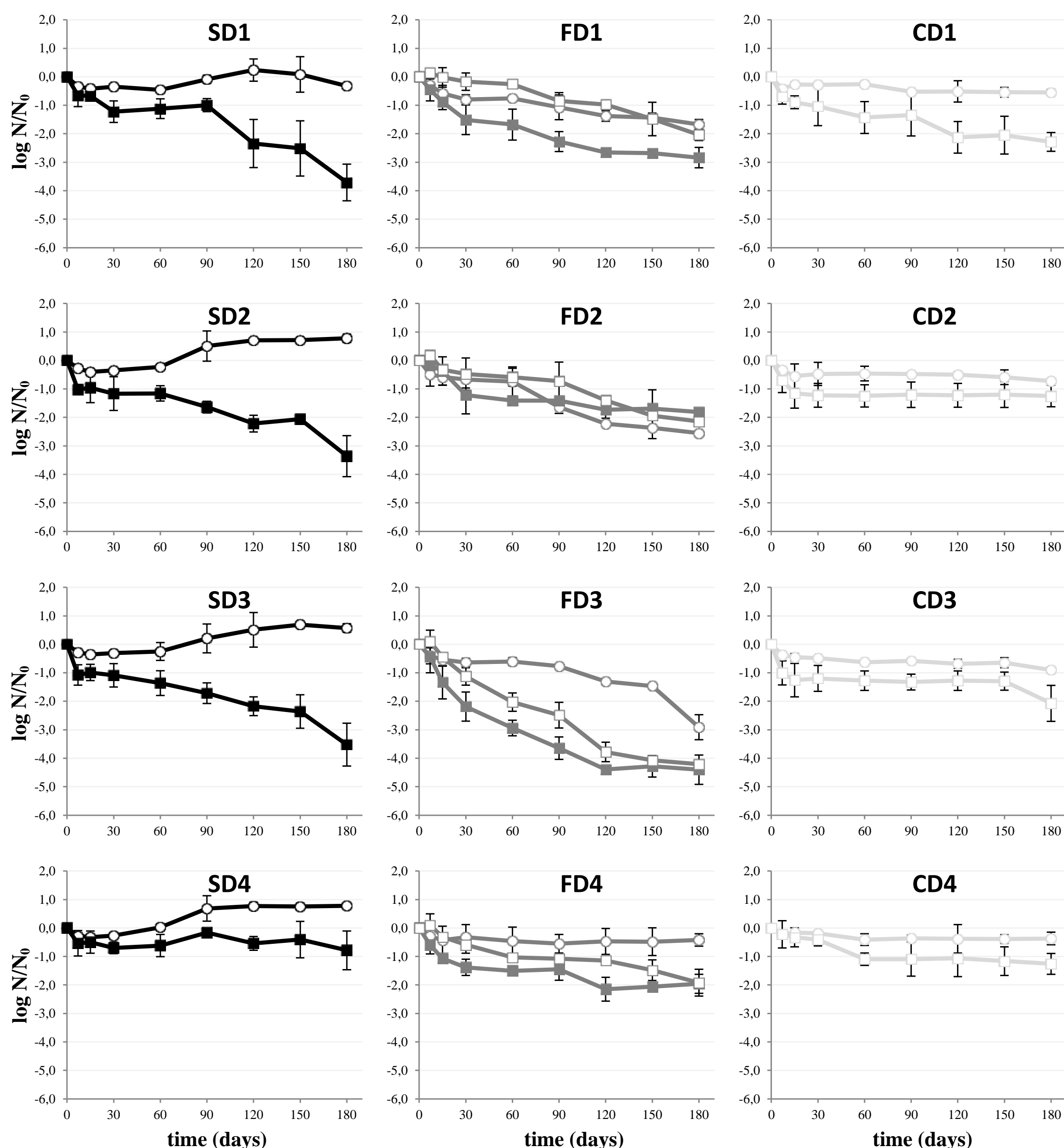

Figure 6.1. Survival of *L. plantarum* 299v incorporated in RSM (open circle), in orange powder with 10 DE maltodextrin (closed square) or in orange powder without 10 DE maltodextrin (open square) after spray drying (**SD**), freeze drying (**FD**) or convective drying (**CD**) and during 180 days of storage in different conditions: room temperature in the presence of light with a_w of 0.03 (**1**); room temperature in the absence of light with a_w of 0.03 (**2**); room temperature in the presence of light with a_w of 0.11 (**3**) and at 4 ºC in the presence of light with a_w of 0.03 (**4**).

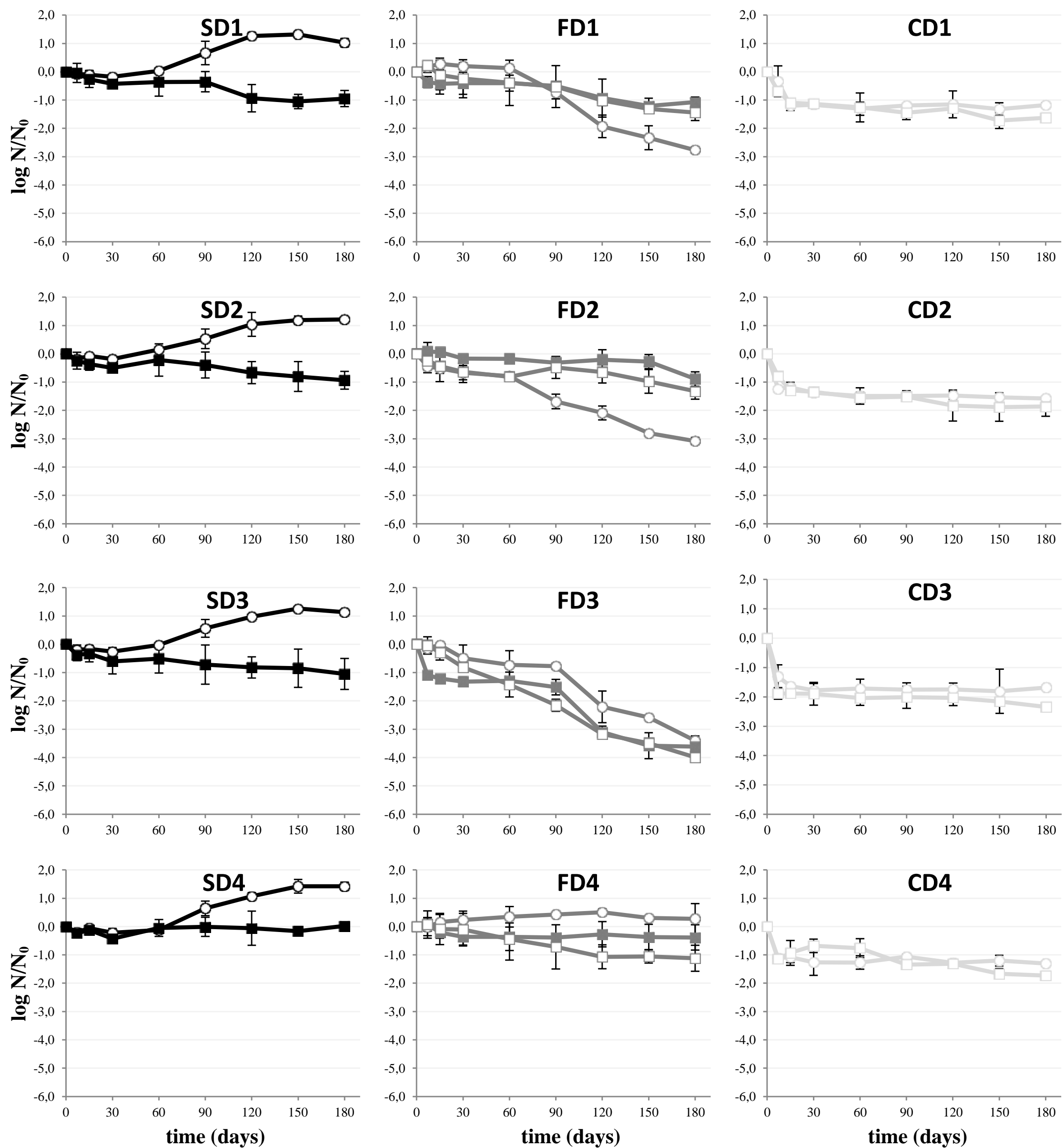

Figure 6.2. Survival of *P. acidilactici* HA-6111-2 incorporated in RSM (open circle), in orange powder with 10 DE maltodextrin (closed square) or in orange powder without 10 DE maltodextrin (open square) after spray drying (**SD**), freeze drying (**FD**) or convective drying (**CD**) and during 180 days of storage at different conditions: room temperature in the presence of light with a_w of 0.03 (**1**); room temperature in the absence of light with a_w of 0.03 (**2**); room temperature in the presence of light with a_w of 0.11 (**3**) and at 4 ºC in the presence of light with a_w of 0.03 (**4**).

RSM was chosen as a control since many authors have already demonstrated its ability to protect the cells of different LAB during spray drying and subsequent storage (Ananta *et al*., 2005; Golowczyc *et al*., 2011b; Teixeira *et al*., 1995b). In a study conducted by Jagannath *et al*. (2010), the authors observed a loss of viability of about 2 log cycles in cells of different *Lactobacillus* spp. dried in RSM by freeze drying and further losses of about 2 log cycles after 60 days of storage at 4 °C. After drying of apple cylinders impregnated with *L. casei* spp. *rhamnosus* CECT 245 by convective drying, Betoret and collaborators (2003) observed the same survival in samples impregnated with probiotic inoculated either in apple juice or in milk.

For *L. plantarum* 299v cells incorporated into orange powder (Figure 6.1) and stored at room temperature with controlled a_w values of 0.03 in the presence (graphs SD1, FD1 and CD1) or absence of light (graphs SD2, FD2 and CD2), survival was lower for cells dried by spray drying (-3.7 and -3.4 log unit reductions, respectively). In the convective drying technique, survival was higher with the exception of storage at room temperature with light, wherein there were no significant differences (p=0.42764) among the cells dried by this technique (graph CD1; -1.3 log units reduction) and the cells dried in orange juice without 10DE maltodextrin by freeze drying (graph FD1; -1.8 log unit reduction). Despite these differences in survival obtained from various drying techniques, it is important to note that the techniques, for which survival was higher during storage, were not always the best during the drying process itself. This is the case for convective drying, in which large reductions during the process provided low counts of *L. plantarum* 299v after 180 days of storage (1.9×10^5 and 1.7×10^4 cfu/g after storage at room temperature with and without light, respectively). In contrast, the large number of cells remaining viable after

spray drying provided that, even after high losses during storage, the number of cells was maintained in the order of 10^6 cfu/mL.

Comparing the same storage conditions for *P. acidilactici* HA-6111-2 (Figure 6.2), there were no significant differences (p>0.05) in survival of cells dried by each of the three techniques and stored at room temperature in the presence of light (graphs SD1, FD1 and CD1), with only about 1 log cycle reduction. Also during storage at room temperature in the dark (graphs SD2, FD2 and CD2), reductions in viable cell counts were minimal for spray drying and freeze drying (p=0.7438). Again, despite the high survival obtained after 180 days of storage, the number of cells was around 10^4 cfu/g for convective drying, while for spray- and freeze drying was higher than 10^8 cfu/mL. Although no significant differences (p>0.05) were obtained for the presence/absence of light in the storage of *P. acidilactici* HA-6111-2 cells, the same was not observed for *L. plantarum* 299v, where reductions were lower in the absence of light, reaching 1 log cycle of difference for convective- and freeze drying (cells dried in orange juice with 10DE maltodextrin). Exposure to light during storage is known to be detrimental to the survival of dried probiotic cultures (Tripathi and Giri, 2014).

Significant differences (p<0.05) between cells stored at room temperature with controlled a_w values of 0.11 and the storage conditions previously discussed were only observed for freeze dried cells. For both LAB, freeze dried in orange juice with or without 10DE maltodextrin, log reductions were about 4 log units (graphs FD3). The number of surviving freeze dried cells (>10^5 cfu/mL for *L. plantarum* 299v and >10^7 cfu/mL for *P. acidilactici* HA-6111-2) was higher than the number of surviving cells dried by the convective technique (approximately 10^4 cfu/g for both LAB) obtained after 180 days at this storage condition. The survival of different dried LAB cultures stored under different

relative humidities has been previously investigated. After spray drying, Golowczyc *et al.* (2011a) found that survival of *Lactobacillus kefir* was higher at both values of a_w (0 and 0.11) during storage. Nevertheless, in contrast to that obtained in this study for freeze drying, Castro *et al.* (1995b) considered the controlled a_w value of 0.11 as being the best storage condition to maintain the survival of freeze dried *Lactobacillus bulgaricus*.

For both LAB at a temperature of 4 °C, log reductions were low, but significant differences (p>0.05) were only obtained for the spray drying technique as compared to all other storage conditions. For *L. plantarum* 299v cells, it was for spray- and convective drying (Fig. 1, graphs SD4 and CD4) that the survival was greater (-0.8 and -1.3 log cycle, respectively) in this storage condition, which means counts of $1.4x10^9$ cfu/mL for spray dried cells and of $1.8x10^5$ cfu/g for convective dried cells. Cells of *P. acidilactici* HA-6111-2 (Figure 2, graphs SD4 and FD4) survived better after spray drying (with no reduction, which means cell counts of $2.5x10^9$ cfu/mL) and freeze drying in orange juice with 10 DE maltodextrin (-0.4 log cycle, with cell counts of $1.6x10^{11}$ cfu/mL). The greater survival of different microorganisms at low storage temperatures has been demonstrated following spray drying (Teixeira *et al.*, 1995b), freeze drying (Castro *et al.*, 1995), and convective hot air drying (Rêgo *et al.*, 2013).

Maltodextrin is essential for the spray drying process, for orange juice as it would not be possible to recover any powder, but the same is not true for freeze drying. Omitting this drying aid allowed the formation of a powder, although the resulting powder was more compact and difficult to handle. After freeze drying of orange juice, Kramer and collaborators (1988) found a hygroscopic powder that was prone to agglomerate. As for a possible increase in survival due to the presence of maltodextrin, there were no significant differences (p>0.05) among the cells dried in orange juice with or without 10DE

maltodextrin for both *P. acidilactici* HA-6111-2 as *L. plantarum* 299v (except for storage at room temperature with light, where the reductions were lower for dried cells without maltodextrin, Figure 6.1 graph FD1; $p<0.05$). Champagne *et al*. (1996) tested the influence of different polymers on the stability of various freeze dried LAB. To a base protective medium, the authors added 10% of 10 DE maltodextrin and, after 12 months under different storage conditions, despite having found a large variability between strains, they also found no significant differences ($p>0.05$) in survival of cells dried with or without maltodextrin.

For a product to be considered probiotic, the viability of a probiotic culture should not only be maintained in amounts of 10^6-10^7 cfu/g or mL during the storage time of the product, but also after ingestion, they should be able to survive the conditions of the gastro-intestinal tract of the consumer (FAO/WHO, 2002). Both LAB survived the simulated GIT with less than 1 log cycle reduction (Barbosa *et al*., 2015), but after being dried in orange juice and taking into account that the cells still have the GIT conditions to overcome, it is important that the chosen drying technique allows the survival of the largest possible number of cells.

The color parameters L^*, a^*, b^*, chroma and hue angle for the powders obtained by each drying technique and at 0, 90 and 180 days of storage are presented in Table 6.3.

Table 6.3. Colorimetric results of the orange dried powders for each drying method and during 180 days of storage

Method	Days after drying	Parameter				
		*L**	*a**	*b**	Chroma	Hue angle (º)
Spray drying [MD]	0	64.44 ± 3.78	-1.12 ± 0.11	6.98 ± 0.89	7.07 ± 0.87	-79.60 ± 1.52
	90	65.27 ± 6.67	-1.13 ± 0.10	6.84 ± 0.74	6.93 ± 0.74	-79.94 ± 1.81
	180	72.11 ± 2.83	-1.20 ± 0.12	5.42 ± 0.59	5.55 ± 0.57	-77.55 ± 1.83
Freeze drying [MD]	0	71.15 ± 0.83	-1.71 ± 0.05	7.16 ± 0.18	7.36 ± 0.17	-76.54 ± 0.36
	90	71.00 ± 0.88	-1.60 ± 0.04	6.22 ± 0.05	6.42 ± 0.04	-75.54 ± 0.47
	180	68.71 ± 1.99	-1.43 ± 0.06	3.90 ± 0.19	4.15 ± 0.18	-69.88 ± 0.94
Freeze drying	0	52.14 ± 0.26	0.65 ± 0.18	21.30 ± 1.43	21.31 ± 1.42	87.37 ± 2.08
	90	51.98 ± 0.02	0.53 ± 0.13	20.77 ± 2.16	20.78 ± 2.16	88.50 ± 0.54
	180	51.13 ± 0.06	0.08 ± 0.06	19.87 ± 0.43	19.87 ± 0.43	89.78 ± 0.18
Convective drying	0	35.52 ± 0.54	7.22 ± 0.49	28.35 ± 0.83	29.26 ± 0.68	75.69 ± 1.35
	90	34.29 ± 0.67	6.67 ± 0.86	26.51 ± 1.85	27.33 ± 2.00	75.91 ± 0.83
	180	28.74 ± 3.23	5.89 ± 1.78	23.12 ± 2.48	23.87 ± 2.84	75.92 ± 2.77

[MD]Orange juice powder with 10 DE Maltodextrin

All the results are represented by the mean ± the standard error of the mean

No significant differences (p>0.0001) were obtained between spray- and freeze drying with maltodextrin for each color parameter. However, significant differences (p<0.0001) were found between these and the remaining techniques. It was to the powders obtained by spray- and freeze drying with maltodextrin that *L** values were higher, indicating a lighter color. The addition of maltodextrin, which is white, has contributed to lighten the powders, and therefore, the values of *a** and *b** were reduced, which contributed to the decrease of chroma and hue angle values. Other authors have already described lighter colors obtained for powders containing maltodextrin (Caparino *et al.*, 2012). The darker powders, with significantly smaller *L** values, were obtained with the convective hot air

drying. Corrêa and colleagues (2011) analyzed the color of *marolo* (*Annona crassiflora*) dehydrated by freeze- and convective hot air drying and also found *L** values lower for convective dried powders. Krokida and collaborators (2001) justified the significant decrease of the *L** parameter and the simultaneous increase of the *a** and *b** parameters of the fruits dried by convective hot air drying as a result of extensive browning reactions such as caramelization of sugars during heating. Only for spray- and freeze drying with maltodextrin powders, the *a** values were negative indicating a tendency to a greenish color, unlike for freeze dried without maltodextrin and convective hot air dried powders wherein the values of *a** were positive indicating powders more red. Despite all the samples having positive *b** values, indicating a tendency to yellowish color, that color was more intense for freeze dried materials without maltodextrin and convective hot air drying, with higher values of *b**, well as higher values of chroma, which is precisely the intensity or color saturation. The hue angle values obtained corresponded to the regions of red (0º) to yellow color (90º), but it was in freeze drying without maltodextrin that these values were higher, with a more attractive yellow color. This is consistent with the study carried out by Krokida *et al*. (2001), who stated that freeze drying avoids changes of color in dried foods compared to other drying techniques. For each technique, no significant differences ($p>0.0001$) were obtained in color parameters during storage, except for the powders obtained by freeze drying with maltodextrin for the parameter *b** and thus to the chroma and hue angle parameters. In theory, the addition of probiotic cultures should not influence the color of the final orange powders. Antunes and collaborators (2013) added a spray dried culture of *Bifidobacterium animalis* subsp. *lactis* BB-12 to an acerola nectar and, after calculating the total difference in color (ΔE^*_{ab}), found no significant differences ($p<0.05$) between the nectar color with or without probiotic added.

Since humans are not able to synthesize vitamin C, it is important that the ingestion of foods supplies a daily dose of about 91 mg/day (INSA, 2014), in order to lower the incidence of some chronic diseases (Valente *et al.*, 2014). Oranges, and orange juice, are a great source of vitamin C, but after being processed, one of the quality parameters of a product is its ability to retain this vitamin, since this usually means that other nutrients are also retained (Uddin *et al.*, 2002). Additionally, as the intention is to produce a probiotic orange juice powder, according to Dave and Shah (1997), the survival of probiotic cultures can be enhanced by the presence of vitamin C. The results of total vitamin C content (DHAA plus AA) determined for the diluted orange juice and the powders obtained with each of the drying techniques are presented in Table 6.4.

Table 6.4. Vitamin C content (mg/100 mL) of diluted orange juice and orange powders obtained after each drying method and during storage

Sample	Days of storage	Concentration (mg/100 mL)			
		DHAA[a]	AA[a]	Total Vit. C[b]	Vit. C loss (%)
Orange juice[c]	—	3.6 ± 0.3	26.1 ± 2.3	29.7 ± 2.0	—
Spray dried[MD]	0	21.7 ± 7.2	6.5 ± 0.4	28.1 ± 7.6	5.4
	90	21.4 ± 4.2	6.4 ± 0.3	27.8 ± 4.0	6.5
	180	20.0 ± 4.7	4.0 ± 0.7	24.0 ± 4.0	19.4
Freeze dried[MD]	0	13.1 ± 0.9	7.9 ± 1.9	21.0 ± 1.4	29.3
	90	11.1 ± 3.6	2.5 ± 0.2	13.6 ± 3.8	54.3
	180	10.6 ± 0.2	2.2 ± 0.1	12.9 ± 0.3	56.7
Freeze dried	0	16.0 ± 0.7	6.2 ± 0.4	22.2 ± 0.3	25.3
	90	13.5 ± 1.5	4.7 ± 0.7	18.5 ± 0.7	37.7
	180	7.1 ± 0.4	2.8 ± 0.4	9.8 ± 0.2	67.0
Convective dried	0	8.2 ± 1.2	5.8 ± 0.8	14.0 ± 1.9	52.9
	90	6.8 ± 2.7	3.4 ± 0.1	10.2 ± 2.6	65.7
	180	3.7 ± 1.5	2.2 ± 0.5	5.9 ± 1.2	80.1

[a]Mean of measurements ± the standard error of the mean

[b]Mean of the sums of DHAA and AA measurements ± the standard error of the mean

[c]Orange juice diluted to 1.5 ºBrix

[MD]Orange juice powder with 10DE Maltodextrin

The content of vitamin C of orange juice, diluted to the same total soluble solids of 1.5% (w/v), was determined to calculate the content of vitamin C lost after drying. For all the drying methods, significant differences were obtained ($p<0.05$) for AA content between orange juice and orange powders. For DHAA also significant differences were obtained ($p<0.05$), except for convective drying ($p=0.2887$). It is clear that AA content of orange juice is higher than DHAA content and after drying the reverse occurs. Indeed, the DHAA is the first compound formed in the oxidative degradation of AA (Chang *et al*., 2006) and the higher the temperature used for powder production, the higher the conversion of AA into DHAA (Fracassetti *et al.,* 2013). This was observed for spray dried powders, due to the higher temperatures used in this technique in comparison with the others. Immediately after drying, spray dried powders were those that had smaller losses of total vitamin C (>5%), followed by freeze drying (>20%) and convective hot air drying (>50%). Many authors have already been reported large losses of vitamin C during convective drying; Lin and colleagues (1998) found losses of 62% in convective dried carrot slices and Chang *et al*. (2006) reported losses higher than 50% in convective dried tomato cubes. The temperature of drying and especially the long drying time required are the main causes of so many losses. Also large losses have been described for juices dried by spray drying; Solval and collaborators (2012) reported losses of 19% after spray drying cantaloupe (*Cucumis melo*) juice with inlet temperatures of 170 °C. Freeze drying is considered a gentle technique, since lower temperatures are used in the drying process and many studies reported losses of vitamin C lower for freeze-dried products than in both convective- and spray dried products (Marques *et al*., 2006; Moβhammer *et al*., 2006). This was not verified in the present study for spray dried orange powders and a possible explanation for these results is due to the long drying time required in the freeze drying technique. Generally, the products are dried in 24h and, in this study, several days

were necessary to obtain orange juice powder. Another explanation is that not all authors measure the total amount of vitamin C, but only the amount of ascorbic acid. If we analyze the data in terms of loss of AA, we can also state that the loss is elevated for spray drying.

There were no significant differences ($p>0.05$) in the freeze drying technique when the maltodextrin was used. The losses of vitamin C after drying were similar; however, during storage, these losses were higher for freeze-dried powder without maltodextrin. A slower decomposition of freeze dried ascorbic acid with maltodextrin had already been reported by Hung *et al*. (2007).

Comparing all drying methods, significant differences ($p<0.05$) were obtained for total vitamin C content during storage. It is not new knowledge that ascorbic acid is degraded when exposed to different conditions such as oxygen, temperature or light (Santos and Silva, 2008). However, using the appropriate conditions of storage, AA can remain stable for a long time (Valente *et al*., 2014).

6.4. Conclusions

To produce a probiotic product, such as an orange juice powder, whilst preserving the physical and nutritional characteristics of the product is important, survival of the probiotic cultures is perhaps the most important. Of the three drying techniques studied, spray- and freeze drying were those which resulted in lower losses in the number of viable cells during the drying process. Although during the different storage conditions tested, these two techniques do not always ensure a good survival of both LAB, because of the initial cells number after drying, the number of cells surviving was always greater than those obtained for convective hot air drying. Storage at 4 °C allowed the best survival, however, at room temperature without light, the reductions were also low,

especially for freeze dried *L. plantarum* 299v cells. Despite all techniques having allowed the development of orange powders with low water activities, the inability to dissolve the convective hot air dried powders renders it impossible to produce a probiotic orange juice powder, using the conditions employed in this study. However, with improved conditions to obtain a higher number of surviving cells with this technique it would be possible to use this powdered product for other purposes in the food or hotel industry. Concerning the color, the more appealing powders were obtained by the freeze drying technique without maltodextrin, but the vitamin C retention capacity was higher for spray- and freeze dried powders with maltodextrin. The color of the final product can always be improved with additives and vitamin C retention best ensured by storage conditions. Despite the good results obtained for spray- and freeze drying, it is important to consider the time and cost of production. In this sense, and despite still missing sensory evaluation, using the probiotic *L. plantarum* 299v or the potential probiotic *P. acidilactici* HA-6111-2, spray drying seems to be a useful technique to obtain a probiotic orange juice powder that suits consumers concerned with the consumption of functional products.

CHAPTER 7

Main conclusions

Main Conclusions

Besides the importance of producing food products that are innovative and satisfy the needs and demands of consumers, the quality-price relationship is also extremely important for industries. Spray drying has several advantages like its low production costs and the increased shelf-life, facility of transport and commercialization of the powdered products.

With this study, it was developed a functional orange juice powder by spray drying. The biggest challenge was to ensure the maximum survival of probiotics, assuring that they remained in large numbers at the end of processing and during storage. The first step was the selection of probiotic cultures. These must be safe and have functional, technological and physiological properties. So, commercial probiotic *L. plantarum* 299v, with probiotic characteristics already recognized, and *P. acidilactici* HA-6111-2, isolated from a food matrix and previously characterized by our research group were selected,. It was found that both *P. acidilactici* and commercial probiotic i) did no present any of the virulence factors investigated or important resistances to antibiotics, ii) were functional, with minimal loss of viability after the passage through the simulated GIT conditions and iii) also had technological properties, as both were able to survive during the drying process and remain viable for 60 days of storage.

Using a laboratory scale spray dryer, inlet and outlet air temperatures of 120 °C and 65 °C, respectively, and the proportion of soluble solids of orange juice: drying aid of 0.5: 2 were selected. These conditions allowed, on the one hand, obtaining orange juice powder with low water activity and high yield and, on the other, obtaining a high survival rate during the drying process. Cells stored at 4 °C under uncontrolled water activity survived better than at room temperature and *P. acidilactici* was more resistant at any condition. At 4 °C, whereas *P. acidilactici* dried in orange juice with 10DE maltodextrin or gum

Arabic, had reductions lower than 1 logarithmic cycle, *L. plantarum* demonstrated a high loss of viability (more than 8 logarithmic cycles); at room temperature both LAB did not survive until the end of the storage time. These results led to the need to improve some parameters as storage at room temperature would be ideal. In order to achieve this, various sugars were added in the culture medium used for the growth of bacteria. Again, dried *P. acidilactici* in orange juice with 10DE maltodextrin was more resistant than *L. plantarum* for all the conditions investigated. In general, the highest survival was observed for cells grown in the presence of lactose, followed by glucose. Cells grown in the presence of fructose resulted in the lowest survival rates. When the functional proprieties were evaluated subjecting cells to the conditions of simulated TGI at the end of storage and at selected conditions (cells grown in culture medium with glucose and stored at 4 ºC after spray drying), both LAB were more resistant to simulated acidic conditions of the stomach than of the small intestine. At the end of short (2h) and long (4h) simulated digestions, reductions in the number of viable cells of about 2 logarithmic cycles were observed for both LAB (final cells number of about 10^7 cfu/mL).

Pre-spray drying exposure of cells to different sub-lethal conditions of temperature, acid and presence of hydrogen peroxide was performed in an attempt to improve cell survival during storage. Except during storage at 4 ºC, where no significant differences were obtained ($p>0.05$), survival of *L. plantarum* was enhanced by prior exposure to any of the sub-lethal conditions. On the contrary, cells of *P. acidilactici* not previously exposed to sub-lethal stresses showed higher survival rates. *Lactobacillus plantarum* was more resistant than *P. acidilactici* when cells were exposed to hydrochloric acid. Again, both LAB were more resistant to stomach than to intestine simulated conditions. At the end of both short and long digestions of simulated TGI, viability was not improved by exposure to any of the sub-lethal stresses investigated.

Post-spray drying conditions were investigated by subsequent storage of each dried bacteria in orange juice under different conditions of temperature, light exposure and water activity. For both pre-spray drying conditions tested, both LAB survived better in powders stored at 4 ºC, a_w of 0.03 and absence of light, instead of room temperature, a_w of 0.11 and presence of light, respectively.

Powdered functional orange juice obtained by spray-, freeze- and convective hot air drying were compared in terms of bacterial survival and powder characteristics (dissolution, water activity, color and retention of vitamin C). Spray drying and freeze drying, allowed the highest number of viable cells along storage, powders with better dissolution, color and higher retention of vitamin C. The choice of spray drying rather than freeze drying would be based on the advantages of spray drying regarding the costs, time and easy operation.

Spray drying was considered a suitable technique to develop a powdered probiotic orange juice. This process is species/strain dependent, so the choice of the prerequisites for better survival of incorporated probiotics is crucial and should be examined case by case. The potential probiotic *P. acidilactici* HA-6111-2 demonstrated to be a valid choice due to its high survival rates. *Lactobacillus plantarum* 299v is a very good alternative already available in the market. Important characteristics such as vitamin C retention or good dissolution of powders were also achieved with this technique. After validation at industrial scale, we believe that, despite challenging, this innovative product should be attractive meeting modern consumer demands.

CHAPTER 8

Future work proposals

Future work proposals

Although this study was a good starting point for the development of a functional orange juice powder, there are still unanswered questions which lead to the proposal of the following suggestions for future research:

- The sensory analysis by a trained panel is essential to check the acceptance of this product as regards its aroma, flavor, texture and appearance. According to this analysis, it could be necessary to adjust some parameters to improve the sensorial characteristics of the product.

- To test other conditions that allow high survival rate of probiotics during drying and subsequent storage at room temperature, such as:

  - Pre-spray drying conditions: the addition of other sugars or compounds to the culture medium used for the growth of probiotics.
  - Conditions during spray drying: the addition of other protective carriers to the drying medium and the best proportion of solids content.
  - Post-spray drying conditions: the production of an efficient packaging in terms of light and gas permeability and type of materials.

- To assess the cellular damage that occurs during the various drying steps and during storage in order to select the protection strategies in a more careful and targeted way.

- The scale up of this product and its validation at industrial scale is necessary to proceed with the production of such a product.

- Since *P. acidilactici* HA-6111-2 proved to be so resistant, it would be interesting to validate its probiotic potential by testing other functional properties such as, for example, the ability to adhere to mucosal surface and the ability to reduce serum cholesterol. The maintenance of these probiotic properties should be subsequently assessed during drying and storage in orange juice.

- The *in vivo* validation of survival ability through the gastro-intestinal tract and the health benefits obtained by the intake of this functional product.

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