Characterization and Genetic Improvement of Lactobacilli for Application in Probiotic Dairy Products
# Contents

1. General introduction ................................................................................. 19

2. Materials and Methods ............................................................................. 25
   2.1 Bacterial cultures and growth conditions .................................................. 25
   2.2 Media ........................................................................................................ 28
   2.3 Additives ................................................................................................... 28
   2.4 Isolation of lactic acid bacteria................................................................. 30
   2.5 Mini-scale isolation of chromosomal DNA from lactobacilli ....................... 30
      2.5.1 Reagents .......................................................................................... 30
      2.5.2 Procedure ........................................................................................ 31
   2.6 Plasmid Miniprep for gram positive bacteria ............................................. 31
      2.6.1 Reagents .......................................................................................... 32
      2.6.2 Procedure ........................................................................................ 32
   2.7 NucleoSpin plasmid quickpure protocol for *E. coli* (Macherey & Nagel, Germany) ........................................................................................................... 33
      2.7.1 Procedure ........................................................................................ 33
   2.8 ARDRA ..................................................................................................... 34
      2.8.1 DNA preparation .................................................................................. 34
      2.8.2 Enzymatic amplification of rDNA .......................................................... 34
      2.8.3 Restriction analysis .............................................................................. 34
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.9</td>
<td>Pulsed Field Gel- Electrophoresis (PFGE)</td>
<td>34</td>
</tr>
<tr>
<td>2.9.1</td>
<td>Cultivation of cells:</td>
<td>34</td>
</tr>
<tr>
<td>2.9.2</td>
<td>Insert preparations:</td>
<td>35</td>
</tr>
<tr>
<td>2.9.3</td>
<td>Tray preparation:</td>
<td>35</td>
</tr>
<tr>
<td>2.9.4</td>
<td>Preparation of the lysozyme solution:</td>
<td>35</td>
</tr>
<tr>
<td>2.9.5</td>
<td>Proteinase- K digest :</td>
<td>35</td>
</tr>
<tr>
<td>2.9.6</td>
<td>Digestion with restriction enzymes:</td>
<td>36</td>
</tr>
<tr>
<td>2.9.7</td>
<td>Pulse-Field Gel Electrophoreses with inserts:</td>
<td>36</td>
</tr>
<tr>
<td>2.10</td>
<td>Acid tolerance test</td>
<td>36</td>
</tr>
<tr>
<td>2.11</td>
<td>Bile salt tolerance test</td>
<td>36</td>
</tr>
<tr>
<td>2.12</td>
<td>Bile salt hydrolase activity assay</td>
<td>37</td>
</tr>
<tr>
<td>2.13</td>
<td>Antimicrobial activity test</td>
<td>37</td>
</tr>
<tr>
<td>2.14</td>
<td>Antibiotic susceptibility test:</td>
<td>37</td>
</tr>
<tr>
<td>2.15</td>
<td>Polymerase chain reaction (PCR)</td>
<td>38</td>
</tr>
<tr>
<td>2.15.1</td>
<td>PCR reaction:</td>
<td>38</td>
</tr>
<tr>
<td>2.15.2</td>
<td>Purification of PCR product:</td>
<td>38</td>
</tr>
<tr>
<td>2.16</td>
<td>Molecular weight markers</td>
<td>41</td>
</tr>
<tr>
<td>2.17</td>
<td>AGAROSE GEL ELECTROPHORESIS</td>
<td>41</td>
</tr>
<tr>
<td>2.17.1</td>
<td>Reagents</td>
<td>41</td>
</tr>
<tr>
<td>2.18</td>
<td>Molecular cloning and other gene manipulation techniques</td>
<td>41</td>
</tr>
<tr>
<td>2.19</td>
<td>Transformation</td>
<td>42</td>
</tr>
</tbody>
</table>
2.31 Southern blot and DNA-DNA hybridization ........................................... 51
  2.31.1 Reagents for Southern hybridization ................................................. 51
  2.31.2 Blotting .......................................................................................... 53
  2.31.3 Labeling of DNA probes .................................................................. 53
    2.31.3.1 Labeling procedure – DIG-labeled DNA probes ............................ 53
    2.31.3.2 Estimation of the efficiency of labeling ........................................ 54
    2.31.3.3 Detection of labeled DNA ......................................................... 54
  2.31.4 Hybridization of probe to DNA ....................................................... 55
    2.31.4.1 Pre-hybridisation: ................................................................. 55
    2.31.4.2 Denaturation of probe: ............................................................ 55
    2.31.4.3 Washing .................................................................................. 55
    2.31.4.4 Stringent washing .................................................................... 55
    2.31.4.5 Blocking ................................................................................ 56
    2.31.4.6 CSPD incubation ...................................................................... 56
    2.31.4.7 Antibody incubation ................................................................. 56
    2.31.4.8 Removal of excess antibody ...................................................... 56
    2.31.4.9 CSPD incubation ...................................................................... 56
    2.31.4.10 Detection of hybridization signal .............................................. 56
  2.32 SDS-PAGE .......................................................................................... 57
  2.32.1 Reagents .......................................................................................... 57
  2.32.2 Procedure ....................................................................................... 58
    2.32.2.1 Preparation of phage extracts .................................................... 58
    2.32.2.2 Preparation of cell extracts ....................................................... 58
    2.32.2.3 Preparation of the SDS gel ....................................................... 58
2.32.2.4 Preparation of sample ................................................................. 59
2.32.2.5 Running of sample on SDS-PAGE ............................................. 59
2.32.2.6 Staining of the gel ....................................................................... 59
2.32.2.7 Destaining of gel ........................................................................... 59
2.33 Autolysis in buffer system ................................................................. 60
2.34 Prophage curing of *L. gasseri* BfEL92075 .................................... 60
2.34.1 Induction of strain *L. gasseri* with MC in the presence of anti-phage serum ................................................................................... 60
2.34.2 Selection of the colonies ................................................................. 60
2.34.3 Re-induction of derivatives ......................................................... 60
2.34.4 Spot test ......................................................................................... 61
3 Chapter I .............................................................................................. 62
Isolation and Identification of some Lactic acid bacteria by using different Molecular biological methods ................................................................. 62
3.1 Introduction ...................................................................................... 62
3.2 Results and Discussion ..................................................................... 64
3.2.1 Isolation of lactic acid bacteria ...................................................... 64
3.2.2 Identification of bacterial isolates from Egyptian fermented dairy products (first part) ................................................................. 64
3.2.2.1 Identification of isolates using API 50 CHL system .................. 64
3.2.3 ARDRA-PCR identification: .......................................................... 65
3.2.3.1 DNA preparation ........................................................................... 66
3.2.3.2 Enzymatic amplification of rDNA .............................................. 66
3.2.3.3 Restriction analysis of the 16S-23S rDNA amplicon ............... 67
<p>| 3.2.4 | Pulsed Field Gel- Electrophoresis (PFGE) Genotyping | 68 |
| 3.2.5 | Cloning and sequencing of 16S-23S rRNA genes | 69 |
| 3.3 | Identification of bacterial isolates from Egyptian fermented dairy products (second part of isolates) | 70 |
| 3.3.1 | PCR of 16S-23S rDNA | 70 |
| 3.3.2 | ARDRA using enzymes HaeIII &amp; HinfI | 71 |
| 3.3.3 | Cloning and sequencing of 16S-23S or 16S rRNA genes | 73 |
| 3.4 | Detection and comparison of the biodiversity of intestine bacteria from feces of twin babies | 75 |
| 3.4.1 | Identification of isolates of intestine bacteria from babies’ feces by PCR with genus specific primers | 75 |
| 3.4.1.1 | PCR for the isolates from feces of babies with genus specific primers | 75 |
| 3.4.1.2 | PCR with isolates from babies’ feces using universal primers | 77 |
| 3.4.2 | Differentiation of babies’ feces isolates by ARDRA | 78 |
| 3.4.2.1 | ARDRA of Lactobacillus Isolates from babies’ feces | 78 |
| 3.4.2.2 | Allocation of the isolates from babies’ feces by ARDRA | 78 |
| 3.4.2.3 | Sequencing of selected isolates | 80 |
| 4 | Chapter II | 82 |
| 4.1 | Introduction | 82 |
| 4.2 | Results and discussion | 84 |
| 4.2.1 | Acid tolerance | 84 |
| 4.2.2 | Bile tolerance | 88 |</p>
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2.3</td>
<td>Antimicrobial activity</td>
<td>95</td>
</tr>
<tr>
<td>4.2.4</td>
<td>Antibiotic susceptibility</td>
<td>100</td>
</tr>
<tr>
<td>4.2.4.1</td>
<td>Susceptibility to inhibitors of cell wall synthesis</td>
<td>100</td>
</tr>
<tr>
<td>4.2.4.2</td>
<td>Susceptibility to inhibitors of protein synthesis</td>
<td>100</td>
</tr>
<tr>
<td>4.2.4.3</td>
<td>Susceptibility to Inhibitor of nucleic acid synthesis</td>
<td>101</td>
</tr>
<tr>
<td>5</td>
<td>Chapter III</td>
<td>105</td>
</tr>
</tbody>
</table>

Cloning of a conjugated bile salt hydrolase gene (cbh) from *Lactobacillus plantarum* in *Lactobacillus* /*E. coli* shuttle vectors and expression of this gene in different *Lactobacillus* strains. .................................................. 105

5.1 | Introduction | 105
5.2 | Results and discussion | 106
5.2.1 | Detection of genes (cbh) encoding bile salts hydrolase activity | 106
5.2.2 | Cloning of the cbh gene into *E. coli* vectors and expression in *E. coli* | 107
5.2.3 | DNA sequencing | 108
5.2.4 | Expression of cbh in *E. coli* | 108
5.2.5 | Cloning of the cbh into *Lactobacillus* / *E. coli* shuttle vectors | 108
5.2.5.1 | Cloning and transformation of cbh gene into *E. coli* | 108
5.2.6 | Expression of cbh in *Lactobacillus* | 112
5.2.6.1 | Transformation of pS3A and pS3B into *Lactobacillus casei* LK1 | 112
5.2.6.2 | Transformation into *Lactobacillus gasseri* | 113
5.2.7 | Construction of a new shuttle vector with a chloramphenicol resistance gene as a selective marker | 113
5.2.7.1 | Cloning of the cat-194 gene as a blunted ended fragment into the Ncol site of pL9-1 and pL9-3 | 115
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2.7.2</td>
<td>Orientation detection of the <em>cat</em> 194-gene into plasmid pL11</td>
<td>117</td>
</tr>
<tr>
<td>5.2.8</td>
<td>Cloning of <em>cbh</em> in pGKV2</td>
<td>117</td>
</tr>
<tr>
<td>5.2.9</td>
<td>Transformation and expression of <em>cbh</em> in <em>Lactobacillus</em></td>
<td>119</td>
</tr>
<tr>
<td>5.2.10</td>
<td>Determination of bile salts tolerance</td>
<td>123</td>
</tr>
<tr>
<td>5.2.11</td>
<td>Use of the <em>cbh</em> gene from <em>Lactobacillus plantarum</em> BfEL 92122 as a food grade indicator gene</td>
<td>124</td>
</tr>
<tr>
<td>6</td>
<td>Chapter IV</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td><strong>Lysogeny and autolysis of lactobacilli. Analysis of temperate phage of <em>Lactobacillus gasseri</em> BfEL 92075</strong></td>
<td></td>
</tr>
<tr>
<td>6.1</td>
<td>Introduction</td>
<td>126</td>
</tr>
<tr>
<td>6.2</td>
<td>Results and discussion</td>
<td>126</td>
</tr>
<tr>
<td>6.2.1</td>
<td>Lysogeny and autolysis in <em>Lactobacillus</em> sp.</td>
<td>126</td>
</tr>
<tr>
<td>6.2.2</td>
<td>Bacteriophage Lbgas1: Morphology and genome structure</td>
<td>129</td>
</tr>
<tr>
<td>6.2.3</td>
<td>Phage DNA restriction analysis</td>
<td>130</td>
</tr>
<tr>
<td>6.2.4</td>
<td>Comparative analysis of restriction patterns</td>
<td>132</td>
</tr>
<tr>
<td>6.2.5</td>
<td>SDS-PAGE</td>
<td>133</td>
</tr>
<tr>
<td>6.2.6</td>
<td>Southern blot and hybridization</td>
<td>134</td>
</tr>
<tr>
<td>6.2.7</td>
<td>PCR analysis for the <em>holin-lysin</em> genes of <em>phi Lbgas1</em></td>
<td>136</td>
</tr>
<tr>
<td>6.2.8</td>
<td>Pro-phage curing from <em>L. gasseri</em> BfEL 92075</td>
<td>137</td>
</tr>
<tr>
<td>6.2.9</td>
<td>Southern blot and DNA-DNA hybridization for *phi Lbgas1, L. gasseri BfEL 92075 and its prophage-cured derivatives</td>
<td>139</td>
</tr>
<tr>
<td>6.2.10</td>
<td>Spot test</td>
<td>140</td>
</tr>
<tr>
<td>7</td>
<td>Summary and conclusion</td>
<td>141</td>
</tr>
</tbody>
</table>
Zusammenfassung und Schlussfolgerungen ........................................... 143
References ............................................................................................. 145
ACKNOWLEDGMENTS ........................................................................ 159
Curriculum vitae ...................................................................................... 160
Lebenslauf .............................................................................................. 161
## Tables list

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>A number of health benefits of probioticsa</td>
<td>20</td>
</tr>
<tr>
<td>Table 2</td>
<td>Selection criteria for probiotic strains</td>
<td>21</td>
</tr>
<tr>
<td>Table 3</td>
<td>Bacterial strains used in this study</td>
<td>25</td>
</tr>
<tr>
<td>Table 4</td>
<td>Antibiotics and additives used in this study</td>
<td>29</td>
</tr>
<tr>
<td>Table 5</td>
<td>Plasmids used in this study</td>
<td>29</td>
</tr>
<tr>
<td>Table 6</td>
<td>Antimicrobial agents and associated interpretative zone diameters for disc diffusion antibiotic susceptibility testing</td>
<td>38</td>
</tr>
<tr>
<td>Table 7</td>
<td>List of primers used</td>
<td>39</td>
</tr>
<tr>
<td>Table 8</td>
<td>PCR programs used in this study</td>
<td>40</td>
</tr>
<tr>
<td>Table 9</td>
<td>Blunting of DNAs for blunt –end ligations</td>
<td>49</td>
</tr>
<tr>
<td>Table 10</td>
<td>Dephosphorylisation of blunted vectors</td>
<td>50</td>
</tr>
<tr>
<td>Table 11</td>
<td>Preparation of serial dilutions of probe and control DNA</td>
<td>54</td>
</tr>
<tr>
<td>Table 12</td>
<td>Preparation of the SDS gel</td>
<td>58</td>
</tr>
<tr>
<td>Table 13</td>
<td>Preparation of the SDS sticky gel (7%)</td>
<td>59</td>
</tr>
<tr>
<td>Table 14</td>
<td>API 50 CHL Identification of four lactobacilli isolates from Egyptian cheese</td>
<td>65</td>
</tr>
<tr>
<td>Table 15</td>
<td>List of identified isolates</td>
<td>74</td>
</tr>
<tr>
<td>Table 16</td>
<td>Summery of identified isolates from babies’ feces</td>
<td>81</td>
</tr>
<tr>
<td>Table 18</td>
<td>Antimicrobial activity of <em>Lactobacillus</em> strains against eight indicator strains</td>
<td>99</td>
</tr>
<tr>
<td>Table 19</td>
<td>List of transformed strains</td>
<td>121</td>
</tr>
<tr>
<td>Table 20</td>
<td>Segregational stability of the recombinant plasmid</td>
<td>122</td>
</tr>
</tbody>
</table>
Table 21: Lysogeny and autolysis of *Lactobacillus* sp. .............................................128

Table 22: Dimensions* of phage Lbgas1 ........................................................................130

Table 23: Estimation of *phi* Lb gas1 genome size ........................................................132
Figures list

Fig. 1: FAO/WHO guidelines for the evaluation of probiotics in food .........................22

Fig. 2: The two predominant bacterial modifications of bile salts ...............................23

Fig. 3: Morphology of four lactobacilli isolates from Egyptian cheese .......................65

Fig. 4: Chromosomal DNA of some Lactobacillus sp and isolates A, C, D and E ........66

Fig. 5: PCR amplicons of 16S-23S rRNA genes from Lactobacillus type strains and Lactobacillus isolates .................................................................67

Fig. 6: ARDRA Profiles with Hae III (A) and Hinf I (B) of some Lactobacillus isolates and type strains ..............................................................68

Fig. 7: PFGE profiles of Lactobacillus strains with restriction enzyme Ascl (a) and NotI (b) .......................................................................................................69

Fig. 8: Clones of 16S-23SDNA fragment of some Lactobacillus isolates .................70

Fig. 9: PCR amplicons of 16S-23S rRNA genes from bacterial isolates numbers 1-28. .........................................................................................................71

Fig. 10: PCR amplicons of 16S-23S rRNA genes from bacterial isolates numbers 28-46. ........................................................................................................71

Fig. 11: HaeIII restriction patterns of 16S-23Sr RNA of isolates numbers 1-46 ...........72

Fig. 12: HinfI restriction patterns of 16S-23SrRNA of isolates numbers 1-46 ............72

Fig. 13: PCR amplicons of 16S-23S r RNA genes from selected isolates between numbers 1-27. ..............................................................................................73

Fig. 14: PCR amplicons of 16S r RNA gene from selected isolates between numbers 28-46. ..............................................................................................73
Fig. 15: PCR of the babies Feces isolates with the *Lactobacillus* specific Primers LactoF and LactoR. .......................................................... 76

Fig. 16: PCR of *Lactobacillus* reference strains with the primers LactoF/LbLMA1. ...................................................................................... 76

Fig. 17: PCR of babies’ feces isolates with *Lactobacillus* specific primers LactoF and LbLMA1. ........................................................................................ 76

Fig. 18: PCR of babies’ feces isolates (28-40A with *Lactobacillus* specific primers LactoF and LbLMA1. .............................................................................. 77

Fig. 19: PCR of babies’ feces isolates (1-20B with *Lactobacillus* specific primers LactoF and LbLMA1 ............................................................................. 77

Fig. 20: PCR of babies’ feces isolates (21-40B with *Lactobacillus* specific primers LactoF and LbLMA1 ............................................................................. 77

Fig. 21: ARDRA (Primer UP68/UP69) with TaqI for some isolates from baby’s (A) feces .......................................................................................................... 78

Fig. 22: ARDRA (Primer UP68/UP69) with TaqI enzyme of some isolates from babies feces. ..................................................................................................... 79

Fig. 23: ARDRA (Primer UP68/UP69) with TaqI enzyme of some isolates from babies feces. ..................................................................................................... 79

Fig. 24: ARDRA (Primer UP68/UP69) with TaqI enzyme of some isolates (B) from babies feces. .......................................................................................... 79

Fig. 25: ARDRA (Primer UP68/UP69) with TaqI enzyme of some isolates (B) from babies feces. .......................................................................................... 79

Fig. 26: The survival of *Lactobacillus* ssp. (log$_{10}$ cfu/ml) in MRS at different pH values during incubation at 37 °C for 90 min. .......................................................... 85

Fig. 27: Effect of different concentrations of ox-gall bile salts on the growth of lactobacili ........................................................................................................ 89
Fig. 28: Bile salt hydrolase activity as detected by plate assay method on MRS supplemented with TDCA or GDCA.................................................................95

Fig. 29: Antimicrobial activity of Lactobacillus strains against Staphylococcus aureus BfEL 91161 and Staphylococcus epidermis BfEL 91112.........................97

Fig. 30: Antimicrobial activity of Lactobacillus strains against pathogenic bacteria after changing the lactobacilli overnight culture growth conditions. ..........98

Fig. 31: Lactobacillus susceptibility to inhibitors of cell wall synthesis ..................102

Fig. 32: Lactobacillus susceptibility to inhibitors of protein synthesis..................103

Fig. 31: Lactobacillus susceptibility to inhibitors of nucleic acid synthesis...........104

Fig. 32: PCR amplification of cbh gene from Lactobacillus plantarum (BfEL92122). ........................................................................................................107

Fig. 33: Plasmids isolated from transformants obtained after cloning of cbh gene of L. plantarum BfEL 92122 into vectors pGEM-T-easy.................................107

Fig. 34: Expression of cbh gene in E. coli.............................................................108

Fig. 35: Selection of positive clones on TDCA- LB agar medium ......................109

Fig. 36: Isolation of plasmids from selected positive transformants...................109

Fig. 37: Conformation of the presence of the cbh gene fragment in the constructed plasmid pS3. .................................................................110

Fig. 38: Orientation of the cloned cbh gene by using HindIII restriction enzyme. ...110

Fig. 39: Construction of pS3A and pS3B .................................................................111

Fig. 40: Expression of cbh from plasmids pS3A and pS3B after transformation into Lactobacillus casei LK1 with plasmids........................................112

Fig. 41: Isolation of clones DNA from Lactobacillus casei LK1.........................112

Fig. 42: Map of pGK12.........................................................................................113
Fig. 43: Double digest of pS3A with SacI and PvuI. ................................................114
Fig. 44: Expression of cbh gene into pL9 plasmids. ................................................114
Fig. 45: Orientation of the cloned 3.7kb SacI fragment by NotI restriction enzyme. 115
Fig. 46: Physical maps of plasmids pL9-1 and pL9-3 ..............................................115
Fig. 47: Blunting of pL9-1 and cat-194 gene fragment ends ..................................116
Fig. 48: Expression of the cbh gene in E. coli easy pore clones transformed with ligation mix 11............................................................116
Fig. 49: Restriction of pL11 with Bvel restriction enzyme. ................................117
Fig. 50: Orientation of cat194 gene in pL11 .............................................................117
Fig. 51: Restriction map of pGKV2 (Kok et al., 1985) ..............................................118
Fig. 52: Transformation and expression of the cbh gene cloned in pGKV2 in E. coli Nova-Blue ............................................................118
Fig. 53: EcoRI digest of L14 Clones (plasmid pL14)..............................................118
Fig. 54: Isolation of recombinant plasmids carrying the cbh gene from Lactobacillus plantarum BfEL 92122 after transformation into Lactobacillus strains. ....................................................................................119
Fig. 55: EcoRI digest of plasmids isolated from L. gasseri transformed with plasmid pL14. ...........................................................................................................120
Fig.56: Expression of cbh gene which constructed in different plasmids and transformed into Lactobacillus sp. ....................................................................................120
Fig. 57: Growth and survival of L. gasseri and its transformant L. gasseri (pL11) in presence of GDCA.........................................................................................................123
Fig. 58: Growth and survival of L. casei and its transformant L. casei (pL11) in presence of GDCA.........................................................................................................124
Fig. 59: Growth and survival of *L. rhamnosus* and its transformant *L. rhamnosus* (pL11) in presence of GDCA. ........................................................................................................... 124

Fig. 60: Transformation of *E. coli* Nova-Blue with the plasmid pL11 and plating onto LB agar medium supplemented with GDCA without any antibiotics A: after 24 hours incubation; B: after 48 hours incubation. ........................................... 125

Fig. 61: Isolation of plasmid pL11 from *E. coli* transformants. ........................................... 125

Fig. 62: Autolysis of Lactobacillus sp. .................................................................................. 128

Fig. 63: Effect of the MC concentration on induction of *L. gasseri* BfEL 92075 ........... 129

Fig. 64: Electron micrographs of phi Lbgas1 (Inducible bacteriophage from strain *Lactobacillus gasseri* BfEL 92075) ........................................................................................................... 129

Fig. 65: Analysis Restriction profiles of the temperate phage phi Lbgas1 total DNA into 1.5% agarose gel (A) and 0.6% agarose gel (B). .................................................. 131

Fig. 66: DNA fingerprints of phi Lbgas1 and phi ADH. .................................................. 133

Fig. 67: SDS-PAGE for phi Lbgas1 and phi ADH. ............................................................. 134

Fig. 68: Southern Blot Hybridization of phi Lbgas1 DNA and *L. gasseri* BfEL 92075.......................................................... 135

Fig. 69: Southern Blot Hybridization of phi Lbgas1 and phi ADH. ................................ 135

Fig. 70: PCR amplicons of holin-lysin cassette. .............................................................. 136

Fig. 71: Restriction analysis of holin-lysin gene fragments. ............................................ 136

Fig. 72: Selection of *Phi Lbgas1*-cured derivatives of *L. gasseri* after MC treatment in the presence of anti-phage serum. ........................................................... 137

Fig. 73: Induction of *L. gasseri* and its derivatives with 0.2µg MC/ml. ......................... 138

Fig. 74. ARDRA for the lysogenic strain *L. gasseri* BfEL 92075 and its non-inducible derivatives using HaeIII and HinfI restriction enzymes. ......................... 138
Fig. 75: Analysis of protein patterns for lysogenic Strain and its derivative. ............139

Fig. 76: Southern blot hybridization of \( \phi \) Lbgas1, \( Lb. \ gasseri \) and its putatively cured derivatives. .............................................................139

Fig. 77: Spot test for lysogenic strain and \( L. \ gasseri \) BfEL 92075 and one of its prophage-cured derivatives BfEL 92075-1 using \( \phi \) Lbgas1 .........................140
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APS</td>
<td>Ammonium per sulphate</td>
</tr>
<tr>
<td>ARDRA</td>
<td>Amplified Ribosomal DNA Restriction Analysis</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>°C</td>
<td>Grad Celsius</td>
</tr>
<tr>
<td>ddNTP</td>
<td>Didesoxynucleotide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylen-Diamin-Tetra acetic acid</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>GDCA</td>
<td>Glycodeoxycholic acid</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo base pair</td>
</tr>
<tr>
<td>l</td>
<td>Liter</td>
</tr>
<tr>
<td>MC</td>
<td>Mitomycin C</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>milli Molar</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed field gel electrophoresis</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>spp.</td>
<td>Species (Plural)</td>
</tr>
<tr>
<td>subsp.</td>
<td>Subspecies</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetat-EDTA</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-Borat-EDTA</td>
</tr>
<tr>
<td>TDCA</td>
<td>Taurodeoxycholic acid</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N',N',N','N'-Tetramethylethylendiamin</td>
</tr>
<tr>
<td>u</td>
<td>unit</td>
</tr>
<tr>
<td>μ</td>
<td>Micro</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>Vol</td>
<td>Volume</td>
</tr>
</tbody>
</table>
1 General introduction

All dairy fermentations are based on usage of lactic acid bacteria (LAB) for acidification and development of flavors. Taxonomically, LAB are divided into 11 genera, but only four, *Lactobacillus*, *Streptococcus*, *Lactococcus* and *Leuconostoc* are commonly used in dairy starter cultures (Hassen and Frank, 2001). Lactobacilli, a large and diverse group of LAB, and have been isolated from many sources including all parts of the human GI tract (Greene & Klaenhammer, 1994). *Lactobacillus* cells are typically rod-shaped with sizes ranging from 0.5-1.2 x 1.0-10 μm; under certain growth conditions they can look almost coccoid-like. They are nonsporing cells, Gram-positive, catalase negative, mainly thermophillic bacteria. The optimal growth temperature is between 30-40°C. They are also aciduric with a pH optimum of 5.5-5.8. The genus *Lactobacillus* has been divided into three groups based on fermentation end-products. Group 1: obligately homofermentative lactobacilli, e.g. *L. delbrückii* subsp. *lactis*, *L. delbrückii* subsp. *bulgaricus*, *L. helveticus*, *L. acidophilus*, *L. gasseri* and *L. johnsonii*; Group 2: facultative heterofermentative lactobacilli, e.g. *L. casei*, *L. paracasei* subsp. *paracasei*, *L. rhamnosus*, and *L. plantarum* and Group 3: obligately heterofermentative lactobacilli, e.g. *L. brevis*, *L. fermentum*, *L. kefir* and *L. reuteri*. Some lactobacilli are used as probiotics, e.g. *L. acidophilus*, *L. johnsonii*, *L. gasseri*, *L. casei*, *L. rhamnosus*, *L. plantarum* (Abe et al., 1995; Ishibashi and Yamazaki, 2001). Probiotics have been defined as “live microbial food supplements which beneficially affect the host by improving microbial balance of the intestine” Fuller, 1989; Sanders, 1998; Tannock, 1999). Most probiotics are lactic acid bacteria (LAB) (Lee and Salminen, 1995) and probiotic products are considered to be safe and have GRAS (Generally Regarded As Safe) status. There are differing degrees of evidence supporting the confirmation of such effects. Table 1 summarized a number of potential health benefits provided by probiotic bacteria.
<table>
<thead>
<tr>
<th>Target health benefit</th>
<th>Probiotic</th>
<th>Postulated Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lactase intolerance</strong></td>
<td>Lactase positive strains</td>
<td>- Bacterial lactase acts on lactose in the small intestine</td>
</tr>
</tbody>
</table>
| **Prevention of diarrhoea and resistance to enteric**     | *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* BB-12            | - Adjuvant effect increasing antibody production  
| **pathogenic**                                            |                                                                           | - Systemic immune effect  
|                                                           |                                                                           | - Colonization resistance  
|                                                           |                                                                           | - Alteration of intestinal conditions to be less favorable for pathogenicity (pH, SCFA, bacteriocins) |
| **Helicobacter pylori infection and complications**       | *L. johnsonii* La1; *L. gasseri* OLL 2716                                  | - Growth inhibition  
| (B gastritis, peptic ulcers and gastric cancer)           |                                                                           | - Decreasing urease enzyme activity necessary for the pathogen to remain in the acidic environment of the stomach |
| **Inflammatory diseases and bowel syndromes**            |                                                                          | - The intestinal microflora plays a critical role in inflammatory conditions in the gut, and potentially probiotics could remediate such conditions through modulation of the microflora. |
| **Anti-colon cancer* effect**                            | *L. casei* Shirota; *L. rhamnosus* GG and LC-705 and bifidobacteria ; *Propionibacterium* | - Modify the gut flora leading to decreasing glucuronidase and carcinogen levels e.g. carcinogenic aflatoxin in the lumen |
| **Mucosal immunity**                                     | *L. casei* Shirota; *B. lactis* HN019 and *L. rhamnosus* HN001             | - Modify immune parameters; increased natural killer (NK) activity; enhance the innate immune response;  
|                                                           |                                                                           | - Strengthening of non-specific and antigen-specific defense against infection and tumors  
|                                                           |                                                                           | - Adjuvant effect in antigen-specific immune responses  
|                                                           |                                                                           | - Regulating/influencing Th1/Th2 cells, production cytokines |
| **Allergy**                                               | *L. rhamnosus* GG; *B. lactis* BB-12                                      | - Modulate the immune response and prevent onset of allergic diseases.  
|                                                           |                                                                           | - Prevention of antigen translocation into blood stream |
| **Cardiovascular disease**                               | lactobacilli                                                               | - Assimilation of cholesterol by bacterial cell  
|                                                           |                                                                           | - Alteration of activity of bile salt hydrolase enzyme  
|                                                           |                                                                           | - Antioxidative effect |
| **Urogenital tract disorders** (Bacterial vaginosis (BV); Yeast vaginitis; Urinary tract infections) | *Lactobacillus acidophilus*                                                | - Adhesion to urinary and vaginal tract cells  
|                                                           |                                                                           | - Competitive exclusion;  
|                                                           |                                                                           | - Production hydrogen peroxide |
| **Use of probiotics newborn children**                   | Many probiotic products                                                    | - Probiotic microorganisms could become primary colonizers that remain long-term |

*a* Compiled from Sanders & Huis in’t Veld, 1999; Report of FAO/WHO, 2001; Ezendam *et al.*, 2005;  
*The Consultation of FAO/WHO was not convinced that there is sufficient proof of a correlation between probiotics and specific anti-cancer effects.*
It is irrational to expect that one strain of any probiotic bacteria can fulfill all of the aforementioned potential health benefits (Gilliland, 2001).

For selection and establishment of probiotic strains, there are many of selection criteria listed in Table 2.

Table 2: Selection criteria for probiotic strains

<table>
<thead>
<tr>
<th>Appropriateness</th>
</tr>
</thead>
<tbody>
<tr>
<td>i. Accurate taxonomic identification</td>
</tr>
<tr>
<td>ii. Normal inhabitant of the species targeted: human origin for human probiotics</td>
</tr>
<tr>
<td>iii. Nontoxic, nonpathogenic, GRAS status</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Technological suitability</th>
</tr>
</thead>
<tbody>
<tr>
<td>iv. Amenable to mass production and storage: adequate growth, recovery, concentration, freezing, dehydration, storage, and distribution</td>
</tr>
<tr>
<td>v. Viability at high cell densities</td>
</tr>
<tr>
<td>vi. Stability of desired characteristics during culture preparation, storage, and delivery</td>
</tr>
<tr>
<td>vii. Provides desirable organoleptic qualities (or no undesirable qualities) when included in foods or fermentation processes</td>
</tr>
<tr>
<td>viii. Genetically stable</td>
</tr>
<tr>
<td>ix. Genetically amenable</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Competitiveness</th>
</tr>
</thead>
<tbody>
<tr>
<td>x. Capable of survival, proliferation, and metabolic activity at the target site in vivo</td>
</tr>
<tr>
<td>xi. Resistant to bile</td>
</tr>
<tr>
<td>xii. Resistant to acid</td>
</tr>
<tr>
<td>xiii. Able to compete with the normal microflora, including the same or closely related species; potentially resistant to bacteriocins, acid, and other antimicrobials produced by residing microflora</td>
</tr>
<tr>
<td>xiv. Adherence and colonization potential preferred</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Performance and functionality</th>
</tr>
</thead>
<tbody>
<tr>
<td>xv. Able to exert one or more clinically documented health benefits (e.g. lactose tolerance)</td>
</tr>
<tr>
<td>xvi. Antagonistic toward pathogenic / cariogenic bacteria</td>
</tr>
<tr>
<td>xvii. Production of antimicrobial substances (bacteriocins, hydrogen peroxide, organic acids, or other inhibitory compounds)</td>
</tr>
<tr>
<td>xviii. Immunostimulatory</td>
</tr>
<tr>
<td>xix. Antimutagenic</td>
</tr>
<tr>
<td>xx. Anticarcinogenic</td>
</tr>
<tr>
<td>xxi. Production of bioactive compounds (enzymes, vaccines, peptides)</td>
</tr>
</tbody>
</table>

Source: Klaenhammer and Kullen, (1999)
For evaluation of newly discovered probiotics, the scheme of FAO/WHO (Fig. 1) may be useful (FAO/WHO, 2002).

**Fig. 1: FAO/WHO guidelines for the evaluation of probiotics in food**
Phenotype characterization (e.g. fermentation profiles, temperature, pH tolerance) is not satisfactory for identification of closely related species. Genetic analysis based on accumulating information of ribosomal RNA sequences release a developing source for comparative identification of probiotic bacteria (Klaenhammer and Kullen, 1999). Phylogenetic analysis of sequence-based typing system using conserved regions of the ribosomal RNA operon is the most powerful tool for identification of probiotic bacteria (O’Sullivan, 1999). ARDRA and PFGE are in addition considered as powerful techniques for comparative identification. Chapter I in the present study was specified for identification of LAB using molecular methods e.g. ARDRA, PFGE, and sequencing of the 16S rRNA gene to achieve accurate identification as the main criterion for selection as a probiotic.

If the desirable action needs that bacteria must survive and grow in the gastrointestinal tract, then characteristics that enable them to survive and grow under these conditions must be measured e.g. bile salt as well as pH tolerance. In chapter II of this study, characterization of Lactobacillus strains concerning selection criteria for probiotics such as acid and bile salts tolerance, antimicrobial activity as well as antibiotic susceptibility as a safety factor was performed.

For the ability of bacteria to establish themselves or grow in the intestine, it is important to consider the bile salt tolerance of selected strains (Gilliland, 2001). Therefore, resistance to bile salts is an important characteristic for the selection and establishment of a probiotic strain (Moser and Savage, 2001; Dussurget et al., 2002). There are many theories, which explain the mechanism of bile salts tolerance (see introduction of chapter III). Generally bile salts are deconjugated first by intestinal bacteria before they are further metabolized, Fig. 2 (Batta et al., 1990; Hill 1995).

![Fig. 2: The two predominant bacterial modifications of bile salts](image)

R indicates the amino acid glycine; GCA: glycocholic acid; CA: cholic acid; DCA: deoxycholic acid (adapted from De Boever and Verstraete, 1999)
Therefore, chapter III of the actual study is concerning with screening of *Lactobacillus* species for bile salt hydrolase activity and cloning, transformation and expression of a responsible gene in different *Lactobacillus* strains.

Many other factors must also be considered for probiotic bacteria to survive in the product and after consumption in the consumer’s gastrointestinal tract. These include the physiologic state of the probiotic organisms added; the physical conditions of product storage, the chemical composition of the product to which the probiotics are added, as well as interactions of the probiotics with the starter cultures (Heller, 2001). In addition, the inhibitory effect of bacteriophages and autolysis during industrial fermentation should be also considered. The greatest economic losses of dairy fermentations are caused by these obligate dairy starter cultures parasites. Therefore, chapter IV in the present study is focused on lysogeny and autolysis of lactobacilli caused by bacteriophages or their enzymes.

The aim of this study was to evaluate putative probiotic lactobacilli isolated from different sources. The following goals were set:

I) Isolation and identification of some lactobacilli by using molecular biological methods was performed.

II) Characterization of *Lactobacillus* strains through probiotic selection criteria (acid tolerance, bile salts resistance and antimicrobial activity). Also, a safety assessment of some *Lactobacillus* isolates.

III) Cloning, transformation and expression of a conjugated bile salt hydrolase gene (*cbh*) in different Lactobacillus strains.

IV) Lysogeny and autolysis of lactobacilli as well as analysis of a template phage of *Lactobacillus gasseri*
2 Materials and Methods

2.1 Bacterial cultures and growth conditions

Lactic acid bacteria, *Escherichia coli* strains and phages used in this study are listed in Table 3 A, B, C. *Lactobacillus* were grown in MRS medium with glucose or lactose at 37°C. *E. coli* strains were routinely grown in LB medium at 37°C. Stocks from all strains were preserved at -80°C in 30% glycerol. The respective recombinant strains were propagated and maintained as above but with recommended antibiotic supplements (Table 4).

Table 3: Bacterial strains used in this study

A) Lactic acid bacterial strains

<table>
<thead>
<tr>
<th>No</th>
<th>Strains</th>
<th>Source</th>
<th>Synonym</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Lactobacillus acidophilus</em></td>
<td>BfEL 92015</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td><em>Lactobacillus johnsonii</em></td>
<td>BfEL 92170</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>Lactobacillus amylovorus</em></td>
<td>BfEL 92203</td>
<td>DSM 20552</td>
</tr>
<tr>
<td>4</td>
<td><em>Lactobacillus brevis</em></td>
<td>BfEL 92026</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td><em>Lactobacillus brevis</em></td>
<td>BFEL 92027</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td><em>Lactobacillus gasseri</em></td>
<td>BfEL 92075</td>
<td>DSM 20243</td>
</tr>
<tr>
<td>7</td>
<td><em>Lactobacillus gasseri</em> BfEL 92075-1</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td><em>Lactobacillus gasseri</em> BfEL 92075-2</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td><em>Lactobacillus gasseri</em> NCK102-ADH</td>
<td>Raya <em>et al.</em>,1989</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td><em>Lactobacillus gasseri</em> NCK102</td>
<td>Raya <em>et al.</em>,1989</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td><em>Lactobacillus plantarum</em></td>
<td>BfEL 92122</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td><em>Lactobacillus rahmnosus</em></td>
<td>BfEL 92149</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>Strains</td>
<td>Source</td>
<td>Synonym</td>
</tr>
<tr>
<td>----</td>
<td>-------------------------------</td>
<td>--------------</td>
<td>---------------</td>
</tr>
<tr>
<td>13</td>
<td><em>Lactobacillus casei</em> LK1</td>
<td>Meyer-Barton, 1995</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td><em>Lactobacillus acidophilus</em></td>
<td>BfEL 92006</td>
<td>ATCC10697</td>
</tr>
<tr>
<td>15</td>
<td><em>Lactobacillus johnsonii</em></td>
<td>BfEL 92204</td>
<td>DSM 20553</td>
</tr>
<tr>
<td>16</td>
<td><em>Lactobacillus plantarum</em></td>
<td>BfEL 92265</td>
<td>DSM 20174</td>
</tr>
<tr>
<td>17</td>
<td><em>Lactobacillus rhamnosus</em></td>
<td>BfEL 92146</td>
<td>DSM 20178</td>
</tr>
<tr>
<td>18</td>
<td><em>Lactobacillus brevis</em></td>
<td>BfEL 92113</td>
<td>DSM 20197</td>
</tr>
<tr>
<td>19</td>
<td><em>Lactobacillus reuteri</em></td>
<td>BfEL 92126</td>
<td>DSM 20016</td>
</tr>
<tr>
<td>20</td>
<td><em>Lactobacillus delb. subsp. bulgaricus</em></td>
<td>BfEL 92067</td>
<td>ATCC 12278</td>
</tr>
<tr>
<td>21</td>
<td><em>Lactobacillus delb. subsp. lactis</em></td>
<td>BfEL 92237</td>
<td>DSM20072</td>
</tr>
<tr>
<td>22</td>
<td><em>Lactobacillus plantarum</em> (A)</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td><em>Lactobacillus plantarum</em> (C)</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td><em>Lactobacillus plantarum</em> (D)</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td><em>Lactobacillus plantarum</em> (E)</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Lactobacillus fermentum IS1</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>Lactobacillus plantarum IS2</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Lactococcus <em>lactis</em> IS5</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>Streptococcus <em>thermophilus</em> IS6</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>Lactococcus <em>lactis</em> IS7</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>Lactococcus <em>lactis</em> IS8</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>Lactococcus <em>lactis</em> IS9</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>Lactobacillus <em>plantarum</em> IS13</td>
<td>This study</td>
<td></td>
</tr>
</tbody>
</table>
Materials and Methods

<table>
<thead>
<tr>
<th>No</th>
<th>Strains</th>
<th>Source</th>
<th>Synonym</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td><em>Lactobacillus fermentum</em> IS1</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>35</td>
<td><em>Lactobacillus fermentum</em> IS15</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td><em>Lactobacillus fermentum</em> IS16</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td><em>Enterococcus saccharinimus</em> IS8</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td><em>Lactobacillus fermentum</em> IS12</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td><em>Leuconostoc mesenteroides</em> IS14</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td><em>Lactobacillus paracasei</em> ISO1</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>41</td>
<td><em>Lactobacillus rhamnosus</em> ISO2</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td><em>Lactobacillus paracasei</em> ISO3</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td><em>Lactobacillus rhamnosus</em> ISO4</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>44</td>
<td><em>Lactobacillus rhamnosus</em> ISO5</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td><em>Lactobacillus rhamnosus</em> ISO6</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td><em>Lactobacillus rhamnosus</em> ISO7</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td><em>Enterococcus gallinarum</em> ISA1</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td><em>Enterococcus saccharolyticus</em> ISA2</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td><em>Enterococcus saccharolyticus</em> ISA3</td>
<td>This study</td>
<td></td>
</tr>
</tbody>
</table>

B) *E. coli* Genotype Source

<table>
<thead>
<tr>
<th>No</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F', ara D139 (ara, leu)7697, lacX74, galU, galK, mcrA -(mrrhsdRMS-mcrBC), rpsL, dor, 80dlacZ M15, endA1, nupG, recA1.</td>
<td>Eurogentec, Belgium</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>F' traD36 lacIΔ(lacZ)M15 pro A+ B+ / e14- (McrA)Δ(lac-proAB)thi gyrA96 (Nal') endA1 hsdR17(rk mK') relA1 supE44 recA1 (1)</td>
<td>Stratagene,</td>
</tr>
</tbody>
</table>
Materials and Methods

3. **Novablue**
   endA1 hsdR17(rK12– mK12+) supE44 thi-1
   recA1 gyrA96 relA1 lacF'[proA+B+ lacIqZ
   lacZM15::Tn10 (TcR)]
   Novagen, C)

### C) Phages

<table>
<thead>
<tr>
<th>Phage</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ФLbgas1</td>
<td>Temperate, isometric, contractile tail, 44kb, pac-type</td>
<td>This study</td>
</tr>
<tr>
<td>ФADH</td>
<td>Temperate, isometric, 44kb, cos-type</td>
<td>Raya <em>et al.</em>, 1992</td>
</tr>
</tbody>
</table>

2.2 **Media**

**LB- Medium (E. coli):**
- Pepton from casein 10.0 g/l
- Yeast extract 5.0 g/l
- NaCl 5.0 g/l
- pH 7.0 ±0.2

**MRS-Medium (Lactobacillus):**
- Pepton from casein 10.0 g/l
- Beef extract 8.0 g/l
- Yeast extract 4.0 g/l
- Glucose 20.0 g/l
- K$_2$HPO$_4$ 2.0 g/l
- Di-ammonium hydrogen citrate 2.0 g/l
- Sodium acetate 5.0 g/l
- MgSO$_4$, 7H$_2$O 0.2 g/l
- MnSO$_4$, 5H$_2$O 0.04 g/l
- Tween80 1.0 g/l

For solid media, 15 g agar per liter of broth media were added

2.3 **Additives**

Antibiotics and other additives used for the media during this study
Table 4: Antibiotics and additives used in this study

<table>
<thead>
<tr>
<th>Antibiotics &amp; Additives</th>
<th>Final concentration in medium for E. coli</th>
<th>Final concentration in medium for Lactobacillus</th>
<th>Dissolving agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>200 µg/ml</td>
<td>-</td>
<td>H₂O dest.</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>150 µg/ml</td>
<td>5-10 µg/ml</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>15 µg/ml</td>
<td>5-10 µg/ml</td>
<td>Ethanol</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>12.5 µg/ml</td>
<td>-</td>
<td>50% Ethanol</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>50 µg/ml</td>
<td>-</td>
<td>H₂O dest</td>
</tr>
<tr>
<td>IPTG</td>
<td>0.5 mM</td>
<td>-</td>
<td>H₂O dest</td>
</tr>
<tr>
<td>X-Gal</td>
<td>40 µg/ml</td>
<td>-</td>
<td>DMF&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>-</td>
<td>0.1-1.0 µg/ml</td>
<td>H₂O dest</td>
</tr>
<tr>
<td>Ox- bile</td>
<td>-</td>
<td>0.1-3%</td>
<td>H₂O dest</td>
</tr>
<tr>
<td>TDCA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.5%</td>
<td>0.5%</td>
<td>H₂O dest</td>
</tr>
<tr>
<td>GDCA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.5%</td>
<td>0.5%</td>
<td>H₂O dest</td>
</tr>
</tbody>
</table>

<sup>a</sup>) Dimethylformamid; <sup>b</sup>) Taurodeoxycholic acid; <sup>c</sup>) Glycodeoxycholic acid

Table 5: Plasmids used in this study

<table>
<thead>
<tr>
<th>No</th>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pBluescript II SK+ (pBSK)</td>
<td>ColE1, am&lt;sup&gt;′&lt;/sup&gt;, lacZ, α-complementation</td>
<td>Stratagene, Germany</td>
</tr>
<tr>
<td>2</td>
<td>PGEM-T-easy vector</td>
<td>ColE1, am&lt;sup&gt;′&lt;/sup&gt;, lacZ, α-complementation</td>
<td>Promaga, USA</td>
</tr>
<tr>
<td>3</td>
<td>pCAT</td>
<td>cat194 cloned in pGEMT Easy (Promega, Germany)</td>
<td>A. Geis (personal Communication)</td>
</tr>
</tbody>
</table>


2.4 Isolation of lactic acid bacteria

Lactic acid bacteria were isolated from different sources as some Egyptian dairy products (Laban rayb, Kariesh and mish cheese) as well as from four faeces samples from two twin babies at the age of 3 and 4 months. The isolation was performed by standard microbiological procedures. As selective media MRS-agar was used for isolation of LABs from the dairy products and AMC agar for the isolation from babies’ faeces. The incubation was performed at 37°C under anaerobic conditions. Once single colonies were obtained they were inoculated in MRS broth or TPY broth and after growth, the cultures were diluted and tested for purity on a suitable agar medium. In total, 66 isolates were obtained from Egyptian dairy products and 40 isolates from each baby. The purity of the active cultures was also ascertained by microscopic examination.

2.5 Mini-scale isolation of chromosomal DNA from lactobacilli

According to Leenhouts et al., 1990

2.5.1 Reagents

1M DL-thereonin
Materials and Methods

Solution A: (20% sucrose; 10 mM Tris-HCl (pH 8.0); 50 mM NaCl.
5 mg/ml lysozyme
10% SDS
1500 U/ml mutanolysin
20 mg/ml proteinase K
5 mg/ml RNase A
phenol
chloroform / isoamylalcohol (chl/iaa-24:1)
3M Na-Ac (pH 5.2)
1XTE Buffer : (10 mM Tris (pH 8.0); 1 mM EDTA)

2.5.2 Procedure

Overnight cultures of lactobacilli were diluted (1%) in MRS broth containing 40 mM DL-threonin and incubated at 37°C until O.D\textsubscript{620nm}=0.6. Cells from 5-10 ml growing cultures were collected in 2 ml eppendorf tubes. The pellets were resuspended in 1 ml solution A, 20 µl lysozyme, and 20µl mutanolysin were added. The resuspended cells were frozen at -80°C for 30 min, thawed and incubated at 37°C for 30 min. 20 µl proteinase K and 50 µl were added, and incubation was continued at 60°C until the mixture was clear. 500 µl phenol and 500 µl of chloroform:isoamyl alcohol (24:1) were added and mixed by frequent inversions. The samples were centrifuged at 5 min and the aqueous phase was transferred to a new tube and extracted with 500µl of chloroform : isoamyl alcohol (24:1) 2 times. The DNA was precipitated by adding 50µl 3M NaAc and one volume of isopropanol. The DNA pellet thus obtained was recovered after centrifugation at 14,000 rpm, washed with 70 percent ethanol and dried at RT for 10 min. DNA was finally dissolved in 150 µl TE buffer (pH 8.0) supplemented with 5 µl RNase A.

2.6 Plasmid Miniprep for gram positive bacteria

As protocol of Macherey & Nagel; Modified by S. Lick (personal communication)
2.6.1 Reagents

Solutions A1, A2, A3, A4, AW and AE (included in the Nucleospin plasmid kit)

Mutanolysin solution : 5000 U/ml in double distilled H$_2$O
Proteinase K solution : 100 mg/ml in double distilled H$_2$O
Lysozyme solution : 50 mg/ml in double distilled H$_2$O
Phenol : Saturated with 3 M NaCl
Chloroform/isoamyl alcohol (24:1; volumetric)

2.6.2 Procedure

-Cells from 3 to 6 ml culture propagated to OD$_{620}$=0.6-0.8 were collected in 2 ml Eppendorf tube by centrifugation. The cell pellets were washed with sterile D.D.W, recentrifuged and resuspended in 250µl solution A1. Solutions of lysozyme (25 µl) and mutanolysin (5 µl) were also added. The cells can be stored at -80°C.

-The cells were incubated at 37°C for 1 h.

-Solution A2 (250 µl) was added. The cells were mixed (for not more than 5 min) by inversion until the cells lysed and the solution was clear.

-Ice cold solution A3 (300 µl) was added and the contents mixed and placed on ice for 5 min.

-The tubes were centrifuged (full speed) for 12 min and the upper phase was transferred to a fresh Eppendorf tube (2 ml).

- 25 µl proteinase K was added, followed by incubation at 37°C for 15 to 30 min.

-Equal volumes of phenol and chloroform : isoamylalcohol (24:1) were added to the upper phase. The contents were mixed well by inversion.

-The tubes were centrifuged (full speed) for 5 min.

-The clear upper phase was transferred to a purification column centrifuged at full speed for 1 min.

-The flow through in the collection tube was discarded.

-Solution AW (500 µl) at 50°C was added to the column and centrifuged. The flow through in collection tube was discarded.
Solution A4 (600 µl) was added and centrifuged (full speed, 1 min). The flow through in the collection tube was discarded.

- Finally, the purification column was transferred to a fresh Eppendorf (1.5 ml) tube. Solution AE (60 µl) was added to the column and DNA was eluted by centrifugation and stored at 4 °C or –20 °C until further use.

- An aliquot of 5 to 10 µl of the plasmid DNA was run on Agarose (0.8%) gel to ascertain the concentration and purity of the DNA.

2.7 NucleoSpin plasmid quickpure protocol for *E. coli* (Macherey & Nagel, Germany)

2.7.1 Procedure

- The overnight grown culture (1 to 5 ml) was harvested and centrifuged at 11,000 x g for 30 sec at 4 °C to pellet the bacterial cells. The supernatant was then discarded.
- The pellet was carefully resuspended in 250 µl of buffer A1.
- 250 µl of buffer A2 was added to the suspension. The content was gently mixed by inverting the tube 6 to 8 times and incubated at room temperature for 5 min.
- An aliquot of 300 µl of buffer A3 was added to the suspension and mixed gently by inverting the tube 6 to 8 times.
- The suspension was centrifuged at 11,000 x g in a microcentrifuge for 5 min at room temperature.
- NucleoSpin column was placed in a fresh 2.0 ml collection tube and the supernatant was loaded into the spin column. The supernatant was then centrifuged at 11,000 x g for 1 min at room temperature and the flow through was discarded.
- 450 µl of buffer AQ (with ethanol) was added to the NucleoSpin column and centrifuged at 11,000 x g for 3 min at room temperature. The flow through was discarded.
- The NucleoSpin column was placed in a fresh 1.5 ml microcentrifuge tube. The DNA was eluted from the columns with 50 to 100 µl of buffer AE (5 mM Tris-HCl, pH 8.5).
2.8 ARDRA

2.8.1 DNA preparation

Total DNA was prepared according to as aforementioned

2.8.2 Enzymatic amplification of rDNA

For PCR, universal primers UP68 and UP69 (Vaneechoutte et al., 1992) were used to amplify a fragment (approx. 2.4 Kb) encompassing the 16S, the spacer region and part of the 23S rDNA. Reactions were carried out in sterile 0.2 ml tubes with final reaction volume of 50µl. Reaction mixture contained of 2X PCR mix [Tag DNA polymerase (recombinant) in reaction buffer, MgCl2 and dNTPs (dATP, dCTP, dGTP, dTTP)] 25 µl, 10 pmol of each primer, and 22.5 µl of sterile H2O and 1.9 µl of target DNA. PCR amplification was carried out in a Mastercycler 5333 (Eppendorf, Hamburg, Germany) where tubes were heated to 93°C for 5min and amplification was started. PCR conditions were 1 min. at 92°C, 1:30 min. at 58°C and 2:20 min. at 72°C for 35 cycle, completed by a terminal elongation step for 10 min at 72°C

2.8.3 Restriction analysis

Restriction digests were carried out in volumes of 20 µl containing incubation buffer, 10U of restriction enzyme and 2-5 µl of PCR product at appropriate temperatures. Restriction fragment patterns were analyzed by agarose- gel –electrophoresis

2.9 Pulsed Field Gel- Electrophoresis (PFGE)

The PFGE was carried out for some isolates and some type strains as follows:

2.9.1 Cultivation of cells:

Fresh over night culture were diluted (1%) in 5 ml of MRS broth containing 20mM DL- Threonin and cells were grown till OD$_{620}$ 0.5-1 (not higher than OD 1). Cells were harvested by centrifugation in a 2 ml Eppendorf tube and washed 2 times with 0.05M EDTA pH8.5. Cell pellets were kept at -20°C till used.
2.9.2 Insert preparations:

100µ 0.05M EDTA pH8.5 was added to each pellet and the cells were resuspended carefully. For the inserts, low melted agarose 1% in 0.05M EDTA (pH8.5) was dissolved and stored at 50ºC till used. 500µ 1% agarose was filled in 2 ml Eppendorf tubes (placed in 50ºC water-bath) and 75-100µ of cell suspensions were added, mixed carefully and placed in 50ºC water-bath ( Turbidity of the solutions should be between 4 to 5 according to McFarland-Standard; Biomerieux).

2.9.3 Tray preparation:

The tray (Bio-Rad system) was rinsed well with d. d. water and cleaned with ethanol, screwed well and placed on the table, the lower side in front. 500µl of the agarose-cell suspension was poured into two insert-slots (each 250µ). The tray was placed for 10min at -20ºC for polymerization. The inserts were transferred in 2 ml Eppendorf tubes containing 1.5 ml lysozyme solution and digested at 37ºC for 24 hrs.

2.9.4 Preparation of the lysozyme solution:

1.67µl N-Lauroylsarcosine (stock solution 30%)
2.0 mg Lysozyme
20 U Mutanolysin
1000µl 0.05 M EDTA pH 8.5

2.9.5 Proteinase K digest :

NDS-Buffer

10 µl 1M Tris HCl pH 8.0
100 µl 10% SDS
890 µl 0.05 M EDTA pH 8.5

Proteinase K solution: 1.5 mg Proteinase K + 1 ml NDS buffer.

The inserts were moved into 2 ml Eppendorf tubes containing 1.5 ml of proteinase K solution and incubated at 50ºC. After 20-30 min the Eppendorf tubes were opened and closed for equalization of pressure and the incubation was continued for at least 24 hrs.
After Proteinase-K digest, inserts were washed with 0.05 M EDTA, pH 8.5 for 30 min at room temperature (6-7 times). Inserts were stored in 0.05 M EDTA, pH 8.5 at 4°C.

2.9.6 Digestion with restriction enzymes:
- 2 stripes, each 3 mm width were cut from the inserts, transferred into Eppendorf tubes filled with 300µl TE-buffer and incubated at 4°C for 30 min.
- The TE-buffer was replaced by 200µl of RC-buffer (180µl TE-buffer + RC buffer 10X) were added, the strips were incubated at 4°C for 30 min.
- For digestion, RC-buffer was replaced by 200µl of RCE (180µl TE-buffer + RC buffer 10X +10U Enzyme). The samples were incubated at 37°C overnight.

2.9.7 Pulsed field gel electrophoreses with inserts:
- 1.1% low melt-agarose in 0.5x TBE-buffer was prepared and cooled down to 50°C
- The gel-trayer was cleaned with ethanol, a comb with 10 slots was placed and 100 ml of the previous gel was poured into the tray.
- After solidification of the agarose the comb was removed and the slots were filled with TBE-buffer.

- The gel strips were placed into the bottom of slots, the TBE-buffer was removed carefully by suckling out with a tissue and the inserts were fixed by pouring 0.8% low melt agarose into each slot.
- PFGE was performed at 175V, 24h, at 14°C.
- After running time, the gel was stained in ethidium bromide and was checked by UV and photographed.

2.10 Acid tolerance test
Acid tolerance was evaluated by growing the strains in MRS broth adjusted to acidic pH 3.5 and 2 by adding HCl and nonacidified MRS broth pH 6.5 and incubating at 37°C for 90 min. Samples were collected during incubation period intervals 0, 30, 60, 90 min and plate counts were done using MRS agar and the pour plate technique.

2.11 Bile salt tolerance test
Overnight cultures were diluted into 10 ml MRS broth medium containing different concentration (0, 0.1, 0.3, 0.5, 1, and 3%) of the ox-gall bile salts, TDCA or GDCA,
respectively. Samples were incubated at 37°C and growth was measured by following the O.D$_{620\text{nm}}$ using the photometer Nanocolor 250D (Macherey-Nagel, Dürem, Germany).

2.12 Bile salt hydrolase activity assay
The *Lactobacillus* strains were tested for bile salt hydrolase activity with a plate assay on MRS agar supplemented with 0.5% sodium salts of taurodeoxycholic acid (TDCA) or 0.5% sodium salts of glycodeoxycholic acid (GDCA) (Scott, 2001). Overnight *Lactobacillus* cultures were diluted and plated onto MRS agar containing TDCA or GDCA. The plates were incubated anaerobically at 37 °C for 48h. cbh activity was indicated by deoxycholic acid precipitate around the colonies.

2.13 Antimicrobial activity test
The antimicrobial activity test was performed by an agar diffusion test. The indicator strains were subcultured in tryptic soy broth. 100µl of indicator bacteria were plated on Muller-Hinton agar plates. Plates were air dried for 15 min and discs impregnated with 10µl of lactobacilli cultures or 30 µl of lactobacilli spent medium were positioned on them. After incubation at 37°C for 24 hrs, the diameter of inhibition zones was measured. (Chang et al., 2001)

2.14 Antibiotic susceptibility test:
Antibiotic susceptibility tests of *Lactobacillus* sp. were performed using the disk diffusion method according to the National Committee for Clinical Laboratories Standard (NCCL1997). Overnight cultures were prepared in MRS broth and freshly diluted 1:10 in MRS broth; 0.1ml of each diluted culture was plated onto Mueller-Hinton agar plates (Chang et al., 2001) as standard medium for diffusion tests. The plates were allowed to stand for 30min at RT prior to dispensing antibiotic-containing discs using the Oxoid disc dispensing system. The plates were incubated anaerobically at 37°C for 24h. Results (average of two determinations) were expressed in terms of resistance (R); moderate susceptibility (MS) and susceptibility (S); according to the interpretative standards described by Charteris et al., (1998) (Table 6).
Table 6: Antimicrobial agents and associated interpretative zone diameters for disc diffusion antibiotic susceptibility testing

<table>
<thead>
<tr>
<th>Group</th>
<th>Antimicrobial agents</th>
<th>Interpretative zone diameters (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Name</td>
<td>disc conc. (µg)</td>
</tr>
<tr>
<td>I- Inhibitors of cell wall synthesis</td>
<td>Ampicillin</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Cefotaxime</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Vancomycin</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Methicillin</td>
<td>5</td>
</tr>
<tr>
<td>II- Inhibitors of protein synthesis</td>
<td>Gentamycin</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Kanamycin</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Streptomycin</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Tetracycline</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Chloramphenicol</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Erythromycin</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Lincomycin</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Neomycin</td>
<td>30</td>
</tr>
<tr>
<td>III- Inhibitors of nucleic acid synthesis</td>
<td>Ofloxacin</td>
<td>5</td>
</tr>
</tbody>
</table>

* Susceptibility expressed as resistance (R); moderate susceptibility (MS) or susceptibility (S)

2.15 Polymerase chain reaction (PCR)

Polymerase chain reaction process was performed using a thermocycler (Mastercycler 5333) Eppendorf AG- Germany. All primers used were listed in Table 7 and all PCR programs were listed in Table 8.

2.15.1 PCR reaction:
- 26 µl deionised water
- 1µl (10 pmol) primer1
- 1µl (10 pmol) primer1
- 20µl PCR master-mix

2.15.2 Purification of PCR product:
All PCR products were purified by QIA quick spin kits pursued from QIAGEN GmbH – Germany, before using in ligation reactions.
## Table 7: List of primers used

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>$T_m$ (°C)</th>
<th>Target Gene</th>
<th>Primer sequence (5’ 3’)</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>UP68</td>
<td>66.1</td>
<td>16S-23SrRNA</td>
<td>TGG CTC AGA TTG AAC GCT GGC GGC</td>
<td>2400</td>
<td>Vaneechoutte et al., 1992</td>
</tr>
<tr>
<td>UP69</td>
<td>66.8</td>
<td>DG74</td>
<td>CCT TTC CCT CAC GGT ACT GGT</td>
<td>1600</td>
<td>Greisen et al., 1994</td>
</tr>
<tr>
<td>DG74</td>
<td>60.3</td>
<td>16S rRNA</td>
<td>AGG AGG TGA TCC AAC CGC A</td>
<td></td>
<td>Carroll et al., 2000</td>
</tr>
<tr>
<td>16SF</td>
<td>58.8</td>
<td>cbh1</td>
<td>TGG GAG AGT TTG ATC CTG GCT C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cbh1</td>
<td>61.9</td>
<td>cbh</td>
<td>GAA GAA TTC GCT ATT GAA GCA ATC ACG</td>
<td>1392</td>
<td>This study*</td>
</tr>
<tr>
<td>cbh2</td>
<td>61.9</td>
<td>hol-lys1</td>
<td>GAA GAA TTC AAA CCC AAC TTA CCA TCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hol-lys1</td>
<td>58.4</td>
<td>hol-lys2</td>
<td>CGC AAG AAG GTT AAA CGG TTA G</td>
<td>1477</td>
<td>This study*</td>
</tr>
<tr>
<td>hol-lys2</td>
<td>59.8</td>
<td>LactoF</td>
<td>TGG AAA CAG RTG CTA ATA CCG</td>
<td>231-233</td>
<td>Byun et al., 2004</td>
</tr>
<tr>
<td>LactoF</td>
<td>56.9</td>
<td>LactoR</td>
<td>GTC CAT TGT GGA AGA TTC CC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LactoR</td>
<td>57.3</td>
<td>LbLMA1</td>
<td>CTC AAA ACT AAA CAA AGT TTC</td>
<td>1600</td>
<td>Duberner et al., 2002</td>
</tr>
<tr>
<td>LbLMA1</td>
<td>50.1</td>
<td>Im26-f</td>
<td>GAT TCT GGC TCA GGA TGA ACG</td>
<td>1417</td>
<td>Kaufmann et al., 1997</td>
</tr>
<tr>
<td>Im26-f</td>
<td>59.8</td>
<td>Im3-r</td>
<td>CGG GTG CTN CCC ACT TTG ATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Im3-r</td>
<td>57.6</td>
<td>St1</td>
<td>TTA TTT GAA AGG GGC AAT TGC T</td>
<td>279</td>
<td>Furet et al., 2004</td>
</tr>
<tr>
<td>St1</td>
<td>54.7</td>
<td>St2</td>
<td>GTG AAC TTT CCA CTC TCA CAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>St2</td>
<td>57.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*R* = A, G  
*Primers were designed using OMIGA sequence analysis or DS gene software. All primers were delivered from MWG –biotech company Germany.
Table 8: PCR programs used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>cbh1/ cbh2</th>
<th>hol-lys1/ hol-lys2</th>
<th>Up68/ Up69</th>
<th>DG74/16SF</th>
<th>Im26-f/Im3-f</th>
<th>LactoF/ LactoR</th>
<th>LactoF/ LbLMA</th>
<th>St1/ St2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
<td>94°C</td>
<td>93°C</td>
<td>93°C</td>
<td>94°C</td>
<td>95°C</td>
<td>95°C</td>
<td>94°C</td>
</tr>
<tr>
<td></td>
<td>2 min</td>
<td>2 min</td>
<td>5 min</td>
<td>5 min</td>
<td>5 min</td>
<td>15 min</td>
<td>5 min</td>
<td>5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>94°C</td>
<td>92°C</td>
<td>92°C</td>
<td>94°C</td>
<td>95°C</td>
<td>95°C</td>
<td>94°C</td>
</tr>
<tr>
<td></td>
<td>30 sec</td>
<td>30 sec</td>
<td>1 min</td>
<td>1 min</td>
<td>1 min</td>
<td>15 sec</td>
<td>20 sec</td>
<td>1 min</td>
</tr>
<tr>
<td>Annealing</td>
<td>57°C</td>
<td>54°C</td>
<td>58°C</td>
<td>55°C</td>
<td>60°C</td>
<td>64°C</td>
<td>54°C</td>
<td>55°C</td>
</tr>
<tr>
<td></td>
<td>40 sec</td>
<td>40 sec</td>
<td>1:30 min</td>
<td>1 min</td>
<td>2 min</td>
<td>1 min</td>
<td>1 min</td>
<td>2 min</td>
</tr>
<tr>
<td>Elongation</td>
<td>72°C</td>
<td>72°C</td>
<td>72°C</td>
<td>72°C</td>
<td>72°C</td>
<td>72°C</td>
<td>72°C</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>1:20</td>
<td>2:20 min</td>
<td>2 min</td>
<td>2 min</td>
<td>30 sec</td>
<td>2 min</td>
<td>2 min</td>
</tr>
<tr>
<td>Total cycles</td>
<td>31</td>
<td>31</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>39</td>
<td>31</td>
<td>40</td>
</tr>
<tr>
<td>Final elongation</td>
<td>72°C</td>
<td>72°C</td>
<td>72°C</td>
<td>72°C</td>
<td>72°C</td>
<td>72°C</td>
<td>72°C</td>
<td>72°C</td>
</tr>
<tr>
<td></td>
<td>10 min</td>
<td>10 min</td>
<td>10 min</td>
<td>10 min</td>
<td>10 min</td>
<td>3.5 min</td>
<td>7 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Holding temperature</td>
<td>4°C</td>
<td>4°C</td>
<td>4°C</td>
<td>4°C</td>
<td>4°C</td>
<td>4°C</td>
<td>4°C</td>
<td>4°C</td>
</tr>
</tbody>
</table>
2.16 Molecular weight markers

- \( \lambda \)StyI DNA containing the molecular weight bands of 19329, 7743, 6223, 4254, 3472, 2690, 1882, 1489, 925, 421 bp.
- DNA 100 bp ladder (Fermentas) was also used as a marker.

2.17 AGAROSE GEL ELECTROPHORESIS

Analysis of chromosomal and plasmid DNAs, PCR amplicons, restriction enzymes digests, was performed by agarose gel electrophoresis. Agarose gel (0.6% - 1.5%) was prepared by dissolving the appropriate quantities of agarose in 1x TAE buffer (pH 8.0) in a microwave oven. Ethidium bromide stock solution was added directly to molten agarose solution at a concentration of 0.5 \( \mu \)g/ml before pouring the gel. Before loading the samples were mixed with 6x loading buffer. After electrophoresis DNA fragments in the agarose gel containing ethidium bromide were visualized and photographed using a Gel Doc™ XR system and quantity one software (Bio Rad).

2.17.1 Reagents

**Tris acetate-EDTA (TAE) buffer (pH 8)**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/100 ml (10X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>48.4</td>
</tr>
<tr>
<td>Na(_2)-EDTA</td>
<td>7.45</td>
</tr>
<tr>
<td>NaCH(_3)COOH x 3H(_2)O (Na-acetat)</td>
<td>6.9</td>
</tr>
</tbody>
</table>

pH adjusted to pH 8.0 with acetic acid.

**Sample buffer**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>g/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5% bromophenol blue</td>
<td>0.05</td>
</tr>
<tr>
<td>66% glycerol (w/v)</td>
<td>47.6 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>52.4 ml</td>
</tr>
</tbody>
</table>

**Staining solution**

Working solution of EtBr was prepared by adding 1 ml of stock solution of EtBr (0.5 mg/ml) to one litre of distilled water to give a final concentration of 0.5 \( \mu \)g / ml.

2.18 Molecular cloning and other gene manipulation techniques

All the molecular biology techniques which include ligation, cloning, restriction digestion, purification of inserts by DNA gel extraction kits (MinElute, Qiagen), PCR
Materials and Methods

product purification (MinElute, Qiagen), plasmid minipreps, construction of plasmids etc. were performed according to protocols given by Sambrook et al., (2001) or the instructions of manufacturers.

2.19 Transformation

2.19.1 Preparation of competent cells of E. coli
Competent cells of E. coli were prepared according to Sambrook et al., (2001) with modifications as follows:
- 5 ml of overnight culture were diluted in fresh 250 ml LB broth in a 1L flask. The culture was incubated at 37°C with vigorous shaking on a rotary shaker until OD at 620 nm reached 0.3-0.4.
- The cells were harvested by centrifugation at 8,000 rpm for 10 min at 4°C. All other steps were carried out aseptically on ice. The medium was poured off and the pellet was resuspended in ice-cold sterile d. d. water.
- The cells were recovered by centrifugation at 8,000 rpm for 10 min at 4°C and resuspended in 400 ml cold 10% glycerol, then collected by centrifugation at 8,000 rpm for 10 min at 4°C.
- The cells pellet was resuspended in ice cold 20 ml GYT medium (10% glycerol + 0.125% yeast extract + 0.25 tryptone), harvested by centrifugation and resuspended in 1 ml GYT, then divided in 40 µl aliquots in eppendorf tubes and frozen directly in liquid nitrogen. Finally, the cells were stored in at -80°C for further use.

2.19.2 Electroporation of E. coli (easy pores) with a Gene pulser (BioRad)
- 40 µl of competent E. coli easy pores cells were mixed with 2µl ligation mix (DNA) in a microcenterfuge tube placed into ice bath
- The cell-DNA mixture was transferred to a cold 0.1 cuvette and placed in the Gene Pulser cuvette holder.
- Cells were treated with one pulse with the Gene Pulser set at 1.25kV and 25µlF. The pulse controller was set at 200 Ω.
- The cells were immediately diluted with 950µl of SOC medium, then transferred to a test tube and incubated at 37°C for 1 hr.
- The cells were plated onto LB agar medium containing 100µg Ampicillin (Amp) 120µg IPTG and 40µg x-gal per ml.
- The plates were incubated at 37°C overnight.
- Transformants were identified by blue/white selection.
2.19.3 Transformation using heat shock (e.g. Nova-Blue)

- 5 µl of the ligated DNA was added to 100 µl of competent cells and mixed gently by flicking. Positive control was also set up to check for transformation efficiency. The mix was incubated on ice for about 30-45 min.

- The tubes were then transferred to water bath maintained at 42°C and held for exactly 90 sec. The tubes were rapidly transferred to ice and allowed to chill for 1-2 min.

- To the above mix, 900 µl of LB broth was added and mixed.

- The tubes were incubated at 37°C for one hour on a rotary shaker to allow the bacteria to recover and express the antibiotic resistance. After incubation, 200 µl of transformation mix were spread evenly on LB agar plates with ampicillin (100 µg / ml) and incubated at 37°C for 16-20 hrs.

- The transformants obtained on LB agar plates with ampicillin were inoculated into LB broth containing ampicillin and incubated in shaking incubator at 37°C for 12-16 hrs after which the cells were harvested and used for plasmid isolation.

2.19.3.1 Reagents for blue and white screening

X-Gal (5-bromo 4-chloro-3-indolyl-β-D-galactopyranoside)

0.5 g dissolved in 5 ml of DMF (dimethyl formamide).

0.1 M IPTG (Isopropyl-1-thio-β-D-galactopyranoside)

2.20 Electroporation of Lactobacillus strains

Electroporation of L. casei and L. gasseri was performed according a protocol of B. Henrich (personal communication) and method of Varmanen et al., 1998 was used for L. rhamnosus with modifications as summarized in following flow charts.
2.20.1 Preparation of *L. casei* competent cells

An over-night culture diluted (1:10) in MRS

Incubation at 37°C until O.D_{620} 2.2-2.8

Centrifugation (8000 rpm / 4°C)

2X washing with the same volume cold EPB buffer*

Centrifugation (8000 rpm / 4°C)

Re-suspending the pellet in 1/6 volume cold EPB buffer*

Distribution 100ul in cold 1.5 ml Epp. caps

Frozen in liquid Nitrogen and immediately storage at (-80°C)

*EPB buffer:

0.5 M Sucrose

1 mM MgCl₂

7 mM potassium phosphate buffer pH7.2
2.20.2 Electroporation of *L. casei*

100μl *L. casei* competent cells + 5-10μl dialysed plasmid DNA

- Electroporation 1.25 KV, 25μF, 200Ω / 0.1cm
- 900μl GMRS medium contains 3ng Em/ml
- Incubation at 37°C for 3-5 hrs
- Plating onto MRS agar medium contains 10μg/ml Em
- Anaerobic incubation at 37°C / 48-72 hrs

*Note: In the case of using the chloramphenicol, less incubation time was used.*
2.20.3 Preparation of *L. gasseri* competent cells

2 ml over-night culture + 100 ml pre-warmed GMRS

↓

Incubation at 37°C until \( O.D_{620} \) 0.5

↓

Centrifugation (8000rpm / 4°C)

↓

2X washing with 100 ml cold 3.5X SMEB buffer*

↓

Centrifugation (8000rpm / 4°C)

↓

Re-suspending in 5 ml cold 3.5X SMEB buffer*

↓

Distribution 100 μl in cold 1.5 ml Epp. caps

↓

Freezing in liquid N2 and immediately storage at (-80°C)

*3.5X SMEB buffer:

952 ml 1M sucrose

3.5 ml 1M MgCl\(_2\)

44.5 ml H\(_2\)O
2.20.4 Preparation of *L. rhamnosus* competent cells

2 ml over-night culture + 100ml pre-warmed GMRS supplemented with 2% glycine

Incubation at 37°C until OD\textsubscript{620} 0.3-0.4

Centrifugation (8000rpm at RT)

2X washing with 100ml electroporation buffer*

Centrifugation

Re-suspending in 1 ml of the same buffer* and place in ice

Distribution 100μl in cold 1.5 ml Epp. caps

Freezing in liquid N2 and immediately storage at (-80°C)

* **Electroporation buffer:**

0.5 M Sucrose

7mM potassium phosphate (pH7.4)

1mM MgCl\textsubscript{2}
2.20.5 Electrotransformation of \textit{L. gasseri} and \textit{L. rhamnosus}

100\mu l \textit{L. rhamnosus} competent cells + 10\mu l dialysed plasmid DNA

\hspace{1cm} \begin{align*} & \text{1min in ice} \\ & \text{Electroporation (1 KV - 0.1cm pre-cooled cuvette - 25\mu F -200}\ \Omega \end{align*}

\hspace{1cm} \begin{align*} & \text{1.5ml GMRS medium contains 3ng/ml Cm or 3ng/ml Em} \\ & \text{Incubation at 37\degree C for 3-4 hrs} \\ & \text{Plating with 3ml soft agar onto GMRS agar medium containing 5 -10\mu g/ml Cm or Em} \\ & \text{An aerobic incubation at 37\degree C / 48-72 hrs} \end{align*}

Note: In the case of using the chloramphenicol, less incubation time was used

2.21 Analysis of Transformants

\textit{E. coli} or \textit{Lactobacillus} transformants isolated from respective growth media plates with appropriate antibiotic were subjected to plasmid minipreps. The minipreps were analysed on agarose gel for the recombinant plasmid vector and the presence of desired insert in the vector was checked by digestion and nucleotide sequencing (whenever necessary).

2.22 Segregational stability of the recombinant plasmids

The stable maintenance of the plasmids pL11 and pL14 in \textit{Lactobacillus} ssp under non-selective conditions was determined. Cultures were transferred daily into fresh MRS broth without antibiotics. Every 7 days cultures were plated onto MRS agar with
and without antibiotics. The differences between the total count on MRS agar and on MRS containing antibiotics indicated the stability of plasmids.

2.23 Nucleotide sequencing

Nucleotide sequencing of the clones produced in this study was done at MWG Biotech, German, using universal sequencing primers. Sequence analysis was carried out by using Molecular Biology softwares such as DNASIS, DS gene etc.

2.24 Construction of clones

Constructions of various recombinant plasmids are described in Chapter III (Results and Discussion).

2.24.1 Cloning and Expression of the cbh gene in E. coli

PCR amplicon of the cbh gene was purified and cloned into pGEM-T-easy vector as a PCR fragment directly and after digestion with EcoRI, and into pBluescriptII SK+ (pBSK). The ligation mixes were transformed into electro-competent cells E. coli easy pores. Transformants were detected by blue/white screening.

2.24.2 Blunting and dephosphorylation of DNA

The DNAs digested with restriction enzymes were blunted using T4 polymerase as shown in Table 9 and the blunted vectors were dephosphorylated using SAP (Shrimp Alkaline phosphatase-Table 10). All reactions were carried out according to instructions of suppliers. (Fermentas or Biolabs)

| Table 9: Blunting of DNAs for blunt–end ligations

<table>
<thead>
<tr>
<th>Blunting Reaction</th>
<th>Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vector</td>
</tr>
<tr>
<td>5X Reaction buffer</td>
<td>4µl</td>
</tr>
<tr>
<td>DNA</td>
<td>10µl</td>
</tr>
<tr>
<td>2mMdNTP mix</td>
<td>1µl</td>
</tr>
<tr>
<td>T4 DNA polymerase</td>
<td>1µl</td>
</tr>
<tr>
<td>D. D. water</td>
<td>4µl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20µl</strong></td>
</tr>
</tbody>
</table>
This reaction mix was incubated at room temperature for 5 min and the reaction was stopped by heating at 70°C for 10 min. For blunting of the PCR product the mixture was incubated at 37°C for 5 min then, at 12°C for 15 min and the reaction was stopped by heating at 75°C for 10 min.

Table 10: Dephosphorylation of blunted vectors

<table>
<thead>
<tr>
<th>Dephosphorylation Reaction</th>
<th>Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction buffer</td>
<td>5µl</td>
</tr>
<tr>
<td>Blunted DNA (vector)</td>
<td>28µl</td>
</tr>
<tr>
<td>SAP (Shrimp Alkaline phosphatase)</td>
<td>1µl</td>
</tr>
<tr>
<td>D. D. water</td>
<td>16µl</td>
</tr>
<tr>
<td>Total</td>
<td>50µl</td>
</tr>
</tbody>
</table>

This reaction mix was incubated at 37°C for 30 min and the reaction was stopped by heating at 65°C for 15 min.

2.25 Induction of Lysogenic Strains

*Lactobacillus* strains propagated in MRS medium at 37°C were treated with mitomycin C (MC) at different concentrations. The lysates were filtered through 0.45µm Millipore membranes and checked with electron microscope.

2.26 UV Induction

The cells were suspended in 0.1 M MgSO₄ and pumped through a quartz tube (internal diameter 1.3 mm length 75cm) placed under a laboratory UV lamp (*Neve et al.*, 1998).

2.27 Isolation of page particles

The bacteriophage particles from strain *L. gasseri* BfEL 92075 were isolated from one liter of MC-induced lysate and purified through CsCl density gradient centrifugation according to procedure described by *Sambrook et al.*, (2001).
2.28 Phage morphology

Dialyzed phage concentrates were stained with 2% (w/v) uranyl acetate as described before (Neve et al., 1989). Electron micrographs were taken in an EM300 electron microscope (Fei-philips, Eindhoven, Netherlands) at an accelerating voltage of KV (Neve and Teuber, 1989).

2.29 Phage and bacterial DNA purification

Phage DNA was extracted from purified phage particles by proteinase K and phenol-chloroform method of Sambrook et al., (2001). Isolation of chromosomal bacterial DNA was performed as described above.

2.30 Restriction enzyme analysis

Cleavages with restriction enzymes were carried out according to instructions of suppliers (Fermentas or Biolabs). Digests were loaded onto 0.6% and 1.5% agarose gels. The gel pictures were applied to computer software Quantity One, version 4.5.2 (BioRad) and fragments size were estimated. In addition, heating of digests at 74°C for 5 min was done according to Quiberoni et al., (2003)

2.31 Southern blot and DNA-DNA hybridization

Southern blot and hybridization were performed after agarose gel electrophoreses of DNA digests according to a modified protocol of the DIG-system, Roche, Germany.

2.31.1 Reagents for Southern hybridization

DIG-DNA labeling kit (Roche)

- Hexanucleotide mix (10X conc.)
- DIG DNA labeling mix (10X conc.)
- Klenow enzyme

250 mM HCl solution

- 21 ml 37 °C HCl, up to 1000 ml D.D.W
- Autoclaved at 121 °C for 20 min

Blotting transfer buffer

- 0.4 M NaOH
Materials and Methods

**SSC-buffer**
- 3 M NaCl
- 300 mM Na-citrate
- pH 7.0
- Autoclaved

**20% SDS:** pH 7.2 (adjusted with conc. HCl).

**Wash buffer (room temperature)**
- 2X SSC
- 0.1% SDS

Five ml of 20% SDS was added to 100 ml of 20X SSC and the volume made up to 1000 ml with sterile D.D.W

**Wash buffer (68°C):**
- 0.1% SSC
- 0.1% SDS

Five ml of 20% SDS was added to 5 ml of 20X SSC and the volume made up to 1000 ml with D.D.W

**4 M LiCl:** Lithium chloride (16.96 g) was dissolved in a final volume of 100 ml D.D.W and autoclaved.

**200 mM EDTA:** EDTA (7.45 g) was dissolved in 80 ml D.D.W. The pH was adjusted to 8.0 and the volume was completed up to 100 ml. The solution was autoclaved.

**Buffer 1 (10X):** 0.1 M Malic acid; 0.15 M NaCl; pH 7.5.

Malic acid (116.07 g) and NaCl (87.66 g) were dissolved in 800 ml D.D.W. The pH was adjusted to 7.5 with NaOH pellets. The volume was made up to 1000 ml with D.D.W and the solution was autoclaved.

**Buffer 1 (1X):** Buffer 1 (10X) and distilled water were mixed in the ratio of 100:900.

**Wash Buffer:** Three ml of Tween 20 was made up to 1000 ml with Buffer 1 (1X).

**Buffer 2 (10X):** Fifty grams of blocking powder was dissolved in 450 ml buffer 1 (1X) at 70°C for 1 h. The volume was made upt0 500 ml with buffer 1 (1X). The solution was autoclaved and stored at −20°C in the ratio 100:900.

1 M NaCl (autoclaved).

1 M Tris (pH 9.5, autoclaved).

**Buffer 3:** Solutions of 1 M NaCl and 1 M Tris (pH 9.5) were mixed in the ratio 100:900. Buffer 3 was freshly prepared and not autoclaved.

DIG Easy Hybridization Solution (Roche).
CSPD (Roche): Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2’-(5’-chloro) tricyclo decan}-4-yl) phenyl phosphate.

Anti-Digoxigenin-AP (Polyclonal sheep anti-digoxigenin, Fab-fragments, conjugated to alkaline phosphatase; Roche)

2.3.1.2 Blotting

The southern blotting was performed using a modified protocol described in the DIG System, Roche. The gel was immersed in 250 mM HCl with slight shaking for only 5 min and washed in distilled water for 1 min. Blotting was done overnight with freshly prepared 0.4 M NaOH. The membrane was dried on filter paper and DNA was fixed by baking at 120°C for 30 min. The membrane was stored at 4°C until further use.

2.3.1.3 Labeling of DNA probes

For labeling the DNA to be used as probes in hybridization experiments, the DIG DNA labeling Mix (10X conc., Roche Diagnostics, GmbH) was used.

2.3.1.3.1 Labeling procedure – DIG-labeled DNA probes.

One to three micrograms of DNA in a maximum of 15 µl volume was denatured by heating for 10 min followed by chilling quickly on ice/salt mixture.

The following were added to the freshly denatured DNA on ice:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexanucleotide mix, 10X conc.</td>
<td>2 µl</td>
</tr>
<tr>
<td>DIG DNA labeling mix, 10X conc.</td>
<td>2 µl</td>
</tr>
<tr>
<td>Klenow enzyme</td>
<td>1 µl</td>
</tr>
<tr>
<td>Sterile D.D.W</td>
<td>Volume made up to 20 µl</td>
</tr>
</tbody>
</table>

- The contents were mixed and spun shortly in a Microcentrifuge.
- The contents were incubated overnight at 37°C.
- The reaction was stopped by adding 2 µl of 200 mM EDTA (pH 8.0).
- The DNA was reprecipitated by adding 2 µl of 4 M LiCl followed by the addition of 60 µl of ice-cold ethanol. The samples were stored at -20°C for 2 h or overnight or at – 70°C for 30 min.
- The contents were centrifuged at 4°C for 15 min (14,000 rpm).
- The supernatant was discarded and the pellet (invisible) washed with 100 µl of 70% ethanol.
- The DNA was air-dried, dissolved in TE (pH 8.0) and stored at −20°C until further use.

### 2.3.1.3.2 Estimation of the efficiency of labeling

The amount of labeled probes prepared by random priming method was estimated by comparing method. In this method, a series of dilutions prepared from the DIG-labeled probe were spotted directly on a membrane Table 11. For comparison, known concentrations of a DIG-labeled control nucleic acid were spotted in parallel on the same membrane.

**Table 11: Preparation of serial dilutions of probe and control DNA**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Procedure</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (1:20)</td>
<td>2 µl DNA + 38 µl TE-buffer, pH 8.0</td>
<td>0.25 ng</td>
</tr>
<tr>
<td>2 (1:200)</td>
<td>5 µl (1:20) + 45 µl TE</td>
<td>25 pg</td>
</tr>
<tr>
<td>3 (1:2000)</td>
<td>5 µl (1:200) + 45 µl TE</td>
<td>2.5 pg</td>
</tr>
<tr>
<td>4 (1:20000)</td>
<td>5 µl (1:2000) + 45 µl TE</td>
<td>0.25 pg</td>
</tr>
<tr>
<td>5 (1:200000)</td>
<td>5 µl (1:20,000) + 45 µl TE</td>
<td>0.025 pg</td>
</tr>
<tr>
<td>6 (1:2000000)</td>
<td>5 µl (1:20,000) + 45 µl TE</td>
<td>0.0025</td>
</tr>
</tbody>
</table>

One microlitre of each dilution was spotted in a line on a nylon membrane. In parallel, the corresponding dilutions of control DNA were spotted.

The DNA was fixed by baking at 120°C for 30 min in a hot air oven.

### 2.3.1.3.3 Detection of labeled DNA

The membrane was incubated for a short time in buffer 1 containing 0.3% Tween 20.

**Blocking:** The membrane was shaken at room temperature in buffer 2 for 10 min.

**Incubation with antibody (anti-digoxigenin):**

Five milliliters of buffer 2 was taken in a shallow disposable polystyrene tray. One microlitre of antibody (anti-digoxigenin) was added and mixed. The membrane was submerged fully in this antibody solution and manually shaken for 10 min.

**Removal of excess antibody:**

The excess antibody was removed by shaking the membrane vigorously in 50 ml buffer 1, containing Tween 20 (0.3%), for 5 min. This washing was repeated twice.

**Incubation with CSPD:**
Membrane was laid in a fresh hybridization bag. Alternatively, two adjacent ends of a pair of rectangular transparent sheets (larger size than the membrane) were heat-sealed and the membrane placed inside. Five microlitre of CSPD was added to 500 µl of buffer 3 and mixed. The CSPD solution was added to the membrane and spread uniformly by stretching over the hybridization bag. The membrane (in hybridization bag) was incubated for 5 min at room temperature.

The membrane was removed from the hybridization bag and dried on a filter paper. The membrane was sealed in a fresh hybridization bag or between two transparency sheets and incubated at 37°C for 15 min.

**Detection of hybridization signal:** The membrane was placed over an X-ray film in dark and left for 30 min. Subsequently, the X-ray film was developed by shaking in a developing solution for 2 to 3 min, washed with distilled water and fixed by shaking in a fixing solution for 2 to 3 min (or until bands appear). The processed X-ray film was later washed with tap water and dried.

2.31.4 Hybridization of probe to DNA

2.31.4.1 Pre-hybridisation:
The hybridization buffer with DIG (20 ml) was forewarmed at 42°C.

2.31.4.2 Denaturation of probe:
The probe was denatured by cooking for 10 min and chilled immediately in ice bath for 2 to 5 minutes. Subsequently, the denatured probe was mixed with the hybridization buffer (60 ml). The nylon membrane was placed in the glass bottle with the hybridization buffer and incubated overnight at 42°C in a hybridization chamber.

2.31.4.3 Washing
The nylon membrane was removed from the hybridization chamber and washed in wash buffer (2XSSC, 0.1% SDS) at room temperature for 5 minutes. This step was repeated twice with fresh wash buffer.

2.31.4.4 Stringent washing
The membrane was washed for 15 min in wash buffer (0.1XSSC, 0.1% SDS) at 68°C by shaking. This step was repeated twice. The membrane was equilibrated for 15 min in buffer 1 containing 0.3 per cent Tween-20.
2.31.4.5  Blocking

The membrane was incubated in buffer 2 with shaking at room temperature for 2 hr.

2.31.4.6  CSPD incubation

The membrane was laid in a hybridization bag. Alternatively, two adjacent sides of a pair of rectangular transparency sheets larger than the size of membrane were heat-sealed and the membrane placed inside. 20µl of CSPD was mixed in 2 ml of buffer 3. The CSPD solution was added into the hybridization bag and was evenly spread over the membrane by stretching over the hybridization bag.

2.31.4.7  Antibody incubation

Two microlitre of antibody was mixed with 20 ml of buffer 2 in a disposable polystyrene tray. The membrane was placed in the antibody solution and shaken for 30 min.

2.31.4.8  Removal of excess antibody

The membrane was washed in 500 ml of buffer 1 containing 0.3 per cent Tween 20 with vigorous shaking for 15 min. This step was repeated with fresh buffer 1 containing 0.3 per cent Tween 20. Membrane was equilibrated in buffer 3 for 5 min.

2.31.4.9  CSPD incubation

The membrane in the hybridization bag was incubated at room temperature for 5 min and then dried on a filter paper. The membrane was sealed in a hybridization bag or between a pair of transparency sheets and incubated at 37°C for 15 min.

2.31.4.10  Detection of hybridization signal

The membrane was placed over an X-ray film in dark and left for 30 min or more. Subsequently, the X-ray film was developed by shaking in a developing solution for 2 to 3 min, washed with distilled water and fixed by shaking in a fixing solution for 2 to 3 min (or until bands appear). The processed X-ray film was later washed with tap water and dried.
2.32 SDS-PAGE

SDS-PAGE of phage proteins was performed as previously described by Fayerd et al., (1993) with some modifications. SDS gel electrophoresis for total protein of bacterial cells was performed using the discontinuous buffer system of Laemmli, (1970). The samples were subjected to electrophoroses in 12% or 15% polyacrylamide gel at 10-15 mA and stained with commassie brilliant blue R250 (Fluka).

2.32.1 Reagents

TES
- 1 ml 10X TE
- 4 ml H$_2$O
- 5 ml 1M sucrose

Lysozyme
- 50 mg/ml

Mutanolysin
- 5000 U/ml

Solution A
- 29% Polyacrylamide
- 1% N,N'-bis-methylene acrylamide
- Ammonium per sulfate 10%
- 1 M Tris HCl pH (8.8)
- 1.5 M Tris HCl pH (6.8)
- 10% SDS
- TEMED

Sample buffer
- 0.0625 Tris HCl pH (6.8)
- 2% SDS
- 10% Glycerol
- 5% 2-Mercaptoethanol
- 0.001% Bromophenol blue dye

10X Running buffer (pH 8.3)
- Tris HCl 30.25 g
- Glycine 144.13 g
- SDS 10 g
Materials and Methods

Distilled water up to 1000 ml

**Destaining Solution**

- Methanol 300 ml
- Acetic acid glacial 150 ml
- Double distilled water 1550 ml

### 2.32.2 Procedure

#### 2.32.2.1 Preparation of phage extracts

Dialysed phage concentrates were disintegrated by boiling for 10 min in sample buffer (Laemmli, 1970)

#### 2.32.2.2 Preparation of cell extracts

An overnight culture was inoculated into fresh medium and grown to an OD_{620} of 0.6 to 0.8 (3-4 h). The cells were collected and weighed. Approximately 250 mg of cells were suspended in 100 µl TES with 20 µl lysozyme and 5 µl mutanolysin and incubated at 37 °C for 30 min. SDS (5-10 µl) was added and the contents were mixed until they appeared clear. The contents could be stored at −20 °C for 1-2 days.

#### 2.32.2.3 Preparation of the SDS gel

The resolving gel (12% or 15%) was prepared by adding the ingredients as follows in Table 12 for 50 ml gel:

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>12%</th>
<th>15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
<td>20 ml</td>
<td>25 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>16.5 ml</td>
<td>11.5 ml</td>
</tr>
<tr>
<td>1.5 M Tris HCl (pH 8.8)</td>
<td>12.5 ml</td>
<td>12.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>20 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td>Ammonium per sulphate</td>
<td>0.5 µl</td>
<td>0.5 µl</td>
</tr>
</tbody>
</table>

The ingredients were mixed, taking care to avoid bubble formation, and poured into the gel assembly quickly. Water was carefully laid on top of the gel and the gel was allowed to polymerize for 40 min. The water was later drained before adding the sticky gel solution (7%). The 7% gel was prepared as shown in Table 13.
Table 13: Preparation of the SDS sticky gel (7%)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Amounts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
<td>4.5 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>12.5 ml</td>
</tr>
<tr>
<td>1.5 M Tris HCl (pH 8.8)</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>20 µl</td>
</tr>
<tr>
<td>Ammonium per sulphate (APS)</td>
<td>0.2 µl</td>
</tr>
</tbody>
</table>

The sticky gel solution was poured over the resolving gel as soon as possible and a comb was fixed in the sticky gel. The gel was allowed to set and stored refrigerated until further use.

2.32.2.4 Preparation of sample

Ten microlitres of 4x sample buffer was added to 30 µl of the cell extract and the samples were cooked by placing in boiling water for 4 min.

2.32.2.5 Running of sample on SDS-PAGE

After removal of the comb, the wells were filled with 1x running buffer. Samples (up to 25 µl) were loaded into the wells. Electrophoresis was run at 10 mA until the sample entered the resolving zone and then the current was increased to 15 mA. The electrophoresis was stopped as soon as the sample front reached the end of the gel.

2.32.2.6 Staining of the gel

The gel was carefully removed and stained in Coomassie brilliant blue solution for 5-6 h.

2.32.2.7 Destaining of gel

Excess dye was removed from the gel by repeated washings in destaining solution, until clear bands appeared.
2.33 Autolysis in buffer system

Autolysis in buffer system was performed as previously described (Niiskasaari, 1989; Yokoi et al., 2004). Overnight cultures were inculcated into fresh MRS medium and incubated at 37°C. Bacterial cells were harvested at OD$_{620}$ 1.2 and washed once with Ringer’s solution. The cells were resuspended in 10mM Tris pH 7 and incubated at 37°C. Lysis was monitored by measuring the OD using Nanocolor 250 D photometer (Macherey-Nagel, Germany).

2.34 Prophage curing of *L. gasseri* BfEL92075

2.34.1 Induction of strain *L. gasseri* with MC in the presence of anti-phage serum

Overnight culture from *L. gasseri* in LMRS broth medium containing 5 % anti-phage serum was incubated at 37°C. Five ml fresh LMRS broth medium containing 5 % anti-phage serum were inoculated 1.5% with the overnight culture, incubated at 37°C until OD$_{620}$ 0.2-0.3; 5 ml fresh LMRS broth medium containing 5 % anti-phage serum and 0.2µg /ml MC were added and the sample was incubated until lysis occurred.

2.34.2 Selection of the colonies

From the lysed culture, 30µl were plated onto LMRS agar medium containing 0.1µg /ml MC and the plates were incubated anaerobically at 37°C. 25 colonies were picked up by teeth picks and streaked in parallel onto LMRS agar and LMRS agar containing 0.2µg MC/ml. The plates were anaerobically incubated at 37°C for 72h. All the eight colonies grown on the LMRS agar containing 0.2µg MC/ml were inoculated into LMRS broth supplemented with 0.2µg MC/ml and after growth regrown in LMRS broth overnight at 37°C.

2.34.3 Re-induction of derivatives

The eight derivatives of *L. gasseri* 92075 were induced with 0.2µg MC/ml. 2% overnight culture was inoculated into 5 ml LMRS and incubated at 37°C until OD$_{620}$ 0.2-0.3, then fresh 5 ml LMRS containing MC was added. The final concentration of MC was 0.2µg/ml.
2.34.4 Spot test

To confirm the successful prophage-curing a spot test with a phi Lbgas1 lysate was performed. MRS was prepared as aforementioned but without autoclaving. The medium was instead sterilized by filtration through a 0.45µm filter and kept at 4°C until use. Before use, 1% agar was added, and the agar was sterilized by boiling in microwave for 3-5 min (Lillehaug, 1997). The ready to use agar contained 10mM CaCl₂, 1% lactose and 0.2-0.25% glycine.

**Soft agar:** 100 ml MRS + 0.3 g agar were sterilized in Microwave oven for 3 min, and then CaCl₂ and glycine were added to the final concentrations 10mM and 0.2-0.25 %, respectively.

**Procedure:**
- Overnight cultures (grown anaerobically at 37°C) were diluted in fresh MRS broth (10 ml, 1%) and incubated in a water-bath at 37°C until OD₆₂₀ about 0.5.
- 0.3 ml of the growing culture, 0.1 ml 40mM CaCl₂, 0.1 ml 7.1% glycine and 0.1 ml 10% skim milk were mixed with 3 ml soft MRS agar and immediately poured onto MRS agar plates.
- 20µl of serial dilutions of dialyzed phage Lbgas1 lysate (10⁻¹ to 10⁻⁷), were spotted onto the culture soft agar layer and incubated at 37°C for 24h.
3 Chapter I

Isolation and Identification of some Lactic acid bacteria by using different Molecular biological methods

3.1 Introduction

Lactic acid bacteria (LAB) are widely distributed in the nature, occurring naturally as indigenous micro-flora in raw milk. LAB are Gram-positive bacteria that play an important role in many food and feed fermentations, especially in the production of fermented dairy products and many other foods and drinks. LAB used in the dairy industry are representatives of the genera *Lactobacillus*, *Lactococcus*, *Pediococcus* and *Leuconostoc*. The lactic acid fermentation, which these bacteria carry out, has been known for long and has been applied by humans for making different foodstuffs. For many centuries, LAB have served to provide an effective form of natural preservation. In addition, they strongly determine flavour, texture and frequently the nutritional value of food and feed products (*Bettache and Mebrouk, 2004*). They should possess stable fermentation characteristics and be resistant to bacteriophages (*Lee, 1996*). *Lactobacillus* is one of the most important genera of lactic acid bacteria (LAB), especially because members of this genus play a major role in the human and animal gastrointestinal tract, as well as in the production of many foods, feeds and beverages (*Guarneri et al., 2001*). Lactobacilli have a worldwide industrial use as starters in the manufacturing of fermented dairy products. Moreover, some *Lactobacillus* strains have probiotic characteristics and are therefore included in fresh fermented products or used as encapsulated health products, such as freeze-dried powder. The use of some *Lactobacillus* strains as probiotics is based on studies which show, that these species belong to the normal intestinal flora and that the strains have beneficial effects on human and animal health (*Marteau and Rambaud, 1993; Salminen, et. al 1996*). Probiotics are live microorganisms which, when administered in sufficient amounts, award health benefits to the host. Probiotic actions, which confer human health benefits (e.g. modulating immune system function, reducing host colonization by pathogens and enhancing lactose digestion in lactose maldigesters), have been reviewed (*Schiffrin and Blum, 2001; Marteau et al., 2002*). Previous studies have indicated that certain probiotic activities are strain-
specific (Lee et al., 1993; Gupta et al., 1996; Jacobsen et al., 1999) and thus, identification of probiotics to the strain level is essential. This conclusion is supported by FAO/WHO guidelines (http://www.fao.org/es/ESN/food/foodandfood_probio_en.stm) for the use of probiotics in food which specify that commercial probiotics be identified to the strain level. Further, the ability to identify specific probiotic strains provides manufacturers a useful quality control. Usually, the identification of lactobacilli has been based on fermentation of some sugars, morphology, and Gram staining, and these methods are still used. However, in recent years, taxonomy has changed significantly with the increasing knowledge of the genomic structure and phylogenetic relationships between Lactobacillus spp. (Klein et al., 1998; Stiles and Holzapfel, 1997; Vandamme et al., 1996). The identification of some Lactobacillus species by biochemical methods alone is not reliable (Charteris et al., 1997; Schleifer et al., 1995). Probiotic health products can contain, perhaps due to the lack of good identification methods, Lactobacillus species other than those declared on the product specifications. A rapid and reliable species-specific identification method, e.g., by PCR, is needed. Identification of lactobacilli at the strain level is important for their industrial use. Biotechnology industry needs tools to monitor, e.g., the use of patented strains or to distinguish proven probiotic strains from natural isolates in the host gastrointestinal tract (Soile et al., 1999). Modern sensitive and reliable nucleic-acid-based methods for species-specific detection of microorganisms, as 16S and 23S rDNA sequencing, are now routinely used for the identification of bacteria (Ludwig and Schleifer, 1994; Stackebrandt and Goebel, 1994). Genetic differentiation of strains belonging to a number of LAB species such as Streptococcus thermophilus (Salzano et al., 1993), Lactobacillus spp. (Dykes and von Holy, 1994; Johansson et al., 1995) Enterococcus spp. (Moschetti et al., 1995), Leuconostoc mesenteroides (Villani et al., 1997) has been effectively performed by DNA digestion with restriction enzymes, combined with pulsed-field gel electrophoresis (PFGE) or conventional electrophoresis. Molecular typing based on polymorphisms of rRNA genes and their intergenic spacer regions and facilitated by restriction analysis of these genes after amplification by polymerase chain reaction (PCR), has shown prospective as a taxonomic technique, since ribosomal DNA is both ubiquitous and highly conserved (Moschetti et al., 1997). Genotypic methods used for strain-typing are typically PCR methods e.g., Amplified Ribosomal DNA-Restricion Analysis (ARDRA) or variations of this method. In PFGE, rare-cutting
endonucleases enzymes are used and large genomic fragments are separated electrophoretically. PFGE can be used as a primary method for strain differentiation, although some probiotic strains from different sources shared highly similar PFGE patterns (Yeung et al., 2004). Aims of this study were the isolation and identification of some lactic acid bacteria from different sources of Egyptian fermented dairy products by using different methods for identification. In addition, the biodiversity of intestinal bacteria of twins babies was determined. These twins were, due to a disease of one of them, were fed in different ways. The healthy one was nourished with mother breast milk, the other one with formula milk. Influences of the different food on the intestinal microflora were analysed.

3.2 Results and Discussion

3.2.1 Isolation of lactic acid bacteria

Lactic acid bacteria were isolated from different Egyptian dairy products as Laban rayb, Kariesh and Mish cheese as well as from four faeces samples from twins babies at the age of 3 and 4 months. Isolation was performed by standard microbiological procedures (see Materials and Methods).

3.2.2 Identification of bacterial isolates from Egyptian fermented dairy products (first part)

Different identification methods were used to identify the unknown isolates from Egyptian fermented dairy products. Only four isolates out of 30 were morphologically Lactobacilli as shown by phase contrast microscopy (Fig. 3). For identification of these four lactobacilli, biochemical identification, ARDRA, PFGE, cloning and sequencing of 16S-23Sr RNA genes were used. Another strategy was performed for the identification of the rest of isolates. ARDRA technique was used to divide the isolates into groups and at least one isolate from each group was chosen for cloning and sequencing of 16S r DNA gene.

3.2.2.1 Identification of isolates using API 50 CHL system

Biochemical identification of four isolates from Egyptian cheese was performed with an API 50 CHL kit. The identification results given by API 50 CHL system version 5.0
database are shown in Table 14. For three isolates (A, C, and E), good identification levels were obtained, and identification level of one isolate (D) was regarded as acceptable due to different fermentation reactions. Isolates A, C and E were identified as *Lactobacillus brevis* with percentages of identification of 84.5% and the isolate D was identified as *Lactobacillus plantarum* with 91.5%.

![Fig. 3: Morphology of four lactobacilli isolates from Egyptian cheese](image)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Identification by API 50 CHL (identification comment)</th>
<th>% ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td><em>L. brevis</em> (good)</td>
<td>84.5%</td>
</tr>
<tr>
<td>C</td>
<td><em>L. brevis</em> (good)</td>
<td>84.5%</td>
</tr>
<tr>
<td>D</td>
<td><em>L. plantarum</em> (acceptable)</td>
<td>91.5%</td>
</tr>
<tr>
<td>E</td>
<td><em>L. brevis</em> (good)</td>
<td>84.5%</td>
</tr>
</tbody>
</table>

*a* Identification by the API 50 CHL kit and the profile status by using the API web software database.

### 3.2.3 ARDRA-PCR identification:

Identification of isolates by biochemical identification methods like (API) is for LABs with limited value, whereas ARDRA is an efficient identification method (*Vaneechoutte et al., 1992*). ARDRA is a reliable and fast method for routines
identification of strain isolates on species level with only minimal instrument equipment needs (Hoppe-Seyler et al., 2003). ARDRA was used to differentiate species of Lactobacillus. Molecular typing of the rDNA genes and the intergenic spacer region can be used as taxonomic marker since ribosomal DNA is ubiquitous and contains highly conserved sequences. In this study, ARDRA was performed for four isolates from Egyptian cheese and some Lactobacillus strains from the collection of the institute for microbiology, BfEL (Kiel, Germany), as L. gasseri BfEL92075, L. plantarum BfEL 92122, L. rhamnosus BfEL 92149. Also some Lactobacillus type and reference strains, as L. plantarum DSM 20174T, L. pastorianus DSM 20197, L. johnsonii DSM 20553, L. gasseri DSM 20243, L. zeae DSM 20178T, L. reuteri DSM 20016T, L. delb. ssp lactis BfEL 3046/62, L. delb.ssp bulgaricus ATCC 12278, L. delb.ssp lactis ATCC 10697 were used for comparison.

3.2.3.1 DNA preparation

Fig. 4 shows the electrophoresis analysis chromosomal DNA of the isolates and some of Lactobacillus type strains.

3.2.3.2 Enzymatic amplification of rDNA

For PCR, universal primers UP68/UP69 (Vaneechoutte et al. 1992) were used to amplify a fragment of approx. 2.4 kb, encompassing the 16S, the spacer region and part of the 23S rDNA. PCR was carried out in a Eppendorf Mastercycler with an annealing temperature at 58ºC (Fig. 5).
3.2.3.3 Restriction analysis of the 16S-23S rDNA amplicon

Restriction digests of the 16S-23s rDNA amplicon were carried out with restriction enzymes HaeIII and HinfI. Restriction fragment patterns were analyzed by agarose gel electrophoresis Fig 6a and b. The restriction patterns of the four isolates A, C, D and E and L. plantarum BfEL 92122 were identical to the restriction patterns of type strain L. plantarum DSM 20174\textsuperscript{T}. ARDRA also revealed that L. gasseri BfEL 92075 had the same restriction profile as type strain L. gasseri DSM 20243\textsuperscript{T}. L. rhamnosus BfEL 92149 did not correlate with any of the Lactobacillus type and reference strains in digests with HaeIII, but showed an identical pattern as type strain L. reuteri DSM 20016\textsuperscript{T} when digested with HinfI. The restriction profiles of L. delb. ssp. lactis BfEL 3046 / 62 matched those of L. delb. ssp. lactis ATCC 10697\textsuperscript{T}. L. plantarum DSM 20174\textsuperscript{T} did not match any of the other Lactobacillus type and reference strains. L. pastorianus DSM 20197, L. zeae DSM 20178\textsuperscript{T}, L. delb. ssp. bulgaricus ATCC 12278, L. delb. ssp. lactis ATCC 10697 also showed species-specific restriction pattern. Only the very related strains L. gasseri DSM 2024\textsuperscript{T} and L. johnsonii DSM 20553 showed identical restriction patterns. In conclusion, ARDRA is an accurate and reliable method capable of distinguishing among many of Lactobacillus species except for narrow related species L. gasseri and L. johnsonii.
Fig. 6: ARDRA Profiles with Hae III (A) and Hinf I (B) of some Lactobacillus isolates and type strains.


3.2.4 Pulsed Field Gel Electrophoresis (PFGE) Genotyping

PFGE allows clear identification at the strain levels. Ascl and NotI restriction fragment patterns of total DNA of Lactobacillus isolates A, C, D and E, identified as Lactobacillus plantarum by ARDRA, were compared to those of L. plantarum BfEL 92122 and type strains L. plantarum DSM 20174T. With Ascl (Fig. 7a) we could discriminate five different genomic fingerprints for all L. plantarum strains (isolates A, C, D, E and L. plantarum BfEL 92122 and type strain L. plantarum DSM 20174T) and a completely different genomic fingerprint for L. zeae DSM 20178T as a negative control. The L. plantarum isolate (A) and the type strain L. plantarum DSM 20174T had an identical Ascl PFGE profiles similar to the L. plantarum BfEL 92122 pattern. The genomic fingerprint of L. plantarum isolates C, D and E were very similar except
for the differences in fragments between > 48.5 - < 97 kb. We conclude that *Lactobacillus* isolates A, C, D and E were different, but narrowly related strains of *L. plantarum*.

![PFGE profiles of Lactobacillus strains with restriction enzymes AscI (a) and NotI (b).](image)

**Fig. 7:** PFGE profiles of *Lactobacillus* strains with restriction enzyme AscI (a) and NotI (b).

- **M:** low range PFG marker (Biolabs)
- **lane 1:** *L. zeae* DSM 20178
- **lane 2:** *L. plantarum* BbEL 92122
- **lane 3:** *L. plantarum* DSM 20174
- **lane 4:** Isolate A
- **lane 5:** Isolate C
- **lane 6:** Isolate D
- **lane 7:** Isolate E

### 3.2.5 Cloning and sequencing of 16S-23S rRNA genes

The determination of 16S and 23S rRNA gene sequences is considered as one of the bases of modern microbial taxonomy. For confirmation of the previous identification of lactobacilli isolates, PCR products of the 16S-23S rRNA genes were amplified using universal primers UP68 and UP69 and cloned into pEGM-T-easy
vector and transformed into *E. coli* JM109. Proper transformants were selected by blue/white colony screening. Plasmid DNA of transformants were isolated and analysed by agarose gel electrophoresis (Fig. 8).

![Fig. 8: Clones of 16S-23SDNA fragment of some Lactobacillus isolates](image)

(C): PGEM-T-easy vector; **clones 1**: L. *plantarum* BfEL 92122; **clones 2**: L. *zeae* DSM 20178; **clones 3**: L. *rhamnosus* BfEL 92149; **clones 4**: L. *plantarum* DSM 20174T; **clones 5**: Isolate A; **clones 6**: Isolate C; **clones 7**: Isolate D; **clones 8**: Isolate E.

One plasmid from each group was sequenced, with T7 and SP6 promoters primers. The sequences were searched at National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) for similarities in the Gen-Bank database. The obtained data after BLAST analysis revealed that the similarities of isolates A, C, D and E with *L. plantarum* were 88%, 99%, 96% and 96%, respectively. *L. plantarum BfEL 92122* and *L. rhamnosus BfEL 92149* had similarities with *L. plantarum* (98%) and *L. casei* (96%), respectively. *L. plantarum DSM 20174* and *L. zeae DSM 20178* matched with *L. plantarum* (98%) and *L. casei* (97%), respectively.

### 3.3 Identification of bacterial isolates from Egyptian fermented dairy products (second part of isolates)

Another strategy was performed for identification of the rest of isolates (46 isolates), ARDRA technique was used to divide the isolates into groups, within the same restriction profile. At least one isolate from each group was chosen for cloning and sequencing of 16S r DNA gene.

#### 3.3.1 PCR of 16S-23S rDNA

For PCR, universal primers UP68/UP69 (*Vaneechoutte et al. 1992*) were used to amplify the fragment (approx. 2.4 kb) encompassing the 16S rDNA, the spacer region and part of the 23S rDNA. PCR was carried out in a Gene-Amp-system with an annealing temperature at 58°C. The obtained amplicons of approx. 2.4 kb are shown in Fig. 9 and 10.
3.3.2 ARDRA using enzymes HaeIII & HinfI

Restriction digests of the 16S-23s rDNA amplicons were carried out with the restriction enzyme HaeIII (Fig. 11) and HinfI (Fig. 12). Restriction fragment patterns were analyzed by agarose gel electrophoresis. The ARDRA technique with HaeIII and HinfI divided the isolates into some clusters, summarized in Table 2. At least one isolate from each group was chosen for accurate identification by sequencing 16S or 16S-23S rRNA genes.
The restriction profiles of isolates 1-27 (Fig 11 and 12) were identical for isolates 1, 3 and 5 and for isolates 2, 4, 7, 8, 9 and 10, while they similar for isolates 12 and 13. Isolates 15, 16, and 17, as well as the group of isolates 19, 20, 21, 22, 22, 23, 24 and 25 showed identical profiles, isolates 26 and 27 had a similar profile. The restriction profiles of isolates 6, 11, 14, 18 were unique and did not match to any of other profiles (Table 2). From these groups, 10 isolates (1, 2, 6, 11, 12, 14, 15, 18, 19, and 26) were chosen for accurate identification. PCR applied with primers UP68 and UP69 to amplify a fragment (approx. 2.4 kb) encompassing the 16S, the spacer region and part of the 23S rDNA, Fig. 13.
The restriction profiles of isolates 28-46 (Fig. 11 and 12) revealed two groups: the first group contained isolates 28, 31, 32, 33, 37, 38; the second one included isolates 29, 39, 40, 41, 42, 43, 44, 45, 46. The restriction profiles also disclosed that isolate number 30 and isolate number 35 had unique profile. At least one isolate from each group was chosen for cloning and sequencing of a part of 16S r RNA gene (isolates 29, 30, 32, 35, 39, and 40), Fig. 14.

\[\text{Fig. 13: PCR amplicons of 16S-23S r RNA genes from selected isolates between numbers 1-27.} \]
\[\text{M: λStyI; lane 1: isolate 1; lane 2: isolate 2; lane 3 isolate 6; lane 4: isolate 11; lane 5: isolate 12; lane 6: isolate 14; lane 7: isolate 15; lane 8: isolate 18; lane 9: isolate 19; lane 10: isolate 26.} \]

\[\text{Fig. 14: PCR amplicons of 16S r RNA gene from selected isolates between numbers 28-46.} \]
\[\text{M: λStyI; lane 1: isolate 29; lane 2: isolate 30; lane 3: isolate 32; lane 4: isolate 35; lane 5: isolate 39; lane 6: isolate 40.} \]

### 3.3.3 Cloning and sequencing of 16S-23S or 16S rRNA genes

Universal primers UP68/UP69 were used for amplification of 16S - 23S rRNA genes isolates 1, 2, 6, 11, 12, 14, 15, 18, 19 and 26 and universal primers 16SF/DG74 for the 6 isolates 29, 30, 32, 35, 39 and 40. PCR-fragments were cloned into pSt Blue I vector (Novagen) or pDrive vector (Qiagen) and transformed into *E. coli* (Nova Blue) competent cells. Proper transformants were selected by blue/white colony screening
and subjected to sequencing of the cloned fragments. The data in Table 15 summerize the result of identification after searching the sequences at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) for similarity in the Gen-Bank database. The obtained data after BLAST analysis revealed similarities of 99%.

Table 15: List of identified isolates

<table>
<thead>
<tr>
<th>Group</th>
<th>Isolates numbers with identical restriction profiles</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 = 3 = 5</td>
<td><em>Lactobacillus fermentum</em> IS1</td>
</tr>
<tr>
<td>2</td>
<td>2 = 4 = 7 = 8 = 9 = 10</td>
<td><em>Lactobacillus plantarum</em> IS2</td>
</tr>
<tr>
<td>3</td>
<td>12 = 13</td>
<td><em>Lactococcus lactis</em> IS5</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td><em>Streptococcus thermophilus</em> IS6</td>
</tr>
<tr>
<td>4</td>
<td>15 = 16 = 17</td>
<td><em>Lactococcus lactis</em> IS7</td>
</tr>
<tr>
<td></td>
<td>19 = 20 = 21 = 22 = 22 = 23 = 24 = 25</td>
<td><em>Lactococcus lactis</em> IS8</td>
</tr>
<tr>
<td>5</td>
<td>26 = 27</td>
<td><em>Lactococcus lactis</em> IS9</td>
</tr>
<tr>
<td>6</td>
<td>28 = 31 = 32 = 33 = 37 = 38</td>
<td><em>Lactobacillus plantarum</em> IS13</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lactobacillus fermentum</em> IS11</td>
</tr>
<tr>
<td>7</td>
<td>29 = 39 = 40 = 41 = 42 = 43 = 44 = 45 = 46</td>
<td><em>Lactobacillus fermentum</em> IS15</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lactobacillus fermentum</em> IS16</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>--NI--</td>
</tr>
<tr>
<td>9</td>
<td>11</td>
<td>--NI--</td>
</tr>
<tr>
<td>10</td>
<td>18</td>
<td><em>Enterococcus saccharinimus</em> IS8</td>
</tr>
<tr>
<td>11</td>
<td>30</td>
<td><em>Lactobacillus fermentum</em> IS12</td>
</tr>
<tr>
<td>12</td>
<td>35</td>
<td><em>Leuconostoc mesenteroides</em> IS14</td>
</tr>
</tbody>
</table>

= identical      ---NI--- Not Identified,   Strains marked bold were used for sequencing.
3.4 Detection and comparison of the biodiversity of intestine bacteria from feces of twin babies

For the differentiation of the unknown isolates from feces of twin babies in the available work the molecular-biological methods PCR and ARDRA were used. Isolates of the baby nourished with mother's milk were named with the letter A, and the letter B was used for isolated from the twin, which was due to a disease, nourished with bottle formula. Numbers 1-20 were used to label isolates taken at the age of three months and numbers 21-40 for those collected from feces at the age of four months.

3.4.1 Identification of isolates of intestine bacteria from babies' feces by PCR with genus specific primers

For identification of the unknown isolates from babies feces on genus level, genus-specific primers lm26/Im3, for *Bifidobacterium*, LactoF/LactoR for *Lactobacillus* as well as, primers St1/St2 for *S. thermophilus* were used. Moreover, PCR with the universal primers UP68/UP69 for 16S-23S rDNA and primers DG74/16SF for 16S rDNA were also applied.

3.4.1.1 PCR for the isolates from feces of babies with genus specific primers

Amplification of the 16S rDNA with the *Bifidobacterium* specific primer-pair Im26 and Im3 ([Kaufmann et al. 1997](#)) resulted in five positive isolates. These positive isolates were named 12A, 12B, 16A, 17A and 19A all with a fragment size of 1417 bp (data not shown). Primers LactoF and LactoR ([Roy et al. 2004](#)) were used to investigate the remaining 75 unknown isolates. PCR resulted in amplicons with a fragment size of approx. 230 bp for all used isolates, including negative controls of 17A and *S. thermophilus* as showed in Fig. 15.
Fig. 15: PCR of the babies’ Feces isolates with the *Lactobacillus* specific Primers LactoF and LactoR.

M: 100bp-Ladder; lanes 1-13: isolates 1A-13A; lane 14: *L. gasseri* (DSM 20243); lanes 15 & 16: *St. thermophilus*; lane 17: *B. breve* 17A; lane 18: Blank.

A change of the annealing temperature did not improve the specificity of the primer pair. Therefore, a primer combination consisting of primer LactoF as forward primers and the *Lactobacillus* specific reverse primer LbLMA1 (*Dubernet et. al* 2002) were used, resulting in PCR products with sizes of approx. 1600 bp. (Fig. 16). However, the negative controls 17A and two *S. thermophilus* isolates also resulted in a clear signal of appropriate size. An increase of the annealing temperature by 2 °C improved the specificity of the PCR. Even at the improved conditions the negative control 17A still showed a weak signal of comparable size (Fig. 18). All reference strains except DSM 20197, as well as the isolates 1-2A, 4-6A, 8-10A, 13-14A, 22-23A, 26A and 36A revealed strong PCR signals. Amplification with isolates 3A, 7A, 11A, 15A, 20A, 24-25A, 27-28A and 37A as well as 1B, 18-20B and 30B led to weak signals of appropriate size. All other isolates were negative. The experiments with primers LactoF and LbLMA1 are presented in Fig. 16, 17, 18 and 19a and b.

Fig. 16: PCR of *Lactobacillus* reference strains with the primers LactoF/LbLMA1.

M: λStyI; lane 1: *L. reuteri* (DSM 20016); lane 2: *L. pastorianus* (DSM 20197); lane 3: *L. zeae* (DSM 20178); lane 4: *L. gasseri* (DSM 20243); lanes 5&8: *L. delbrueckii* ssp. *lactis* (DSM 20072, ATCC 10697); lane 6: *L. johnsonii* (DSM 20553); lane 7: *L. bulgaricus* (ATCC 12278); lane 9: *L. plantarum* ssp. *plantarum* (DSM 20174); lane 10: blank sample.

Fig. 17: PCR of babies’ feces isolates with *Lactobacillus* specific primers LactoF and LbLMA1.
To investigate the presence of *Streptococcus* in the feces micro-flora the *S. thermophilus* specific primers St1 and St2 were used. None of tested isolates revealed positive signals (data not shown).

The identification of *Lactobacillus* on genus level was more difficult compared to the *Bifidobacteria*, since primers LactoF and LbLMA1 appeared to be less specific.

### 3.4.1.2 PCR with isolates from babies’ feces using universal primers

All isolates, except 12A, 12B, 16A, 17A, and 19A were subjected to PCR with universal primers UP68/UP69. All samples yielded positive signals. Amplicons of approx. 2400 bp in size including 16S r RNA gene, intergenic spacer region and a part of 23S r RNA gene were obtained (*Vaneechout et al.*, 2002). PCR with the universal primer DG74 and 16SF was performed to amplify the 16S r RNA genes for sequencing. All isolates revealed positive signals with an amplicon size of 1536 bp.
3.4.2 Differentiation of babies’ feces isolates by ARDRA

3.4.2.1 ARDRA of Lactobacillus Isolates from babies’ feces

PCR products obtained with primers LactoF and LbLM1 were not suitable for ARDRA. Therefore amplicons of 16S rRNA gene, intergenic spacer region and a part of 23S rRNA gene with size approx. 2400 bp, obtained with universal primers UP68/UP69 were used for ARDRA. Restriction analysis with enzymes HaeIII, HhaI, HinfI and TaqI was applied. Restriction-digests with HhaI showed two different patterns. The restriction profiles of isolates 1-2A, 4-6A, 8A, 13A, 14A, 22-23A, 26A and 36A were identical to the restriction profile of the reference strain \textit{L. zeae} (DSM 20178\textsuperscript{T}). None of the reference strains matched with isolates 9A and 10A. Digest with HinfI achieved three different restriction profiles. Isolates 9A and 13A had identical patterns, which were similar to the pattern of \textit{L. gasseri} (DSM 20243\textsuperscript{T}) as well as \textit{L. johnsonii} (DSM Z 20553). The restriction profile of the isolate 10A did not match with any of the reference strains. The remaining isolates matched with \textit{L. zeae} (DSM Z 20178\textsuperscript{T}). ARDRA restriction profile of with TaqI were identical to the reference strain \textit{L. zeae}, DSM 20016\textsuperscript{T} indicating that all these isolates are closely related to this bacterial species. Isolate 9A did not match with the restriction profiles of any of the reference strains. 9A, 10A and 13A could not be differentiated unambiguously by ARDRA.

3.4.2.2 Allocation of the isolates from babies’ feces by ARDRA

With exception of the isolates identified as \textit{Bifidobacterium} no further isolates identified by PCR as \textit{Lactobacillus} could be differentiated clearly according the past criteria. Therefore ARDRA with TaqI was performed for 75 isolates. For isolates from baby A (Fig. 21, 21 and 23) two, and for the isolates from baby B (Fig. 24 and 25) three different restriction profiles were found.

![Fig. 21: ARDRA (Primer UP68/UP69) with TaqI for some isolates from baby’s (A) feces.](image)

\textbf{M}: 100bp-Ladder; lane 1: isolate 1A; lane 2: isolate 2A; lane 3: isolate 4A; lane 4: isolate A5; lane 5: isolate A6; lane 6: isolate A8; lane 7: isolate A9; lane 8: isolate 10A; lane 9: isolate 13A; lane 10: isolate 14A; lane 11: isolate 22A; lane 12: isolate 23A; lane 13: isolate 26A; lane 14: isolate 36A.
Fig. 22: ARDRA (Primer UP68/UP69) with TaqI enzyme of some isolates from babies feces.
M: 100bp-Ladder; lane 1: isolate 3A; lane 2: isolate 11A; lane 3: isolate 15A; lane 4: isolate 18A; lane 5: isolate 20A; lane 6: isolate 24A; lane 7: isolate 31A; lane 8: isolate 32A

Fig. 23: ARDRA (Primer UP68/UP69) with TaqI enzyme of some isolates from babies feces.
M: 100bp-Ladder; lane 1: isolate 7A; lane 2: isolate 21A; lane 3: isolate 25A; lanes 4-7: isolates 27A-30A; lanes 8-10: isolates 33A-35A; lanes 11-14: isolates 37A-40A.

Fig. 24: ARDRA (Primer UP68/UP69) with TaqI enzyme of some isolates (B) from babies feces.
M: 100bp-Ladder; lane 1: isolate 1B; lane 2: isolate 9B; lane 3: isolate 18B; lane 4: isolates 19B; lane 5: isolates 20B; lane 6: isolate 21B; lane 7: isolates 22B; lane 8: isolates 23B

Fig. 25: ARDRA (Primer UP68/UP69) with TaqI enzyme of some isolates (B) from babies feces.
M: 100bp-Ladder; lanes 1-7: isolates 2B-8B; lane 8: isolate 10B; lane 9: isolate 11B; lanes 10-14: isolates 13B-17B; lanes 15-31: isolates 24B-40B.
ARDRA with TaqI allowed dividing the 75 isolates into three groups.


Group 2: 9A, 11A, 33A; 4B, 7B, 18B, 29B.


3.4.2.3 Sequencing of selected isolates

For identification, sequencing of the 16S rDNA of the following selected isolates 1A, 2A, 10A, 13A and 15A from group 1, 9A and 11A from group 2 and 13B, 19B and 33B from Group 3 were performed. PCR amplicons of the 16S rDNA amplified with primers DG74 and 16SF rDNA were cloned in the vector pStBlue-1 and approx. 1000 bp from the 1536 bp fragment was sequenced. The receiving data were compared with those in data bases summarized 16S rDNA sequences. The isolates 2A, 10A, 13A, 15A from the group 1 and the isolate 11A from group 2 were identified as *L. rhamnosus*. The isolates 1A (group1) and 9A (group 2) identified as *L. paracasei*. 13B, 19B and 33B (from group 3) were identified as *Enterococcus gallinarum* and *Enterococcus saccharolyticus*, respectively (Table 16).
<table>
<thead>
<tr>
<th>Isolate</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td><em>Lactobacillus paracasei</em> ISO1</td>
</tr>
<tr>
<td>2A</td>
<td><em>Lactobacillus rhamnosus</em> ISO2</td>
</tr>
<tr>
<td>9A</td>
<td>Lactobacillus paracasei ISO3</td>
</tr>
<tr>
<td>10A</td>
<td><em>Lactobacillus rhamnosus</em> ISO4</td>
</tr>
<tr>
<td>11A</td>
<td><em>Lactobacillus rhamnosus</em> ISO5</td>
</tr>
<tr>
<td>13A</td>
<td><em>Lactobacillus rhamnosus</em> ISO6</td>
</tr>
<tr>
<td>15A</td>
<td><em>Lactobacillus rhamnosus</em> ISO7</td>
</tr>
<tr>
<td>13B</td>
<td><em>Enterococcus gallinarum</em> ISA1</td>
</tr>
<tr>
<td>19B</td>
<td><em>Enterococcus saccharolyticus</em> ISA2</td>
</tr>
<tr>
<td>33B</td>
<td><em>Enterococcus saccharolyticus</em> ISA3</td>
</tr>
</tbody>
</table>

Significant differences in the microflora of the two babies may be due to the nutrition. Only Lactobacilli were isolated from the infant A, who was fed with breast milk and several *Enterococcus* sp. from infant B, who was fed with Milk formula. These results may demonstrate that human milk is considered to be an important factor affecting infants gut microflora composition and breast feeding can be a significant source of *Lactobacilli* in the infant gut ([Martin et. al., 2005](#)).
4 Chapter II

Phenotypic characterization of some Lactobacilli

4.1 Introduction

Products available on the market that contain probiotics are one reason for the increases consumption of fermented milk. (Chumchalova et al., 2004). Probiotics are defined to be live microorganisms, which exert a positive effect on the health and the well-being of the host (Ouwehand and Salminen, 1998 and Klingberg et al., 2005). Probiotic bacteria should survive passage through the upper part of the gastrointestinal tract. High tolerances to low pH and bile salts concentrations corresponding to the conditions of the human gastrointestinal tract (GIT) have therefore been considered as important selection criteria (Tuomola et al., 2001; Klingberg et al., 2005). Probiotic bacteria are exposed to acid-stress in the stomach with its low pH (pH3.5 or lower) (Barada, 1991). Before passing to intestinal tract, they must therefore survive transit through the stomach (Dunne et al., 2001; Martin et al., 2005. In the present study we considered survival at pH 3.5 as a criterion for acid tolerance, although acid tolerance until up to pH 2 was tested to detect highly acid tolerant strains. Acid tolerance was evaluated by growing strains in MRS broth adjusted to acidic pH 3.5 and 2 and non-acidified MRS broth pH 6.5 at 37°C for 90 min, since the food transit time through the human stomach is about 90 min (Barada, 1991; Chou and Weimer, 1999).

Bile tolerance, another important criterion for selection of probiotic strains, enables them to survive, grow and exert their actions in the small intestine. Strains that are able to grow and metabolize in the presence of physiological levels of bile salts should logically be more likely to survive intestine transit. Although the degree of bile tolerance required for maximum growth of the microorganisms in the intestine tract is unknown, it is important to select those strains having a high degree of bile salt tolerance.
Concentration of 0.3% oxgall closely approximates the bile levels found in the gastrointestinal tract (Gilliland et al., 1984; Goldin and Gorbach, 1992; McAuliffe et al., 2005).

Also, antimicrobial activity against pathogenic bacteria is considered as one of the important criteria for the selection of probiotic strains. Lactic acid bacteria produce various antimicrobial compounds such as organic acids and their derivatives (phenyl lactic acid and 4-hydroxyl-phenyl-lactic acid, Lavermicocca et al., 2000), diacetyl, hydrogen peroxide, and bacteriocins or bacteriocidal proteins during lactic fermentation (Anderssen et al., 1998; Sholeva et al., 1998; Ouwehand, 1998; Zhennai, 2000; Oyetayo et al., 2003; Savadogo et al., 2004).

Lactobacilli strains, are frequently used for production of fermented milks with enhanced probiotic effect, may provide strong antagonistic effect towards Gram-negative bacteria, Gram-positive bacteria and fungi (Suskovic et al., 1997a,b). Lactobacillus species are considered as part of the normal microflora of the gastrointestinal and female genital tract (Avlami et al., 2001). Lactobacilli are widely used as probiotics or in starter cultures. They have the potential to serve as hosts of antibiotic resistance genes with the risk of transferring the genes in other lactic acid bacteria and/or pathogenic bacteria. In recent years, studies on the selection for and the dissemination of antibiotic resistances have focused on clinically relevant bacterial species. Recently, it was speculated that food bacteria may also act as reservoirs of antibiotic resistance genes (Hummel et al., 2007; Mathur and Singh, 2005).

There is little information regarding presence of antibiotic resistance genes in starter strains and their potential to transfer the resistance genes to pathogens (Mathur and Singh, 2005). Reduced susceptibility to certain antibiotics is not an unusual feature of food isolated microorganisms including starter and probiotic cultures. Many of these resistances are due to complex intrinsic features such as cell wall structure or metabolic properties (Kastner et al., 2006). The antibiotic susceptibility of Lactobacillus species has received little attention (Charteris et al., 1998) in this regard; antibiotic susceptibility testing of lactic acid bacteria may be advisable in a number of instances e.g. for checking the biosafety of potentially probiotic isolates and genetically modified strains used in biotechnology (Huys et al., 2002).
The objectives of the present study were the evaluation and characterization of the *Lactobacillus* strains, which identified in the previous chapter (11 from Egyptian fermented dairy products, 7 from baby’s faeces in addition to, 9 from BfEL culture collection) and from through probiotic selection criteria (acid tolerance, bile salts resistance and antimicrobial activity). Also, a safety assessment of some *Lactobacillus* isolates was done. To achieve that, we studied susceptibility of 26 *Lactobacillus* to 13 antibiotics including inhibitors of cell wall synthesis, protein synthesis and nucleic acid synthesis.

### 4.2 Results and discussion

#### 4.2.1 Acid tolerance

Twenty-two strains were tested for tolerance to low pH for up to 90 min (Fig 26). According to Lan-szu and Bart, (1999), strains that showed little or no reduction in colony-forming units per millilitre of culture were considered to be candidates for selection of acid-tolerant strains. Nearly all strains investigated survived very well 90 min incubation at pH 3.5 (Figs. 26-(1-22). These results are in agreement with those of other authors (Chou and Weimer 1999; Xanthopoulos et al 2000 and Lin and Chin 2000). At pH 2 significant differences in acid resistance were observed between different strains. CFUs of *Lactobacillus fermentum* IS12, *L. rhamnosus* ISO7 and *L. johnsonii* BfEL92170 decreased only one logarithmic unit after 90 min at pH 2, those of *L. fermentum* IS16 and *L. brevis* BfEL 92027 by two logarithmic units. The cell number of strains *L. fermentum* IS11, *L. fermentum* IS15, *L. rhamnosus* ISO4, *L. rhamnosus* ISO5 and *L. amylovorus* BfEL 92203 were reduced by three log units, those of *L. fermentum* IS1, *L. plantarum* IS2 and *L. fermentum* IS16 by four and those of *L. rhamnosus* ISO2, *L. paracasei* ISO3, *L. rhamnosus* ISO6 and *L. gasseri* BfEL92075 by five logarithmic units. *L. plantarum* IS13, *L. paracasei* ISO1, *L. plantarum* BfEL 92122 and *L. acidophilus* BfEL92015 did not survive at pH2 for 90 min. These results are in conform with those represented by Lin and Chin 2000, that most of *Lactobacillus acidophilus* strains did not survive at pH 2.
Fig. 26: Survival of *Lactobacillus* ssp. (log_{10} cfu/ml) in MRS medium at different pH values during incubation at 37 °C for 90 min.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Time (min)</th>
<th>Viable Count (log_{10} cfu/ml)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. fermentum IS16</td>
<td>0 20 40 60 80</td>
<td>10 8 6 4 2</td>
<td>pH 6.5, pH 3.5, pH 2</td>
</tr>
<tr>
<td>L. paracasei IS01</td>
<td>0 20 40 60 80</td>
<td>10 8 6 4 2</td>
<td>pH 6.5, pH 3.5, pH 2</td>
</tr>
<tr>
<td>L. rhamnosus IS02</td>
<td>0 20 40 60 80</td>
<td>10 8 6 4 2</td>
<td>pH 6.5, pH 3.5, pH 2</td>
</tr>
<tr>
<td>L. paracasei IS03</td>
<td>0 20 40 60 80</td>
<td>10 8 6 4 2</td>
<td>pH 6.5, pH 3.5, pH 2</td>
</tr>
<tr>
<td>L. rhamnosus IS04</td>
<td>0 20 40 60 80</td>
<td>10 8 6 4 2</td>
<td>pH 6.5, pH 3.5, pH 2</td>
</tr>
<tr>
<td>L. rhamnosus IS05</td>
<td>0 20 40 60 80</td>
<td>10 8 6 4 2</td>
<td>pH 6.5, pH 3.5, pH 2</td>
</tr>
</tbody>
</table>

Fig. 26: continued
Fig. 26: continued
4.2.2 Bile tolerance

Data in Figs. 27-(1-27) show the growth abilities of twenty-seven Lactobacillus strains in the presence of different concentrations (0.1; 0.3; 0.5; 1 and 3%) of ox-gall bile salts. Most strains grew in the presence of 0.1% bile salts except L. casei BfEL 92040, L. paracasei ISO1 and L. rhamnosus ISO6, which did not grow at any of the bile salt concentrations tested. Most of the strains grow well in to 0.3% bile salts, except L. gasseri BfEL 92075, L. plantarum (A), L. plantarum (C), L. plantarum (D) L. plantarum (E), and L. rhamnosus ISO2. L. plantarum BfEL 92122, L. fermentum IS12, L. fermentum IS15, L. fermentum IS16, L. plantarum IS2, L. plantarum IS13, L. paracasei ISO3, L. rhamnosus ISO4, L. rhamnosus ISO5 and L. rhamnosus ISO7 showed growth up to 0.5% ox–gall bile salts (Figs. 27-(2, 12, 13,14,15,16, 23, 24, 25, 26).
and 27), respectively. Only *L. plantarum* BfEL 92122 was highly resistant to bile salts and able to grow in 3% bile (Fig. 27-2).

Most of *Lactobacillus* strains tested for bile salts hydrolase activity expressed bile salts hydrolase and deconjugated taurine or glycine-bile acid or both of them and produced a precipitate around the colonies on agar medium which containing 0.5% TDCA or 0.5% GDCA (Table 17 and Fig. 28).

![Graphs of different lactobacilli growth](image-url)

**Fig. 27:** Effect of different concentrations of ox-gall bile salts on growth of lactobacilli
Fig. 27: Continued
Fig. 27: Continued
Fig. 27: Continued
Fig. 27: Continued
Table 17: Bile salts hydrolase activity of various *Lactobacillus* strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Bile salts hydrolase activity</th>
<th>TDCA</th>
<th>GDCA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus plantarum</em> BfEL92122</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Lactobacillus brevis</em> BfEL 92026</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em> BfEL92149</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Lactobacillus gasseri</em> BfEL 92075</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em> (A)</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em> (C)</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em> (D)</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em> (E)</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em> BfEL92015</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Lactobacillus johnsonii</em> BfEL 92170</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>Lactobacillus amylovorus</em> BfEL92203</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em> BfEL92040</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Lactobacillus fermentum</em> IS1</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em> IS2</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Lactobacillus fermentum</em> IS11</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Lactobacillus fermentum</em> IS12</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em> IS13</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Lactobacillus fermentum</em> IS15</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Lactobacillus fermentum</em> IS16</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Lactobacillus paracasei</em> ISO1</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em> ISO 2</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Lactobacillus paracasei</em> ISO3</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em> ISO 4</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em> ISO 5</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em> ISO 6</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Lactobacillus rhamnosus</em> ISO 7</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Fig. 28: Bile salt hydrolase activity as detected by plate assay method on MRS supplemented with TDCA or GDCA.

(A): MRS; (B): MRS+0.5% TDCA; (C): MRS+0.5%GDCA; 1: L. plantarum BfEL 92122; 2: L. brevis BfEL 92026; 3: L. rhamnosus BfEL 92149; 4: L. gasseri BfEL 92075; 5: L. plantarum (A); 6: L. plantarum (C); 7: L. plantarum (D); 8: L. plantarum (E); 9: L. acidophilus BfEL 92015; 10: L. johnsonii BfEL 92170; 11: L. amylovorus BfEL 92203; 12: L. casei BfEL 92040.

4.2.3 Antimicrobial activity

Antimicrobial activity screening of 26 lactobacilli strains isolated from traditional Egyptian dairy products, from babies faeces and from the culture collection BfEL–KIEL (see Table 18) against different pathogenic bacteria as indicators was performed. Antimicrobial activities were determined in agar diffusion tests using overnight cultures of the test bacteria and spent media of these cultures at their original pH-values and after adjusting the pH to 7. Inhibition was observed with viable cells only but not with spent media, independently of the pH-value (Fig. 29). However, antimicrobial activities were detected in the growth media after changing
the growth conditions. Lactobacilli cultures were grown anaerobically for about 7 hours (O.D. 0.5-1), then 0.5% of each culture were transferred immediately under aseptic conditions to warmed MRS broth medium and allowed to grow aerobically with slight shaking. These growth conditions were selected to expose the growing cells to stress. The results shown in Table 18 and Fig. 29 indicate that most of the Lactobacillus strains produced antimicrobial substances against some pathogens. Staphylococcus aureus BfEL 91161 and Staphylococcus epidermis BfEL 91112, were inhibited by the most of Lactobacillus strains, which agrees to observations represented by Gulahmadov et al., 2006; Olivares et al., 2006. L. gasseri BfEL 92075, L. brevis BfEL92026, L. plantarum BfEL92122, L. plantarum (A, D, E) and L. casei BfEL92040 showed, in addition, slight inhibitory effects against Enterococcus faecalis BfEL90519 and Listeria monocytogenes. The screening also revealed that L. fermentum IS1 inhibited Enterobacter aerogenes BfEL 9805, Serratia marcescens BfEL 98027, S. aureus BfEL 91161, S. epidermidis BfEL 91112 and E. faecalis BfEL 90519. L. plantarum IS2 and L. fermentum IS11 inhibited the growth of E. aerogenes BfEL 9805, S. marcescens BfEL 98027, and E. faecalis BfEL 90519, L. plantarum IS13 showed inhibitory activity against E. aerogenes BfEL 9805, S. marcescens BfEL 98027, S. aureus BfEL 91161, and E. faecalis BfEL 90519. In addition, L. fermentum IS15 and L. fermentum IS16 were active against E. aerogenes BfEL 9805, S. epidermidis BfEL 91112 and E. faecalis BfEL 90519. L. fermentum IS12 and L. rhamnosus ISO4 only showed activity against E. faecalis BfEL 90519. On the other hand, L. johnsonii BfEL 92170, L. paracasei ISO1, 3; L. rhamnosus ISO2, 5, 6,7 strains did not show any antimicrobial activity against any of the eight indicator pathogens.

Production of a variety of substances by probiotic bacteria with antibacterial properties including organic acids, hydrogen peroxide, bacteriocins are considered important factors for selecting probiotic strains (Rolfe 2000). The inhibitory effect shown by some Lactobacillus strains might be due to the presence of bacteriocins or metabolites similar to them. Many authors already have reported that production of bacteriocins by lactobacilli is relatively common; this fact may contribute to their ability to colonize their habitats and to their competitive edge over other bacteria (González et al. 2007; Garriga et al. 1993). The antibacterial activities seem to be affected by different factors and the activities vary with respect to strength and action spectrum (Olivares et al. 2006; González et al. 2007). Our results showed, that
most of *Lactobacillus* strains had antibacterial effect against *Enterococcus faecalis* BfEL 90519 (Fig. 30). *Enterococcus faecalis* accounts for the majority of enterococcal infections in humans (Tailor *et al.* 1993, Huycke *et al.*; 1998 and Charteries *et al.* 2001).

Fig. 29: Antimicrobial activity of *Lactobacillus* strains against *Staphylococcus aureus* BfEL 91161 and *Staphylococcus epidermis* BfEL 91112

A: 10 µl of lactobacillus broth cultures; B: 10 µl of supernatant without adjusting the pH (actual pH ≈ 4); C: 10 µl of supernatant with adjusting the pH to ≈ 7. 1: *L. gasseri* BfEL 92075; 2: *L. brevis* BfEL 92026; 3: *L. rhamnosus* BfEL 92149; 4: *L. plantarum* BfEL 92122; 5: *L. plantarum* (A); 6: *L. plantarum* (C); 7: *L. plantarum* (D); 8: *L. plantarum* (E); 9: *L. acidophilus* BfEL 92015; 10: *L. johnsonii* BfEL 92170; 11: *L. amylovorus* BfEL 92203; 12: *L. casei* BfEL 92040.
Fig. 30: Antimicrobial activity of *Lactobacillus* strains against pathogenic bacteria after changing the lactobacilli overnight culture growth conditions.
A: *E. coli* BIEL 98082; B: *Enterobacter aerogenes* BIEL 9805; C: *Serratia marcescens* BIEL 98027; D: *Staphylococcus aureus* BIEL 91161; E: *Staphylococcus epidermidis* BIEL 91112; F: *Enterococcus faecalis* BIEL 90519; G: *Salmonella typhimurium*; H: *Listeria monocytogenes*; 1: *L. gasseri* BIEL 92075; 2: *L. brevis* BIEL 92026; 3: *L. rhamnosus* BIEL 92149; 4: *L. plantarum* BIEL 92122; 5: *L. plantarum* (A); 6: *L. plantarum* (C); 7: *L. plantarum* (D); 8: *L. plantarum* (E); 9: *L. acidophilus* BIEL 92015; 10: *L. johnsonii* BIEL 92170; 11: *L. amylovorus* BIEL 92203; 12: *L. casei* BIEL 92040.
Table 18: Antimicrobial activity of *Lactobacillus* strains against eight indicator strains*

<table>
<thead>
<tr>
<th>Lactobacillus strains</th>
<th><em>E. coli</em></th>
<th>Enterobacter aerogenes</th>
<th><em>Serratia marcescens</em></th>
<th><em>Staphylococcus aureus</em></th>
<th><em>Staphylococcus epidermis</em></th>
<th>Enterococcus faecalis</th>
<th><em>Salmonella typhimurium</em></th>
<th>Listeria monocytogenes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. gasseri BfEL 92075</em></td>
<td>8</td>
<td>9</td>
<td>9</td>
<td>15</td>
<td>11</td>
<td>10</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td><em>L. brevis BfEL 92026</em></td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>16</td>
<td>10</td>
<td>10</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td><em>L. rhamnosus BfEL 92149</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. plantarum BfEL 92122</em></td>
<td>9</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td><em>L. plantarum (A)</em></td>
<td>9</td>
<td>10</td>
<td>10</td>
<td>12</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td><em>L. plantarum (C)</em></td>
<td>-</td>
<td>9</td>
<td>10</td>
<td>15</td>
<td>9</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td><em>L. plantarum (D)</em></td>
<td>-</td>
<td>10</td>
<td>9</td>
<td>15</td>
<td>11</td>
<td>10</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td><em>L. plantarum (E)</em></td>
<td>8</td>
<td>9</td>
<td>9</td>
<td>15</td>
<td>10</td>
<td>9</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td><em>L. acidophilus BfEL 92015</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>16</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. johnsonii BfEL 92170</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. amylovorus BfEL 92203</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td>14</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. casei BfEL 92040</em></td>
<td>9</td>
<td>9</td>
<td>10</td>
<td>15</td>
<td>12</td>
<td>11</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td><em>L. fermentum IS1</em></td>
<td>-</td>
<td>9</td>
<td>12</td>
<td>12</td>
<td>10</td>
<td>11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. plantarum IS2</em></td>
<td>-</td>
<td>8</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. fermentum IS 11</em></td>
<td>-</td>
<td>12</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. fermentum IS12</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. plantarum IS13</em></td>
<td>-</td>
<td>11</td>
<td>10</td>
<td>10</td>
<td>-</td>
<td>9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. fermentum IS15</em></td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>10</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. fermentum IS16</em></td>
<td>-</td>
<td>11</td>
<td>-</td>
<td>10</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. paracasei ISO1</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. rhamnosus ISO2</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. paracasei ISO3</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. rhamnosus ISO4</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. rhamnosus ISO5</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. rhamnosus ISO6</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. rhamnosus ISO7</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* The total diameter was measured, including the filter disc (6 mm).
- No inhibition zone around filter disc observed.
4.2.4 Antibiotic susceptibility

Fermented foods may be regarded as important vehicles transferring enormous amounts of living bacteria into the human body. These bacteria may carry antibiotic resistances, which might be transferred to commensal or pathogenic bacteria. The presence of transmissible antibiotic resistance markers of strains is thus an important safety criterion (Hummel et al., 2007). There are no approved standards for testing phenotypic antibiotic susceptibility in most food isolates (Kastner et al., 2006; Danielsen and Wind, 2003).

4.2.4.1 Susceptibility to inhibitors of cell wall synthesis

Fig. 31 shows the susceptibility of 26 lactobacilli strains against inhibitors of cell wall synthesis (ampicillin, cefotaxime, methicillin and vancomycin). All strains were very sensitive to ampicillin. Except *L. rhamnosus* BfEL92149 and *L. plantarum* BfEL92122, which were resistant, most of the strains were also sensitive or moderately susceptible to cefotaxime. In addition, the majority of strains were resistant to vancomycin including all strains isolated from the Egyptian dairy products and 3 strains from infant isolates. Only eight strains were sensitive to vancomycin (*L. brevis* BfEL92026, *L. acidophilus* BfEL92015, *L. johnsonii* BfEL92170, *L. amylovorus* BfEL92203, *L. paracasei* ISO3, *L. rhamnosus* ISO4, *L. rhamnosus* ISO5, *L. rhamnosus* ISO7). Most of the strains were resistant to methicillin including *L. gasseri* BfEL92075, *L. brevis* BfEL92026, *L. plantarum* BfEL92122 and all strains isolated from the Egyptian dairy products and *L. rhamnosus* ISO7 from infant isolates. The rest of strains ranged from moderate sensitive to sensitive.

4.2.4.2 Susceptibility to inhibitors of protein synthesis

Fig. 32 shows the susceptibility of 26 lactobacilli strains against inhibitors of protein synthesis (gentamycin, kanamycin, streptomycin, tetracycline, chloramphenicol, erythromycin, lincomycin and neomycin). Almost all strains were very sensitive to gentamycin, except *L. fermentum* IS1, *L. rhamnosus* ISO5 and *L. rhamnosus* ISO7, which were resistant. Fifteen strains exhibited resistance to kanamycin including five from the infant isolates. Only *L. plantarum* (D) strain was very sensitive to kanamycin; the rest was moderately susceptible. Most of the *Lactobacillus* strains revealed resistance to streptomycin. These comprise five strains from the infant isolates, eight
strains from Egyptian dairy products and three of BfEL strains. *L. amylovorus* BfEL 92203 was highly sensitive to streptomycin. Twelve strains were sensitive, three moderate sensitive and eleven revealed resistance to tetracycline. All investigated strains showed resistance to chloramphenicol except *L. rhamnosus* BfEL 92149 which was moderately susceptible. The same situation was observed for erythromycin. All strains were sensitive excluding *L. fermentum* IS12, which was moderately susceptible. Ten strains were resistant against lincomycin. The rest of the strains ranged from highly to moderate sensitive. Almost the same results were obtained for neomycin where eleven strains were resistant and the rest ranged from highly to moderately sensitive.

### 4.2.4.3 Susceptibility to Inhibitor of nucleic acid synthesis

Fig. 33 shows the susceptibility of 26 lactobacilli strains to inhibitor of nucleic acid synthesis (Ofloxacin). Most strains were resistant to ofloxacin, including all strains isolated from the Egyptian dairy products, most the BfEL strains and two strains from infant isolates. *L. casei* BfEL 92040, *L. rhamnosus* ISO2, 4, 5, 6, 7, *L. paracasei* ISO3 were sensitive; *L. acidophilus* BfEL 92015 was moderately sensitive, respectively. The rest of the infant isolates were sensitive to ofloxacin.

In the present study, all the tested *Lactobacillus* strains were sensitive to the clinically relevant antibiotics ampicillin, erythromycin, chloramphenicol and cefotaxime. These results confirm those reported by Hummel et al., (2007), who found, that most of *Lactobacillus* strains were quite sensitive to these antibiotics. On the other hand, our findings revealed that 88.5% of strains were sensitive to gentamycin. This result is in contrast to observations by Hummel et al., (2007). This difference may be due to different isolation of strains from different environments. Moreover, 69% of strains were resistant to vancomycin. Among the 31% showing sensitivity were *L. acidophilus* and the very related *L. johnsonii*. These results are consistent with those of Hamilton-Miller and Shah, (1998), who found that more than 60% of *Lactobacillus* strains were resistant to vancomycin, while *L. acidophilus* was sensitive. Antibiotic concentrations, used for determination of antibiotic susceptibility, were higher than the values reached in blood serum: e. g. the blood serum concentrations for erythromycin; tetracycline and chloramphenicol were 2-3, 8, 10-15mg/ml, respectively (Hummel et al., 2007; Yao and Moellering, 1995).
Susceptibility determination at concentrations higher than these values is only of academic interest. We conclude that safety evaluation of probiotics used in dairy fermentations should be intensified because the role of probiotics in potentially spreading antibiotic resistance gene is still unclear as is their effect on the host under conditions of antibiotic treatment.

**Fig. 31:** *Lactobacillus* susceptibility to inhibitors of cell wall synthesis (ampicillin, cefotaxime, Methicillin and vancomycin). Scale indicates inhibition zone in mm.
Fig. 32: *Lactobacillus* susceptibility to inhibitors of protein synthesis

(A) gentamycin, kanamycin, streptomycin and tetracycline; (B) chloramphenicol, erythromycin, lincomycin and neomycin. See legend of Fig. 31
Fig. 33: *Lactobacillus* susceptibility to inhibitors of nucleic acid synthesis (Ofloxacin). See legend of fig. 31.
5 Chapter III

Cloning of a conjugated bile salt hydrolase gene (cbh) from *Lactobacillus plantarum* in *Lactobacillus /E. coli* shuttle vectors and expression of this gene in different *Lactobacillus* strains.

5.1 Introduction

Bile salts, the water-soluble end products of cholesterol metabolism in the liver, have amphipathic properties through which they play an important role in the digestive processes. Bile acids are synthesized from cholesterol and conjugated to either glycine or taurine in the liver (*Savage et al., 1995*) and are subsequently stored in the gall bladder. In response to the ingestion of food, bile salts are released into the duodenum helping fat digestion and absorption (*Dussurget et al., 2002*). In addition to their role in digestion, bile salts have antimicrobial properties. Bile salts are amphipathic molecules, which can destroy lipid-containing bacterial and viral membranes. Some resident enteric microflora and intestinal pathogens have evolved mechanisms to resist the detergent action of bile salts by preventing or reduce entry into the cells (*Gunn, 2000*). Other gastrointestinal bacteria of several genera, including *Bacteroides, Clostridium, Enterococcus, Bifidobacterium and Lactobacillus* produce bile salt hydrolases. The first step in bacterial bile salt metabolism is hydrolysis of the amide bond by which the glycine and/or taurine moiety is released from the side chain of the bile salt steroid core. Bile salt hydrolysis, resulting in deconjugated bile salts, is an important bile salt modification mediated by a wide range of colonic anaerobes (*Hill, 1995*). These enzymes are known as conjugated bile salt hydrolase (CBH) or (BSH). Bile salts are biotransformed into a number of metabolites by the intestinal microflora of man and animals (*Hylemon, 1985*). The hydrolysis of conjugated bile acids is one of the most common microbial bile salt biotransformations. There are to two major hypotheses to explain the function of bacterial CBH s. One hypothesis states that bacteria of those species that are able to deconjugated bile salts may be able to use the amino acid taurine as an electron acceptor. Evidence which supports this hypothesis has been obtained for certain *Clostridium* species (*Huijghebaert et al., 1982; Huis In’t Veld and Shortt, 1996*). The second hypothesis states that CBH s decrease the toxicity of conjugated bile acid for bacteria (*Savage, 1992*).
It has been also suggested that CBHs are detergent shock proteins, that protect the bacteria from the toxicity of bile acids in the gastrointestinal tract (Adamowicz et al., 1991; Flahaut, et al., 1996; De Boever et al., 2000; Grill et al., 2000 a,b). Phenotypic and genetic screens revealed that bile salt hydrolase activity was acquired horizontally in lactobacilli. Lactobacilli within the species acidophilus, brevis, buchneri, fermentum, gasseri and plantarum express a variable bile salt hydrolase phenotype. PCR screening within L. acidophilus and L. delbrueckii subsp. bulgaricus also produced variable results (Elkin et al., 2001).

A major consideration in the choice of probiotic strains is its property to survive and establish itself under the conditions of the intestinal environment. Resistance of bile acids is, therefore, an important characteristic for the selection of probiotic strain that enables it to survive and grow and exert its action in intestine (Moser and Savage, 2001; Dussurget et al., 2002).

The aim of this chapter was to screen strains of several Lactobacillus species for bile salt hydrolase activity and to identify the encoding gene for cloning in different shuttle vectors and for transformation in lactobacilli.

5.2 Results and discussion

5.2.1 Detection of genes (cbh) encoding bile salts hydrolase activity

In the previous chapter, it was concluded that most of lactobacilli have bile salts hydrolase activity and can survive and grow in the presence of bile salts. To identify the responsible genes, primers were designed using data available in Gene-Bank and sequence analysis application software (OMIGA). EcoRI restriction sites were added to the primers to facilitate sub-cloning of the cbh gene. Chromosomal DNA of twelve Lactobacillus strains was used for screening and amplification of the cbh genes. Only from one strain - Lactobacillus plantarum BfEL92122- a 1392 bp PCR-amplicon was obtained (Fig. 34).
5.2.2 Cloning of the \( cbh \) and expression in \( E.\ coli \)

The amplified DNA was purified and ligated into pGEM-T-easy vector resulting plasmid pS1. Cloning into pBSK, after digestion with EcoRI yielded plasmid pS2. For transformation, electro-competent cells of \( E.\ coli \) easypores were used. Transformants were identified by blue/white colony screening (Fig. 35 A and B).
5.2.3 DNA sequencing

Inserted DNAs from two plasmids were sequenced using T7 and SP6 specific primers. The sequences were compared with NBCI data. Data revealed that the cbh gene of L. plantarum BfEL 92122 had a similarity of 99% with cbh gene of L. plantarum 80.

5.2.4 Expression of cbh in E. coli

Expression of cbh gene in E. coli easypores was analyzed by streaking transformants harboring plasmid pS1 or pS2 onto LB-agar medium containing 0.5%TDCA and 0.5%GDCA, respectively. Transformants with plasmid pS2 apparently expressed cbh causing deconjugate taurine- and glycine-bile acid forming precipitates on plates, as seen in figures B and C of Fig. 36.

5.2.5 Cloning of the cbh into Lactobacillus / E. coli shuttle vectors

5.2.5.1 Cloning and transformation of cbh gene into E. coli

The cbh amplicon from Lactobacillus plantarum (BfEL 92122) was cloned in pL120 (see Fig. 41) an E. coli-Lactobacillus shuttle vector and transformed into E. coli Nova Blue. Colonies grown on LB medium containing 100μg/ml Amp and 100μg/ml Em
were streaked on LB agar medium containing antibiotics and 0.5% TDCA. Transformants, producing a precipitate (Fig. 37), were chosen for plasmid analysis (Fig. 38). The presence of the \( cbh \) gene fragment was confirmed by EcoRI restriction enzyme (Fig. 39). Orientation of \( cbh \) was determined by HindIII restriction digestion, (Fig. 36). In plasmid pS3A (Fig. 41), \( cbh \) was cloned in the same orientation as the \( mob \) promoter, in pL120 and in plasmid pS3B, \( cbh \) was cloned in the opposite orientation.

Fig. 37: Selection of positive clones on TDCA- LB agar medium

Fig. 38: Isolation of plasmids from selected positive transformants.
\( M: \lambda \) Styl; lanes 1-10: positive clones; \( C: \) control plasmid pL120
Fig. 39: Conformation of the presence of the cbh gene fragment in the constructed plasmid pS3.
M: λ StyI; lanes 1-10: positive clones; C: control plasmid pL120

Fig. 40: Orientation of the cloned cbh gene by using HindIII restriction enzyme.
M: λ StyI; lanes 1-10: positive clones; C: control plasmid pL120.
Fig. 41: Construction of pS3A and pS3B
5.2.6 Expression of \( cbh \) in \( Lactobacillus \)

5.2.6.1 Transformation of pS3A and pS3B into \( Lactobacillus casei \) LK1

The two plasmid constructs pS3A and pS3B, were introduced into electro-competent \( Lactobacillus casei \) LK1 (B. Henrich, personal communication) by electroporation. Transformants were grown on MRS agar medium containing 10µg/ml erythromycin. Colonies were picked up and streaked on MRS agar plates containing 10µg/ml erythromycin and 0.5% TDCA (Fig. 42, 43). Expression of the \( cbh \) was higher in transformants carrying pS3A (\( cbh \) gene in the same direction of \( mob \). promoter) than in those with pS3B (\( cbh \) gene in the opposite orientation of \( mob \). promoter). Presence of proper plasmids was confirmed by agarose gel electrophoresis (Fig. 43).

![Fig. 42: Expression of \( cbh \) from plasmids pS3A and pS3B after transformation into \( Lactobacillus casei \) LK1 with plasmids. Arrows refer to transformants with pL120 as negative control](image1)

![Fig. 43: Isolation of clones DNA from \( Lactobacillus casei \) LK1. M: \( \lambda \) Styl; C: control plasmid; lanes 1-5: positive clones of plasmid pS3A; lanes 6-10: positive clones of plasmid pS3B d pL120](image2)
5.2.6.2 Transformation into *Lactobacillus gasseri*

Several attempts to transform pS3A or pS3B into *Lactobacillus gasseri* using different electroporation conditions and erythromycin concentrations from 10µg/ml to 3µg/ml failed. Selection for erythromycin resistance seems not to be applicable for *L. gasseri*. Therefore, selection for chloramphenicol (Cm) resistance was tested. Transformation of pGK12 (Fig. 44) which contains a Cm-resistance gene, into *L. gasseri* using different electroporation conditions (0.63, 1, 1.25, 1.5, 2 and 2.5KV/cm), resulted in many Cm-resistant clones, which all carried pGK12 (not shown). Therefore, we tried to construct a new shuttle vector with a chloramphenicol resistance gene as a selective marker.

![Fig. 44: Map of pGK12](image)

5.2.7 Construction of a new shuttle vector with a chloramphenicol resistance gene as a selective marker

Plasmid pS3A was digested with SacI and PvuI restriction enzymes (Fig. 45). The resulting 3.7 Kb SacI fragment containing the *cbh*, *repA* genes and the promoter of *mob* was cloned in both orientations into SacI linearised pBluescriptII SK+ (pBSK). *E. coli* Nova Blue competent cells were transformed. Transformants, selected by blue-white selection, were tested for the expression of the *cbh*. Expression was dependent on the orientation (Fig. 46). Plasmids representing both orientations were named pL9-1 and pL9-3, respectively. Clones carrying recombinant plasmid with the *cbh* gene in the opposite direction of lac-promoter of the vector (pL9-1) showed high expression, whereas those with *cbh* gene in the other orientation (pL9-3) didn’t show expression at all (Fig. 47 and 48).
Fig. 45: Double digest of pS3A with Sacl and PvuI.
M: λ StyI; lane 1: pS3A digested with Sacl and PvuI; lane 2: undigested plasmid.

Fig. 46: Expression of cbh gene into pL9 plasmids.
upper left: pL9-1 (cloned fragment in the opposite direction to the lac promoter of pBSK+);
upper right: pL9-3 (cloned fragment in the same direction as the lac promoter of pBSK+);
under left: clone which contains only the vector;
under right: positive control.
5.2.7.1 Cloning of the *cat-194* gene as a blunted ended fragment into the Ncol site of pL9-1 and pL9-3

Plasmid pL9-1 was linearised with Ncol, blunted with T4-polymerase, dephosphorylated and purified (Fig. 49). The chloramphenicol resistance gene from *Staphylococcus aureus* was isolated after digested digestion of pCAT with NotI as a 925 bp fragment and subsequent agarose gel electrophoresis (Fig. 49). After blunt end ligation, *E. coli* easy pores cells were transformed with the ligation mix by electroporation. Chloramphenicol-resistant clones were selected and tested for *cbh*
expression (Fig. 50). One of the clones 11 showing high cbh–activity, was shown to have the cat gene cloned in the same direction as the cbh gene (Fig. 51 and 52).

![Blunting of pL9-1 and cat-194 gene fragment ends](image)

Fig. 49: Blunting of pL9-1 and cat-194 gene fragment ends
M: λ StyI; lane1: cat fragment; lane 2: pL9-1

![Expression of the cbh gene in E. coli easy pore clones transformed with ligation mix 11](image)

Fig. 50: Expression of the cbh gene in E. coli easy pore clones transformed with ligation mix 11. 1-15 different transformants picked up after transformation.
5.2.7.2 Orientation detection of the \textit{cat}194-\textit{gene} into plasmid pL11

Ligation after blunting NotI and Ncol ends created new Ncol sites in pL11. Thus, the \textit{cat} gene can be easily replaced by another antibiotic resistance gene (Fig. 20).

5.2.8 Cloning of \textit{cbh} in \textit{pGKV2}

This vector based on the broad host-range replicon of pWG2, a \textit{Lactococcus lactis} plasmid (Fig. 53) replicates in various Gram-positive and -negative bacteria, as e.g. in \textit{Lactobacillus} and \textit{E. coli}. The \textit{cbh} amplicon was ligated into the single EcoRI–site of the \textit{pGKV2} and transformed into \textit{E. coli} Nova blue chemical competent cells. Transformants were selected on LB-agar plates supplemented with 100µg/ml Em +
5µg/ml Cm, or on plates, containing 0.5% TDCA in addition to antibiotics. On agar plates containing 0.5% TDCA cbh expressing clones were easily recognized by a precipitate around the colonies (Fig. 54). All transformants tested carried a plasmid of the expected size (not shown), which contained a cloned fragment of about 1500bp (Fig. 55).

Fig. 53: Restriction map of pGKV2 (Kok et al., 1985)

Fig. 54: Transformation and expression of the cbh gene cloned in pGKV2 in E. coli Nova-Blue

Fig. 55: EcoRI digest of L14 Clones (plasmid pL14).
M: λ Styl; lane 1: EcoRI digest of pGKV2; lanes 2-9: EcoRI digest of transformants plasmids; lane 10: undigested pGKV2
5.2.9 Transformation and expression of cbh in Lactobacillus

By optimizing the transformation conditions for L. casei and L. gasseri, we succeeded to transform these two strains with the newly constructed plasmids pS3A, pL11 and pL14. In addition, L. rhamnosus was transformed with pL11. Successful transformation was obtained with a Biorad Gene Pulser at electric field strength of 10KV/cm for L. gasseri and L. rhamnosus, and 12.5KV/cm for L. casei, a capacity of 25µF and at 200Ω in 0.1cm cuvettes. To phenotypically express Em or Cm resistance, electroporated cells were incubated for 4-5 hrs at 37°C in MRS medium, supplemented with 3ng/ml Em or Cm, respectively. By applying these conditions, plasmids pS3A, pL11 and pL14 were transformed into L. casei, L. gasseri and L. rhamnosus (Fig. 56 and Table 19). All transformants expressed cbh and were able to deconjugated taurine and glycine-bile acid, resulting in a precipitate from deoxycolic acid around colonies on MRS agar plates, supplemented with 0.5% TDCA or 0.5% GDCA (Fig. 58). EcoRI-digestion of plasmids isolated from transformants confirmed the structural integrity of these plasmids (see e.g. Fig. 57 for L. gasseri).

Segregational stability of plasmids pL11 and pL14 was tested. Plasmid pL11 in L. gasseri showed stability of 60, 25 and 6% after 70,140 and 210 generation, respectively. In L. casei the corresponding values were 56, 15 and 2%, respectively. Plasmid pL14 was less stable and was almost completely lost from L. gasseri after 140 generation (Table 20).
Chapter III

Fig. 57: *EcoRI* digest of plasmids isolated from *L. gasseri* transformed with plasmid pL14.

M: λ Styl; lanes 1 – 3: *EcoRI* digest of pL14 isolated from *L. gasseri* transformants; lane 4: undigested pL14 as a control. Arrow refers to *cbh* gene fragment.

Fig. 58: Expression of *cbh* gene which constructed in different plasmids and transformed into *Lactobacillus* sp.
### Table 19: List of transformed strains

<table>
<thead>
<tr>
<th>Constructed plasmids</th>
<th>Description / relevant phenotype</th>
<th>Transformation</th>
<th>cbh gene Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>E. coli</strong></td>
<td><strong>L. casei</strong></td>
</tr>
<tr>
<td>pS3A</td>
<td>Replicon pSMA53, cbh(^+), Em(^+), Ap(^+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pL9-1</td>
<td>Replicon pSMA53, 3.7 kb SacI fragment (cbh(^+); rep(^+); mob(^+)) cloned in pBluescript IISK+; Ap(^+)</td>
<td>+</td>
<td>NS</td>
</tr>
<tr>
<td>pL11</td>
<td>Replicon pSA53, blunted cat194 gene cloned in pL9-1; Ap(^+); cat(^+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pL14</td>
<td>Replicon pGKV2, cbh(^+) Em(^+); cat(^+)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**NS:** Antibiotic selective marker not suitable  
**NT:** Not tested  
**+:** Transformants obtained
Table 20: Segregational stability of the recombinant plasmid

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-AB</td>
<td>+AB</td>
<td>% b)</td>
<td>-AB</td>
<td>+AB</td>
<td>% b)</td>
</tr>
<tr>
<td>1st week / 50</td>
<td>37.4X10^8</td>
<td>22.5X10^8</td>
<td>60</td>
<td>34.6X10^8</td>
<td>19.3X10^8</td>
<td>55</td>
</tr>
<tr>
<td>2nd week / 100</td>
<td>12X10^8</td>
<td>3X10^8</td>
<td>25</td>
<td>41X10^8</td>
<td>6X10^8</td>
<td>14.6</td>
</tr>
<tr>
<td>3rd week / 150</td>
<td>35X10^8</td>
<td>22.2X10^7</td>
<td>6.34</td>
<td>47.6X10^8</td>
<td>9X10^7</td>
<td>1.9</td>
</tr>
</tbody>
</table>

a) Approximate number of generations  

b) Segregational stability [CFU+AB / CFU–AB X100] %  
+AB; -AB with and without antibiotic
5.2.10 Determination of bile salts tolerance

To evaluate the ability to survive and/or grow in the presence of bile, the parent strains of \textit{L. casei}, \textit{L. gasseri} and \textit{L. rhamnosus} and their transformants carrying \textit{cbh} on plasmid pL11 were compared for survival and growth in MRS medium supplemented with 0.5 or 1\% GDCA. The cell numbers of all wild type strains decreased rapidly after inoculation in the presence of bile salts, whereas transformants of these strains even showed slight increases (2-4 folds) in cell numbers (Figs. 59-61). This clearly indicates that the \textit{cbh} gene was expressed in these \textit{Lactobacillus} strains and that the bile salt hydrolase protects the cells against the detrimental effects of the bile salt. The estimated bile concentration in the gastrointestinal tract is about 0.3\%, significantly lower than the concentrations used in these experiments. From this we suppose, that the introduction and expression of a \textit{cbh} gene will increase the survival of lactobacilli in the gut.

Fig. 59: Growth and survival of \textit{L. gasseri} and its transformant \textit{L. gasseri} (pL11) in presence of GDCA.
Chapter III

Fig. 60: Growth and survival of *L. casei* and its transformant *L. casei* (pL11) in presence of GDCA

Fig. 61: Growth and survival of *L. rhamnosus* and its transformant *L. rhamnosus* (pL11) in presence of GDCA.

5.2.11 Use of the *cbh* gene of *Lactobacillus plantarum* BfEL 92122 as a food grade indicator gene

The *cbh* gene from *Lactobacillus plantarum*, when cloned into appropriate shuttle vectors, was over-expressed in *E. coli* to an extent, that transformants could be identified directly on indicator plates containing bile salts as substrate (see Fig. 62). This was verified by transforming competent *E. coli* Nova-Blue cells either with ligation mixes containing vector and *cbh* gene fragment or with intact recombinant plasmids, carrying the *cbh* gene (Fig. 63). After transformation, the cells were incubated at 37°C for 60 min and plated directly or serially diluted onto LB agar medium supplemented with 0.5% TDCA or GDCA, respectively. Incubation of the plates for 24 to 48h at 37°C, colonies of transformed cells were due to the activity of the bile salt hydrolase, surrounded by a less soluble precipitated of free deoxycholic.
acid. The very obvious phenotype of cbh expressing cells on plates supplemented with conjugated bile salts attempted us to test cbh to be used as selective marker. Fig. 62 shows that cbh acts very well as indicator gene. Therefore, selection based on cbh could not be demonstrated so far (data not shown).

Fig. 62: Transformation of *E. coli* Nova-Blue with the plasmid pL11 and plating onto LB agar medium supplemented with GDCA without any antibiotics A: after 24 hours incubation; B: after 48 hours incubation.

Fig. 63: Isolation of plasmid pL11 from *E. coli* transformants.
M: λ StyI; lane 1: control; lanes 2 & 3: pL11 from positive transformants with antibiotics; lanes 4 & 5: pL11 from positive transformants without antibiotics; lanes 6 & 7: pL11 from positive transformants without antibiotics and with ox-gall.
6 Chapter IV

Lysogeny and autolysis of lactobacilli. Analysis of temperate phage of *Lactobacillus gasseri* BfEL 92075

6.1 Introduction

For effective application of probiotic strains, lysogeny and autolysis are, especially in terms of stability of the cultures, undesirable properties. Although lysogeny is widespread in lactobacilli, relatively little information on this topic is available for many potentially probiotic lactobacilli. Dependent on the species, strains inducible for phage range from 10-80 %, but complete temperate phages are present less frequently (Carminati *et al.*, 1997; Stetter, 1977; Yokokura *et al.*, 1974). Several temperate phages (FSW, A2, Øg1e) have been isolated from Lactobacillus strains used in dairy fermentations (Brüssow, 2001). So far, data on *Lactobacillus gasseri* temperate phages are very rare. Only phi ADH, isolated from *Lactobacillus gasseri* ADH, has been well characterized. It belongs to the morphotype B1 siphovirus with double stranded linear DNA (Raya *et al.*, 1989). Here, we describe a second temperate bacteriophage - phi Lbgas1- from *Lactobacillus gasseri* BfEL 92075. In addition, a method to cure the wild-type strain from its prophage will be presented.

6.2 Results and discussion

6.2.1 Lysogeny and autolysis in *Lactobacillus* sp.

Eleven strains belonging to six Lactobacillus species were examined for autolysis in a standard buffer system (Nilskasaari, 1989; Yokoi *et al.*, 2004) and for induction of prophages after treatment with mitomycin C (MC). Although MC-induced lysis was observed with five strains, intact phage particles were only found in the lysates of *L. gasseri* strains BfEL 92075 and NCK 102-adh by electron microscopy. These phages could be propagated only in the prophage-cured derivatives BfEL 92075-1 and NCK of the parent strains, respectively. *L. gasseri* NCK 102-adh and its prophage-cured derivative NCK 102 (B. Henrich, personal communication) were used as control strains throughout these experiments.

The efficiency of induction of *L. gasseri* BfEL 92075 was dependent on MC- and Ca++-concentration, and on the cell density at the time of MC addition. Best induction in MRS-medium was observed at a low cell density (OD$_{620}$<0.25), MC-concentration of
0.1 to 0.4 µg/ml and a Ca\textsuperscript{++}- concentration of 10 mM. Several attempts to induce the strain by UV irradiation always showed incomplete lysis (data not shown).

Autolysis of bacteria is caused by specific, mostly cell-wall-associated enzymes, peptidoglycan hydrolases. In lysogenic strains induction of an intact or even a defective prophage may simulate autolysis. The lytic system of the prophage is then, at least partially, responsible for the host strain lysis. Autolytic activities of all strains used for induction experiments were determined after transfer of stationary phase bacteria into buffer solution at 37°C. The extent of autolysis differed significantly, revealing a species and strain dependency of the autolytic behaviour. After curing of its prophage (shown later), \textit{L. gasseri} BfEL 92075 lost the autolytic activity almost completely. A non MC-inducible, but not completely prophage-cured derivative of this strain showed autolysis only after prolonged incubation. In addition, large amounts of defective phage particles were detected in the lysates of strongly autolytic \textit{L. brevis} strains, indicating that mainly phage-encoded enzymes were responsible for autolysis in these lactobacilli. Our results were in agreement of those by \textit{Yokoi et al., 2004}, who suggested that phage encoded lysine and bacterial lytic enzymes act additively in autolysis of \textit{L. gasseri} cells. Although NCK102 strain is non-inducible, it still contains lysis genes. As shown below, this result may be explained by presence of \textit{phi Lbgas1} DNA in the chromosomal DNA of \textit{L. gasseri} NCK102-Adh and \textit{L. gasseri} NCK102. The results are summarized in Table 21 and in Fig. 64 and 65.
Table 21: Lysogeny and autolysis of Lactobacillus sp.

<table>
<thead>
<tr>
<th>Lactobacillus. sp.</th>
<th>Strain</th>
<th>MC-induced lysis(^a)</th>
<th>Phage particles(^b)</th>
<th>Autolysis(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. acidophilus</td>
<td>BfEL 92015</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L. amylovorus</td>
<td>BfEL 92203</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L. plantarum</td>
<td>BfEL 92122</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L. brevis</td>
<td>BfEL 92026</td>
<td>-</td>
<td>+d</td>
<td>+++</td>
</tr>
<tr>
<td>L. brevis</td>
<td>BfEL 92027</td>
<td>+</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>L. johnsonii</td>
<td>BfEL 92170</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>L. gasseri</td>
<td>BfEL 92075</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>L. gasseri</td>
<td>BfEL 92075-1(^1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>L. gasseri</td>
<td>BfEL 92075-2(^2)</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
</tr>
<tr>
<td>L. gasseri</td>
<td>NCK 102-adh</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>L. gasseri</td>
<td>NCK 102</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

\(^a\): -) no induction of prophage; +) induction;
\(^b\): +) infectious phage; +d) >99% defective phage particles
\(^c\): -) no lysis after 24h; (+) weak lysis after 24h; +) incomplete lysis after 24h; ++) complete lysis after 2h; +++) complete lysis after 1h.
\(^1\): prophage-cured derivative of L. gasseri BfEL 92075
\(^2\): non MC-inducible derivative of L. gasseri BfEL 92075

Fig. 64: Autolysis of Lactobacillus sp.
Cells were resuspended in autolysis buffer (10mM Tris pH7).
6.2.2 Bacteriophage Lbgas1: Morphology and genome structure

The phage particles obtained after MC-induction of *L. gasseri* BfEL 92075 possessed isometric, hexagonal heads, contractile tails and a base plate fitted with fibres (Fig. 66). The sizes of the prophage substructures are summarized in Table 22.

Fig. 66: Electron micrographs of *phi* Lbgas1 (bacteriophage inducible from *Lactobacillus gasseri* BfEL 92075)
Table 22: Dimensions* of phage Lbgas1

<table>
<thead>
<tr>
<th>Nr.</th>
<th>Head C/C Width</th>
<th>Neck S/S Length</th>
<th>Tail Width</th>
<th>Length</th>
<th>Sheath a Width</th>
<th>Length</th>
<th>Base plate Width</th>
<th>Length</th>
<th>Fiber Width</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>63.5</td>
<td>57.3</td>
<td>12.1</td>
<td>10.1</td>
<td>19.4</td>
<td>176.2</td>
<td>26.1</td>
<td>103.1</td>
<td>27.6</td>
<td>50.9</td>
</tr>
<tr>
<td>2</td>
<td>60.4</td>
<td>55.0</td>
<td>11.7</td>
<td>8.9</td>
<td>20.9</td>
<td>173.6</td>
<td>27.4</td>
<td>106.4</td>
<td>25.1</td>
<td>50.0</td>
</tr>
<tr>
<td>3</td>
<td>64.7</td>
<td>57.9</td>
<td>9.7</td>
<td>10.4</td>
<td>19.2</td>
<td>175.4</td>
<td>25.3</td>
<td>105.8</td>
<td>24.6</td>
<td>39.7</td>
</tr>
<tr>
<td>4</td>
<td>63.5</td>
<td>60.6</td>
<td>12.1</td>
<td>11.1</td>
<td>20.2</td>
<td>179.6</td>
<td>24.5</td>
<td>103.9</td>
<td>23.1</td>
<td>48.0</td>
</tr>
<tr>
<td>5</td>
<td>66.7</td>
<td>53.9</td>
<td>10.7</td>
<td>8.2</td>
<td>21.7</td>
<td>180.9</td>
<td>26.3</td>
<td>100.9</td>
<td>23.0</td>
<td>46.7</td>
</tr>
<tr>
<td>6</td>
<td>60.4</td>
<td>60.2</td>
<td>9.5</td>
<td>8.8</td>
<td>19.3</td>
<td>179.6</td>
<td>24.4</td>
<td>97.8</td>
<td>24.2</td>
<td>46.8</td>
</tr>
<tr>
<td>7</td>
<td>60.1</td>
<td>56.9</td>
<td>9.7</td>
<td>12.2</td>
<td>18.9</td>
<td>178.8</td>
<td>22.8</td>
<td>102.7</td>
<td>21.4</td>
<td>46.3</td>
</tr>
<tr>
<td>8</td>
<td>62.6</td>
<td>59.4</td>
<td>11.3</td>
<td>20.6</td>
<td>179.0</td>
<td>22.9</td>
<td>98.7</td>
<td>38.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>61.3</td>
<td>54.3</td>
<td>18.5</td>
<td>24.2</td>
<td>102.2</td>
<td>50.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>61.7</td>
<td>20.5</td>
<td>22.5</td>
<td>92.3</td>
<td>46.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>19.0</td>
<td>22.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td>26.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Average 62.5 57.3 10.9 10.0 19.8 177.9 24.6 101.4 24.1 46.4

*: after tail contraction  C/C : corner to corner  S/S: side to side; * using analySIS 3.2 software (Soft Imaging SIS, Münster, Germany)

For further analysis and characterisation of phage Lbgas1, DNAs of phi Lbgas1 and phi ADH were isolated as described in Material and Methods. In addition, chromosomal DNAs of L. gasseri BfEL 92075; L. gasseri NCK102-Adh; L. gasseri NCK102 were extracted.

### 6.2.3 Phage DNA restriction analysis

To characterize phi Lbgas1, the extracted phage DNA was digested with the restriction enzymes: EcoRI, HindIII, Sall, Hinfl, SpeI, Nhel, XbaI, SspI, HpalI and EcoRV and the DNA digests were separated on 1.5% and 0.6% agarose gels (Fig 67A and B), respectively.
The genome size of \textit{phi} Lbgas1 was calculated from the DNA restriction patterns by estimating the fragments generated by some restriction enzymes, e.g. EcoRI, Spel and Nhel, which revealed clear fragment profiles on the agarose gel. The estimated genome size was about 44 Kb (see Table 23).

In addition, heating of phage DNA digests at 74°C for 5 min (data not shown) revealed no differences before and after heating, indicating the absence of cohesive ends \textit{(Quiberoni et al., 2003)}. \textit{phi} Lbgas1 may therefore be considered a phage of the \textit{pac}-type.
Table 23: Estimation of \( \phi Lb \) gas1 genome size

<table>
<thead>
<tr>
<th>NO.</th>
<th>Fragment Size (kp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EcoRI</td>
</tr>
<tr>
<td>1</td>
<td>12.300</td>
</tr>
<tr>
<td>2</td>
<td>11.700</td>
</tr>
<tr>
<td>3</td>
<td>7.100</td>
</tr>
<tr>
<td>4</td>
<td>3.800</td>
</tr>
<tr>
<td>5</td>
<td>3.300</td>
</tr>
<tr>
<td>6</td>
<td>3.000</td>
</tr>
<tr>
<td>7</td>
<td>2.750</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>43.950</strong></td>
</tr>
</tbody>
</table>

### 6.2.4 Comparative analysis of restriction patterns

Bacteriophages \( \phi Lbgas1 \) and \( \phi ADH \) DNAs were compared by restriction analysis using endonucleases HindIII, Nhel, Spel and EcoRV. The restriction patterns obtained of \( \phi Lbgas1 \) were different from those of \( \phi ADH \) DNA. Although these DNA fingerprints revealed that phage \( \phi Lbgas1 \) from \( L. \text{gasseri} \) BfEL 92075 and phage \( \phi ADH \) from \( Lactobacillus gasseri \) NCK102- Adh are genetically different, some fragments with identical size were detected. (Fig. 68).
Fig. 68: DNA fingerprints of phi Lbgas1 and phi ADH.

M: λStyl; lane 1: phi Lbgas1+HindIII; lane 2: phi ADH +HindIII; lane 3: phi Lbgas1+NheI; lane 4: phi ADH+NheI; lane 5: Phi Lbgas1+SpeI; lane 6: Phi Adh+SpeI; lane 7: phi Lbgas1+EcoRV; lane 8: phi ADH + EcoRV.

6.2.5 SDS-PAGE

For further characterization of phi Lbgas1, the profile of structural proteins on SDS-PAGE was studied in comparison with phi ADH protein profile (Fig. 69). The protein profile of phi Lbgas1 showed 3 major proteins with estimated molecular masses of about 52, 42 and 21 kDa. This protein profile of phi Lbgas1 is completely different from the protein profile of the cos-type phi ADH, which revealed two major proteins with molecular masses of about 32 and 26 kDa. This result also demonstrates that temperate phi Lbgas1 from strain L. gasseri BFEL 92075 is a pac-type phage. It appear that L. gasseri phages divide into two groups according to their nature of DNA packaging (pac-type e.g. phi Lbgas1 and cos-type e.g. phi ADH) and that this nature is reflected by their structural proteins, as it has been for Streptococcus thermophilus (Le Marrec et al., 1997; Y. Ali, unpublished data).
6.2.6 Southern blot and hybridization

Southern blot and hybridization were performed to prove that phi Lbgas1 is integrated into the genome of its host L. gasseri BfEL 92075. DNA of phi Lbgas1 was hydrolyzed with HindIII or NheI enzymes, labeled and hybridized in a Southern blot to HindIII-digested L. gasseri BfEL 92075 DNA (Fig. 70). The hybridization revealed differences in the restriction patterns, which are expected when comparing phage DNA with prophage DNA (Raya et al., 1992). This proved that L. gasseri strain BfEL 92075 harbors the temperate phi Lbgas1 as a prophage.

The relationship between phi Lbgas1 and phi ADH as well as the occurrence of phi Lbgas1 homologous sequences in L. gasseri strains were studied. Labeled phi Lbgas1 DNA was hybridized to blots of Nhel digested DNA of phi ADH, L. gasseri NCK102-Adh and L. gasseri NCK102 (cured from ADH prophage) DNA. Both temperate phages showed weak homology (Fig.71). However, the chromosomal DNA of L. gasseri NCK102 revealed DNA bands efficiently hybridizing with phi Lbgas1 probe. This homology may due to presence of parts of phi Lbgas1 in the genome of L. gasseri NCK102, or to presence of DNA sequences very similar to phi Lbgas1. This result may explain the autolytic behavior of L. gasseri NCK102 (see 6.2.7), which appears to be caused by hydrolase activities induced from genes on a non-inducible or defective phi Lbgas1 prophage.
Chapter IV

Fig. 70: Southern Blot Hybridization of phi Lbgas1 DNA and L. gasseri BIEL 92075.

lane 1: HindIII digested DNA of phi Lbgas1; lane 2: HindIII digested chromosomal DNA of L. gasseri BIEL 92075; lane 3: marker λStyI as a negative control.

Fig. 71: Southern Blot Hybridization of phi Lbgas1 and phi ADH.

M: λStyI; lane 1: phi Lbgas1; lane 2: phi ADH; lane 3: Chromosomal DNA of L. gasseri BIEL 92075; lane 4: Chromosomal DNA of L. gasseri NCK102-Adh; lane 5: Chromosomal DNA of L. gasseri NCK102. DNA in Lane 1-5 were digested with Nhel.
6.2.7 PCR analysis for the holin-lysin genes of phi Lbgas1

Our results had been revealed phi Lbgas1 homologous sequences in L. gasseri NCK102 DNA and autolytic behavior of this strain. Therefore, we carried out PCR analysis using primers specific for the holin-lysin genes of phi Lbgas1 (Fig. 72). Restriction analyses of the holin-lysin gene amplicons of 1477bp generated were performed using HapII, XbaI and TaqI restriction enzymes. The results in Fig. 73 revealed no differences in restriction pattern for the enzymes between all holin-lysin genes from phi Lbgas1; phi ADH; L. gasseri BfEL 92075; L. gasseri NCK102- Adh. This result confirms that the autolytic behavior of L. gasseri NCK102 (cured from phi ADH) is most likely due to the presence of holin-lysin expressed from phi Lbgas1 DNA sequence present in the chromosomal DNA of L. gasseri NCK102.

**Fig. 72: PCR amplicons of holing-lysin cassette.**
M: λStyI; lane 1: phi Lbgas1; lane 2: phi ADH; lane 3: L. gasseri BfEL 92075; lane 4: L. gasseri NCK102- Adh; lane 5: L. gasseri NCK102

**Fig. 73: Restriction analysis of holin-lysin gene fragments.**
Fragments of the holin-lysin-operon were amplified by PCR with primers (5'-CGCAAGAAGGTAAACCGTTAG-3') and (5'-CCAG-AGAAACATCCAGACG-3'). The resulting 1477bp fragments were digested and analyzed. M: λStyI; lane 1: phi Lbgas1; lane 2: phi ADH; lane 3: L. gasseri BfEL 92075; lane 4: L. gasseri NCK102-Adh; lane 5: L. gasseri NCK102
6.2.8 Pro-phage curing from *L. gasseri* BfEL 92075

*L. gasseri* BfEL 92075 was cured from its prophage *phi* Lbgas1 after MC treatment. Selection of MC-resistant derivatives (Fig.74) was only possible in the presence of phage-specific antibodies during induction and selection. After induction of strain *L. gasseri* with MC in the presence of anti-phage serum, 30 µl of induced culture were plated onto MRS agar plates, surviving cells were streaked in parallel onto LMRS agar and LMRS agar containing 0.2 µg MC/ml. The plates were anaerobically incubated at 37°C for 72h. From 25 colonies only eight were able to grow in the presence of 0.2µg MC/ml. These colonies were inoculated into LMRS broth and grown overnight at 37°C.

![Fig. 74: Selection of *Phi Lbgas1*-cured derivatives of *L. gasseri* after MC treatment in the presence of anti-phage serum.](image)

Re-induction of the eight derivatives with 0.2µg MC/ml revealed that only 3 of them and the control could be induced again. The other five derivatives could no longer be induced by MC (Fig. 75).
Fig. 75: Induction of *L. gasseri* and its derivatives with 0.2µg MC/ml.

**Control:** *L. gasseri* BfEL 92075; **S1-S5:** derivatives of *L. gasseri* isolated after treatment with MC in the presence of phi Lbgas1 antiserum.

To confirm the descent of the non-inducible derivatives from *L. gasseri* BfEL 92075, ARDRA analysis and SDS-page were done. PCR products of 16-23S rDNA genes were restricted with HaeIII and HinfI. The results revealed that *L. gasseri* and its five non-inducible derivatives showed the same ARDRA patterns (Fig. 76). Also when SDS page applied there were no differences between *L. gasseri* and its five non-inducible derivatives. See Fig. 77

Fig. 76. ARDRA for the lysogenic strain *L. gasseri* BfEL 92075 and its non-inducible derivatives using HaeIII and HinfI restriction enzymes.

**M:** λStyl; **lanes 1-5:** non-inducible derivatives of *L. gasseri* BfEL 92075; **lane 6:** lysogenic strain *L. gasseri* BfEL 92075
6.2.9 Southern blot and DNA-DNA hybridization for phi Lbgas1, L. gasseri BfEL 92075 and its prophage-cured derivatives

Southern blot and hybridization were performed to study the relatedness between the temperate phi Lbgas1, its host Lb. gasseri BfEL 92075 and the five non-inducible derivatives (Fig. 77). The hybridization revealed that L. gasseri and two of its five non-inducible derivatives still harbored the temperate phage, while the other three non-inducible derivatives were cured from the prophage.

Fig. 78: Southern blot hybridization of phi Lbgas1, Lb. gasseri and its putatively cured derivatives.
6.2.10 Spot test

To further confirm the prophage-curing derivatives were tested for sensitivity to *phi* Lbgas1 in a spot test (Fig. 78). Clear spots appeared in the three derivatives of *L. gasseri* after infection with a dialyzed *phi* Lbgas1 lysate at dilutions up to $10^{-7}$. Plaques count indicated that the phage *phi* Lbgas1 lysate titer ranged from $10^8$ to $10^9$ PFU per ml. These results indicated the sensitivity of cured derivatives to *phi* Lbgas1.

![Spot test for lysogenic strain and *L. gasseri* BfEL 92075 and one of its prophage-cured derivatives BfEL 92075-1 using *phi* Lbgas1](image)

During our study we faced difficulties to discover plaques formed by *phi* Lbgas1 on prophage-cured strain *L. gasseri* BfEL 92075-1. This may be due to low numbers of intact phages induced by MC treatment, as *phi* Lbgas1 seemed to have an abnormal tail structure (as seen in Fig 66). Most studies so far focused on improvement of plaque formation of virulent phages (Lillehaug, 1997). We optimized the conditions to obtain plaques with *phi* Lbgas1 on *L. gasseri* prophage-cured strain BfEL 92075-1 (Fig.79).

Our isolation of a prophage cured derivative of *L. gasseri* BfEL 92075 will be useful to eliminate the potential occurrence of virulent phages originating from *phi* Lbgas1. It was previously reported that virulent phage Ø FSV had derived from *L. casei* temperate phage Ø FSW (Shimizu-Kadota and Sakurai,1982). Also, Lysogenic starters used in dairy products may be considered as a source of bacteriophage contamination on an industrial scale (Quiberoni *et al.*, 2003; Shimizu-Kadota and Sakurai, 1982). Prophage curing improved *L. gasseri* under two aspects: i) it
eliminated the potential of virulent phages originating from \( \text{phi} \) Lbgas1, and ii) it eliminated the lysis gene from \( L. \text{gasseri} \) genome. We consider both aspects to be of advantage for a strain to be applied as probiotic.

7 Summary and conclusion

Lactobacilli have a worldwide industrial use as starters in the manufacturing of fermented dairy products. Moreover, some \( \text{Lactobacillus} \) strains have probiotic characteristics leading to increasing the use of lactobacilli in fermented food products. Increasing of probiotic lactobacilli in food products and nutritional supplements underscores the need to evaluate and correctly identify these useful bacteria.

The aims of this study were to evaluate putative probiotic lactobacilli isolated from different sources as fermented milk products from Egypt and faeces from two new born children (twins) for establishment of probiotic strains, to clone and transform a bile salt hydrolase gene for genetic improvement of \( \text{Lactobacillus} \) strains to act as probiotics. Chapter I of the present study deals with for identification of LAB using molecular methods, like e.g. ARDRA, PFGE, and sequencing of the 16S rRNA gene, to achieve accurate identification for selection of probiotics. The objectives of chapter II were evaluation and characterization of 26 \( \text{Lactobacillus} \) strains through probiotic selection criteria (acid tolerance, bile salts resistance and antimicrobial activity). Also, a safety assessment of some \( \text{Lactobacillus} \) isolates was done. We report a survey of susceptibility of 26 \( \text{Lactobacillus} \) against 13 antibiotics including inhibitors of cell wall synthesis, protein and nucleic acid synthesis.

Resistance to bile salts is one of the important criteria for selection and establishment of probiotic strains with good health benefits. Most of \( \text{Lactobacillus} \) strains showed some resistance to bile salts. In chapter III, we describe that conjugated bile salt-hydrolase gene (\( \text{cbh} \)) is responsible for de-conjugation of bile acids. The \( \text{cbh} \)-gene and its regulatory sequences (promoter, ribosomal-binding site and terminator) from highly bile salts resistance \( \text{Lactobacillus plantarum} \) BIEL 92122 strain were amplified by PCR, cloned into \( \text{E. coli} \) vectors. The \( \text{cbh} \) was so strongly expressed in \( \text{E. coli} \) that transformants could be identified directly on indicator plates containing bile salts as a substrate, indicating that the \( \text{cbh} \) gene might be generally useable as a food-grade indicator gene. \( \text{Cbh} \) was recloned into several \( \text{E. coli/Lactobacillus} \) shuttle vectors. Electrotransformation of \( L. \text{gассери, L. casei} \) and \( L. \text{rahmносus} \) strains with these
recombinant plasmids and expression of \( cbh \) gene significantly improved resistance against ox-gall, glycodeoxycholic- and taurodeoxycholic acid. For probiotic bacteria to survive in the product and after consumption in the consumer’s gastrointestinal tract, inhibitory effect of bacteriophages and autolysis during industrial fermentation should be also considered. Therefore, chapter IV focused on lysogeny and autolysis of lactobacilli caused by bacteriophages or their enzymes and the characterization of a bacteriophage, \( \phi \) \( \text{Lbgas1} \) from \( \text{L. gasseri} \). The establishment of a prophage cured derivative of this strain is reported.

Finally, we propose for completion of characterization prospective in vivo studies on survival of Lactobacillus strain in animal models (like e.g. Gottingen minipigs). These studies may then form the basis for clinical evaluation the potential of the selected strains. health properties
8 Zusammenfassung und Schlussfolgerungen


Kapitel I dieser Arbeit befasst sich mit der exakten Identifizierung von 26 Lactobacillus-Isolaten mittels molekularbiologischer Methoden wie ARDRA, PFGE und Sequenzierung von 16S rRNA Genen.

In Kapitel II werden probiotische Eigenschaften dieser Stämme, wie Säure- und Gallensalztoleranz sowie deren antibakteriellen Aktivitäten und ihre Empfindlichkeiten gegenüber 13 Antibiotika beschrieben.

Wichtig für das Überleben probiotischer Bakterien im Produkt und im Verdauungstrakt des Konsumenten sind deren Verhalten gegenüber Bakteriophagen sowie ihre autolytischen Aktivitäten. In Kapitel IV werden Fragen zur Lysogenie und Autolyse einiger Lactobacillen Stämme beantwortet. Im Rahmen dieser Untersuchungen wurde ein temperenter Bacteriophage, phiLbgas1, aus L. gasseri isoliert und charakterisiert, sowie ein prophagenfreies Derivat dieses Stammes hergestellt.

References


References

89 (4):553-563.


**Huijghebaert, S. M.; Mertens, J. A. and Eyssen, H. J. (1982)** Isolation of a bile salt


References

Biotechnology VEH, B. Lee (Ed), New York: 219-290.


Lick, S. Personal Communication. Federal Research Centre for Nutrition and Food, Institute of Microbiology, Kiel, Germany.


Raya, R. R.; Fremaux, C.; De Antoni, G. L. and Klaenhammer, T. R. (1992) Site-Specific Integration of the Temperate Bacteriophage Φ adh into the *Lactobacillus gasseri* Chromosome and Molecular Characterization of the Phage (attP) and Bacterial (attB) Attachment Sites. J. Bacteriol. 174;17: 5584-5592

Characterization of the temperate bacteriophage phi adh and plasmid transduction in *Lactobacillus acidophilus* ADH. Appl Environ Microbiol., 55(9): 2206-2213


**Sanders, M. E. and Huis in’t Veld, J. (1999)** Bringing a probiotic-containing functional food to the market: microbiological, product, regulatory and labeling issues. Antonie van Leeuwenhoek 76: 293–315


ACKNOWLEDGMENTS

My endless thanks are to GOD for conciliation and providing patience.
I would like to thank the Egyptian government, ministry of higher education and Benha University for providing me a full scholarship and financial support during my accommodation in Germany.
I sincerely thank and appreciate my supervisor Prof. Dr. K. J. Heller for giving me the opportunity to do my Ph.D. in the Institute for Microbiology, BfEL, Kiel and for his efforts, helping, fruitful discussion, kindness and generous advice during my work in this research.
I owe a great deal of thanks to Dr. A. Geis for his benefit suggestions, constructive criticism, invaluable assistance and guidance, fruitful discussion, kindness, and allowing me a lot of his time.
I would like to express my deep thanks to Dr. H. Neve for his real help and useful scientific suggestions during my work. My deep thanks to Dr. W. Bockelmann.
I greatly appreciate the assistance provided by Frau Claudia Zinke for helping in identification of my babies’ isolates.
Special thanks to my colleague Jochen Dietrich for his standing beside me in the critical moments and offering assistance. My thankful to my colleagues Yahya Ali, and Mazhar Mohamed for their kindness and help.
My grateful thanks to Frau Veronika Wind for her spontaneous assistance and providing technical facilities during this work.
I am thankful to Frau Inka Lammertz, Frau Vera Meiners, Frau Angela Back, Frau G. Paasch and Here Niels Rosch for their spontaneous assistance I would like to express my deepest thanks to Frau Annegret Laudy and Frau Britta Kaminski.
The warmest feelings are extending to my wife Eman, and my sons Mohamed, Omar and Abe El-Rahman for their valuable moral support in the progress of my research.
Last, and by no means least, my heartfelt thanks to all staff in Food Science Department in Faculty of Agriculture Moshtoher, Benha University, Egypt.
11 Curriculum vitae

Name: Elsayed Ismail
Date of Birth: 24 August 1973
Birth place: Benha, Qaliubiya, Egypt
Nationality: Egyptian

Education:
1980 – 1985 Elementary school, in Atrieb Primary School, Benha, Egypt
1986 – 1988 Primary school, in Abo Bakr El-Sediek Primary School, Benha, Egypt
1992– 1995 Bachelor Degree, in the Department of Food science, Faculty of Agriculture Moshtohor, Zagazig University, Benha Branch, Egypt

Research
1996 – 2000 M.Sc. Degree, in the Department of Food science, (Dairying), Faculty of Agriculture Moshtohor, Zagazig University, Benha Branch, Egypt
2003– 2007 Ph.D Degree:
Experimental work was done in the Institute of Microbiology, Federal Research Centre for Nutrition and Food (BfEL) at Kiel.
Final examination took place in Faculty of Agricultural and Nutritional Sciences, Christian Albrecht’s University of Kiel, Germany.
12 Lebenslauf

Name: Elsayed Ismail
Geburtsdatum: 24 August 1973
Geburtsort: Benha, Qaliubiya, Ägypten
Staatsangehörigkeit: Ägypter

Schulbildung:
1989–1991 Schulausbildung, Benha, Ägypten

Studium
1992–1995 Bakkalaureat, an der Abteilung für Lebensmittelwissenschaft der Agrarwissenschaft Fakultät Moshtohor, Zagazig Universität, Benha Branch, Egypt
1996–2000 M.Sc. Grad, an der Abteilung für Lebensmittelwissenschaft der Agrarwissenschaft Fakultät Moshtohor, Zagazig Universität, Benha Egypt
2003–2007 Promotion:

Die experimentellen Arbeiten wurden am Institut für Mikrobiologie, Bundesforschungsanstalt für Ernährung und Lebensmittel in Kiel durchgeführt.

Die Abschlussprüfung fand an der Agrar- und Ernährungswissenschaftlichen Fakultät der Christian-Albrechts-Universität zu Kiel, Deutschland, statt.