

Aus dem Institut für Mikrobiologie der Bundesforschungsanstalt für
Ernährung und Lebensmittel, Kiel

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

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Abbreviations

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

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 thermophilic 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 1: A number of health benefits of probiotics^a

Target health benefit	Probiotic	Postulated Mechanism
Lactase intolerance	Lactase positive strains	-Bacterial lactase acts on lactose in the small intestine
Prevention of diarrhoea and resistance to enteric pathogenic	<i>Lactobacillus rhamnosus</i> GG and <i>Bifidobacterium lactis</i> BB-12	- Adjuvant effect increasing antibody production - Systemic immune effect - Colonization resistance - Alteration of intestinal conditions to be less favorable for pathogenicity (pH, SCFA, bacteriocins)
<i>Helicobacter pylori</i> infection and complications (B gastritis, peptic ulcers and gastric cancer)	<i>L. johnsonii</i> La1; <i>L. gasseri</i> OLL 2716	-Growth inhibition - 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	<i>L. casei</i> Shirota; <i>L. rhamnosus</i> GG and LC-705 and bifidobacteria ; <i>Propionibacterium</i>	-Modify the gut flora leading to decreasing glucuronidase and carcinogen levels e.g. carcinogenic aflatoxin in the lumen
Mucosal immunity	<i>L. casei</i> Shirota; <i>B. lactis</i> HN019 and <i>L. rhamnosus</i> 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	<i>L. rhamnosus</i> GG; <i>B. lactis</i> 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	<i>Lactobacillus acidophilus</i>	-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

<p>Appropriateness</p> <ul style="list-style-type: none"> i. Accurate taxonomic identification ii. Normal inhabitant of the species targeted: human origin for human probiotics iii. Nontoxic, nonpathogenic, GRAS status
<p>Technological suitability</p> <ul style="list-style-type: none"> iv. Amenable to mass production and storage: adequate growth, recovery, concentration, freezing, dehydration, storage, and distribution v. Viability at high cell densities vi. Stability of desired characteristics during culture preparation, storage, and delivery vii. Provides desirable organoleptic qualities (or no undesirable qualities) when included in foods or fermentation processes viii. Genetically stable ix. Genetically amenable
<p>Competitiveness</p> <ul style="list-style-type: none"> x. Capable of survival, proliferation, and metabolic activity at the target site in vivo xi. Resistant to bile xii. Resistant to acid 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 xiv. Adherence and colonization potential preferred
<p>Performance and functionality</p> <ul style="list-style-type: none"> xv. Able to exert one or more clinically documented health benefits (e.g. lactose tolerance) xvi. Antagonistic toward pathogenic / cariogenic bacteria xvii. Production of antimicrobial substances (bacteriocins, hydrogen peroxide, organic acids, or other inhibitory compounds) xviii. Immunostimulatory xix. Antimutagenic xx. Anticarcinogenic xxi. Production of bioactive compounds (enzymes, vaccines, peptides)

Source: **Klaenhammer and Kullen, (1999)**

For evaluation of newly discovered probiotics, the scheme of FAO/WHO (Fig. 1) may be useful (FAO/WHO, 2002).

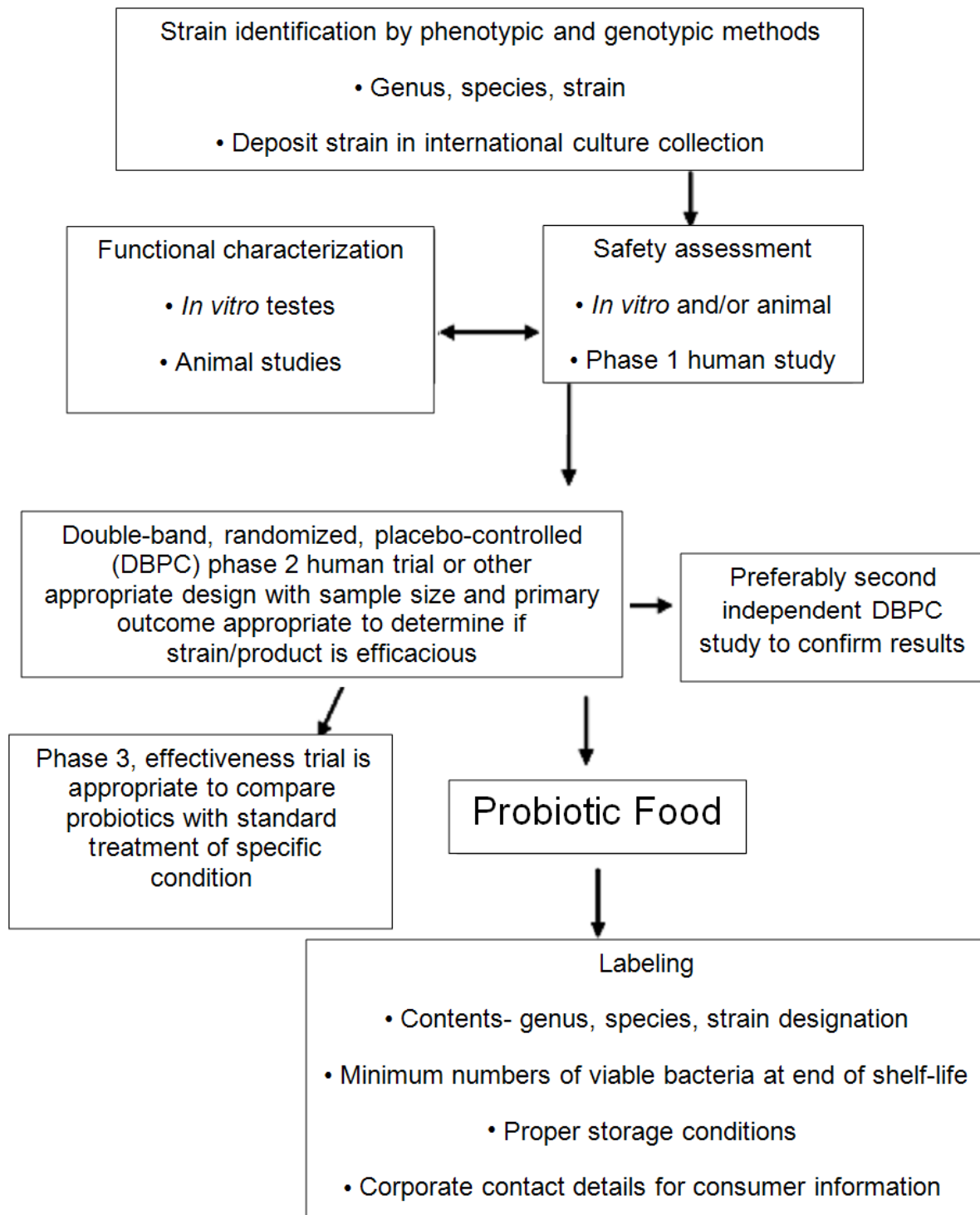


Fig. 1: FAO/WHO guidelines for the evaluation of probiotics in food

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)**.

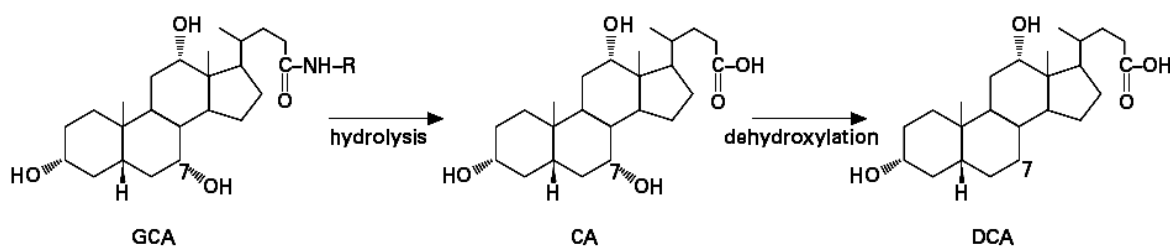


Fig. 2: The two predominant bacterial modifications of bile salts

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

No	Strains	Source	Synonym
1	<i>Lactobacillus acidophilus</i>	BfEL 92015	
2	<i>Lactobacillus johnsonii</i>	BfEL 92170	
3	<i>Lactobacillus amylovorus</i>	BfEL 92203	DSM 20552
4	<i>Lactobacillus brevis</i>	BfEL 92026	
5	<i>Lactobacillus brevis</i>	BFEL 92027	
6	<i>Lactobacillus gasseri</i>	BfEL 92075	DSM 20243
7	<i>Lactobacillus gasseri</i> BfEL 92075-1	This study	
8	<i>Lactobacillus gasseri</i> BfEL 92075-2	This study	
9	<i>Lactobacillus gasseri</i> NCK102-ADH	Raya <i>et al.</i> ,1989	
10	<i>Lactobacillus gasseri</i> NCK102	Raya <i>et al.</i> ,1989	
11	<i>Lactobacillus plantarum</i>	BfEL 92122	
12	<i>Lactobacillus rahmnosus</i>	BfEL 92149	

No	Strains	Source	Synonym
13	<i>Lactobacillus casei</i> LK1	Meyer-Barton, 1995	
14	<i>Lactobacillus acidophilus</i>	BfEL 92006	ATCC10697
15	<i>Lactobacillus johnsonii</i>	BfEL 92204	DSM 20553
16	<i>Lactobacillus plantarum</i>	BfEL 92265	DSM 20174
17	<i>Lactobacillus rahmnosus</i>	BfEL 92146	DSM 20178
18	<i>Lactobacillus brevis</i>	BfEL 92113	DSM 20197
19	<i>Lactobacillus reuteri</i>	BfEL 92126	DSM 20016
20	<i>Lactobacillus delb. subsp. bulgaricus</i>	BfEL 92067	ATCC 12278
21	<i>Lactobacillus delb. subsp. lactis</i>	BfEL 92237	DSM20072
22	<i>Lactobacillus plantarum</i> (A)	This study	
23	<i>Lactobacillus plantarum</i> (C)	This study	
24	<i>Lactobacillus plantarum</i> (D)	This study	
25	<i>Lactobacillus plantarum</i> (E)	This study	
26	<i>Lactobacillus fermentum</i> IS1	This study	
27	<i>Lactobacillus plantarum</i> IS2	This study	
28	<i>Lactococcus lactis</i> IS5	This study	
29	<i>Streptococcus thermophilus</i> IS6	This study	
30	<i>Lactococcus lactis</i> IS7	This study	
31	<i>Lactococcus lactis</i> IS8	This study	
32	<i>Lactococcus lactis</i> IS9	This study	
33	<i>Lactobacillus plantarum</i> IS13	This study	

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

B)	<i>E. coli</i>	Genotype	Source
1.	Easypore	F', ara D139 (ara, leu)7697, lacX74, galU, galK, mcrA -(mrrhsdRMS-mcrBC), rpsL, dor, 80dlacZ M15, endA1, nupG, recA1.	Eurogentec, Belgium
2.	JM109	F'traD36 lacI ^q Δ(lacZ)M15 pro A+ B+ / e14 ⁻ (McrA)Δ(lac-proAB)thi gyrA96 (Nal ^r)endA1 hsdR17(r _k ⁻ m _k ⁺)relA1 supE44 recA1 (1)	Stratagene,

3.	Novablue	endA1 hsdR17(rK12- mK12+) supE44 thi-1 recA1 gyrA96 relA1 lacF'[proA+B+ lacIq \bar{Z} M15::Tn10 (TcR)]	Novagen,
----	----------	--	----------

C) Phages

Phage	Characteristics	Reference
Φ Lbgas1	Temperate, isometric, contractile tail, 44kb, pac-type	This study
Φ ADH	Temperate, isometric, , 44kb, cos-type	Raya <i>et al.</i> , 1992

2.2 Media

LB- Medium (<i>E. coli</i>):	Pepton from casein	10.0 g/l
	Yeast extract	5.0 g/l
	NaCl	5.0 g/l
	pH 7.0 \pm 0.2	
MRS-Medium (<i>Lactobacillus</i>):	Peptone 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

Antibiotics & Additives	Final concentration in medium for		Dissolving agent
	<i>E. coli</i>	<i>Lactobacillus</i>	
Ampicillin	200 µg/ml	-	H ₂ O _{dest.}
Erythromycin	150 µg/ml	5-10 µg/ml	Ethanol
Chloramphenicol	15 µg/ml	5-10 µg/ml	Ethanol
Tetracycline	12.5 µg/ml	-	50% Ethanol
Kanamycin	50 µg/ml	-	H ₂ O _{dest}
IPTG	0.5 mM	-	H ₂ O _{dest}
X-Gal	40 µg/ml	-	DMF ^a
Mitomycin C	-	0.1-1.0 ug/ml	H ₂ O _{dest}
Ox- bile	-	0.1-3%	H ₂ O _{dest}
TDCA ^b	0.5%	0.5%	H ₂ O _{dest}
GDCA ^c	0.5%	0.5%	H ₂ O _{dest}

^a) Dimethylformamid; ^b) Taurodeoxycholic acid; ^c) Glycodeoxycholic acid

Table 5: Plasmids used in this study

No	Plasmid	Description	Source
1	pBluescriptII SK+ (pBSK)	ColE1, am ^r , lacZ, α-complementation	Stratagene, Germany
2	PGEM-T-easy vector	ColE1, am ^r , lacZ, α-complementation	Promega, USA
3	pCAT	<i>cat194</i> cloned in pGEMT_Easy (Promega, Germany)	A. Geis (personal Communication)

4	pL120	Cloning shuttle vector, <i>am^r</i> , <i>em^r</i>	S. Mudadda (personal Communication)
5	pS1	<i>cbh⁺</i> cloned into pGEM-T-easy vector	This study
6	pS2	<i>cbh⁺</i> cloned into pBluescript IISK+ vector	This study
7	pS3A	<i>cbh⁺</i> in pL120, <i>em^r</i> , <i>am^r</i>	This study
8	pL9-1	3.7 kb <i>SacI</i> fragment(<i>cbh⁺</i> , <i>rep⁺</i> ; <i>mob⁺</i>) in pBSK+ ; <i>am^r</i>	This study
9	pL11	blunted <i>Cat194</i> gene in pL9-1; <i>am^r</i> ; <i>cm^r</i>	This study
10	pL14	<i>cbh⁺</i> in pGKV2; <i>em^r</i> ; <i>cm^r</i>	This study

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-threonin

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

5mg/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 $O.D_{620nm}=0.6$. 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 H₂O

Proteinase K solution : 100 mg/ml in double distilled H₂O

Lysozyme solution : 50 mg/ml in double distilled H₂O

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 OD₆₂₀=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.

-450µl 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 OD₆₂₀ 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:

100 μ 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 50°C till used. 500 μ 1% agarose was filled in 2 ml Eppendorf tubes (placed in 50°C water-bath) and 75-100 μ of cell suspensions were added, mixed carefully and placed in 50°C 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. 500 μ l of the agarose-cell suspension was poured into two insert-slots (each 250 μ). The tray was placed for 10min at -20°C for polymerization. The inserts were transferred in 2 ml Eppendorf tubes containing 1.5 ml lysozyme solution and digested at 37°C for 24 hrs.

2.9.4 Preparation of the lysozyme solution:

1.67 μ l N-Lauroylsarcosine (stock solution 30%)

2.0 mg Lysozyme

20 U Mutanolysin

1000 μ l 0.05 M EDTA pH 8.5

2.9.5 Proteinase K digest :

NDS-Buffer

10 μ l 1M Tris HCl pH 8.0

100 μ l 10% SDS

890 μ l 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 50°C. 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_{620nm} using the photometer Nanocolor 250D (Macherey-Nagel, Düren, 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

Group	Antimicrobial agents		Interpretative zone diameters (mm)*		
	Name	disc conc.(µg)	R	MS	S
I- Inhibitors of cell wall synthesis	Ampicillin	10	≤12	13-15	≥16
	Cefotaxime	30	≤14	15-22	≥23
	Vancomycin	30	≤14	15-16	≥17
	Methicillin	5	---	10-13	----
II- Inhibitors of protein synthesis	Gentamycin	10	≤12	----	≥13
	Kanamycin	30	≤13	14-17	≥18
	Streptomycin	10	≤11	12-14	≥15
	Tetracycline	30	≤14	15-18	≥19
	Chloramphenicol	30	≤13	14-17	≥18
	Erythromycin	15	≤13	14-17	≥18
	Lincomycin	15	---	18-20	----
III- Inhibitors of nucleic acid synthesis	Neomycin	30	---	15-17	----
	Ofloxacin	5	≤13	14-18	≥19

* 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) primer2
 20µl PCR master-mix

2.15.2 Purification of PCR product:

All PCR products were purified by QIA quick spin kits purchased from QIAGEN GmbH – Germany, before using in ligation reactions

Table 7: List of primers used

Primer Name	T _m (°C)	Target Gene	Primer sequence(5' 3')	Amplicon size (bp)	Reference
UP68	66.1	16S-23S rRNA	TGG CTC AGA TTG AAC GCT GGC GGC	2400	Vaneechoutte <i>et al.</i> , 1992
UP69	66.8		CCT TTC CCT CAC GGT ACT GGT		
DG74	60.3	16S rRNA	AGG AGG TGA TCC AAC CGC A	1600	Greisen <i>et al.</i> , 1994
16SF	58.8		TTG GAG AGT TTG ATC CTG GCT C		Carroll <i>et al.</i> , 2000
cbh1	61.9	cbh	GAA GAA TTC GCT ATT GAA GCA ATC ACG	1392	This study*
cbh2	61.9		GAA GAA TTC AAA CCC AAC TTA CCA TCG		
hol-lys1	58.4	holin-lysin	CGC AAG AAG GTT AAA CGG TTA G	1477	This study*
hol-lys2	59.8		CCA GAG AAA CAT CTC CAG AGC		
LactoF	56.9	16S rRNA	TGG AAA CAG RTG CTA ATA CCG	231-233	Byun <i>et al.</i> , 2004
LactoR	57,3		GTC CAT TGT GGA AGA TTC CC		
LactoF	56.9	16S rRNA	TGG AAA CAG RTG CTA ATA CCG	1600	Byun <i>et al.</i> , 2004
LbLMA1	50.1		CTC AAA ACT AAA CAA AGT TTC		Duberner <i>et al.</i> , 2002
Im26-f	59.8	16S rRNA	GAT TCT GGC TCA GGA TGA ACG	1417	Kaufmann <i>et al.</i> , 1997
Im3-r	57.6		CGG GTG CTN CCC ACT TTG ATG		
St1	54.7	16S rRNA	TTA TTT GAA AGG GGC AAT TGC T	279	Furet <i>et al.</i> , 2004
St2	57.9		GTG AAC TTT CCA CTC TCA CAC		

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

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

2.16 Molecular weight markers

- λ Styl 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 μ 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 DocTM XR system and quantity one software (Bio Rad).

2.17.1 Reagents

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

Ingredients	g/1000 ml (10X)
Tris base	48.4
Na ₂ -EDTA	7.45
NaCH ₃ COOH x 3H ₂ O (Na-acetat)	6.9
pH adjusted to pH 8.0 with acetic acid.	

Sample buffer

Ingredients	g/100 ml
0.5% bromophenol blue	0.05
66% glycerol (w/v)	47.6 ml
Distilled water	52.4 ml

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 μ 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

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 microcentrifuge 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 25µF. 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)

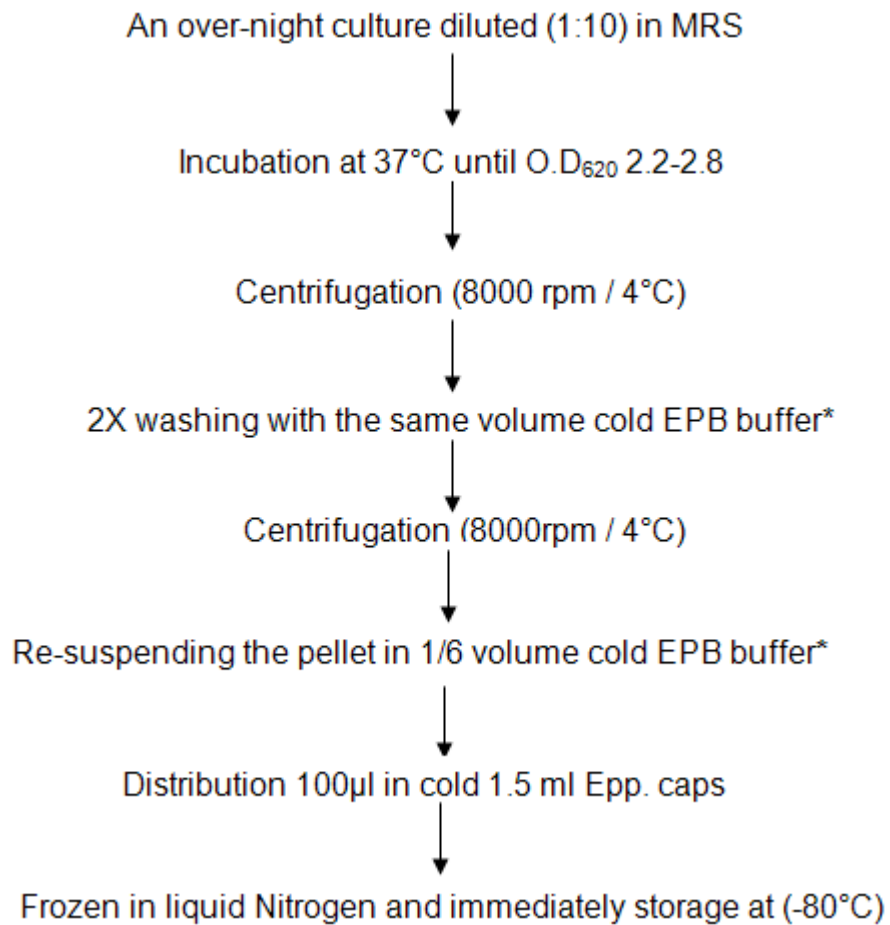
0.5 g dissolved in 5 ml of DMF (dimethyl formamide).

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



*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*



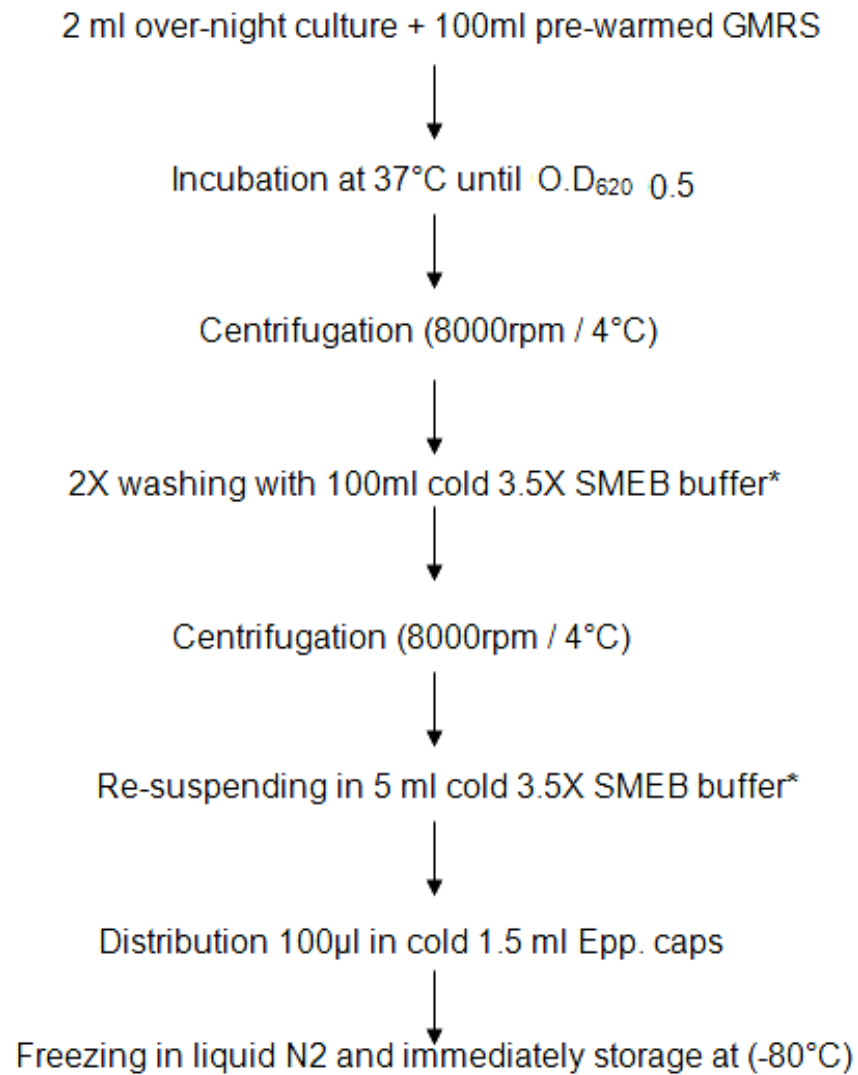
Plating onto MRS agar medium contains 10µg/ml Em



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



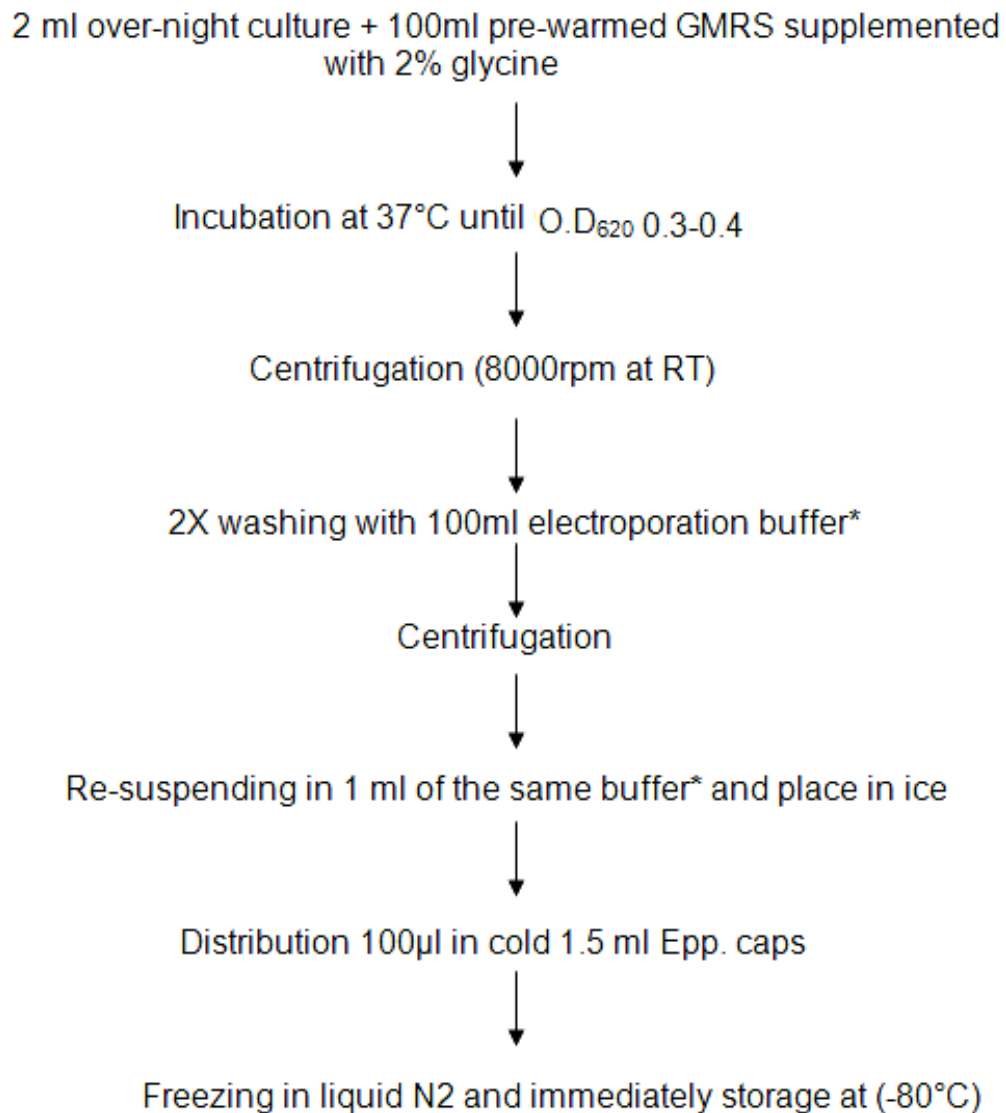
***3.5X SMEB buffer:**

952 ml 1M sucrose

3.5 ml 1M MgCl₂

44.5 ml H₂O

2.20.4 Preparation of *L. rhamnosus* competent cells



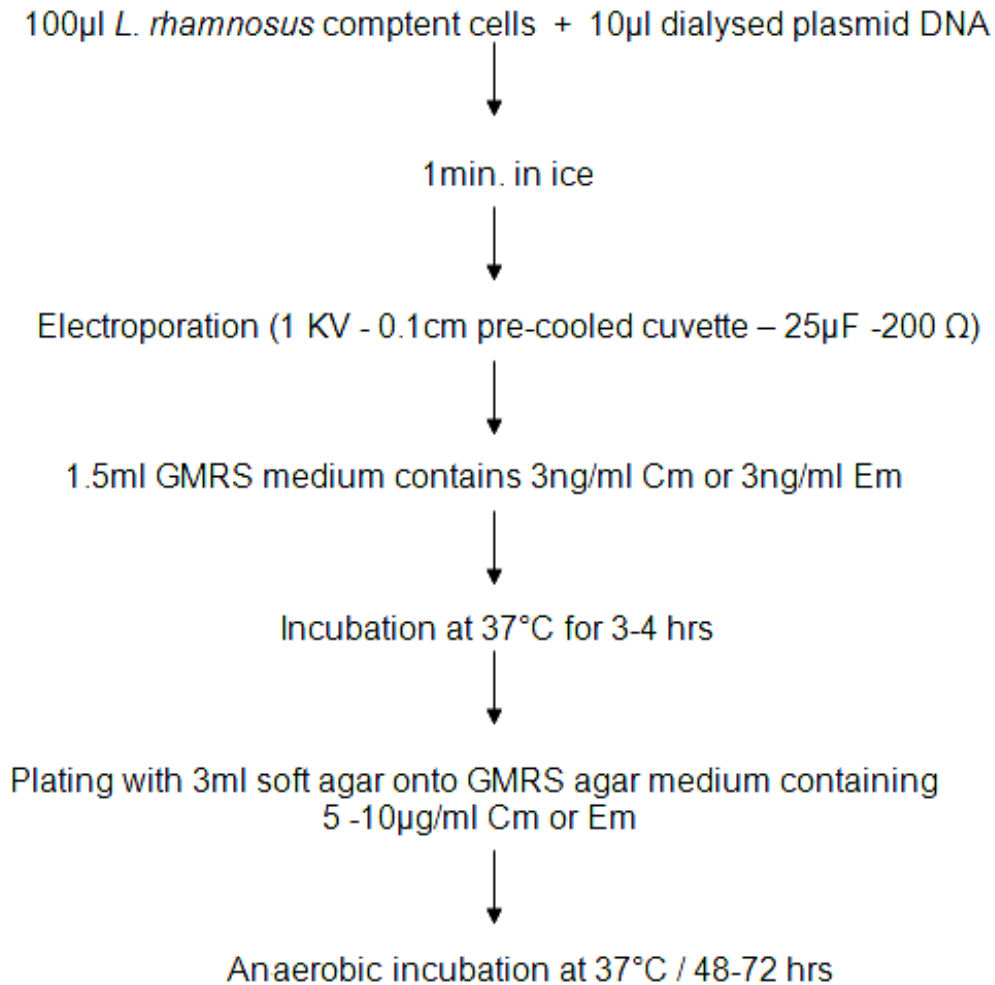
*** Electroporation buffer:**

0.5 M Sucrose

7mM potassium phosphate (pH7.4)

1mM MgCl₂

2.20.5 Electrotransformation of *L. gasseri* and *L. rhamnosus*



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

2.21 Analysis of Transformants

E. coli or *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 *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

Blunting Reaction	Amounts	
	Vector	PCR product
5X Reaction buffer	4µl	4µl
DNA	10µl	5µl
2mMdNTP mix	1µl	1µl
T4 DNA polymerase	1µl	1µl
D. D. water	4µl	9µl
Total	20µl	20µl

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: Dephosphorylisation of blunted vectors

Dephosphorylisation Reaction	Amounts
Reaction buffer	5µl
Blunted DNA (vector)	28µl
SAP (Shrimp Alkaline phosphatase)	1µl
D. D. water	16µl
Total	50µl

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 phage 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 soft ware 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

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 up to 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)

2.31.2 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.

2.31.3 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.

2.31.3.1 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:

Reagent	Volume
Hexanucleotide mix, 10X conc.	2 µl
DIG DNA labeling mix, 10X conc.	2 µl
Klenow enzyme	1 µl
Sterile D.D.W	Volume made up to 20 µl

-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.

2.31.3.2 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

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

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.

2.31.3.3 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 μ l 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 prewarmed 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 electrophoreses 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 H₂O
- 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 |

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 10min 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 μ l TES with 20 μ l lysozyme and 5 μ l mutanolysin and incubated at 37°C for 30 min. SDS (5-10 μ l) 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 12: Preparation of the SDS gel

Ingredients	12%	15%
Solution A	20 ml	25 ml
Distilled water	16.5 ml	11.5 ml
1.5 M Tris HCl (pH 8.8)	12.5 ml	12.5 ml
10% SDS	0.5 ml	0.5 ml
TEMED	20 μ l	20 μ l
Ammonium per sulphate	0.5 μ l	0.5 μ l

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%)

Ingredients	Amounts
Solution A	4.5 ml
Distilled water	12.5 ml
1.5 M Tris HCl (pH 8.8)	2.5 ml
10% SDS	0.2 ml
TEMED	20 μ l
Ammonium per sulphate (APS)	0.2 μ l

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 μ l 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 μ l) 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 (Nilskasaari, 1989; Yokoi *et al.*, 2004). Overnight cultures were inoculated into fresh MRS medium and incubated at 37°C. Bacterial cells were harvested at OD₆₂₀ 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 OD₆₂₀ 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 OD₆₂₀ 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-Restriction 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-23S rRNA 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 rDNA 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%.

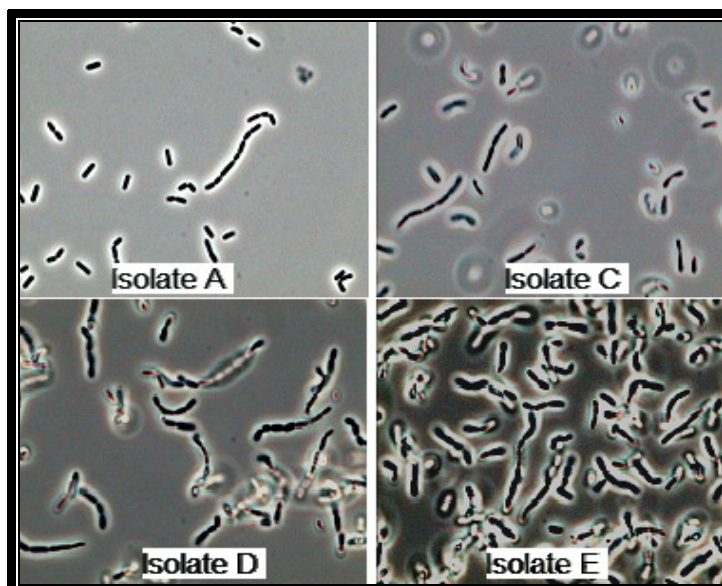


Fig. 3: Morphology of four lactobacilli isolates from Egyptian cheese

Table 14: API 50 CHL Identification of four lactobacilli isolates from Egyptian cheese

Isolate	Identification by API 50 CHL (identification comment) ^a	% ID
A	<i>L. brevis</i> (good)	84.5%
C	<i>L. brevis</i> (good)	84.5%
D	<i>L. plantarum</i> (acceptable)	91.5%
E	<i>L. brevis</i> (good)	84.5%

^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 (Vanechoutte *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 20174^T, *L. pastorianus* DSM 20197, *L. johnsonii* DSM 20553, *L. gasseri* DSM 20243, *L. zae* DSM 20178^T, *L. reuteri* DSM 20016^T, *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.

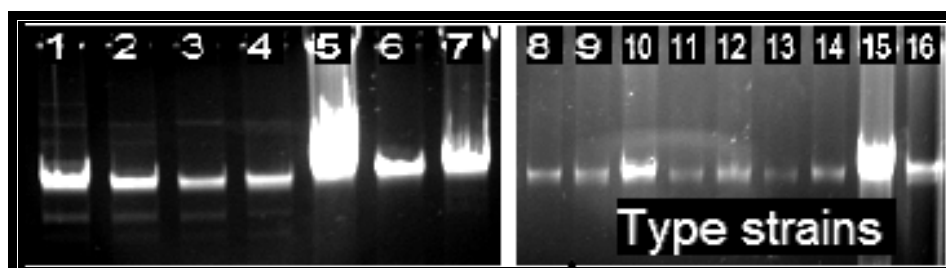


Fig. 4: Chromosomal DNA of some *Lactobacillus* sp and isolates A, C, D and E

lane 1: *L. gasseri* BfEL92075; **lane 2:** *L. plantarum* BfEL 92122; **lane 3:** *L. rhamnosus* BfEL 92149; **lane 4:** isolate A; **lane 5:** isolate C; **lane 6:** isolate D; **lane 7:** isolate E; **lane 8:** *L. reuteri* DSM 20016^T; **lane 9:** *L. pastorianus* DSM 20197; **lane 10:** *L. zae* DSM 20178^T; **lane 11:** *L. gasseri* DSM 20243^T; **lane 12:** *L. johnsonii* DSM 20553; **lane 13:** *L. delb.ssp bulgaricus* ATCC 12278; **lane 14:** *L. delb.ssp lactis* ATCC 10697; **lane 15:** *L. plantarum* DSM 20174^T; **lane 16:** *L. delb. ssp lactis* BfEL 3046/62

3.2.3.2 Enzymatic amplification of rDNA

For PCR, universal primers UP68/UP69 (**Vanechoutte 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 58°C (Fig. 5).

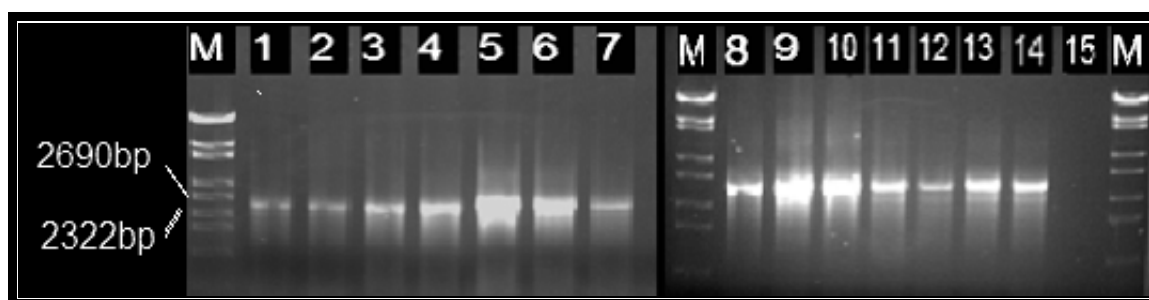
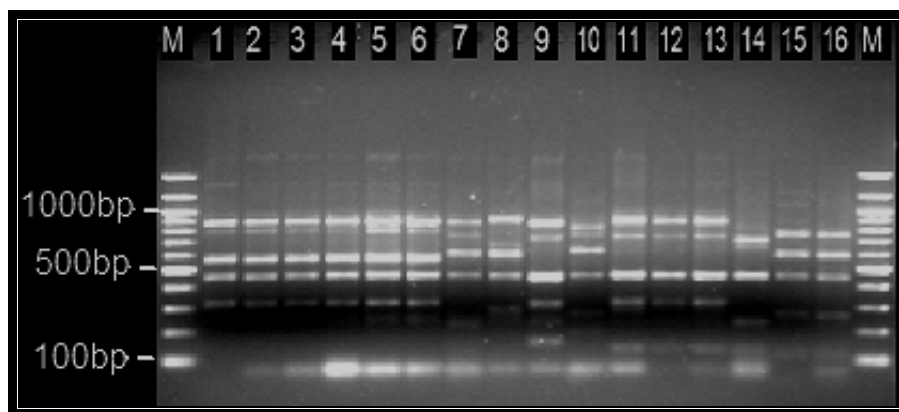


Fig. 5: PCR amplicons of 16S-23S rRNA genes from *Lactobacillus* type strains and *Lactobacillus* isolates.

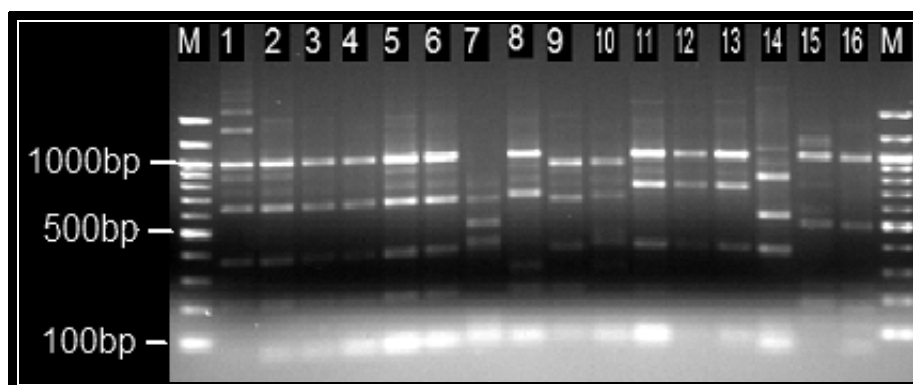
M: λ StyI; **lane 1:** *L. reuteri* DSM 20016^T; **lane 2:** *L. pastorianus* DSM 20197; **lane 3:** *L. zeae* DSM 20178^T; **lane 4:** *L. gasseri* DSM 20243; **lane 5:** *L. johnsonii* DSM 20553; **lane 6:** *L. delb. ssp bulgaricus* ATCC 12278; **lane 7:** *L. delb. ssp lactis* ATCC 10697; **lane 8:** *L. gasseri* BfEL 92075; **lane 9:** *L. plantarum* BfEL 92122; **lane 10:** *L. rhamnosus* BfEL92149; **lane 11:** isolate A; **lane 12:** isolate C; **lane 13:** isolate D; **lane 14:** isolate E; **lane 15:** blank.

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^T. ARDRA also revealed that *L. gasseri* BfEL 92075 had the same restriction profile as type strain *L. gasseri* DSM 20243^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^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^T. *L. plantarum* DSM 20174^T did not match any of the other *Lactobacillus* type and reference strains. *L. pastorianus* DSM 20197, *L. zeae* DSM 20178^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^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*.



(a): Hae III



(b): Hinf I

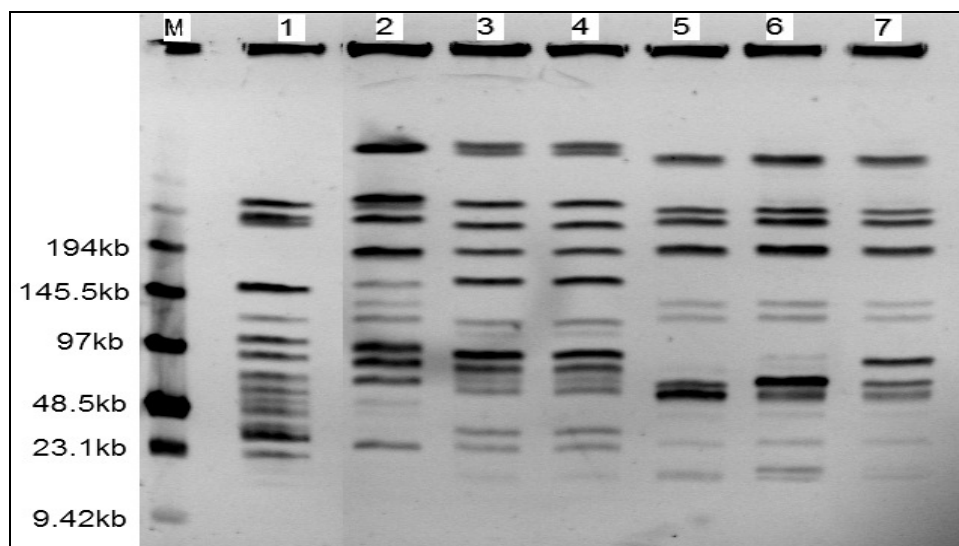
Fig. 6: ARDRA Profiles with Hae III (A) and Hinf I (B) of some Lactobacillus isolates and type strains.

M: 100bp Ladder; **lane 1:** *L. plantarum* DSM 20174; **lane 2:** *L. plantarum* BfEL92122; **lane 3:** isolate A; **lane 4:** isolate C; **lane 5:** isolate D; **lane 6:** isolate E; **lane 7:** *L. pastorianus* DSM 20197; **lane 8:** *L. zeae* DSM 20178; **lane 9:** *L. rhamnosus* BfEL 92149; **lane 10:** *L. reuteri* DSM 20016; **lane 11:** *L. gasseri* BfEL 92075; **lane 12:** *L. gasseri* DSM 20243; **lane 13:** *L. johnsonii* DSM 20553; **lane 14:** *L. delb. ssp bulgaricus* ATCC 12278; **lane 15:** *L. delb.ssp lactis* BfEL 3046/62; **lane 16:** *L. delb.ssp lactis* ATCC 10697^T

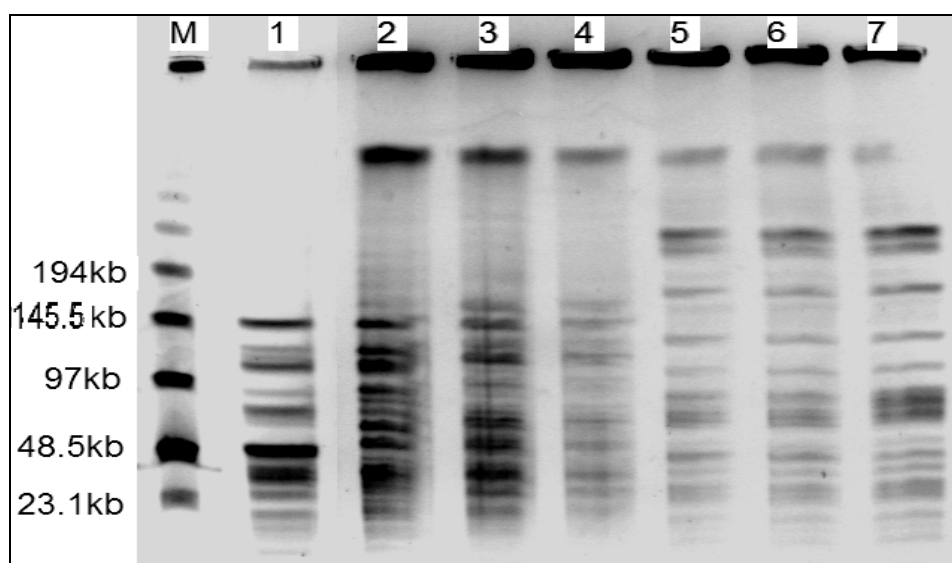
3.2.4 Pulsed Field Gel Electrophoresis (PFGE) Genotyping

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 20174^T. 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 20174^T) and a completely different genomic fingerprint for *L. zeae* DSM 20178^T as a negative control. The *L. plantarum* isolate (A) and the type strain *L. plantarum* DSM 20174^T 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*.



(a) Ascl.



(b) NotI

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

M: low range PFG marker (Biolabs); **lane 1:** *L. zeae* DSM 20178^T; **lane 2:** *L. plantarum* BfEL 92122; **lane 3:** *L. plantarum* DSM 20174^T; **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).

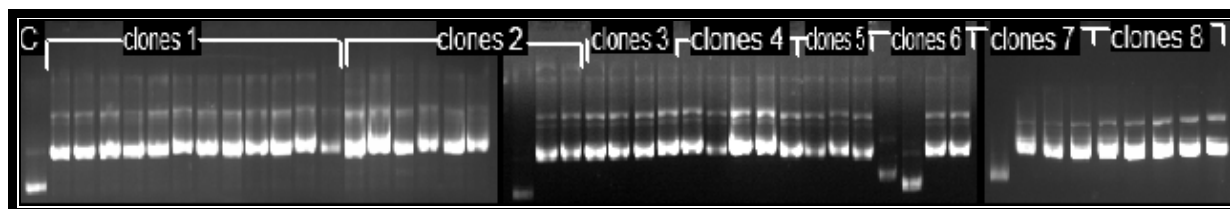


Fig. 8: Clones of 16S-23S rDNA fragment of some *Lactobacillus* isolates

(C): PGEM-T-easy vector; **clones 1:** *L. plantarum* BfEL 92122; **clones 2:** *L. zea* DSM 20178; **clones 3:** *L. rhamnosus* BfEL 92149; **clones 4:** *L. plantarum* DSM 20174^T; **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^T and *L. zea* 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 (Vanechoutte 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.

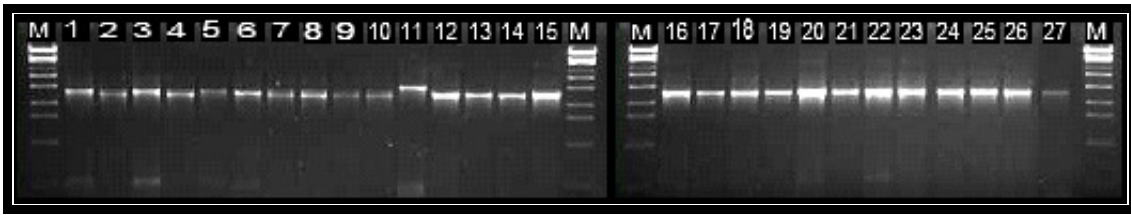


Fig. 9: PCR amplicons of 16S-23S rRNA genes from bacterial isolates numbers 1-28.
M: λ Styl, lanes **1-12:** Kariesh cheese isolates, lanes **13-14 and 15-25:** Laban rayeb isolates
lanes **26&27:** Mish cheese isolates

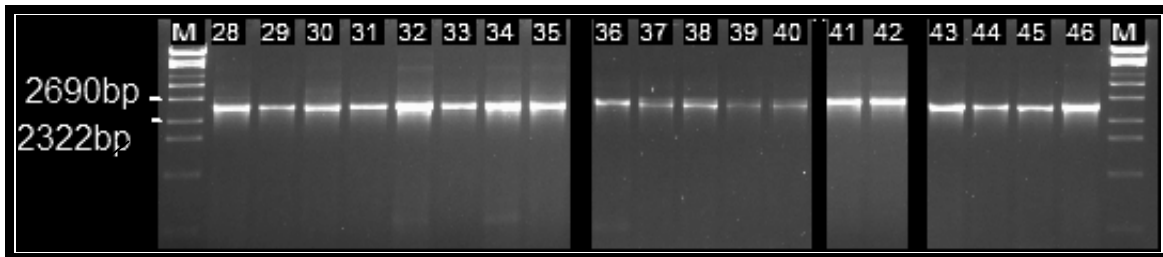
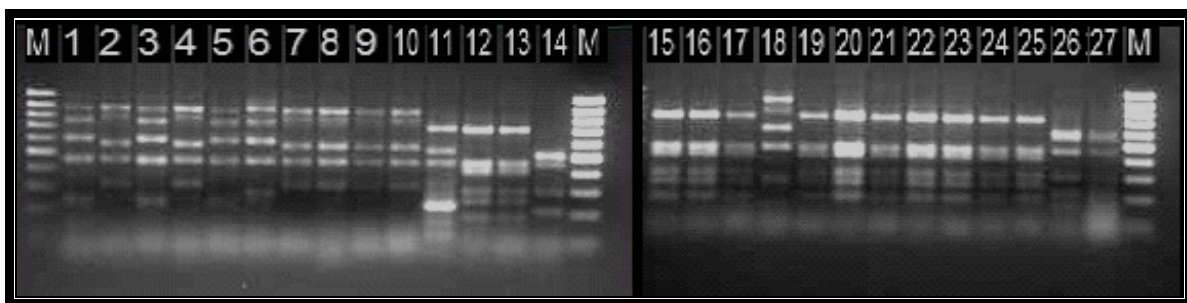


Fig. 10: PCR amplicons of 16S-23S rRNA genes from bacterial isolates numbers 28-46.
M: λ Styl, lanes **28-36:** Kariesh cheese isolates, lanes **36-46:** Laban Rayeb isolates.

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.



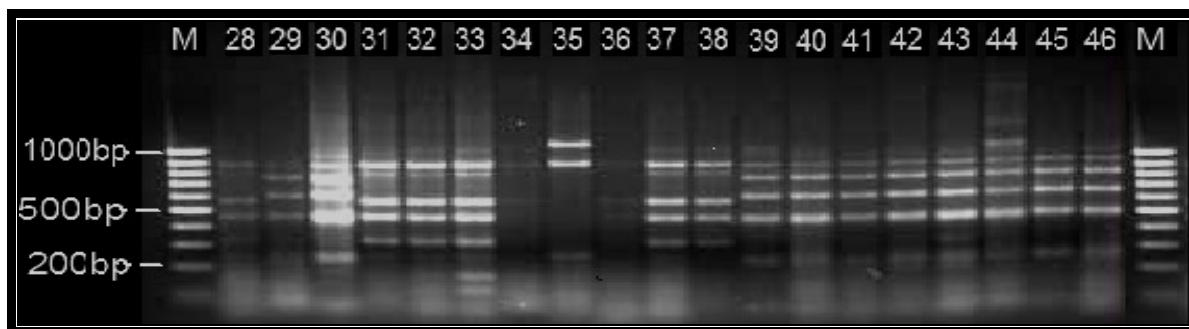


Fig. 11: HaellI restriction patterns of 16S-23Sr RNA of isolates numbers 1-46.

M: 100bp ladder; lanes **1-12:** Kariesh cheese; lanes **13-14 and 15-25:** Rayeb milk lanes **26&27:** Mish cheese; lanes **28-36:** Kariesh cheese isolates; lane **36-46:** Laban Rayeb isolates.

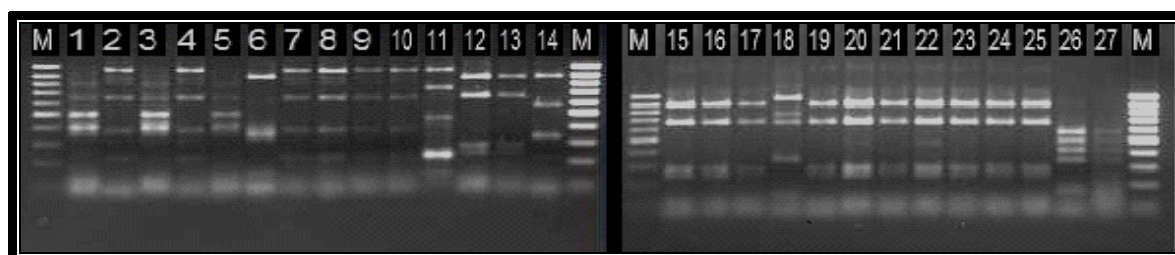


Fig. 12: HinI restriction patterns of 16S-23SrRNA of isolates numbers 1-46.

M: 100bp ladder; **1-12:** Kariesh cheese; lanes **13-14 and 15-25:** Laban Rayeb; lanes **26&27:** Mish cheese; lanes **28-36:** Kariesh cheese isolates; lanes **36-46:** Laban Rayeb isolates.

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.

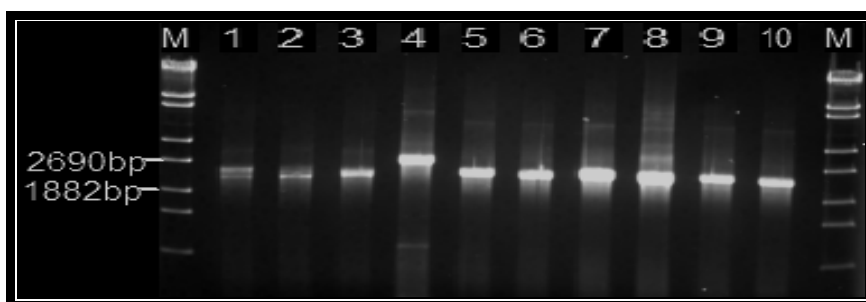


Fig. 13: PCR amplicons of 16S-23S r RNA genes from selected isolates between numbers 1-27.

M: λ Styl; 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.

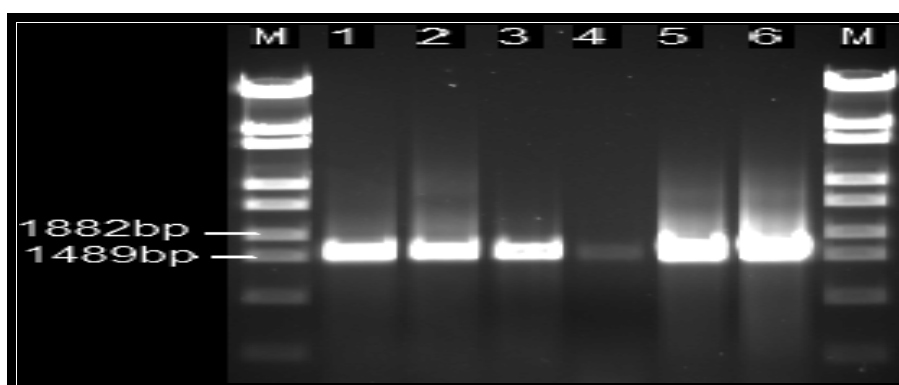


Fig. 14: PCR amplicons of 16S r RNA gene from selected isolates between numbers 28-46.

M: λ Styl; 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 summarize 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

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

= 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 Im26/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.

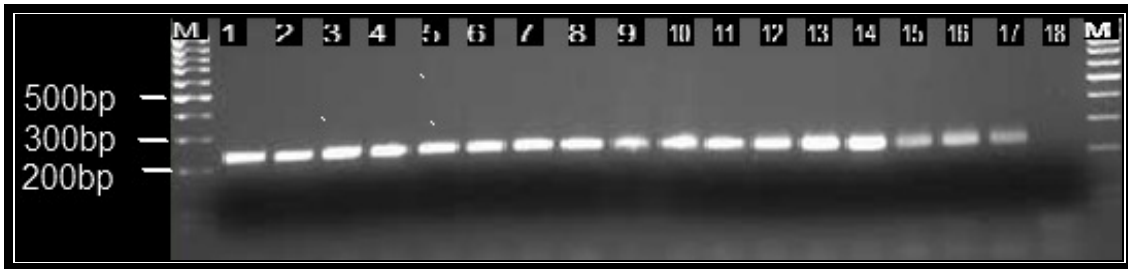


Fig. 15: PCR of the babies Feces isolates with the *Lactobacillus* specific Primers LactoF and LactoR.

M: 100bp-Ladder; lanes **1-13:** isolates 1A-13A; **lane14:** *L. gasseri* (DSM 20243^T); lanes**15 & 16:** *St. thermophilus*; **lane17:** *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.

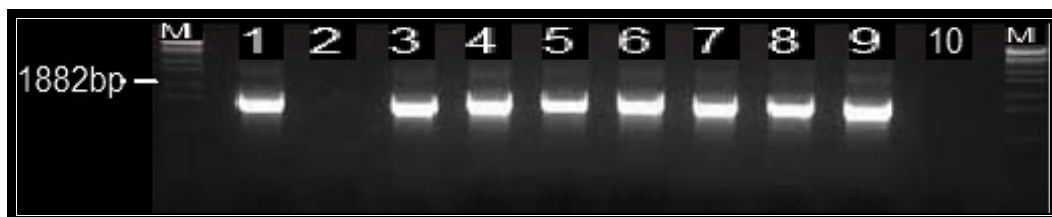


Fig. 16: PCR of *Lactobacillus* reference strains with the primers LactoF/LbLMA1.

M: λStyl; lane **1:** *L. reuteri* (DSM 20016^T); lane **2:** *L. pastorianus* (DSM 20197); lane **3:** *L. zae* (DSM 20178); lane **4:** *L. gasseri* (DSM 20243^T); 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^T); lane **10:** blank sample.

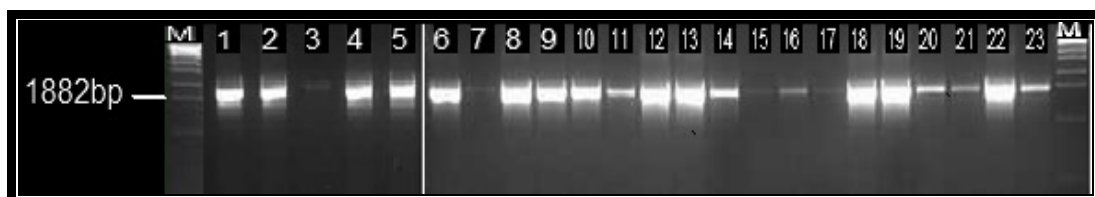


Fig. 17: PCR of babies' feces isolates with *Lactobacillus* specific primers LactoF and LbLMA1.

M: λ Styl; lanes **1-11:** isolates 1A-11A; lanes **12-14:** isolates 13A-15A; lane **15:** isolate 18A; lanes **16-23:** isolates 20A-27A.

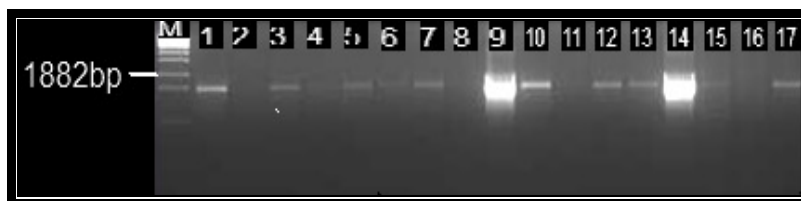


Fig. 18: PCR of babies' feces isolates (28-40A with *Lactobacillus* specific primers LactoF and LbLMA1.

M: λ Styl; lanes **1-13:** isolates 28A-40A; lane **14** *L. gasseri* (DSM 20243^T); lanes **15&16:** *St. thermophilus*; lane **17:** *B. breve* (17A).



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

M: λ Styl; lanes **1-11:** isolates 1B-11A; lane **12:** isolate 13B; lanes **13-19:** isolates 14B-20B; lane **20:** *L. gasseri* (DSM 20243^T); lane **21:** *B. breve* (17A); lanes **22&23:** *St. thermophilus*



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

M: λ Styl; lanes **1-12:** isolates 21B-33A; lanes **13-19:** isolate 34B-40B; lane **20:** *L. gasseri* (DSM 20243^T); lanes **21&22:** *St. thermophilus* lane **23:** *B. breve* (17A).

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 (Vanechout *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 r RNA gene, intergenic spacer region and a part of 23S r RNA 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 *L. zeae* (DSM 20178^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 *L. gasseri* (DSM 20243^T) as well as *L. johnsonii* (DSM Z 20553). The restriction profile of the isolate 10A did not match with any of reference strains. The remaining isolates matched with *L. zeae* (DSM Z 20178^T). ARDRA restriction profile of with TaqI were identical to the reference strain *L. zeae*, DSM 20016^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 *Bifidobacterium* no further isolates identified by PCR as *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.

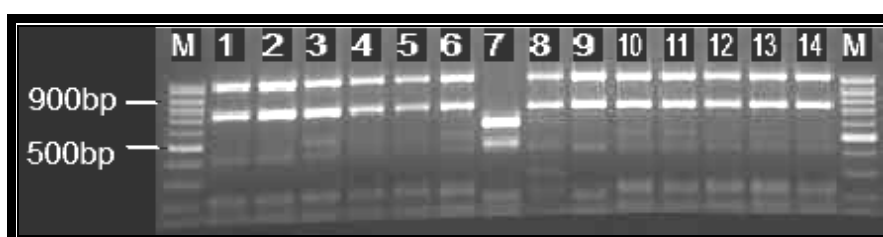


Fig. 21: ARDRA (Primer UP68/UP69) with TaqI for some isolates from baby's (A) feces.

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.

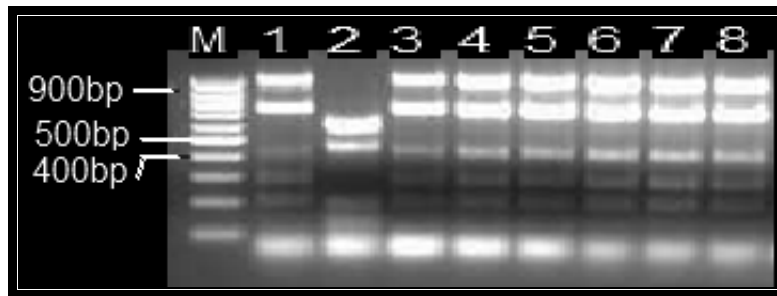


Fig. 22: ARDRA (Primer UP68/UP69) with TaqI enzyme of some isolates from babies feces.

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

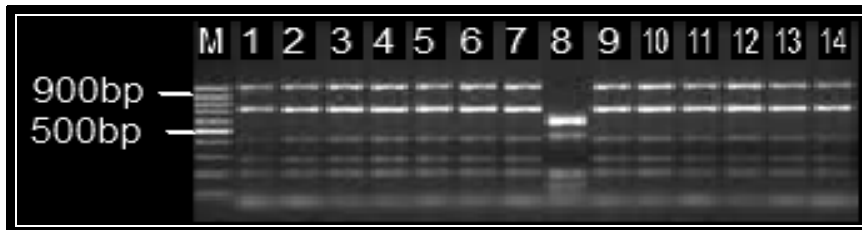


Fig. 23: ARDRA (Primer UP68/UP69) with TaqI enzyme of some isolates from babies feces.

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.

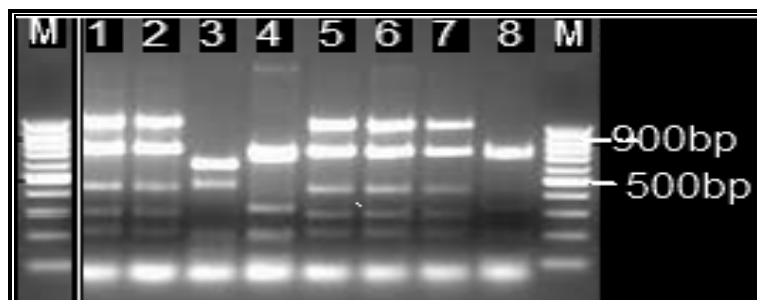


Fig. 24: ARDRA (Primer UP68/UP69) with TaqI enzyme of some isolates (B) from babies feces.

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

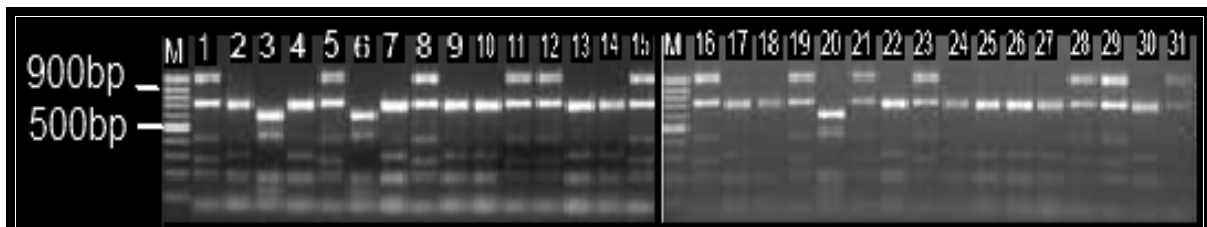


Fig. 25: ARDRA (Primer UP68/UP69) with TaqI enzyme of some isolates (B) from babies feces.

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 1: 1-8A, 10A, 13-15A, 18A, 20-32A, 34-40A; 1-2B, 6B, 9-10B, 14-15B, 20-22B, 24-25B, 28B, 30B, 32B, 37-38B, 40B

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

Group: 3B, 5B, 8B, 11B, 13B, 16-17B, 19B, 23B, 26-27B, 31B, 33-36B, 39B.

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 16: Summary of identified isolates from babies' feces.

Isolate	Identification
1A	<i>Lactobacillus paracasei</i> ISO1
2A	<i>Lactobacillus rhamnosus</i> ISO2
9A	<i>Lactobacillus paracasei</i> ISO3
10A	<i>Lactobacillus rhamnosus</i> ISO4
11A	<i>Lactobacillus rhamnosus</i> ISO5
13A	<i>Lactobacillus rhamnosus</i> ISO6
15A	<i>Lactobacillus rhamnosus</i> ISO7
13B	<i>Enterococcus gallinarum</i> ISA1
19B	<i>Enterococcus saccharolyticus</i> ISA2
33B	<i>Enterococcus saccharolyticus</i> ISA3

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 feeded with breast milk and several *Enterococcus sp.* from infant B, who was feeded 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.

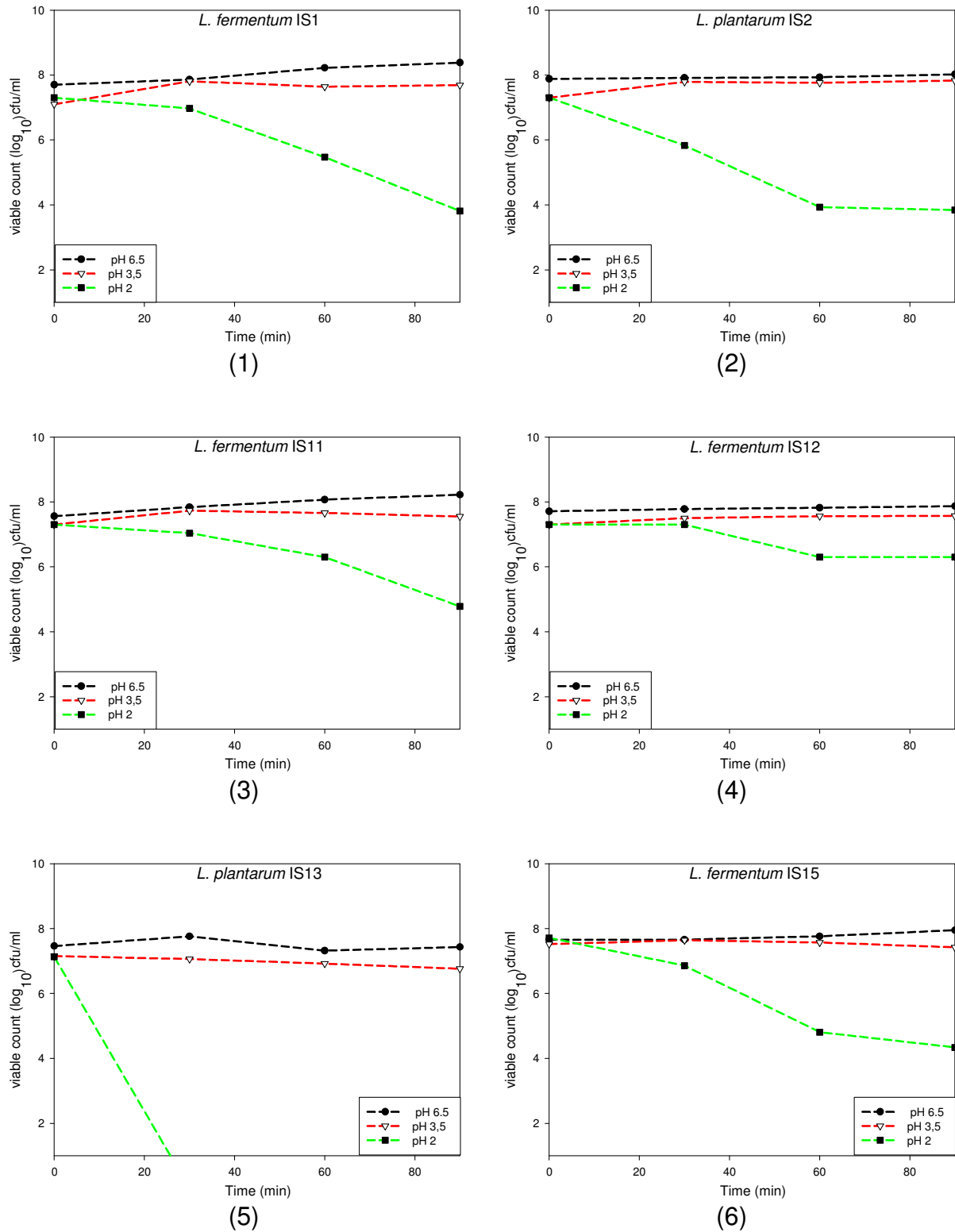
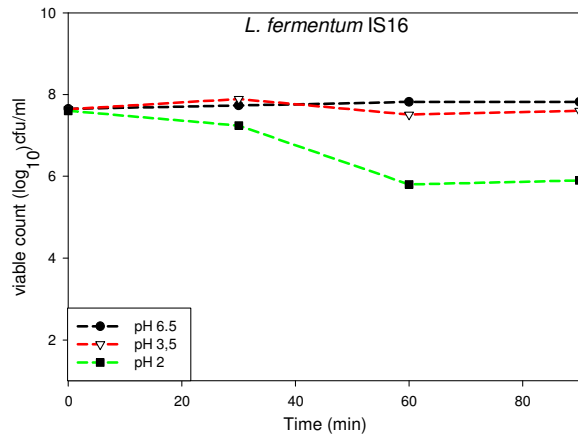
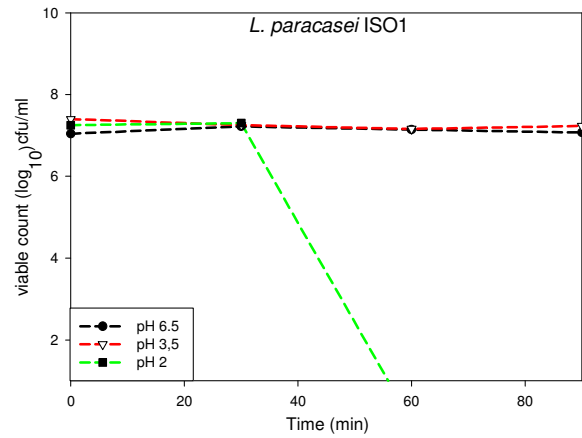


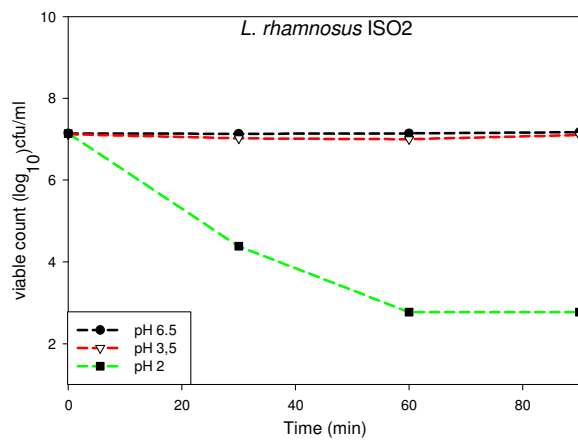
Fig. 26: Survival of *Lactobacillus* spp. (log₁₀ cfu/ml) in MRS medium at different pH values during incubation at 37 °C for 90 min.



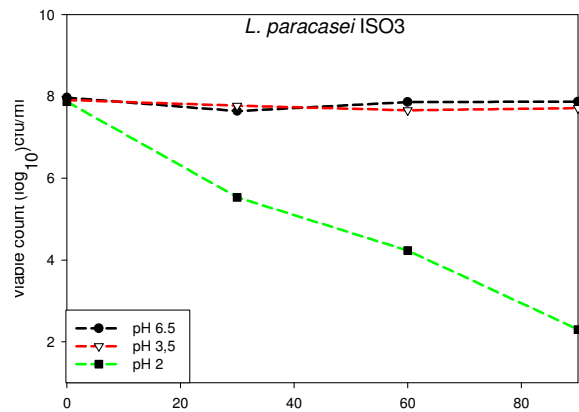
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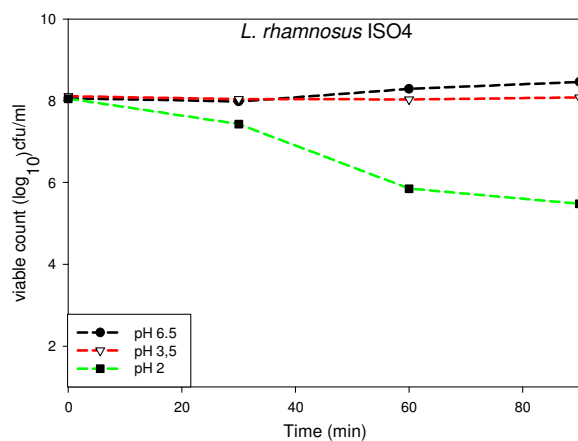
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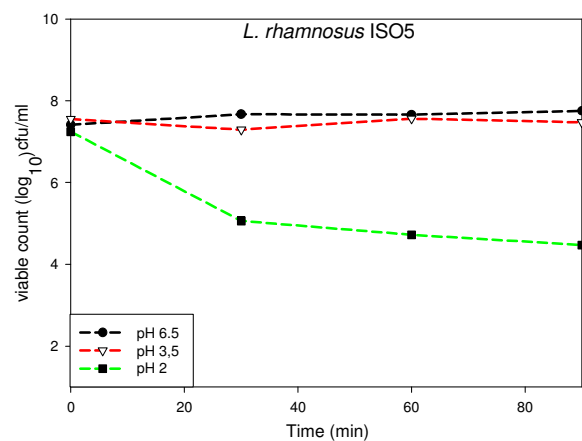
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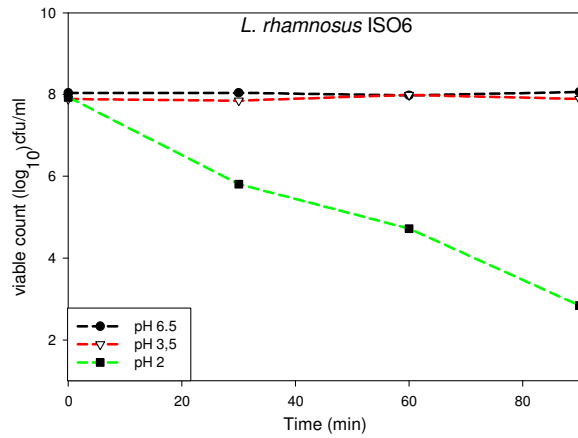


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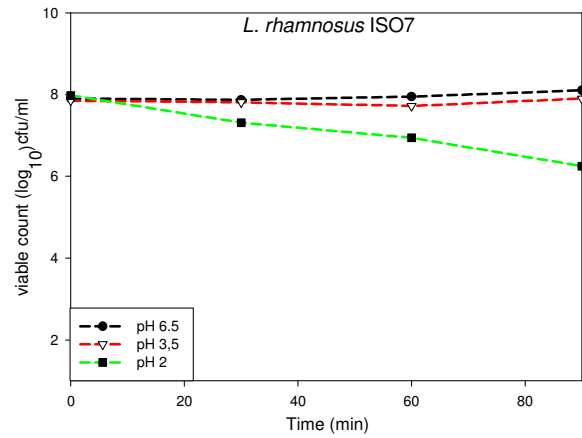


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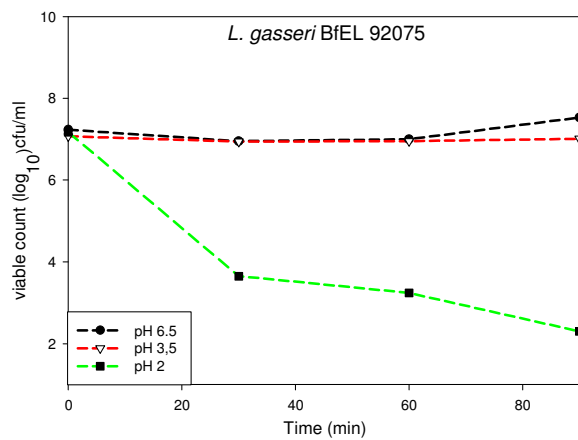
Fig. 26: continued



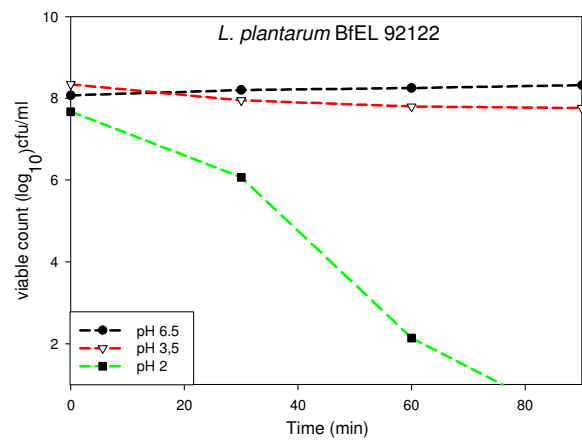
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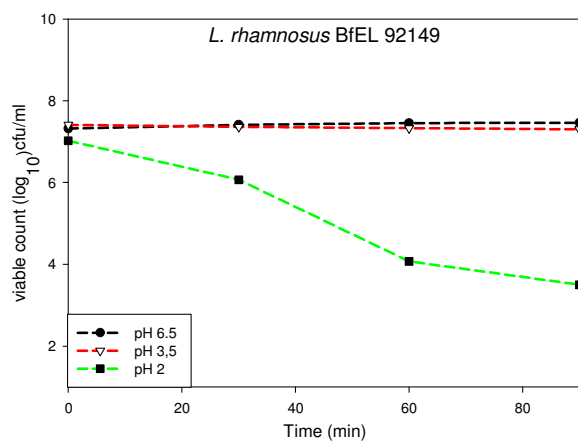
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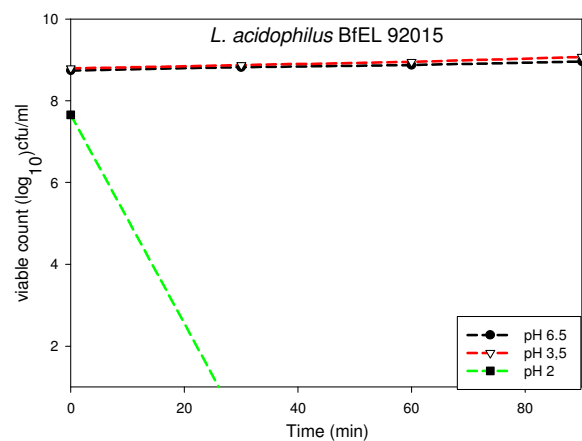
(15)



(16)



(17)



(18)

Fig. 26: continued

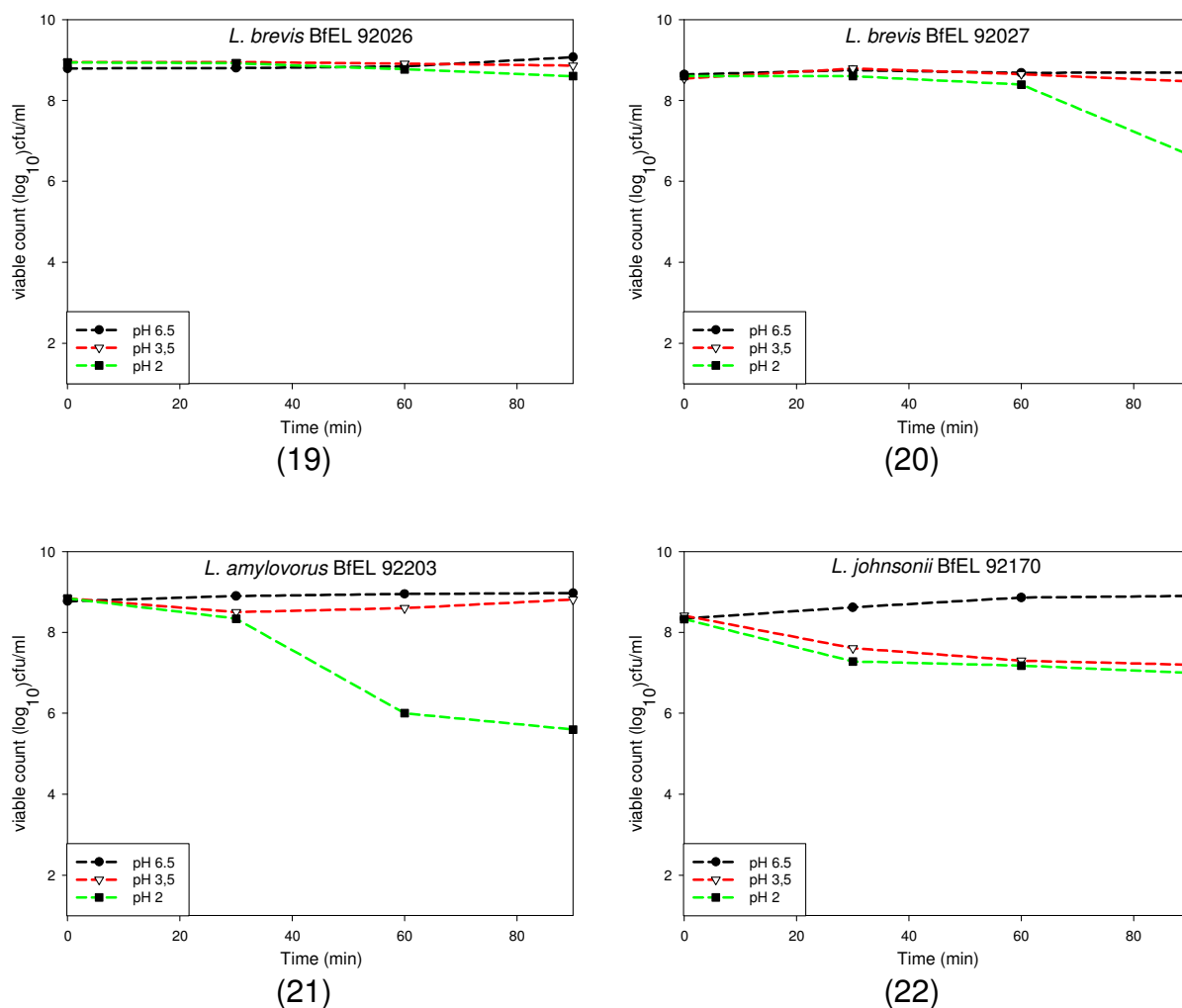


Fig. 26: continued

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

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).

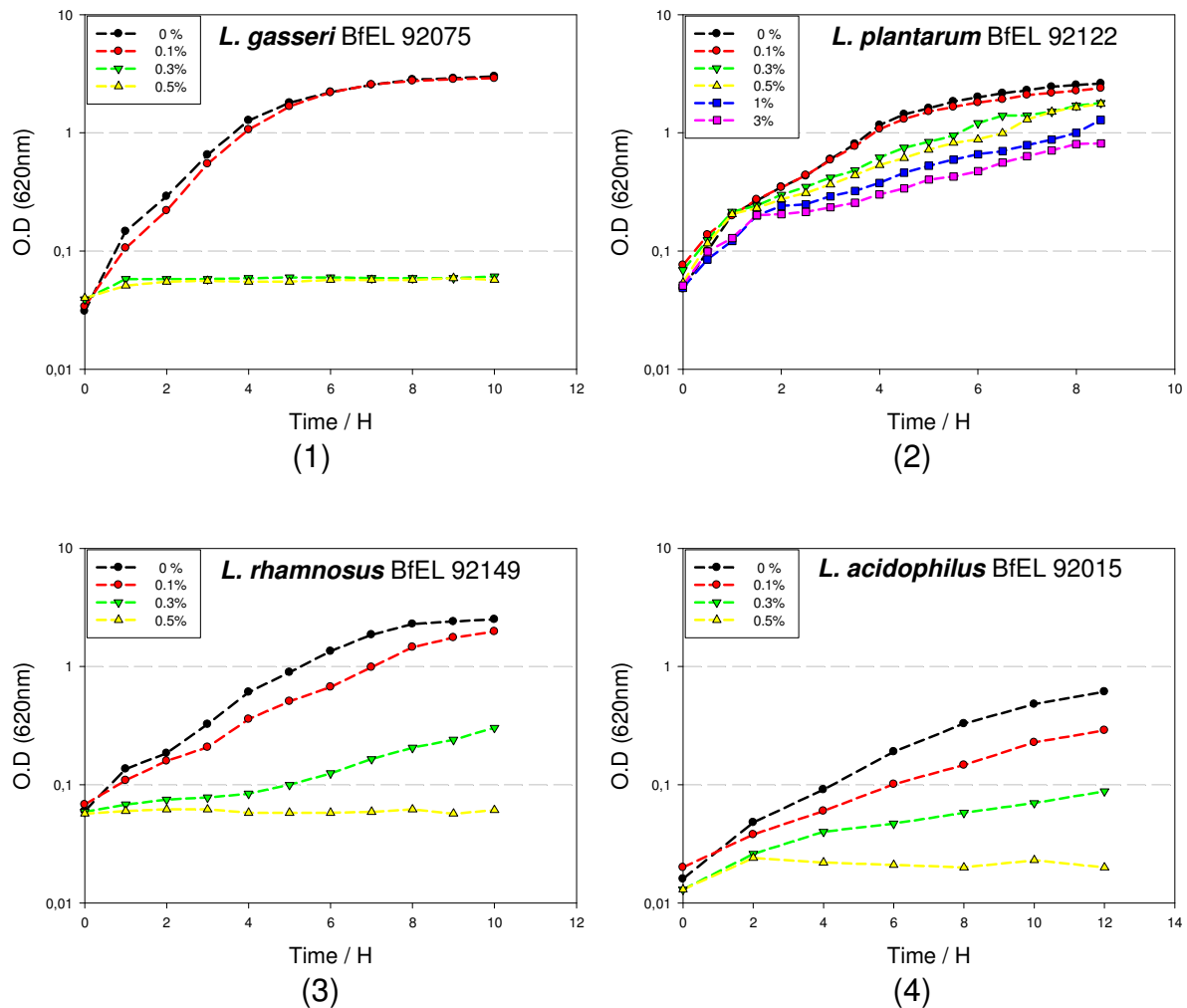


Fig. 27: Effect of different concentrations of ox-gall bile salts on growth of lactobacilli

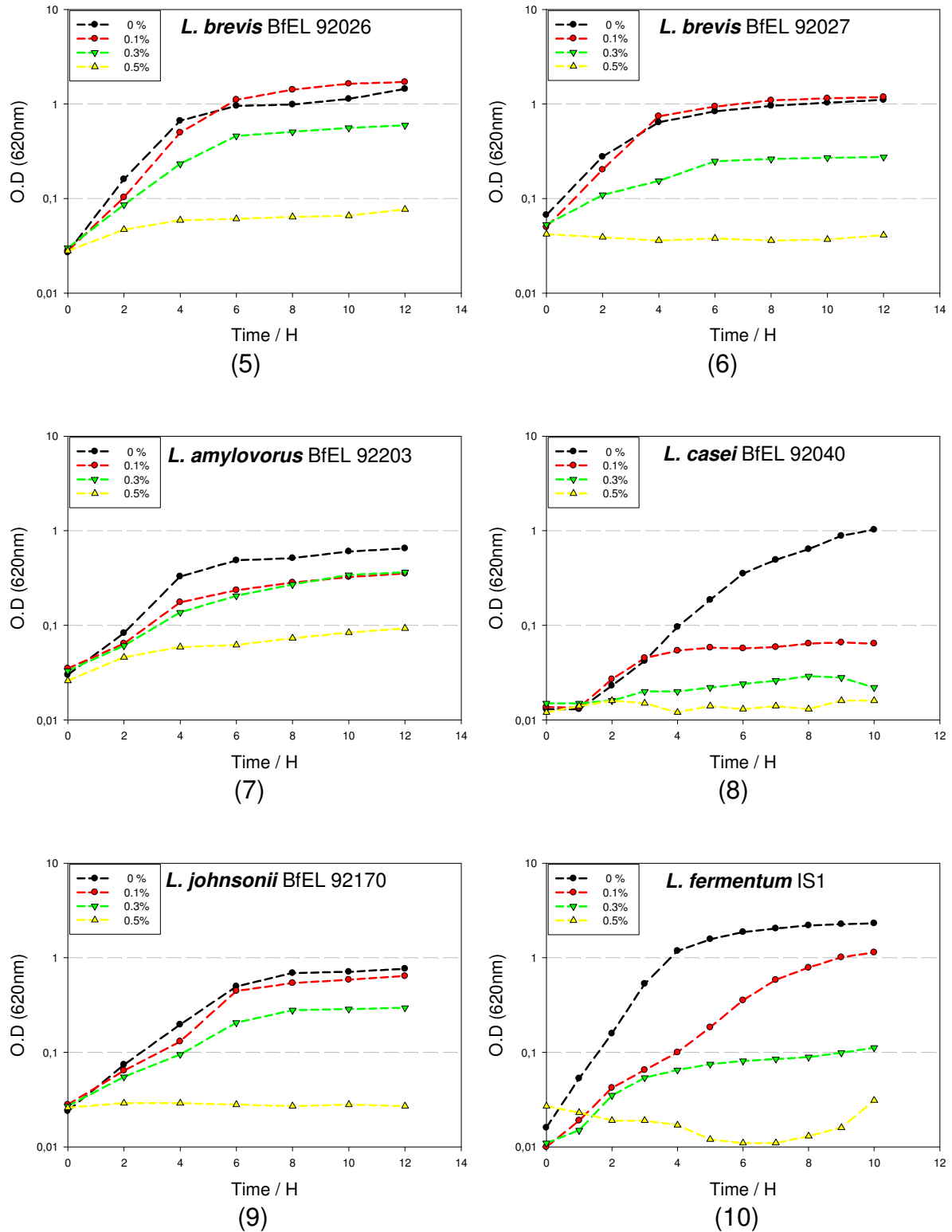


Fig. 27: Continued

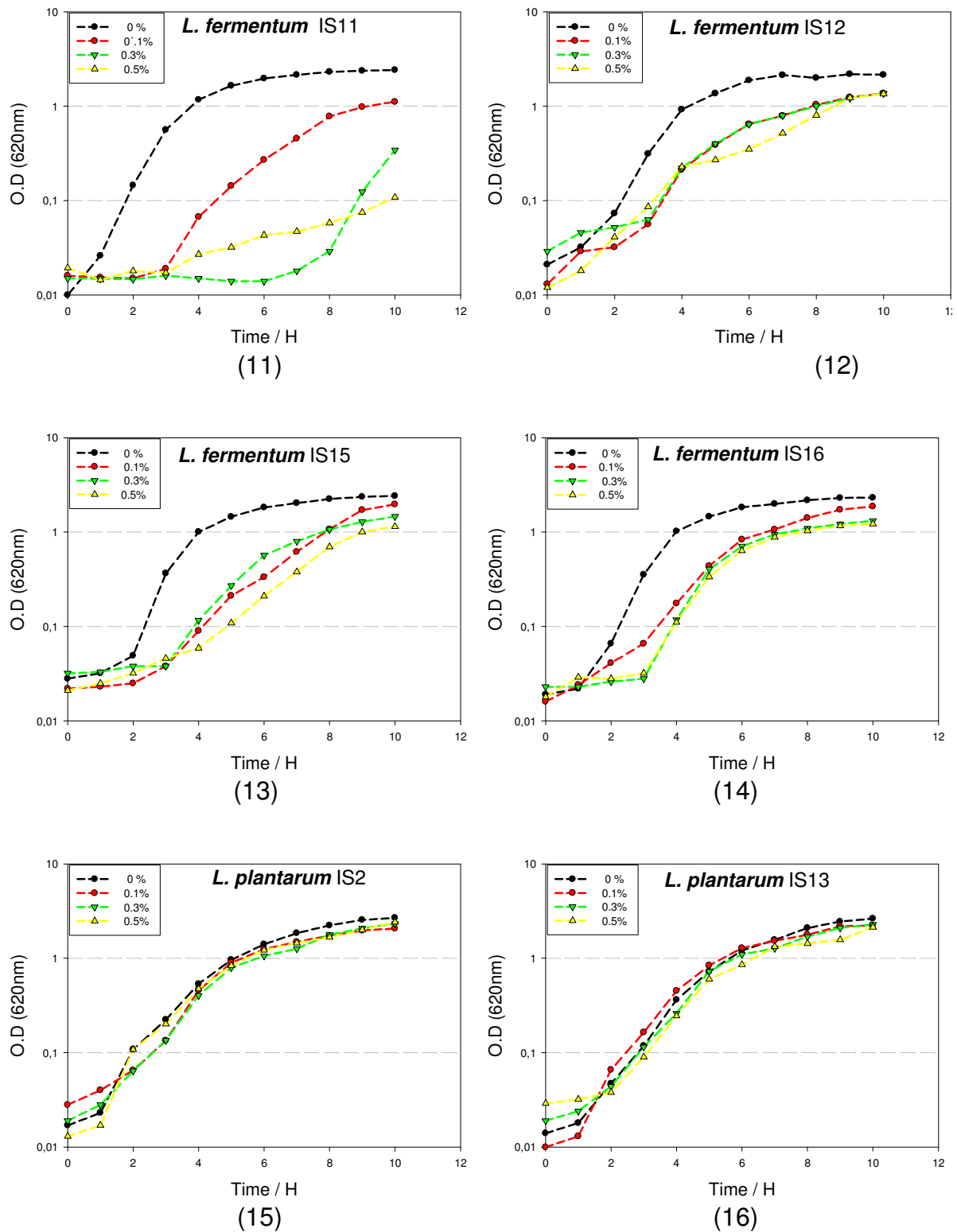


Fig. 27: Continued

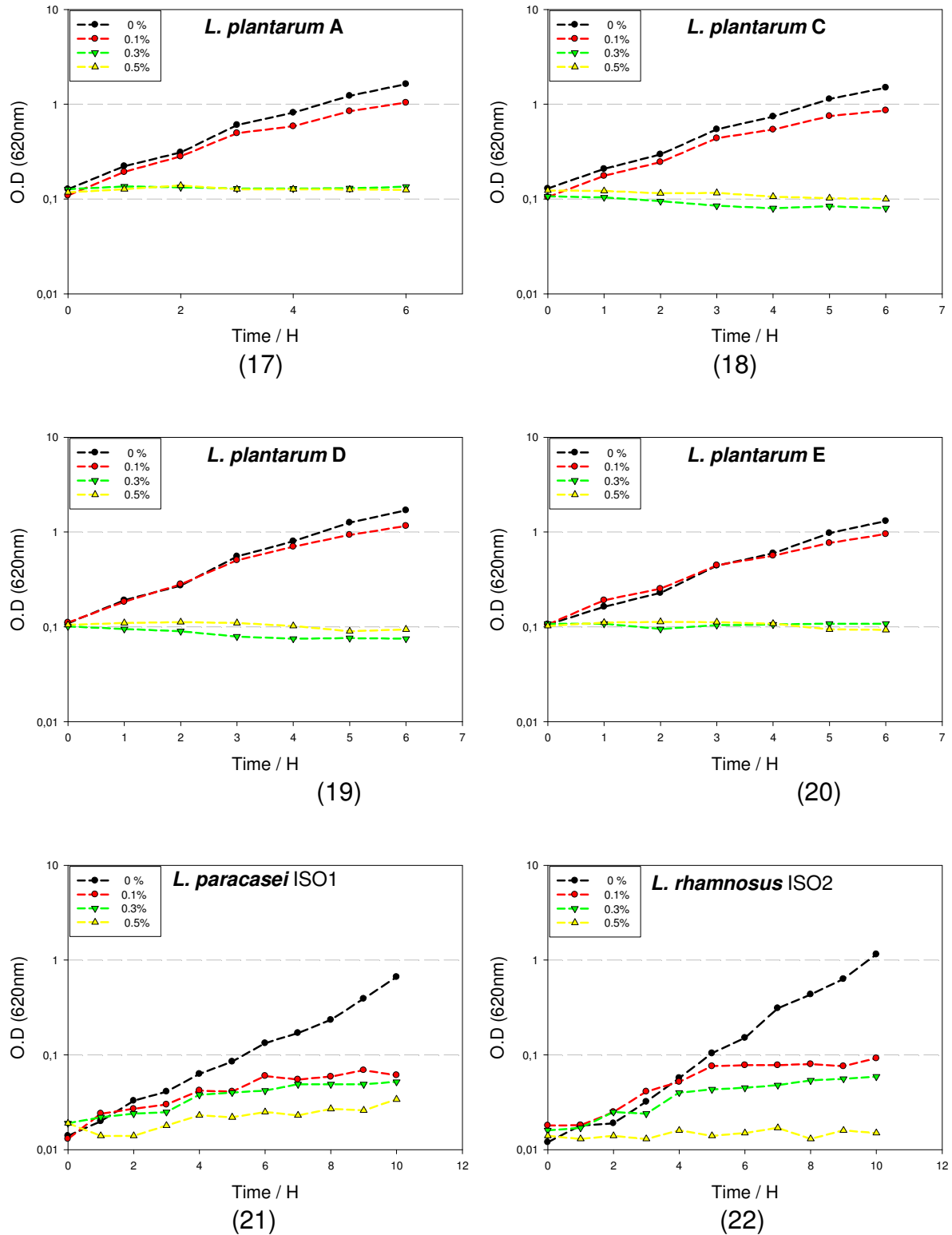


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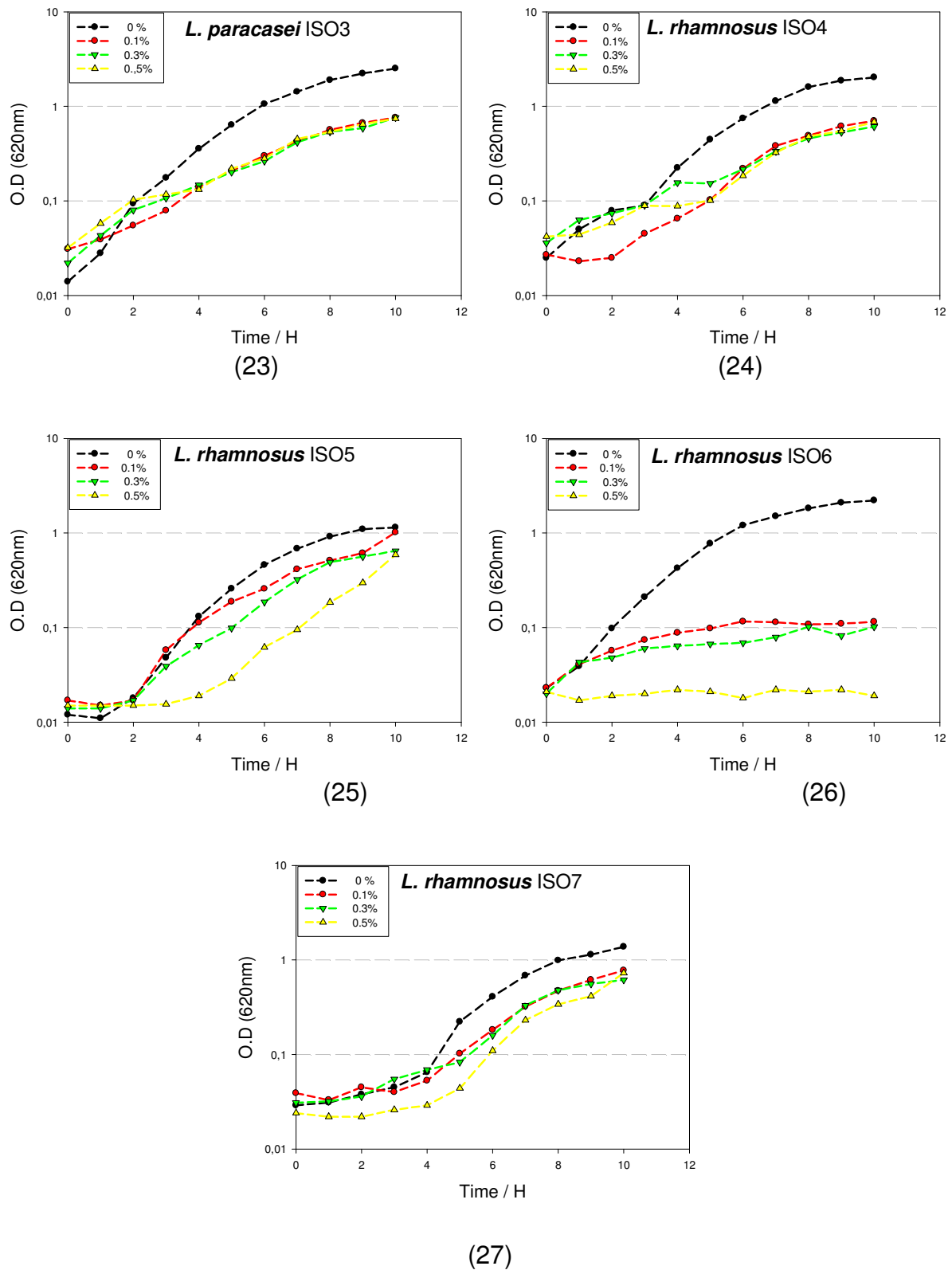


Fig. 27: Continued

Table 17: Bile salts hydrolase activity of various *Lactobacillus* strains

Strains	Bile salts hydrolase activity	
	TDCA	GDCA
<i>Lactobacillus plantarum</i> BfEL92122	+	+
<i>Lactobacillus brevis</i> BfEL 92026	+	+
<i>Lactobacillus rhamnosus</i> BfEL92149	+	+
<i>Lactobacillus gasseri</i> BfEL 92075	-	+
<i>Lactobacillus plantarum</i> (A)	-	+
<i>Lactobacillus plantarum</i> (C)	-	+
<i>Lactobacillus plantarum</i> (D)	-	+
<i>Lactobacillus plantarum</i> (E)	-	+
<i>Lactobacillus acidophilus</i> BfEL92015	+	+
<i>Lactobacillus johnsonii</i> BfEL 92170	+	+
<i>Lactobacillus amylovorus</i> BfEL92203	+	-
<i>Lactobacillus casei</i> BfEL92040	-	+
<i>Lactobacillus fermentum</i> IS1	-	-
<i>Lactobacillus plantarum</i> IS2	-	-
<i>Lactobacillus fermentum</i> IS11	-	-
<i>Lactobacillus fermentum</i> IS12	-	-
<i>Lactobacillus plantarum</i> IS13	-	-
<i>Lactobacillus fermentum</i> IS15	-	-
<i>Lactobacillus fermentum</i> IS16	-	-
<i>Lactobacillus paracasei</i> ISO1	-	-
<i>Lactobacillus rhamnosus</i> ISO 2	-	-
<i>Lactobacillus paracasei</i> ISO3	+	-
<i>Lactobacillus rhamnosus</i> ISO 4	+	-
<i>Lactobacillus rhamnosus</i> ISO 5	-	-
<i>Lactobacillus rhamnosus</i> ISO 6	-	-
<i>Lactobacillus rhamnosus</i> ISO 7	+	+

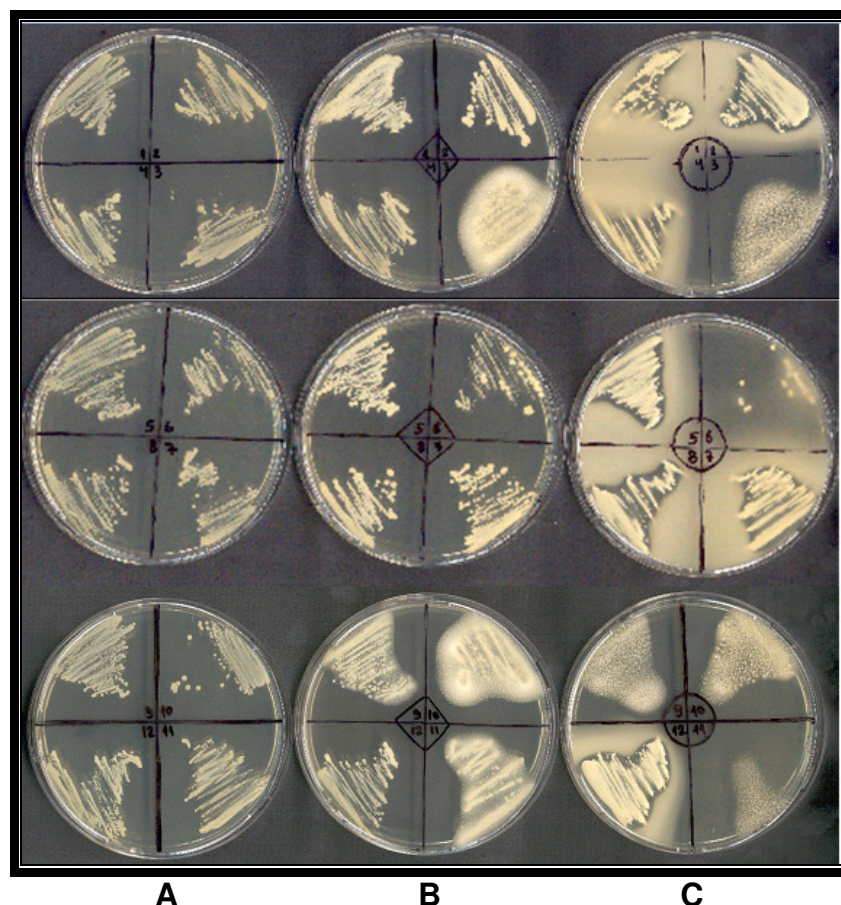


Fig. 28: Bile salt hydrolase activity as detected by plate assay method on MRS supplemented with TDCA or GDCA.

(A): MRS; **(B):** MRS+0.5% TDCA; **(C):** MRS+0.5%GDCA; **1:** *L. plantarum* BfEL 92122; **2:** *L. brevis* BfEL 92026; **3:** *L. rahmnosus* BfEL 92149; **4:** *L. gasserii* 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).

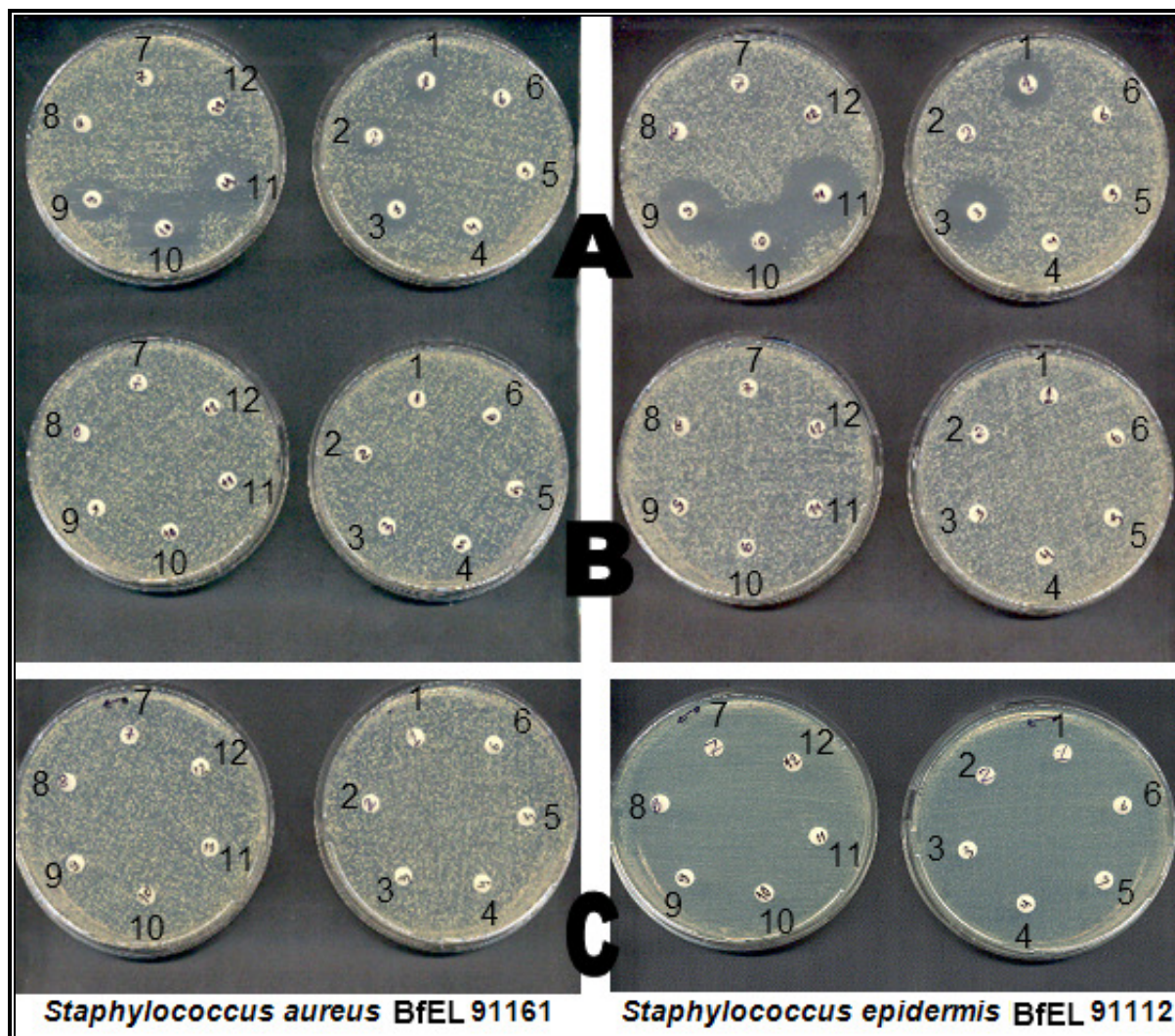


Fig. 29: Antimicrobial activity of *Lactobacillus* strains against *Staphylococcus aureus* BfEL 91161 and *Staphylococcus epidermis* BfEL 91112

A: 10 μ l of lactobacillus broth cultures; **B:** 10 μ l of supernatant without adjusting the pH (actual pH \approx 4); **C:** 10 μ l of supernatant with adjusting the pH to \approx 7. **1:** *L. gasseri* BfEL 92075; **2:** *L. brevis* BfEL 92026; **3:** *L. rahnosus* 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

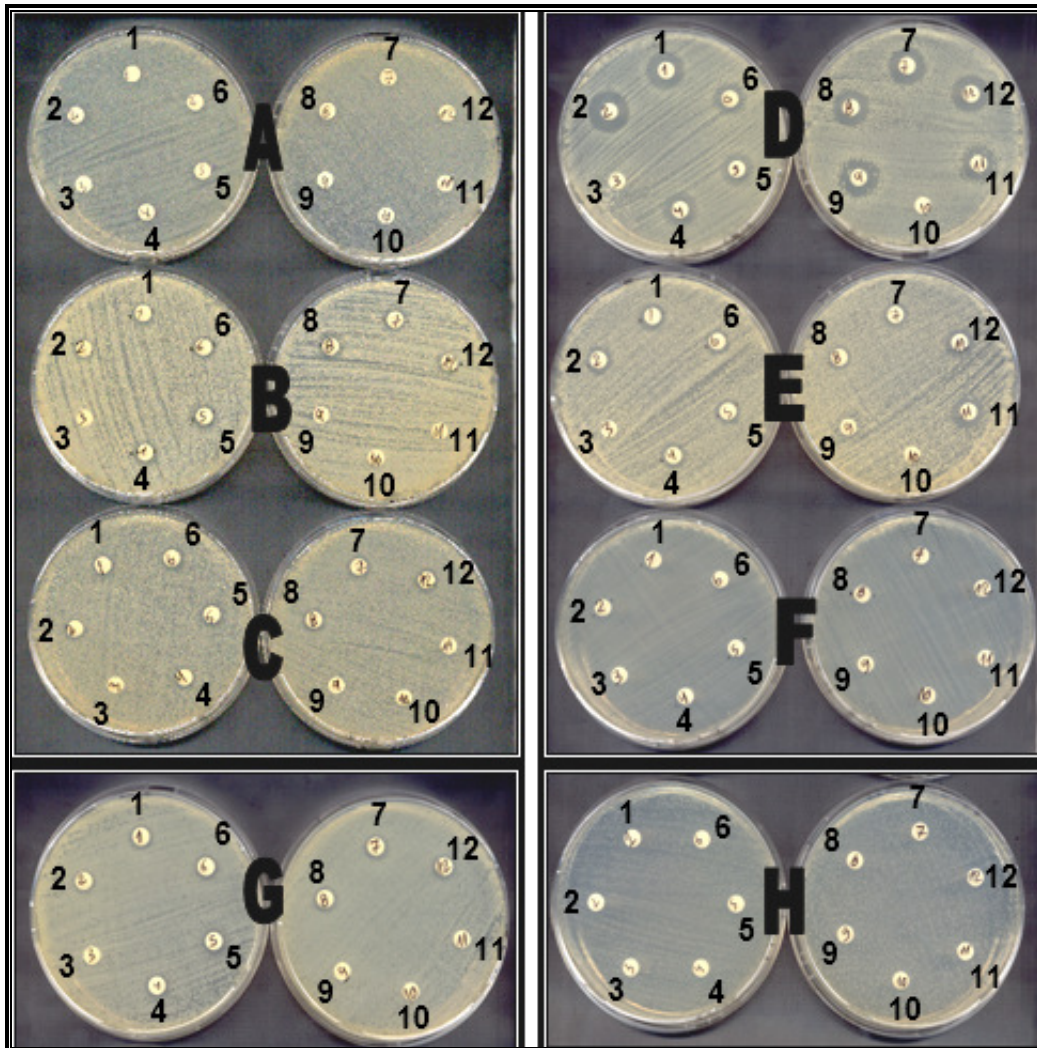


Fig. 30: Antimicrobial activity of *Lactobacillus* strains against pathogenic bacteria after changing the lactobacilli overnight culture growth conditions.

A: *E. coli* BfEL 98082; **B:** *Enterobacter aerogenes* BfEL 9805; **C:** *Serratia marcescens* BfEL 98027; **D:** *Staphylococcus aureus* BfEL 91161; **E:** *Staphylococcus epidermidis* BfEL 91112; **F:** *Enterococcus faecalis* BfEL 90519; **G:** *Salmonella typhimurum*; **H:** *Listeria monocytogenes*; **1:** *L. gasseri* BfEL 92075; **2:** *L. brevis* BfEL 92026; **3:** *L. rahmnosus* 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* BfEL92040.

Table 18: Antimicrobial activity of *Lactobacillus* strains against eight indicator strains*

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

* 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.

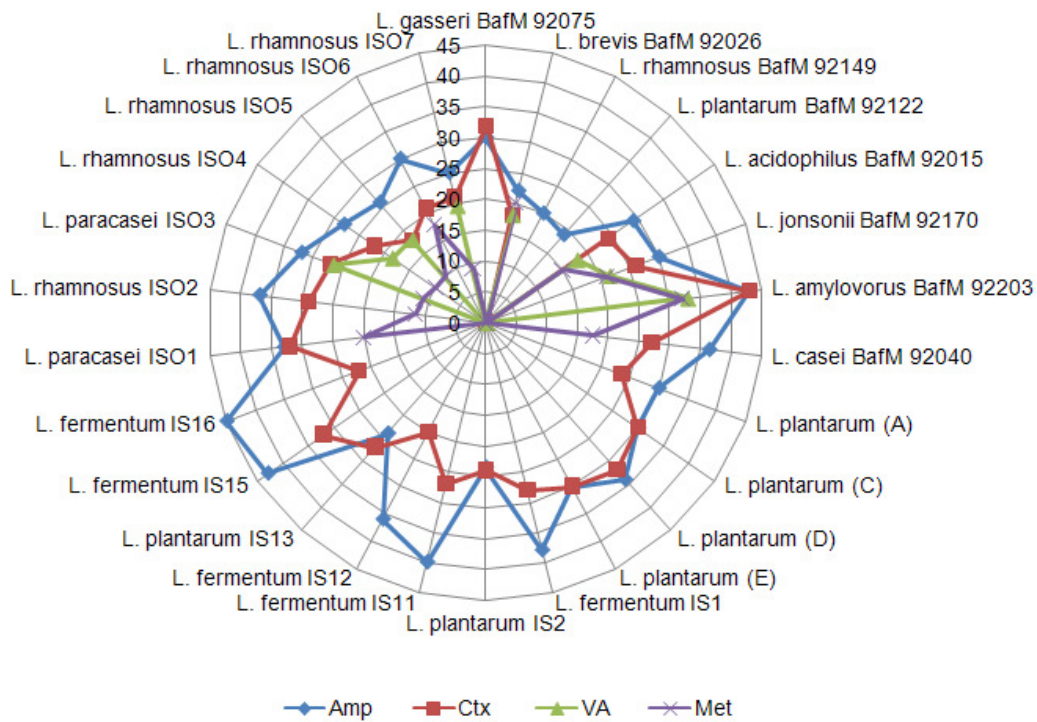
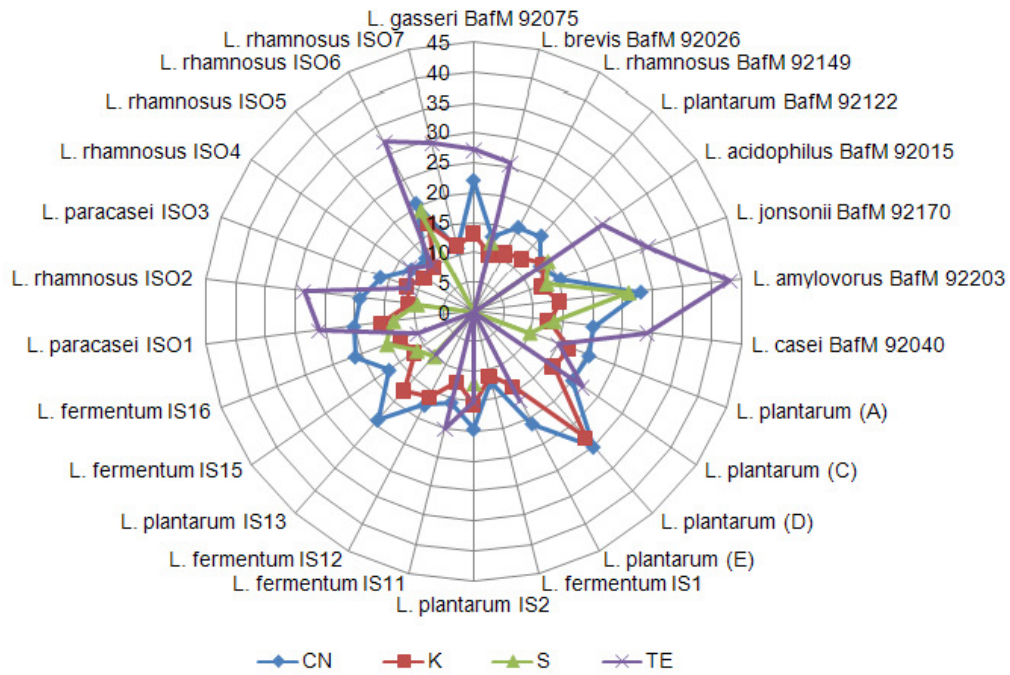
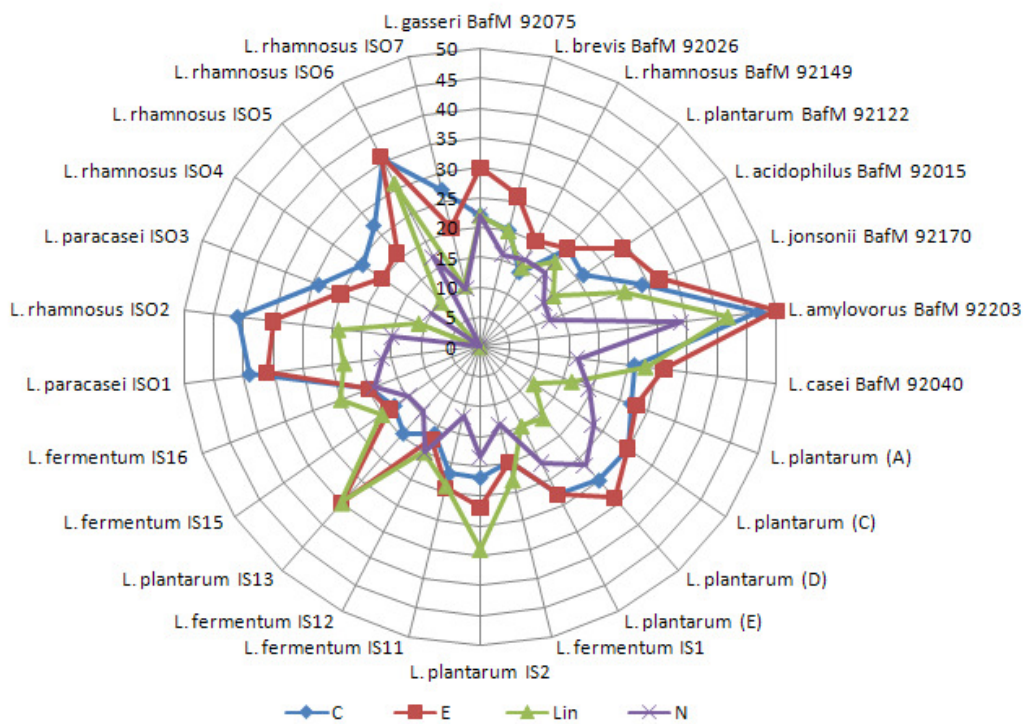


Fig. 31: *Lactobacillus* susceptibility to inhibitors of cell wall synthesis (ampicillin, cefotaxime, Methicillin and vancomycin). Scale indicates inhibition zone in mm.



(A)



(B)

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

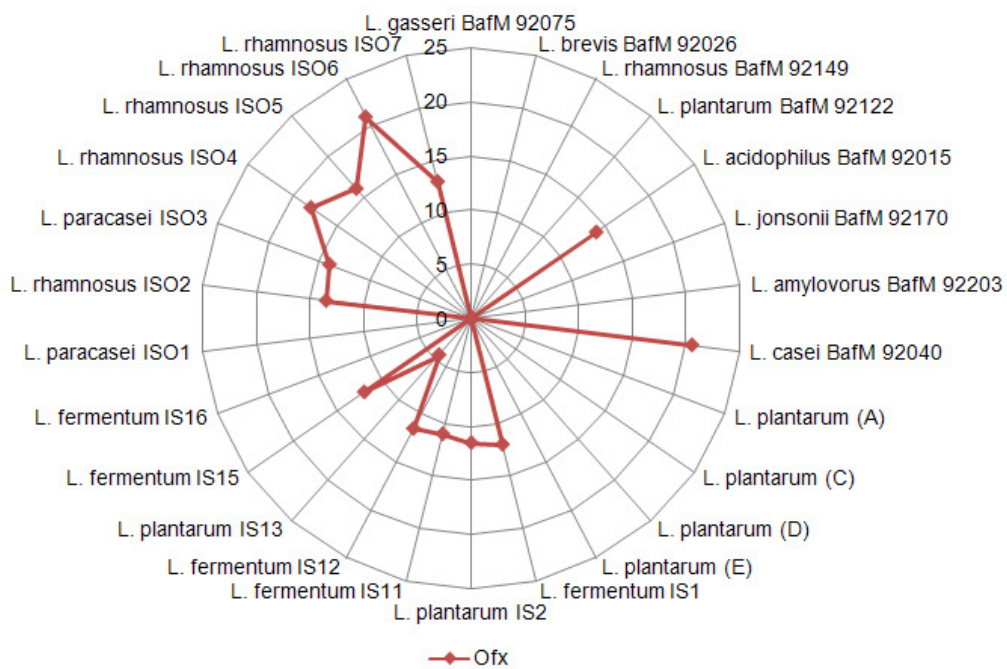


Fig. 33: *Lactobacillus* susceptibility to inhibitors of nucleic acid synthesis (Ofloxacin). See legened of fig. 31

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 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).

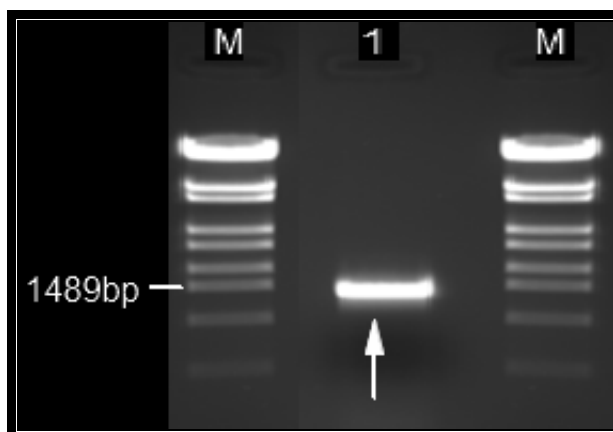
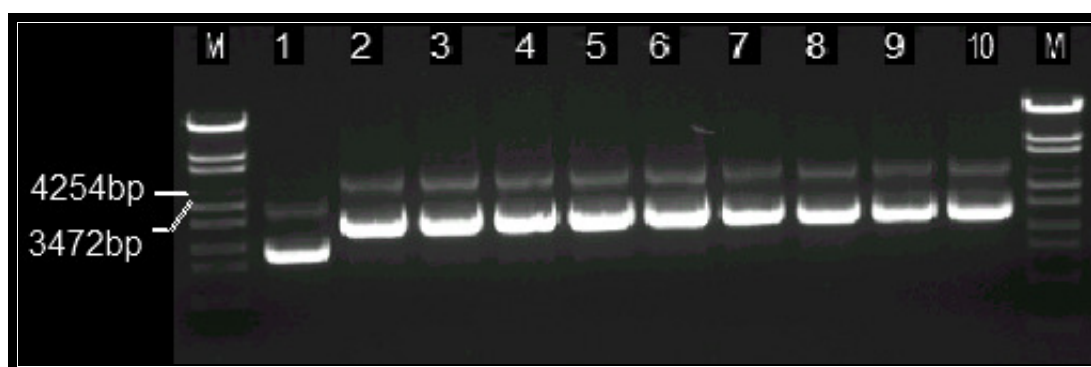


Fig. 34: PCR amplification of *cbh* gene from *Lactobacillus plantarum* (BfEL92122).

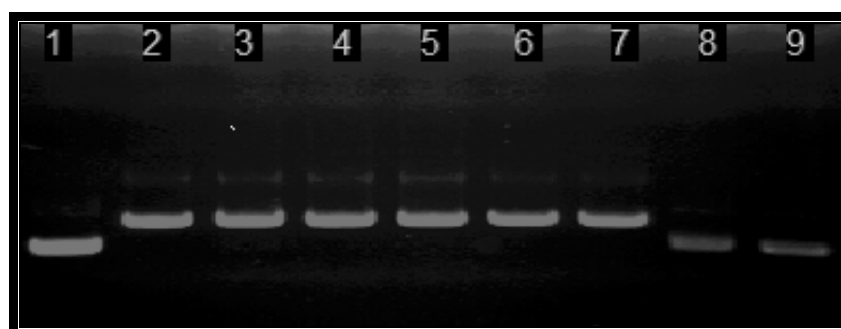
M: λ Styl; lane1: PCR amplicon of *cbh* gene. Arrow indicates *cbh* amplicon

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* easycores were used. Transformants were identified by blue/white colony screening (Fig. 35 A and B).



(A)



(B)

Fig. 35: Plasmids isolated from transformants obtained after cloning of *cbh* gene of *L. plantarum* BfEL 92122 into vectors pGEM-T-easy

(A) and pBluescriptII SK+ (B). **M:** λ Styl; lane 1: control vector; lanes 2-10: clones of *cbh* gene fragment.

5.2.3 DNA sequencing

Inserted DNAs from two plasmids were sequenced using T7 and SP6 specific primers. The sequences were compared with NCBI 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.

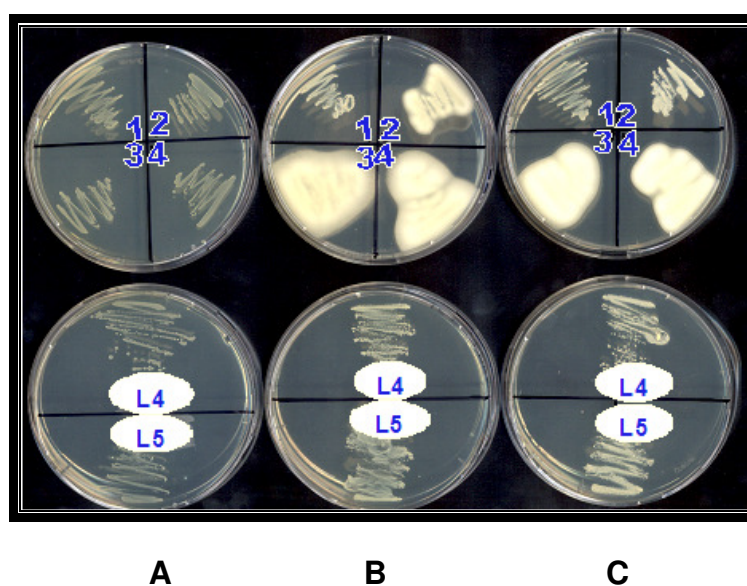


Fig. 36: Expression of *cbh* gene in *E. coli*.
A: LB medium; **B:** LB medium + 0.5% TDCA; **C:** LB medium + 0.5% GDCA; **1&2:** pS1 clones; **3&4:** pS2 clones; **L4:** control carrying only vector pGEM-T-easy; **L5:** control carrying only vector pBSK.

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.

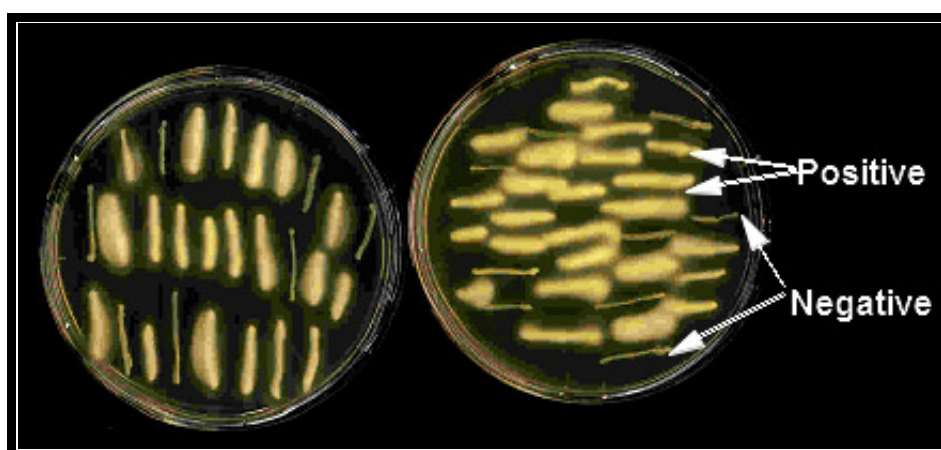


Fig. 37: Selection of positive clones on TDCA- LB agar medium

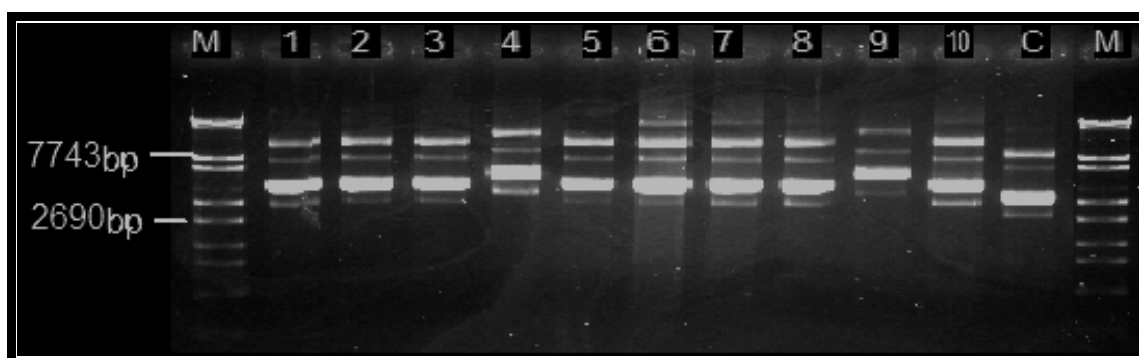


Fig. 38: Isolation of plasmids from selected positive transformants.
M: λ Styl; lanes 1-10: positive clones; C: control plasmid pL120

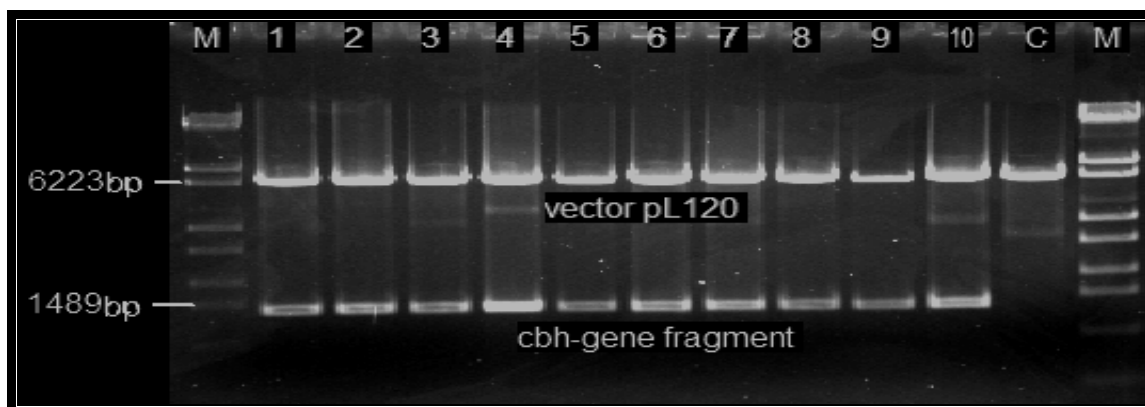


Fig. 39: Confirmation of the presence of the *cbh* gene fragment in the constructed plasmid pS3.

M: λ Styl; lanes 1-10: positive clones; C: control plasmid pL120

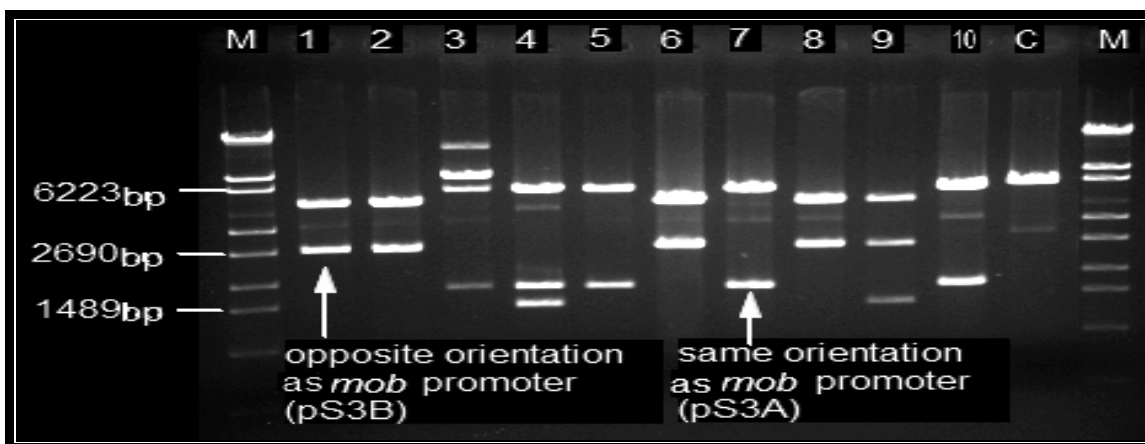


Fig. 40: Orientation of the cloned *cbh* gene by using *HindIII* restriction enzyme.

M: λ Styl; lanes 1-10: positive clones; C: control plasmid pL120.

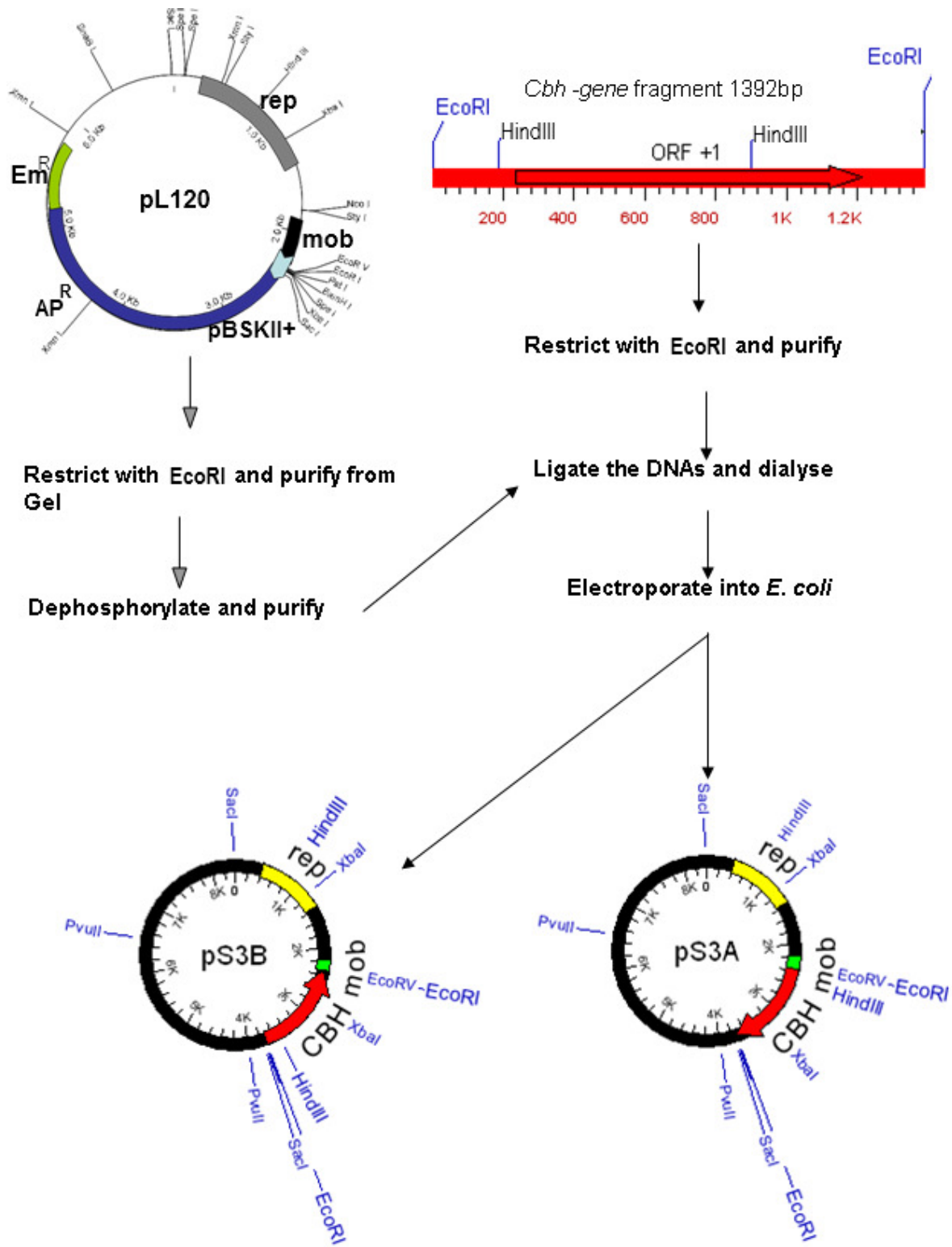


Fig. 41: Construction of pS3A and pS3B

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).

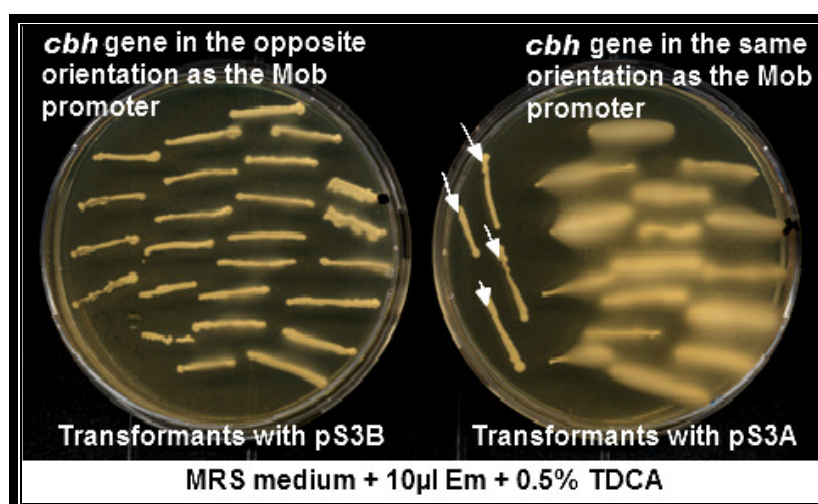


Fig. 42: Expression of *cbh* from plasmids pS3A and pS3B after transformation into *Lactobacillus casei* LK1 with plasmids. Arrows refer to transformants with pL120 as negative control

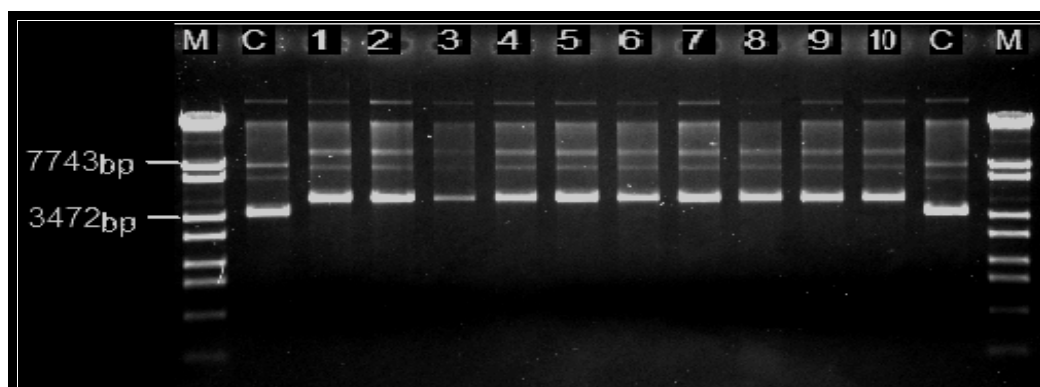


Fig. 43: Isolation of clones DNA from *Lactobacillus casei* LK1
M: λ Styl; **C:** control plasmid; lanes 1-5: positive clones of plasmid pS3A; lanes 6-10: positive clones of plasmid pS3B d pL120

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.

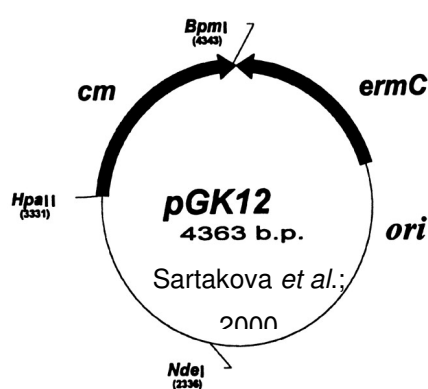


Fig. 44: Map of pGK12

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).

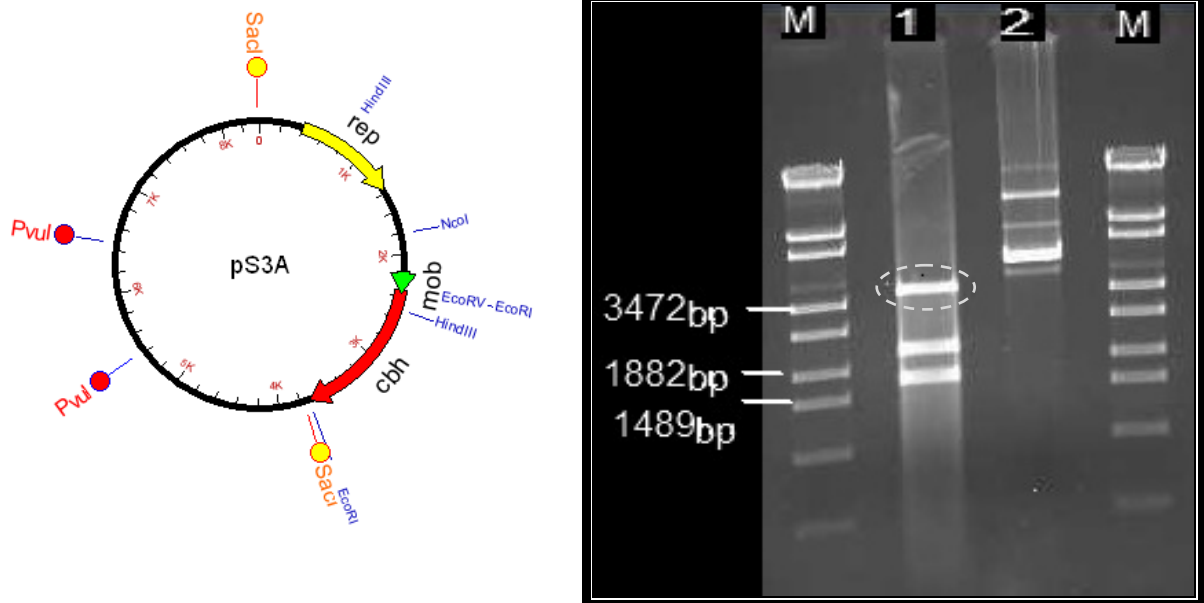


Fig. 45: Double digest of pS3A with SacI and PvuI.

M: λ Styl; lane 1: pS3A digested with SacI and PvuI; lane 2: undigested plasmid

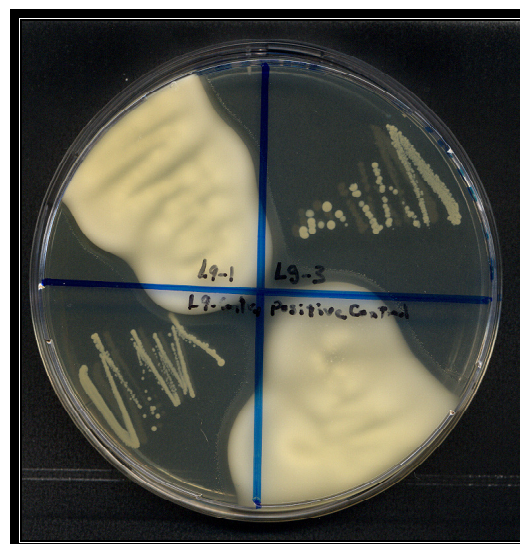


Fig. 46: Expression of *cbh* gene into pL9 plasmids.

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.

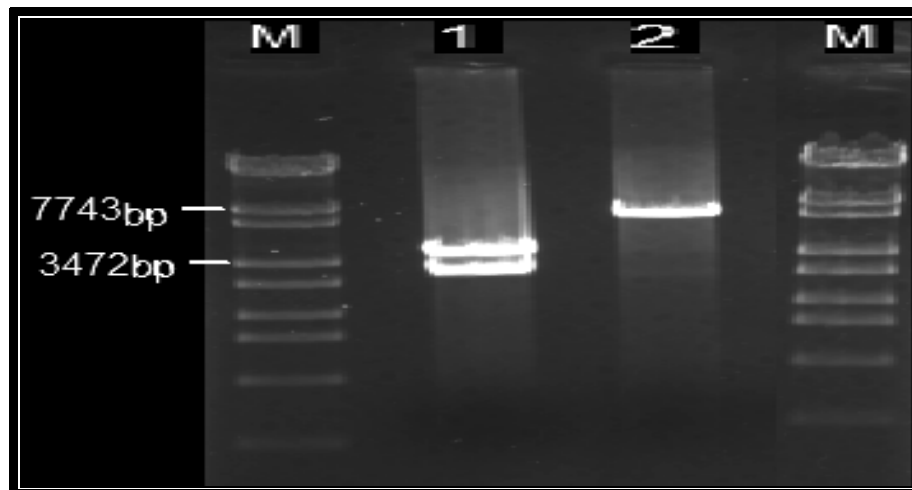


Fig. 47: Orientation of the cloned 3.7kb *SacI* fragment by *NotI* restriction enzyme.

M: λ Styl; lane1: pL9 – 1; lane2: pL9 – 3

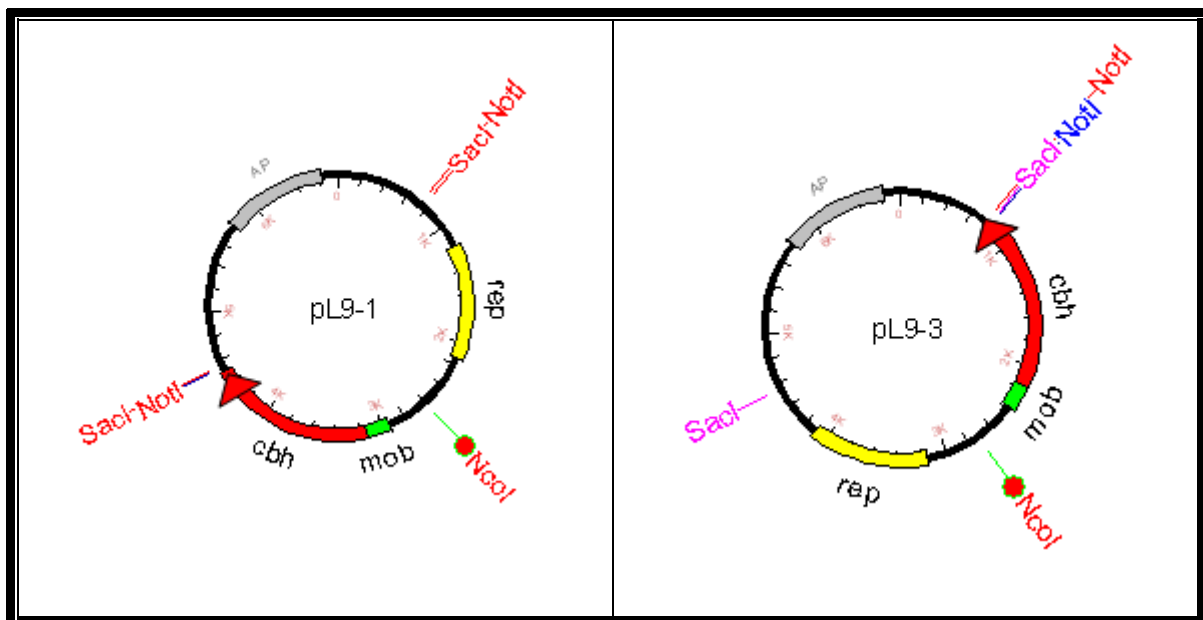


Fig. 48: Physical maps of plasmids pL9-1 and pL9-3

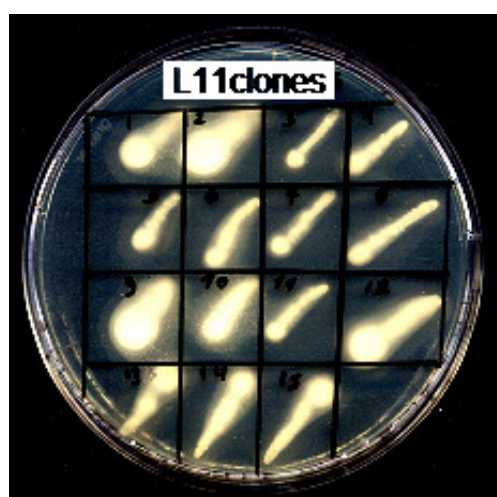
5.2.7.1 Cloning of the *cat-194* gene as a blunted ended fragment into the *NcoI* site of pL9-1 and pL9-3

Plasmid pL9-1 was linearised with *NcoI*, blunted with T4-polymerase, dephosphorylated and purified (Fig. 49). The chloramphenicol resistance gene from *Staphylococcus aureus* was isolated after 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)



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



Medium: Lb + Amp + Cm + TDCA

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.

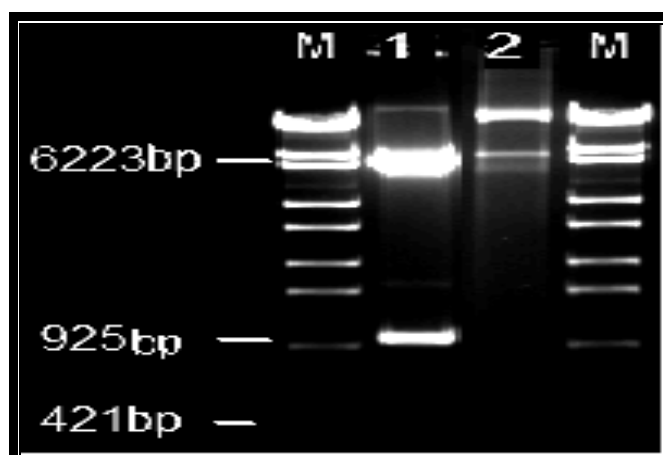


Fig. 51: : Restriction of pL11 with BvuI restriction enzyme.

M λ Styl; lane 1: clone of pL11; lane 15: undigested pL11.

5.2.7.2 Orientation detection of the *cat194*-gene into plasmid pL11

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

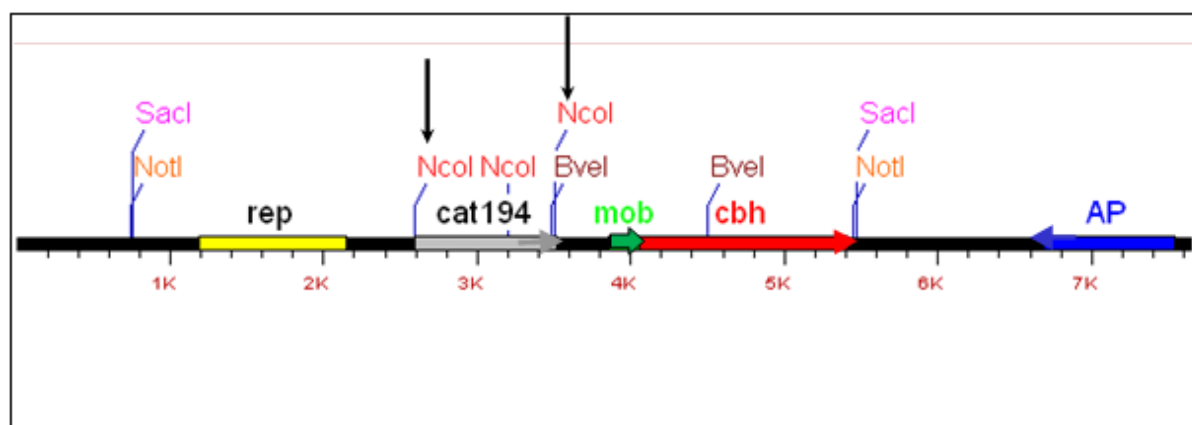


Fig. 52: Orientation of *cat194* gene in pL11

5.2.8 Cloning of *cbh* in pGKV2

This vector based on the broad host-range replicon of pWG2, a *Lactococcus lactis* plasmid (Fig. 53) replicates in various Gram-positive and -negative bacteria, as e.g. in *Lactobacillus* and *E. coli*. The *cbh* amplicon was ligated into the single EcoRI-site of the pGKV2 and transformed into *E. coli* Nova blue chemical competent cells. Transformants were selected on LB-agar plates supplemented with 100 μ g/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).

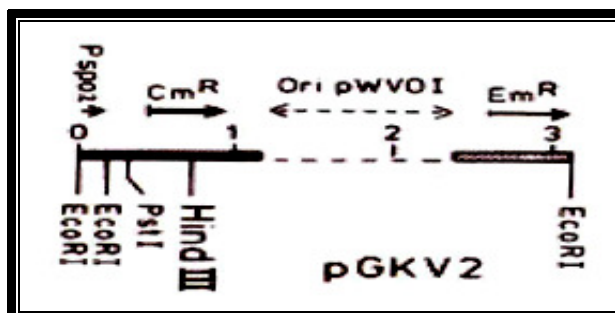


Fig. 53: Restriction map of pGKV2 (Kok et al., 1985)

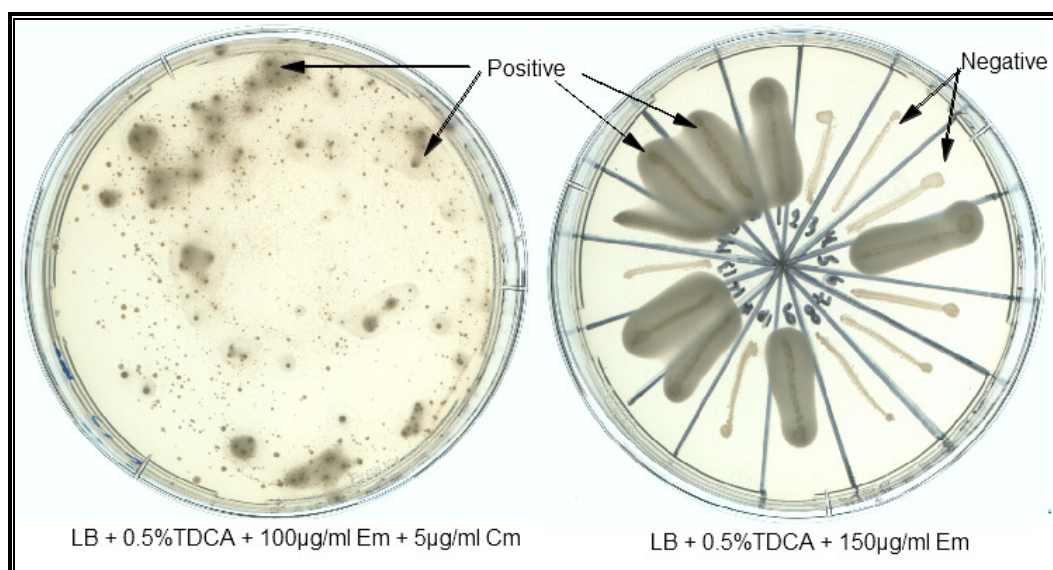


Fig. 54: Transformation and expression of the *cbh* gene cloned in pGKV2 in *E. coli* Nova-Blue

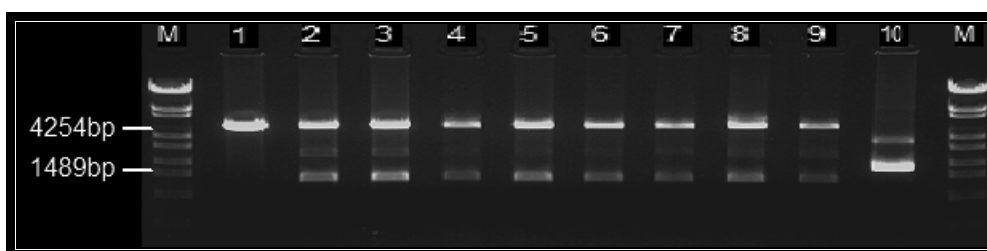


Fig. 55: EcoRI digest of L14 Clones (plasmid pL14).

M: λ Styl; lane1: EcoRI digest of pGKV2; lanes 2-9: EcoRI digest of transformants plasmids; lane10: 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 25 μ F and at 200 Ω 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).

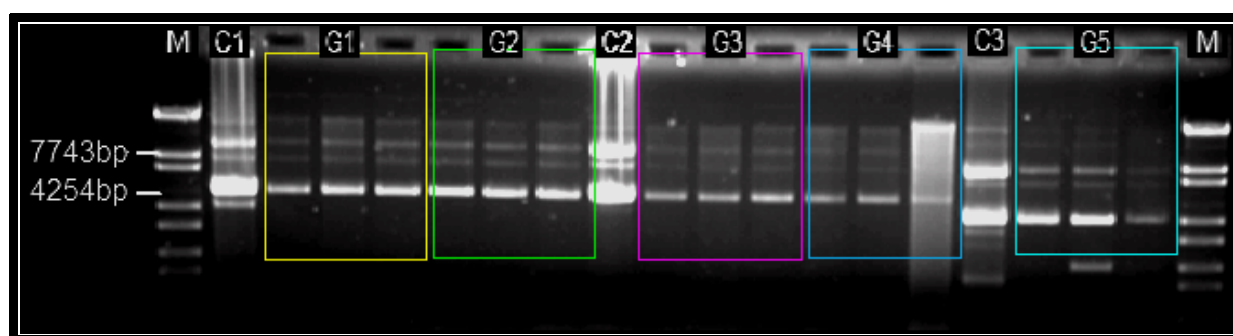


Fig. 56: Isolation of recombinant plasmids carrying the *cbh* gene from *Lactobacillus plantarum* BfEL 92122 after transformation into *Lactobacillus* strains.

M: λ Styl; lane **C1:** pS3A control; lanes **G1:** group of *L. casei* transformed with pS3A; lanes **G2:** group of *L. gasseri* transformed with pS3A; lane **C2:** pL11 control; lanes **G3:** group of *L. casei* transformed with pL11; lanes **G4:** group of *L. gasseri* transformed with pL11; lane **C3:** pL14 control; lanes **G5:** group of *L. gasseri* transformed with pL14

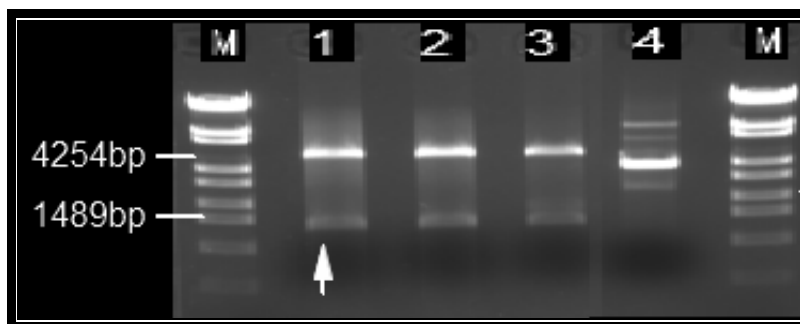


Fig. 57: *EcoRI* digest of plasmids isolated from *L. gasseri* transformed with plasmid pL14.

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.

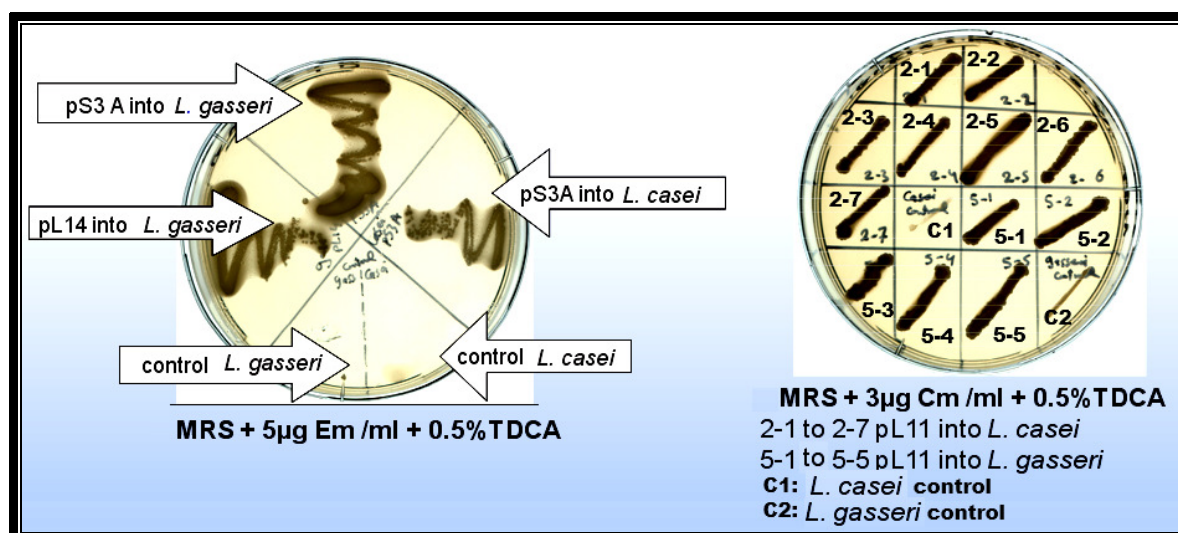


Fig.58: Expression of *cbh* gene which constructed in different plasmids and transformed into *Lactobacillus* sp.

Table 19: List of transformed strains

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

NS: Antibiotic selective marker not suitable NT: Not tested +: transformants obtained

Table 20: Segregational stability of the recombinant plasmid

Time/generation ^{a)}	pL11/ <i>L. gasseri</i>		Stability % ^{b)}	pL11/ <i>L. casei</i>		Stability % ^{b)}	pL14/ <i>L. gasseri</i>		Stability % ^{b)}
	-AB	+AB		-AB	+AB		-AB	+AB	
1st week / 50	37.4X10 ⁹	22.5X10 ⁹	60	34.6X10 ⁹	19.3X10 ⁹	55	39.5X10 ⁸	12.5X10 ⁸	31.6
2^{ed} week / 100	12X10 ⁸	3X10 ⁸	25	41X10 ⁸	6X10 ⁸	14.6	22.5X10 ⁷	2.5X10 ⁶	1.1
3rd week / 150	35X10 ⁸	22.2X10 ⁷	6.34	47.6X10 ⁸	9X10 ⁷	1.9	4X10 ⁶	22X10 ⁷	1.8

^{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 *L. casei*, *L. gasseri* and *L. rhamnosus* and their transformants carrying *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 *cbh* gene was expressed in these *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 *cbh* gene will increase the survival of lactobacilli in the gut.

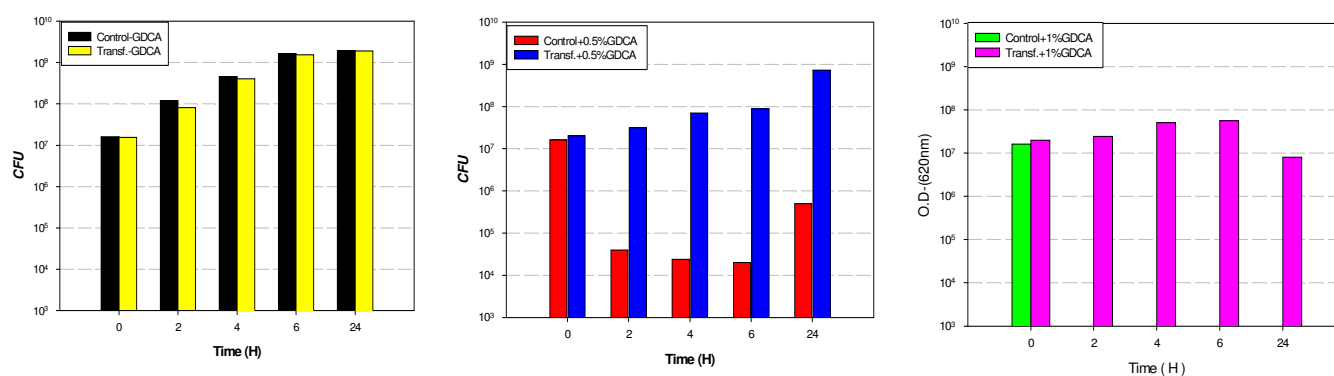


Fig. 59: Growth and survival of *L. gasseri* and its transformant *L. gasseri* (pL11) in presence of GDCA

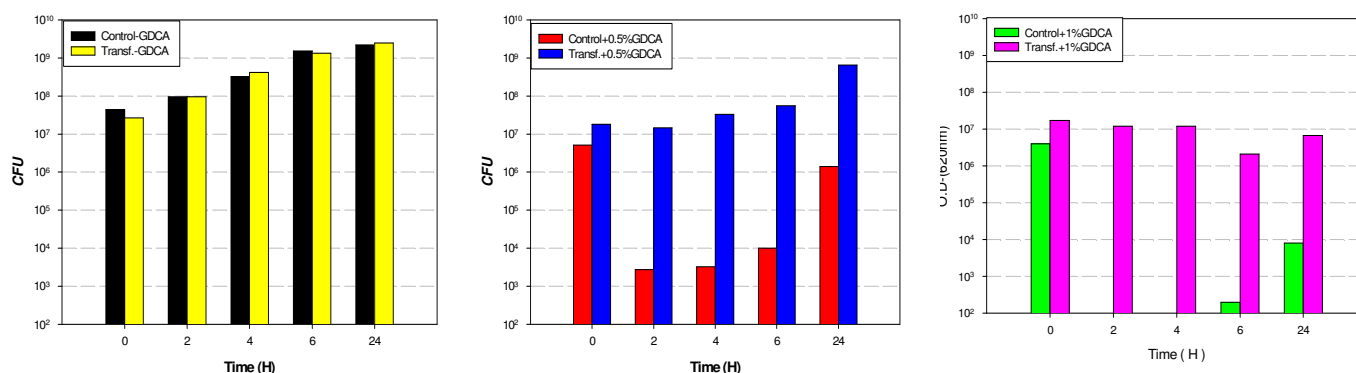


Fig. 60: Growth and survival of *L. casei* and its transformant *L. casei* (pL11) in presence of GDCA

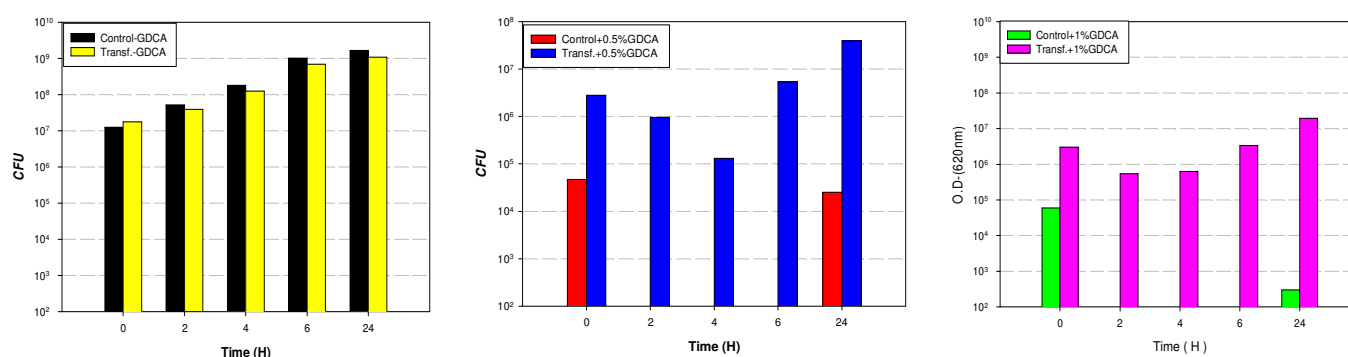


Fig. 61: Growth and survival of *L. rhamnosus* and its transformant *L. rhamnosus* (pL11) in presence of GDCA.

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).

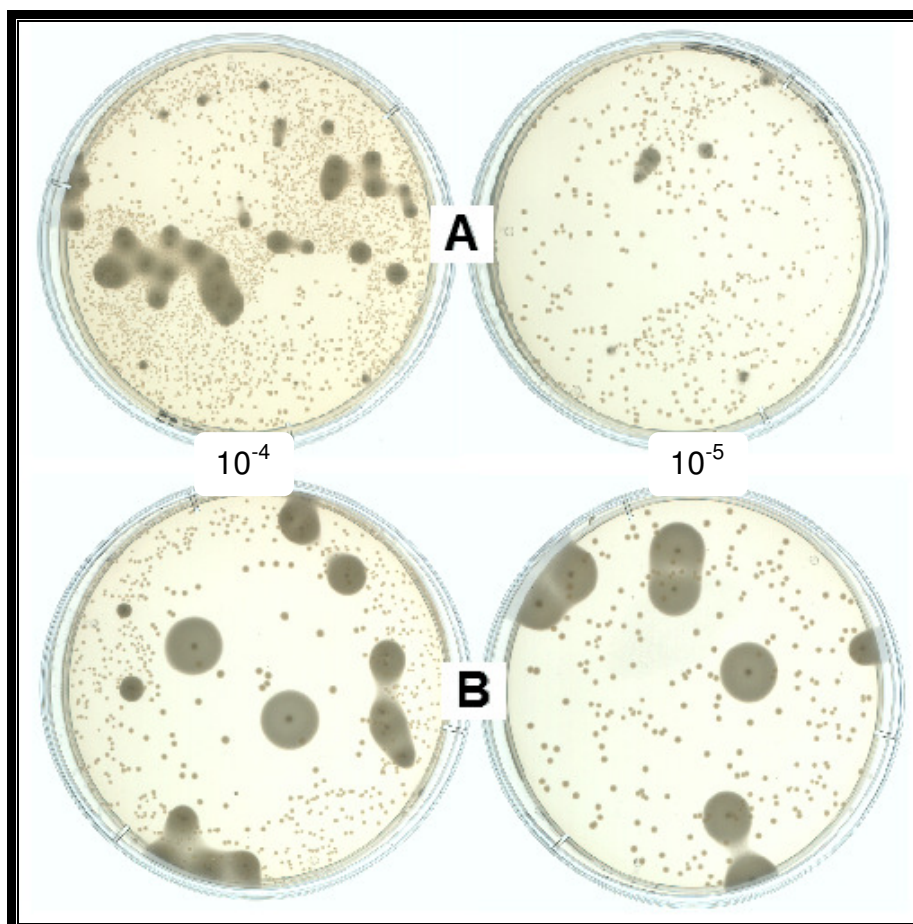


Fig. 62: Transformation of *E. coli* Nova-Blue with the plasmid pL11 and plating onto LB agar medium supplemented with GDCA without any antibiotics **A:** after 24 hours incubation; **B:** after 48 hours incubation.

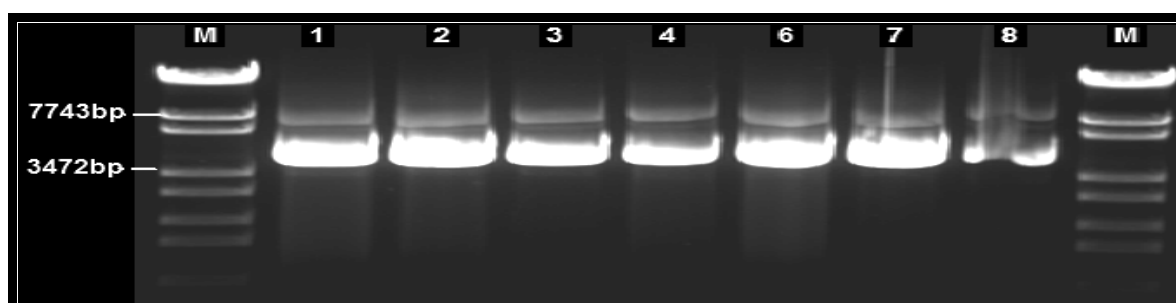


Fig. 63: Isolation of plasmid pL11 from *E. coli* transformants.

M: λ Styl; 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 (OD₆₂₀<0.25), MC-concentration of

0.1 to 0.4 µg/ml and a Ca⁺⁺- 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), *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 *L. brevis* strains, indicating that mainly phage-encoded enzymes were responsible for autolysis in these lactobacilli. Our results were in agreement of those by **Yokoi *et al.*, 2004**, who suggested that phage encoded lysine and bacterial lytic enzymes act additively in autolysis of *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 *phi* Lbgas1 DNA in the chromosomal DNA of *L. gasseri* NCK102-Adh and *L. gasseri* NCK102. The results are summarized in Table 21 and in Fig. 64 and 65.

Table 21: Lysogeny and autolysis of *Lactobacillus* sp.

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

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

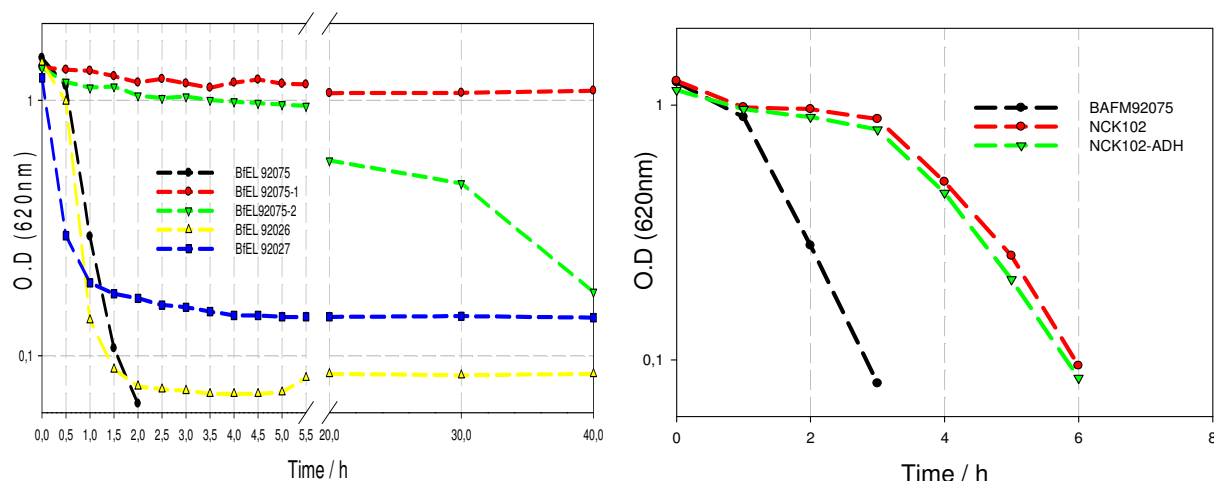


Fig. 64: Autolysis of *Lactobacillus* sp.

Cells were resuspended in autolysis buffer (10mM Tris pH7).

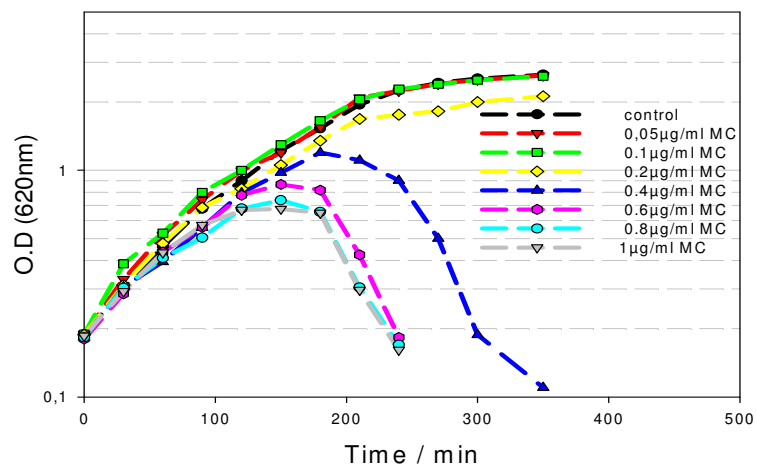


Fig. 65: Effect of the MC concentration on induction of *L. gasseri* BfEL 92075

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.

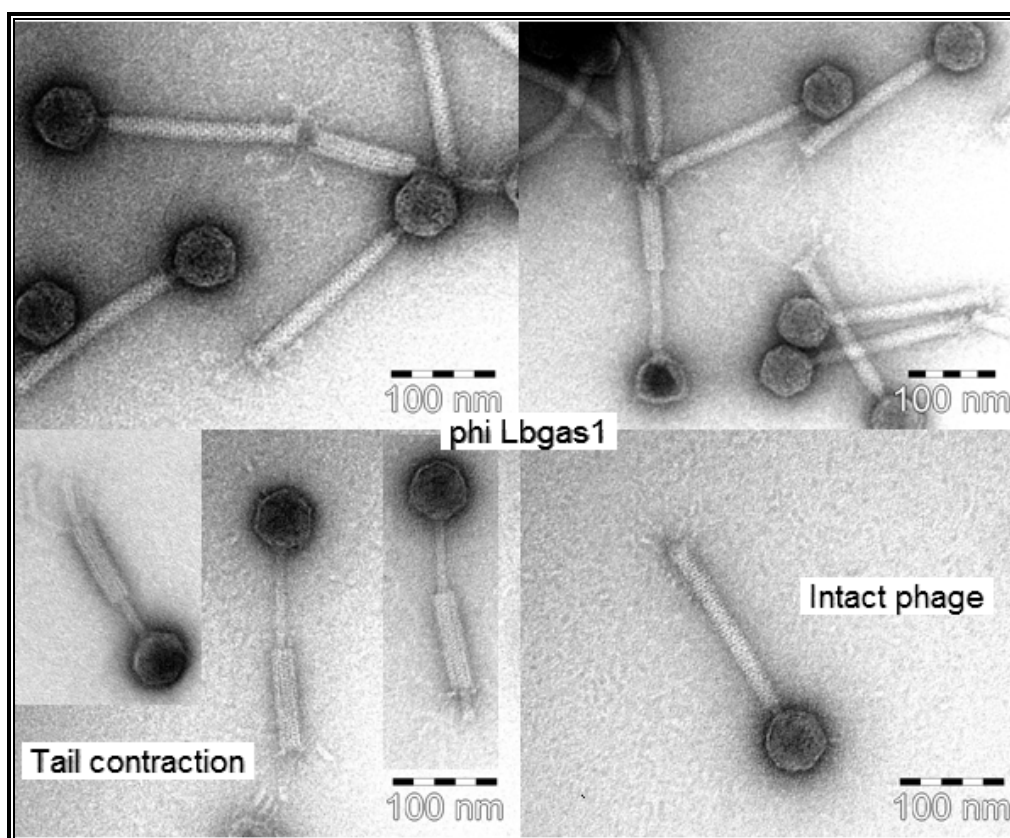


Fig. 66: Electron micrographs of *phi* Lbgas1 (bacteriophage inducible from *Lactobacillus gasseri* BfEL 92075)

Table 22: Dimensions* of phage Lbgas1

Nr.	Head		Neck		Tail		Sheath ^a		Base plate	Fiber
	C/C	S/S	Width	Length	Width	Length	Width	Length	Width	Length
1	63.5	57.3	12.1	10.1	19.4	176.2	26.1	103.1	27.6	50.9
2	60.4	55.0	11.7	8.9	20.9	173.6	27.4	106.4	25.1	50.0
3	64,7	57.9	9.7	10.4	19.2	175.4	25.3	105.8	24.6	39.7
4	63.5	60.6	12.1	11.1	20.2	179.6	24.5	103.9	23.1	48.0
5	66,7	53.9	10.7	8.2	21.7	180.9	26.3	100.9	23.0	46.7
6	60,4	60.2	9.5	8.8	19.3	179.6	24.4	97.8	24.2	46.8
7	60.1	56.9	9.7	12.2	18.9	178.8	22.8	102.7	21,4	46.3
8	62.6	59.4	11.3		20.6	179.0	22.9	98.7		38.9
9	61.3	54.3			18.5		24.2	102.2		50.2
10	61.7				20.5		22.5	92.3		46.6
11					19.0		22.0			
12							26.8			
Average	62.5	57.3	10.9	10.0	19.8	177.9	24.6	101.4	24.1	46.4

^a: 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, HinfI, SpeI, NheI, XbaI, SspI, HpaI and EcoRV and the DNA digests were separated on 1.5% and 0.6% agarose gels (Fig 67A and B), respectively .

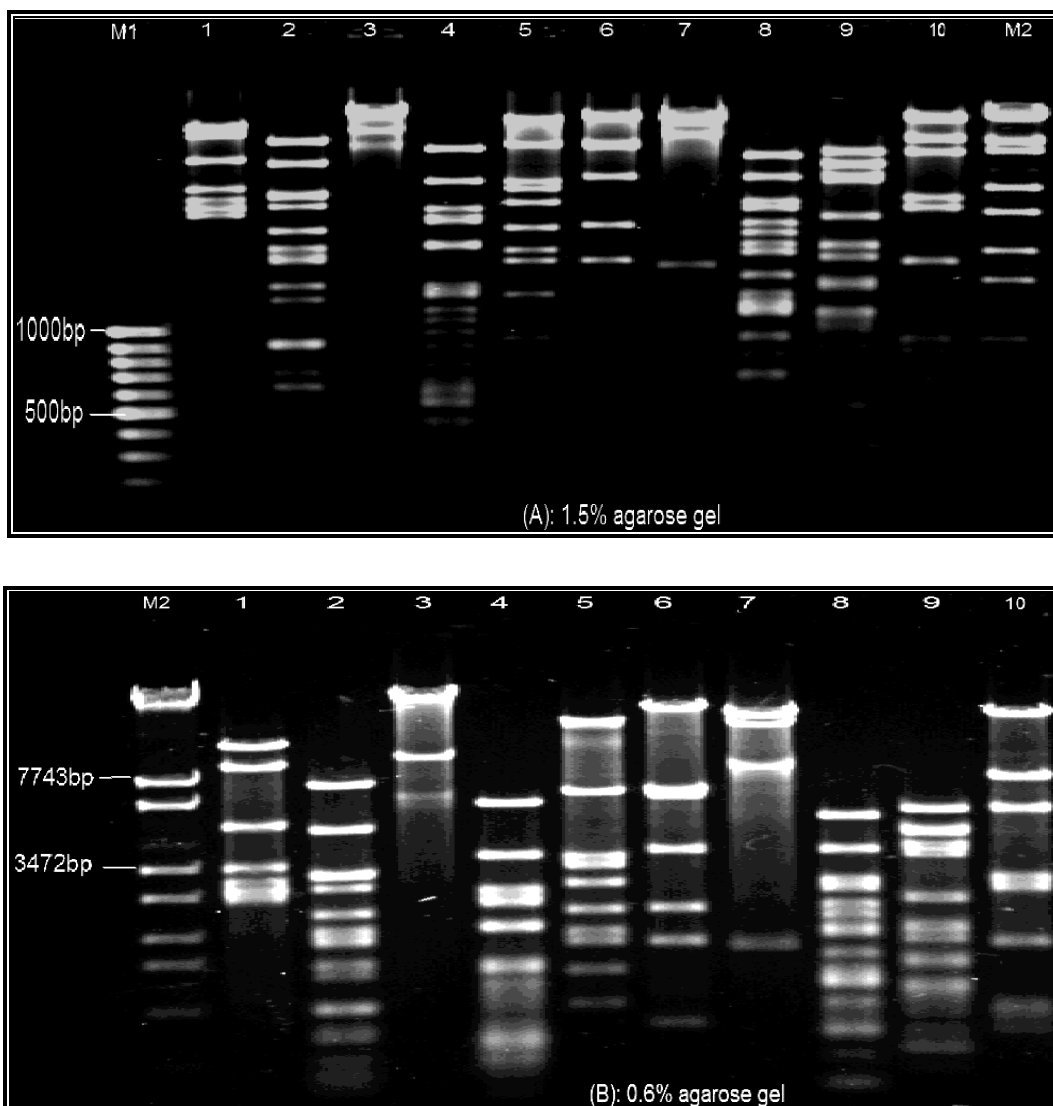


Fig. 67: Analysis Restriction profiles of the temperate phage *phi* Lbgas1 total DNA into 1.5% agarose gel (A) and 0.6% agarose gel (B).

M1:100bp Ladder; **M2:** λ Sty1; **lane 1:** EcoRI; **lane 2:** HindIII; **lane 3:** Sall; **lane 4:** HinfI; **lane 5:** SpeI; **lane 6:** NheI; **lane 7:** XbaI; **lane 8:** SspI; **lane 9:** HpaII; **lane 10:** EcoRV.

The genome size of *phi* Lbgas1 was calculated from the DNA restriction patterns by estimating the fragments generated by some restriction enzymes, e.g. EcoRI, SpeI and NheI, 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 (Quiberoni *et al.*, 2003). *phi* Lbgas1 may therefore be considered a phage of the *pac*-type.

Table 23: Estimation of *phi* Lb gas1 genome size

NO.	Fragment Size (kp)		
	EcoRI	SpeI	NheI
1	12.300	15.500	18.600
2	11.700	8.150	7.900
3	7.100	4.500	7.500
4	3.800	3.950	4.500
5	3.300	3.250	2.600
6	3.000	2.550	1.850
7	2.750	2.100	1000
8	-	1.700	-
9	-	1.400	-
10	-	1.050	-
Total	43.950	44.150	43.950

6.2.4 Comparative analysis of restriction patterns

Bacteriophages *phi* Lbgas1 and *phi* ADH DNAs were compared by restriction analysis using endonucleases HindIII, NheI, SpeI 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. gasseri* BfEL 92075 and phage *phi* ADH from *Lactobacillus gasseri* NCK102- Adh are genetically different, some fragments with identical size were detected. (Fig. 68).

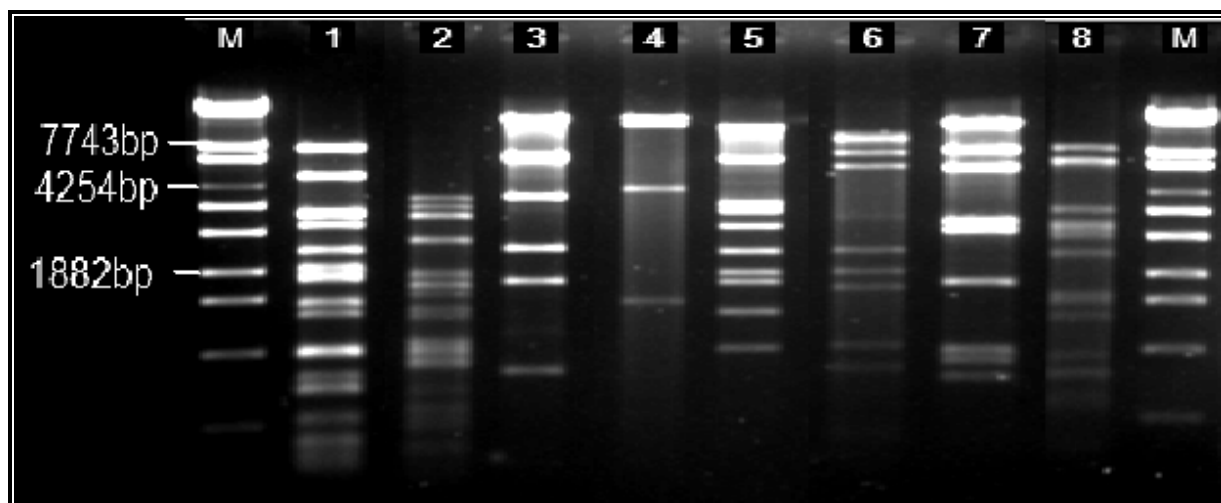


Fig. 68: DNA fingerprints of *phi* Lbgas1 and *phi* ADH.

M: λ Styl; **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 appears 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).

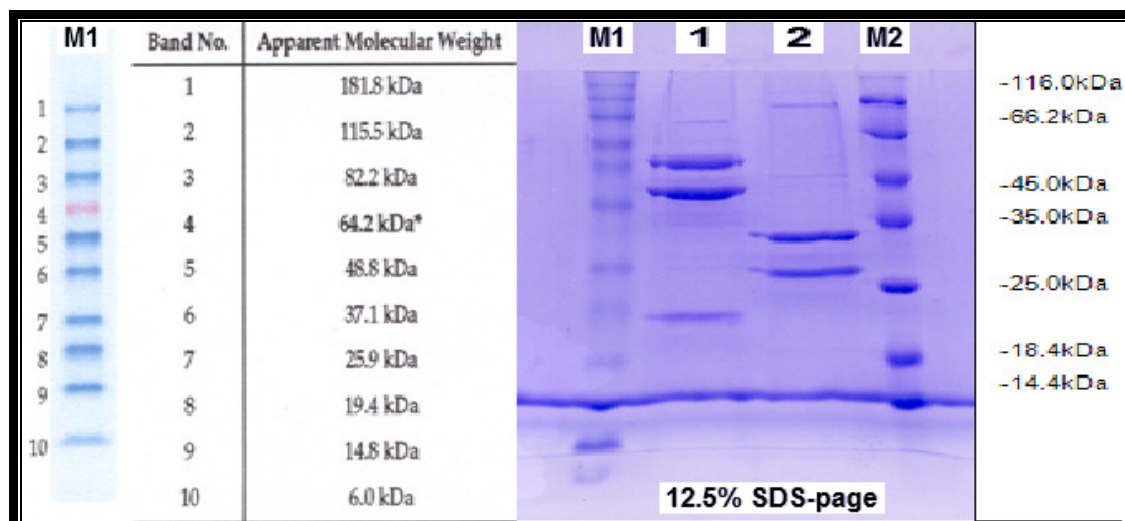


Fig. 69: SDS-PAGE for *phi* Lbgas1 and *phi* ADH.

M1: pre-stained protein ladder (Invitrogen No. 10748-010; **M2:** Protein molecular weight marker (Fermentas SM0431); **lane 1:** *phi* Lbgas1; **lane 2:** *phi* ADH.

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 NheI 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 be 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.

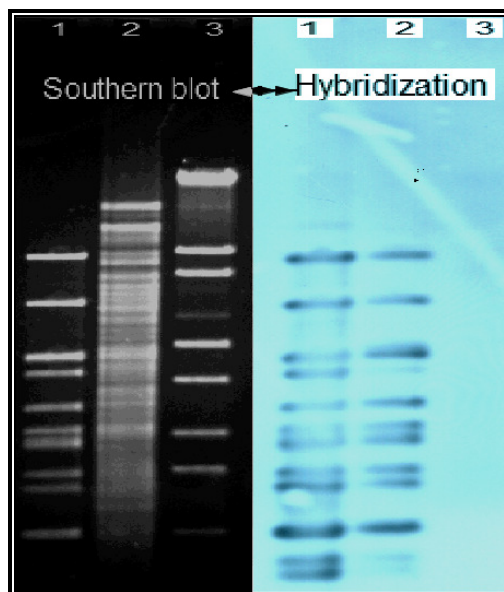


Fig. 70: Southern Blot Hybridization of *phi* Lbgas1 DNA and *L. gasseri* BfEL 92075.

lane1: HindIII digested DNA of *phi* Lbgas1; **lane 2:** HindIII digested chromosomal DNA of *L. gasseri* BfEL 92075; **lane 3:** marker λ Styl as a negative control.

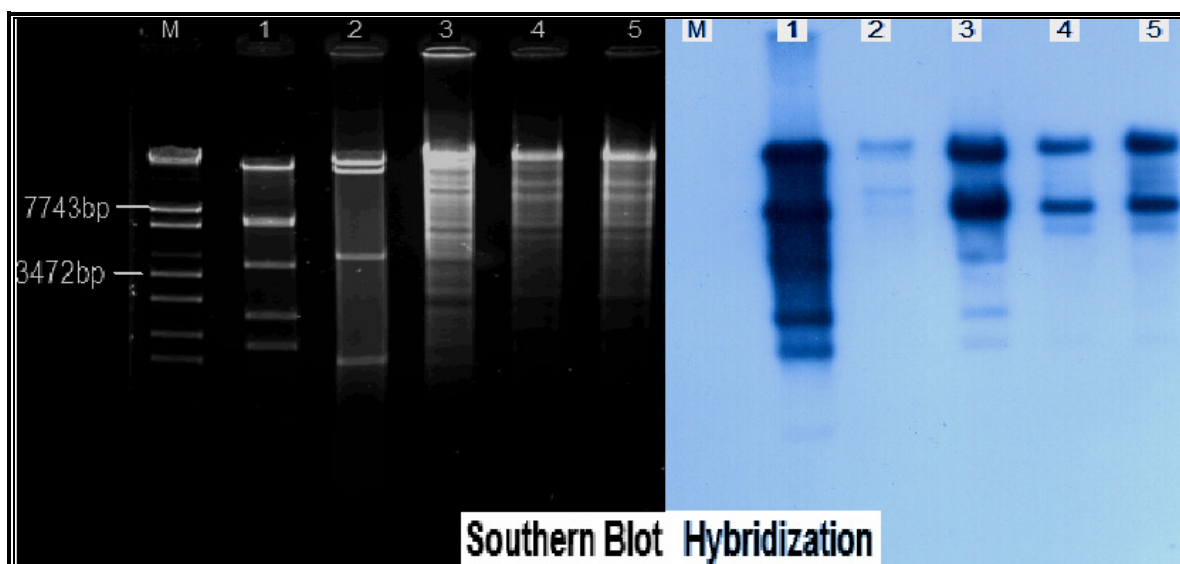


Fig. 71: Southern Blot Hybridization of *phi* Lbgas1 and *phi* ADH.

M: λ Styl; **lane 1:** *phi* Lbgas1; **lane2:** *phi* ADH; **lane 3:** Chromosomal DNA of *L. gasseri* BfEL 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 NheI.

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.

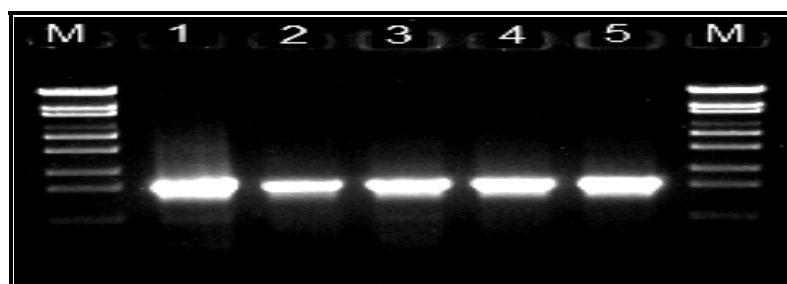


Fig. 72: PCR amplicons of holin-lysin cassette.

M: λ Styl; **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

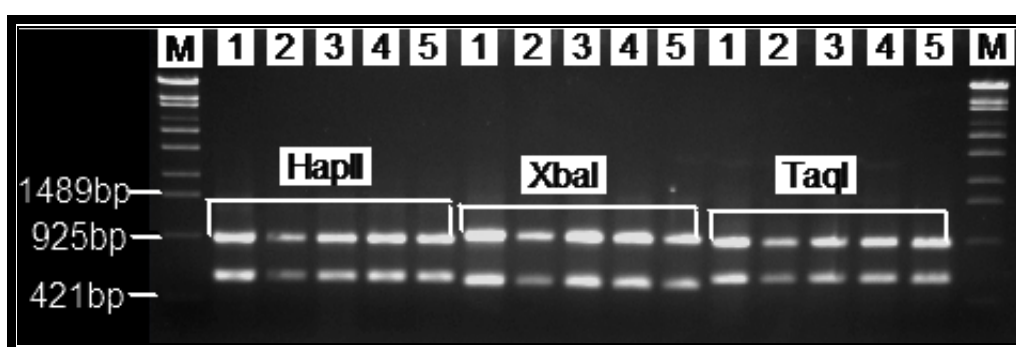


Fig. 73: Restriction analysis of holin-lysin gene fragments.

Fragments of the *holin-lysin*-operon were amplified by PCR with primers (5'-CGCAAGAAGGTTAAACGGTTAG-3') and (5'-CCAG-AGAAACATCTCCAGAGC-3'). The resulting 1477bp fragments were digested and analyzed. **M:** λ Styl; **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 μ l of induced culture were plated onto MRS agar plates, surviving cells were streaked in parallel onto LMRS agar and LMRS agar containing 0.2 μ g 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 0.2 μ g MC/ml. These colonies were inoculated into LMRS broth and grown overnight at 37°C.

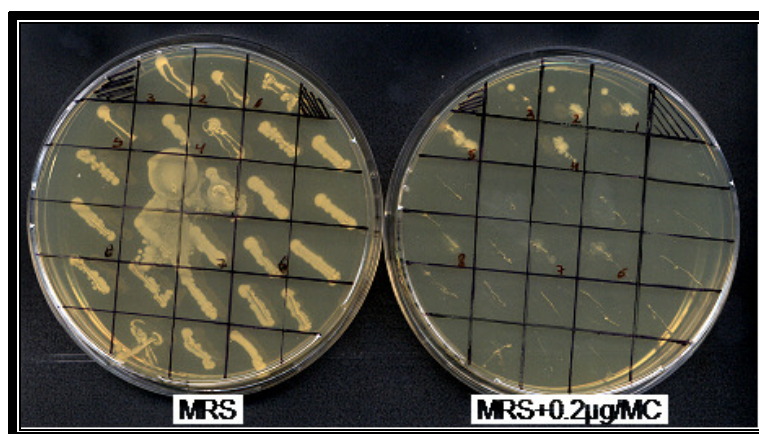


Fig. 74: Selection of *Phi* *Lbgas1*-cured derivatives of *L. gasseri* after MC treatment in the presence of anti-phage serum.

Re-induction of the eight derivatives with 0.2 μ g 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).

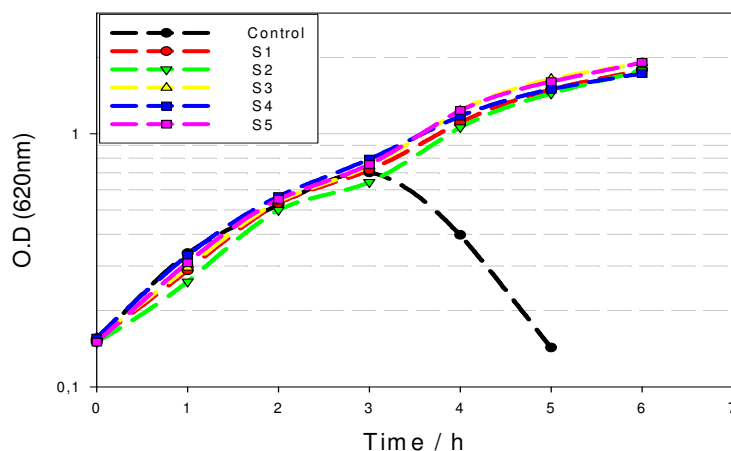


Fig. 75: Induction of *L. gasseri* and its derivatives with 0.2 μ g MC/ml.

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 *Hae*III and *Hin*fl. 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

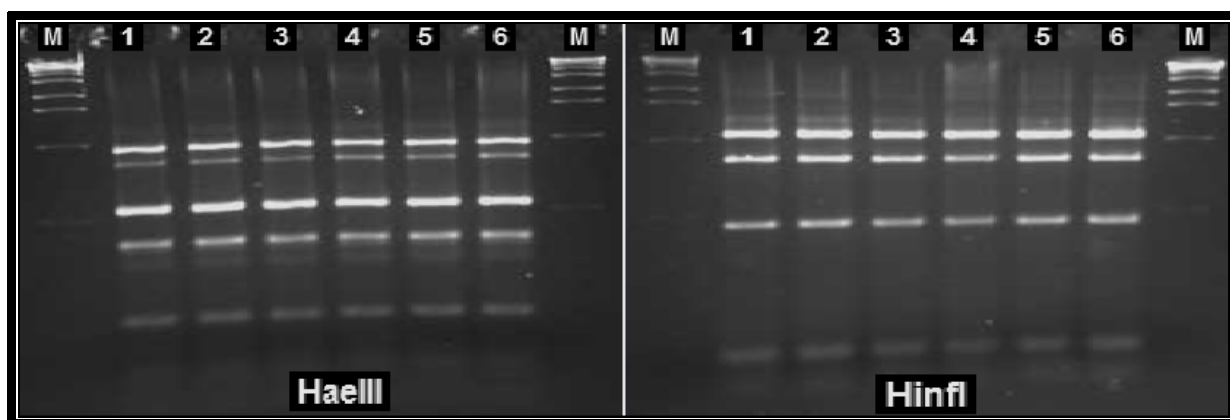


Fig. 76. ARDRA for the lysogenic strain *L. gasseri* BfEL 92075 and its non-inducible derivatives using *Hae*III and *Hin*fl restriction enzymes.

M: λ Styl; **lanes 1-5:** non-inducible derivatives of *L. gasseri* BfEL 92075; **lane 6:** lysogenic strain *L. gasseri* BfEL 92075

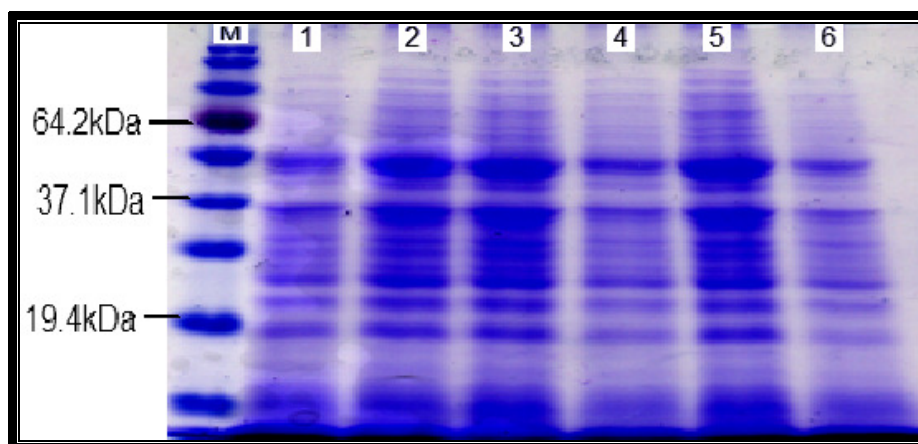


Fig. 77: Analysis of protein patterns for lysogenic Strain and its derivative.
M: Pre-stained protein ladder; **lane 1:** lysogenic strain *L. gasseri* BfEL 92075;
lanes 2-6: non-inducible derivatives of *L. gasseri* BfEL 92075

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.

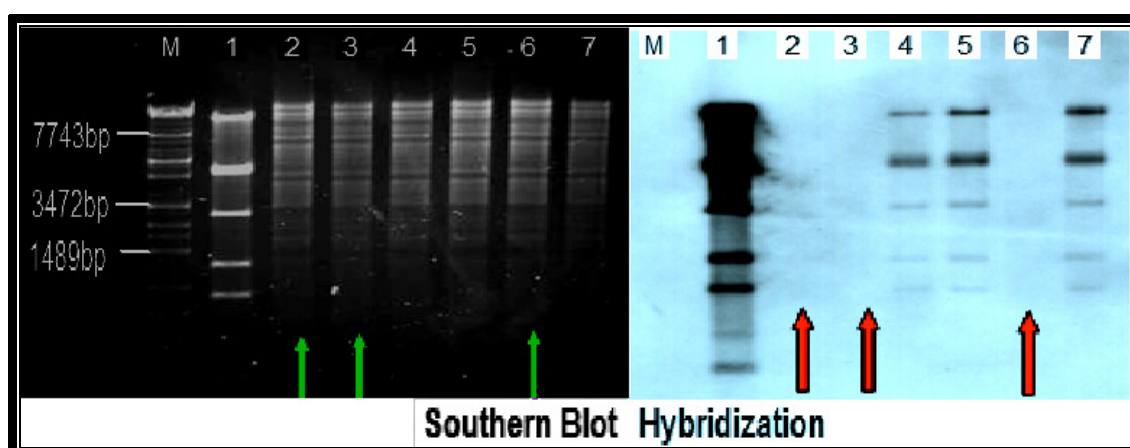


Fig. 78: Southern blot hybridization of *phi* Lbgas1, *Lb. gasseri* and its putatively cured derivatives.

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.

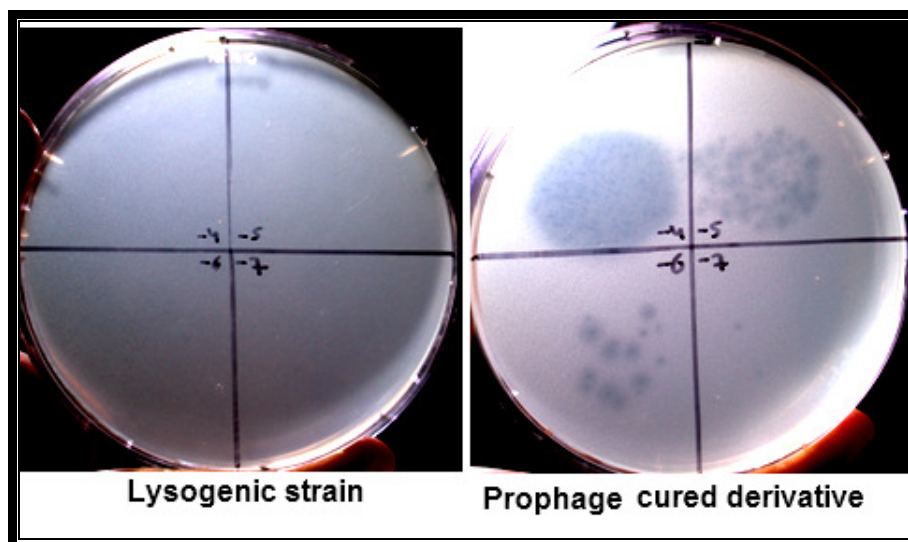


Fig. 79: Spot test for lysogenic strain and *L. gasseri* BfEL 92075 and one of its prophage-cured derivatives BfEL 92075-1 using *phi* Lbgas1

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 \emptyset FSV had derived from *L. casei* temperate phage \emptyset 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 *phi* Lbgas1, and ii) it eliminated the lysis gene from *L. 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 *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 *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 *Lactobacillus* strains through probiotic selection criteria (acid tolerance, bile salts resistance and antimicrobial activity). Also, a safety assessment of some *Lactobacillus* isolates was done. We report a survey of susceptibility of 26 *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 *Lactobacillus* strains showed some resistance to bile salts. In chapter III, we describe that conjugated bile salt-hydrolase gene (*cbh*) is responsible for de-conjugation of bile acids. The *cbh*-gene and its regulatory sequences (promoter, ribosomal-binding site and terminator) from highly bile salts resistance *Lactobacillus plantarum* BfEL 92122 strain were amplified by PCR, cloned into *E. coli* vectors. The *cbh* was so strongly expressed in *E. coli* that transformants could be identified directly on indicator plates containing bile salts as a substrate, indicating that the *cbh* gene might be generally useable as a food-grade indicator gene. *Cbh* was recloned into several *E. coli*/*Lactobacillus* shuttle vectors. Electrotransformation of *L. gasseri*, *L. casei* and *L. rahnmosus* 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* Lbgas1 from *L. gasseri*. The establishment of a prophage cured derivative of this strain is reported.

Finally, we purpose 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

Lactobacillen werden weltweit für die Herstellung fermentierter Lebensmittel benutzt. Neben ihrer Eignung als Säuerungsorganismen besitzen einige Stämme der Lactobacillen probiotische Eigenschaften. Um Bakterien als Probiotica nutzen zu können, ist eine korrekte Identifizierung und Evaluierung dieser Stämme unumgänglich. Das Ziel dieser Studie war es deshalb eventuell probiotische Lactobacillen-Stämme unterschiedlichster Herkunft (fermentierte Milchprodukte aus Ägypten, Fäzes von zwei neugeborenen Zwillingen) zu charakterisieren. Durch Klonierung und anschließender Übertragung eines Gens für eine Gallensalz-Hydrolase wurde zudem versucht, die probiotischen Eigenschaften einiger Stämme von Lactobacillen zu verbessern.

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.

Resistenz gegen Gallensalze ist ein wichtiges Kriterium für die Selektion und die Etablierung von probiotischen Stämmen. Die meisten der untersuchten Stämme zeigten eine gewisse Basistoleranz gegenüber Gallensalzen. Um dies zu erhöhen wurde das Gen *cbh* für eine Gallensalz hydrolase (conjugated bile salt hydrolase) aus *Lactobacillus plantarum* kloniert und in verschiedenen Lactobacillus-Spezies transformiert. Das *cbh*-Gen und seine regulatorischen Sequenzen (Promotor, Ribosomen-Bindungsstelle, Terminator) wurden mittels PCR amplifiziert und in *E. coli* Klonierungsvektoren eingebaut. In *E. coli* wurde das Gen solchermaßen stark exprimiert, dass eine direkte Identifizierung transformierter Zellen möglich war. Das Gen eignet sich daher als ein „Food-Grade“ Indikator Gen. Das *cbh*-Gen wurde anschließend in verschiedenen *E. coli*/Lactobacillus Shuttle-Vektoren rekloniert und in verschiedenen Lactobacillus Spezies (*L. casei*, *L. gasseri*, *L. rhamnosus*) exprimiert. Die Hydrolaseaktivität führte immer zu einer signifikanten Erhöhung der Resistent transformierter Stämme gegen Ochsen-galle sowie Glyco- und Taurodeoxicholsäure.

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.

Für eine abschließende Bewertung der untersuchten Stämme als Probioticum sind in-vivo Studien zum Überleben dieser Stämme in einem tierischen Modell wie z.B. in Göttinger Minischweinen geplant. Die Ergebnisse solcher Studien könnten die Basis bilden für eine anschließende Evaluierung potentieller, gesundheitsfördernder Eigenschaften ausgewählter Stämme.

9 References

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