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DIPLOMARBEIT

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„Impact of spray drying on the cellular activity and culturability of *Bifidobacterium bifidum* Bb12“

verfasst von

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1 Introduction

According to the currently adopted definition by the World Health Organisation (WHO) and the Food and Agriculture Organisation of the United Nations (FAO) probiotics are:
"Live micro organisms which when administered in adequate amounts confer a health benefit on the host"[1]. Lactic acid bacteria (LAB) and bifidobacteria are the most common types of microorganisms used as probiotics[2,3].

Probiotics are generally non-pathogenic microorganisms and are important constituents of the human and animal gut system[4,5].
They are usually known for their beneficial effect on the host by maintaining or improving their intestinal microbial balance[6,7].
Probiotic benefits are:[3,8,9]

- modulation of immune response, allergies
- anti tumur effect
- lowering blood cholesterol
- improving of intestinal flora
- improving immune function and infections prevention
- help at antibiotic associated diarrhoea / constipation by improving faecal properties
- preventing of gastrointestinal tract infection by suppressing harmful bacterial growth

It has to be noted that probiotics also can present disadvantages for the host. Especially if the gut system presents injuries or the immunological system is deficient or suppressed[8].

To exert any beneficial effect, probiotics have to comply with several aspects. They must present technological qualities to be manufactured into formulations without losing viability or activity. Probiotics must survive the gastrointestinal passage in order to fulfil their function in the intestinal tract. It's also important that the concentration of viable and active microorganisms is sufficient high in the host´s gut. The application of "Functional food" like reinforce yoghurts (Yakult®) is intended to increase the number of active probiotic microorganisms[9,10]. The concentration of 10^6-10^8 cfu g^{-1} viable bacteria is recommended by food organisations[11,12].
To achieve this level in probiotic products, it is important to stabilize the microorganisms.

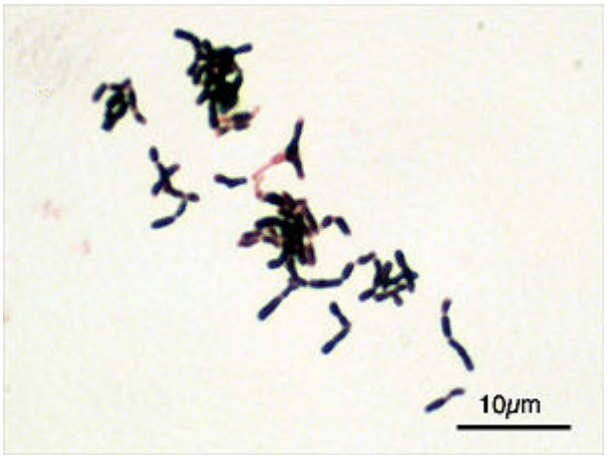


Figure 1: Bifidobacterium;
http://en.wikipedia.org/wiki/Bifidobacterium; accessed April 14th, 2011

Bifidobacterium bifidum Bb12 is a genus of gram-positive, non-motile, often branched anaerobic bacteria inhabiting the gastrointestinal tract and vagina. Bifidobacteria are one of the major genera of bacteria that make up the intestinal flora and is a bacterium found in large quantities in the colon. Bifidobacteria aid in digestion, are associated with a lower incidence of allergies and also prevent some forms of tumour growth. Some species of bifidobacteria such as *Bifidobacterium bifidum* Bb12, *Bifidobacterium lactis* (B94) or *Bifidobacterium longum* (BB536) are used as probiotics[13,14].

1.1 Stabilisation of *B. bifidum* Bb12 by spray drying

There are different methods for dehydration and stabilisation of probiotic microorganisms such as fluid bed drying[15], lyophilisation[16,17] or spray drying[18,19]. Each method exerts different kind of stress on the microorganism to be dried, such as thermal, osmotic or mechanical stress[20].
As the focus of this work was on investigating the impact of spray drying on the viability of microorganisms, only this method will be described in detail.

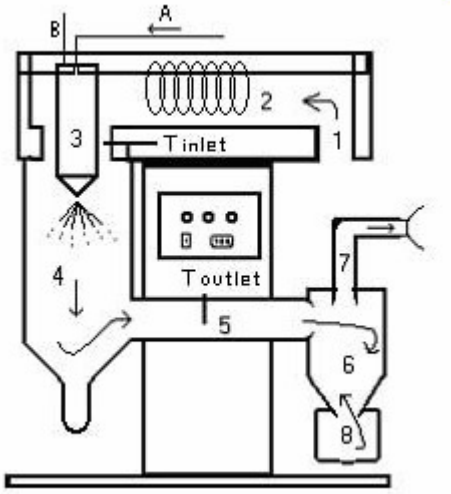
Spray drying is a method of producing a dry powder from a liquid by rapidly drying it with hot air. This is a favourable method of drying large amount of liquid materials such as food (dairy products) and pharmaceuticals (probiotic formulations)[18,21]. One of the advantages of spray drying is a consistent particle size of distribution. Hot air is the drying medium. The droplet size depends on the atomizing air pressure, the spraying rate and of aspiration force. There is a precise balance between these factors in relation to droplet size.

The higher the atomizing air pressure, the smaller the droplets, which makes the aspiration of evaporated water easier.

With help of a pump the solution is transported towards the atomizer. In the atomizer the solution, with help of hot air, is then dispersed through fine holes into drop size. The hot air for drying is usually passed as a co-current flow to the atomiser. The liquid is atomised to droplets which are dried to particles. By aspiration the particles pass a thermometer (Outlet temperature) and are trapped by the cyclone.

The end product is collected in the product chamber or collection vessel. This part of the spray-dryer also has a certain heating level. Unfortunately a big amount of the product adheres to the glass wall of the cyclone.

Laboratory-scale spray dryer (Arrows show the co-current principal of the lab-spray dryer)



A: Solution or suspension to be dried
B: Atomization gas
1: Drying gas
2: Heat element
3: Spray nozzle
4: Drying chamber
5: Part between drying chamber and cyclone
6: Cyclone
7: Outlet channel
8: Collection vessel of product

Figure 2: Laboratory-scale spray dryer;
http://en.wikipedia.org/wiki/Spray_drying; accessed April 14th, 2011

One of the challenges of spray drying is to keep the end product dry as it is very hygroscopic and readily takes up water from the atmosphere. This is done by ensuring that the product powder is gathered in the collection vessel before the system had time to cool off.

During the spray drying, the microorganisms are exposed to different harms. Firstly they are exposed to oxygen, which increases the stress level for an anaerobic microorganism. Moreover, there is a mechanical stress, inflicted by the atomizing step. Last but not least, the

temperature causes a certain stress on probiotic cells, not only during the drying step but also when the powder is collected in the product chamber.
These sources of stress affect the cell viability.

1.2 Methods for investigation of probiotic cell viability

1.2.1 Spreading method

The simplest method to investigate probiotic cell viability is the spread method. This method evaluates the culturability. The bacteria suspension is spread and dispersed on an agar-plate. The plates are put in an incubator for 24 to 48h at 37°C. After this time the colony forming units (CFU) are counted. This method only evaluates living microorganisms with the possibility to reproduce. "Microorganisms which do not form colonies because they are dead, sub lethally damaged, viable but non-culturable, dormant or inactive cannot be counted"[22,23,24].
Moreover, the time needed to form visible colonies is relatively long.

1.2.2 Cellular properties

Cellular properties such as membrane integrity, esterase activity, hydrogen peroxide production, membrane potential or intracellular pH can be investigated.
The bacteria cells are stained with suited fluorescent dyes and subsequently the emitted fluorescence intensity is measured[22]. Commonly there are two methods of investigation: fluorimetry and flow cytometry.

1.2.3 Fluorimetry

Fluorimetry is a photometric measurement, which use the fluorescence emitted by a sample after exposition to light. Light can excite the electrons and provoke the passage to a higher energy level. But the electrons cannot maintain this energy level for long and return to the ground level by emitting photons. These photons are detected and analysed.

1.2.4 Flow cytometry

Flow cytometry is a technology used for counting small particles, especially cells. Cells are suspended in a stream of fluid, which passes through an electronic detector system. It allows the simultaneous analysing of multiparametric physical and/or chemical characteristics[24]. This technology is applied in a number of fields in biology and other life sciences.

1.3 Aim of the thesis:

The aim of this thesis was to investigate the effect of spray drying on the cellular properties and culturability of *Bifidobacterium bifidum* Bb12. In a first step the effects of exposing cells to different temperatures was investigated. In a second step the influence of the spray drying process and the effect of using gum arabic, gelatine, maltodextrin and skimmed milk as protective additives on the cell viability were studied.

While culturability was studied using the spreading method, alterations in cell membrane, esterase activity, hydrogen peroxide production, membrane potential and intracellular pH, were studied after staining the cells with suitable fluorescent dyes using fluorimetry and flow cytometry.

In chapter two materials and methods used in this study will be presented. Chapter three is dedicated to the presentation of the results which will be discussed in chapter four.

2 Materials and Methods

2.1 Material

- *B. bifidum* Bb12: Chr. Hansen A/S (Denmark)
- Reinforced clostridium medium (RCM) (OXOID, Germany)

Yeast extract	3g/l
„Lab Lemco"- Powder	10g/l
Peptone	10g/l
Glucose	5g/l
Soluble starch	1g/l
Sodium chloride	5g/l
Sodium acetate	3g/l
Cysteine hydrochloride	0,5g/l
Agar	0,5g/l

- Reinforced clostridium Agar (RCA) (OXOID)

Yeast extract	3g/l
„Lab Lemco"- Powder	10g/
Peptone	10g/l
Glucose	5g/l
Soluble starch	1g/l
Sodium chloride	5g/l
Sodium acetate	3g/l
Cysteine hydrochloride	0,5g/l
Agar	15g/l

- Phosphate buffer with citric acid (pH 6.8)

$Na_2HPO_4 * 2\ H_2O$	13,70g/l
$NaH_2PO_4 * 2\ H_2O$	3,65g/l

10g of citric acid was dissolved in 250ml distilled water and used to reach a pH value of 6.8 with a pH-meter.

- Insta-Gene® matrix kit by BIO RAD
- Propidium iodide (Sigma- Aldrich; Austria)
- Dihydrorhodamine 123 (Sigma- Aldrich; Austria)
- Fluorescein diacetate (Sigma- Aldrich; Austria)
- Carboxy fluorescein diacetate succinimidyl ester (Sigma- Aldrich; Austria)
- 3,3´- Dihexyloxacarbocyanine iodide (Sigma- Aldrich; Austria)
- Gum arabicum (Sigma-Aldrich; Austria)
- Gelatine (Sigma-Aldrich; Austria)
- Maltodextrin (Sigma-Aldrich; Austria)
- Skimmed milk (Sigma-Aldrich; Austria)

2.2 Equipment

- incubator (Memmert GmbH + Co KG, Germany)
- autoclave Varioklave (Type 400 H+P Labortechnik GmbH, Oberschleißheim, Germany)
- safety bench (DIN 12950 Variolab Mobilien W90 Type SWB Waldner Electronics, Germany)
- Vortex (Heidolph REAX 2000, Germany)
- Petri dishes (Sterilin, Bibby Sterilin Ldt., Stone, Staffs, UK)
- Safe lock tubes 1.5ml (Eppendorf AG, Germany)
- Themomixer comfort (Eppendorf AG, Germany)
- Glass spreader
- pH-meter (HANNA instruments PH211, Microprocessor pH meter)
- centrifuge instrument (Sorvall®instruments, RC5C)
- microplate reader Infinite®2000 (Tecan)
- 96 well polystyrene microplate, transparent (Greiner bio-one)
- Stericup®-GP (0.22µm, polyethersulfone, 500ml, radio-sterilized; Millipore)
- Incubator shaker innova™4000 (New Brunswick Scientific Co. Inc., USA)
- Flow cytometer EPICS®XL-MCL™ (Beckman Coulter, USA)
- AnaeroGen sachets (OXOID)
- Sealed jar
- Tubes with cap-o-test caps
- Spray Dryer (Büchi Mini Spray Dryer B-191, Switzerland)

2.3 Preparation of culture medium

To produce the medium, 38g of the RCM-powder were boiled in 1 litre of distilled water. 10 ml of the dissolved medium were filled in test tubes and were autoclaved at 121°C for 15 minutes. To reduce the risk of contamination the tube were stored at 4°C until use.

2.4 Preparation of phosphate buffer

For the washing step 10ml of phosphate buffer were filled into test tubes and closed with cap-o-test caps. The test tubes were put into the autoclave for 15min at 121°C. They were stored at 4°C until use.

The phosphate buffer was not only used for washing, but also needed for the dilution series. In this case, 9.0 ml of phosphate buffer were filled into test tubes and closed with cap-o-test caps. The test tubes were put into the autoclave for 15min at 121°C. They were stored at 4°C until use.

For tests with the flow cytometry, the phosphate buffer must be particle free.

Therefore, 500ml of phosphate buffer were filtered through a filter with a mesh opening of 0.22 µm under the laminar airflow.

2.5 Growth of bacteria

Bacteria show a four phase growth. The first phase is called the lag phase: After the transfer of bacteria in another media, bacteria do not grow immediately. They need a certain time of physiological adaptation. The second phase is called log or logarithmic phase and is marked by a significant increase in cell number. The third phase is called stationary phase. It is a sort of steady state between viable and death cell bodies. The fourth phase is called decline or death phase. This phase is marked by the death of the bacteria[16,25].

Bacteria have special nutrition needs for their survival, respected in the culture media. There are many different types of culture media, e.g. salts, organic compounds, mineral nutrients, etc. In this study, *B. bifidum* Bb12 was grown in a reinforced clostridium media (RCM), a fluid culture medium, at 37°C for 24h.

Using the laminar airflow, 1ml of the fresh harvested bacteria suspension was transferred to 10ml of RCM and was grown for 24h at 37°C under anaerobic conditions.

2.6 Harvesting the cells

After 24h of growth, the bacteria reached the stationary phase and could be harvested. This was done by separating the cells from the culture media by hydro extraction at 8000rpm over duration of 10min. After this the collected cells were washed twice with phosphate buffer (pH 6.8) and subsequently resuspended in phosphate buffer.

2.7 Effect of heat stress on cellular properties of *B. bifidum* Bb12

Cell viability is generally related to the growth phase. In terms of stress resilience the stationary phase is more favourable due to ended cell growth[26,27].

2.7.1 Heat stress

In order to detect the effect of heat stress on the viability of *B. bifidum* Bb12, samples were stressed using different temperatures over different periods of times. In order to observe cellular characteristics in response of heat stress, fluorimetry and flow cytometry were used after the staining of cells with different fluorescence dyes.

After 24h of growth the cells were harvested and washed twice with phosphate buffer. Then, they were resuspended in 10ml phosphate buffer.

500µl of resuspended cells were filled in safe lock tubes and put into a thermomixer for the following times and temperatures.

60°C: 30sec, 1min, 90sec, 2min, 4min, 6min, 10min
70°C: 30sec, 1min, 90sec, 2min, 4min, 6min, 10min
80°C: 30sec, 1min, 90sec, 2min, 4min, 6min, 10min
90°C: 30sec, 1min, 90sec, 2min, 4min

The stressed samples were further prepared for studying the alterations in culturability and cellular properties.

2.7.2 Culturability

Before the culturability of B.*bifidum* Bb12 could be investigated, a dilution series had to be produced.

For this purpose, 1ml of bacteria suspension was added to 9ml of autoclaved phosphate buffer and mixed on a lab- vortex. (= “dilution 1”). Then 1ml of “dilution 1” was added to another 9ml of phosphate buffer and was vortexed. (=”dilution 2”).

Altogether 7 dilution samples were prepared.

These dilution series were made with freshly harvested cells, heat-stressed cells and spray dried cells.

Freshly harvested cells were used for comparison with heat stressed cells and spray dried cells. These fresh cells were taken directly from the medium, see 2.6.
For the investigation of heat stress, freshly harvested cells were stressed using a thermomixer in a temperature range from 60°C to 90°C for different time intervals. After this procedure, the dilution series was produced.
For the investigation of spray dried cells, freshly harvested cells were dried (see 1.1). After the drying the cells were resuspended in phosphate buffer to produce the dilution series.

2.7.2.1 Determination of colony forming units (CFU)

The agar used for determintation of CFUs was a reinforced clostridial agar (RCA).
To prepare the counting plates, 52,5g of RCA were suspended in 1 litre of distilled water.
To dissolve completely, the suspension must be boiled. The solution was further autoclaved at 121°C for 15min. Afterwards, 10ml of the sterile solution were poured in Petridishes using a "dispersette". The prepared Petri dishes were stored at 4°C until use.

For detecting the CFUs of *B. bifidum* Bb12, 100µl of the bacterial dilution was spread on a RCA Petri dish with a glass spreader and incubated for 42h at 37°C under anaerobic conditions using AnaeroGen® sachets. Thereafter, the colony forming units were counted. All plate counts were carried out in duplicates.

2.7.3 Staining

2.7.3.1 Determination of cellular properties

Alteration in cellular properties was investigated using fluorimetry and flow cytometry after staining the cells with fluorescent dyes.
The impact of heat stress on the membrane permeability, esterase activity and superoxide production was studied by using following fluorescence dyes:

- Propidium iodide (PI) for membrane integrity
- Fluorescein diacetate (FDA) for esterase activity
- Dihydrorhodamine 123 (DHR123) for superoxide production

The samples were measured by using fluorimetrie assay.

The volume of 500µl of stressed sample was incubated at 37°C with 500µl of the working solution of the fluorescent dyes for 15 minutes in the case of PI, and for 1 hour in case of the

other dyes. Light can influence the stability of the fluorescent dyes and cause a falsification of the measured values. To protect the photosensitives dyes against light, the samples were covered with aluminium foil during the measurement.

The impact of spray drying on the membrane permeability, esterase activity superoxide production, cell membrane potential and intracellular pH was studied using following fluorescence dyes:

- Propidium iodide (PI) for membrane integrity
- Fluorescein diacetate (FDA) for esterase activity
- Dihydrorhodamine 123 (DHR123) for superoxide production
- 3,3´-dihexyloxacarbocyanine iodide (DioC6(3)) for cell membrane potential
- Carboxy fluorescein diacetate succinimidyl ester (CFDASE) for intracellular pH

The samples were measured using fluorimetrie assay and flow cytometrie assay.

2.7.3.2 Fluorescent dyes

Propidium iodide [23,28,29,30,31](PI):

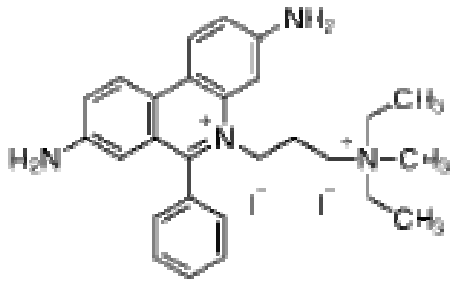


Figure 3: Propidium iodide; http://www.sigmaaldrich.com/catalog/ProductDetail.do?lang=de&N4=P4170|SIGMA&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC, accessed April 14th, 2011

Absorption 535nm
Extinction 617nm

A microorganism needs an intact cyto plasma membrane to survive. The cell membrane works like a barrier between the cytoplasm and the extracellular environment. With this barrier, the DNA is protected from mutagenous agents. Propidium iodide (PI) is an intercalating agent and a fluorescent molecule with a molecular mass of 668.4 Dalton, that can be used to stain DNA.

PI binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4–5 base pairs of DNA. PI is membrane impenetrable and generally excluded from viable cells. PI is commonly used for identifying dead cells in a population. PI is known as mutagen and should be treated very carefully.
A stock solution of 1,5mM PI in distilled water was prepared and stored at 4°C until use. The working solution was made of 1.2µl stock solution in 1ml PB for flow cytometry and 0.9µl/1,5ml for fluorimetry.

Fluorescein diacetate [23,31,32] (FDA):

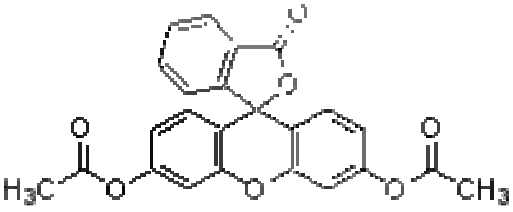


Figure 4: Fluorescein diacetate; http://www.sigmaaldrich.com/catalog/ProductDetail.do?D7=0&N5=SEARCH_CONCAT_PNO|BRAND_KEY&N4=F7378|SIGMA&N25=0&QS=ON&F=SPEC; accessed April 14th,2011

Absorption 494nm
Emission 521nm

Esterase activity is also an indication for the viability of the cells. FDA (MW 416.38) is a non-fluorescent hydrophobic fluorescein derivate that can pass through the cell membrane whereupon intracellular esterase hydrolyzes the diacetate group producing the highly fluorescent product fluorescein. The fluorescein molecules accumulate in cells that possess intact membranes so the green fluorescence can be used as a marker of cell viability. Cells that do not possess an intact cell membrane or an active metabolism will not accumulate the fluorescent product and therefore do not exhibit green fluorescence.
The solid FDA can be dissolved in dimethyl sulfoxid (DMSO) or in acetone. A stock solution with the concentration of 5mg/ml was prepared and stored at 4°C- protected from light.
The working solution was made of 6µl/1.5ml for Fluorimetry and 4µl/ml for Flow cytometry.

Dihydrorhodamine 123[33] (DHR 123):

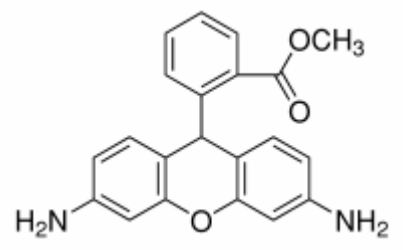


Figure 5: Dihydrorhodamine 123; http://www.sigmaaldrich.com/catalog/ProductDetail.do?lang=de&N4=D1054|SIAL&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC; accessed April 14th, 2011

Absorption 500nm

Emission 536nm

DHR 123 is a reduced form of rhodamine 123, a mitochondrion-selective probe with a strong green fluorescence. DHR 123 is able to penetrate the cells and is oxidized by oxidative species or by cellular redox systems to rhodamine 123. The solid state is soluble in dimethyl sulfoxid (DMSO) and has to be stored at -20°C until use. A working solution is freshly prepared from stock solution is of 5mM in dimethyl sulfoxid (DMSO): For fluorimetry measurements the concentration conducts 1µl/1.5ml phosphate buffer and for the flow cytometeric measurements a concentration of 5µl/ml was use.

3,3´- Dihexyloxacarbocyanine iodide[31,34] (DioC6(3))

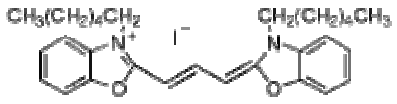


Figure 6: DioC6(3); http://www.sigmaaldrich.com/catalog/ProductDetail.do?lang=en&N4=318426|ALDRICH&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPECi; accessed April 14th, 2011

Absorption 484nm

Emission 501nm

DioC6(3) is a florescent dye used for the staining of a cell's endoplasmic reticulum. Binding to mitochondria and vesicle membranes occurs via the dye's hydrophilic groups.

This carbocyanine derivate can accumulate on hyperpolarized membrane, by translocation into the lipid bilayer and aggregation that decreases their fluorescence. It is used for cell membrane potential measurements in flow cytometry. Cells stained with this dye can only be exposed to light for short periods of time. DioC6(3) is soluble in DMSO. The concentration for the stock solution was 1mM in DMSO. A working solution was freshly prepared in a concentration of 3µl/1.5ml for fluorimetric measurement and 2µl/ml for flow cytometric measurement.

Carboxy fluorescein diacetate succinimidyl ester[35] (CFDA-SE):

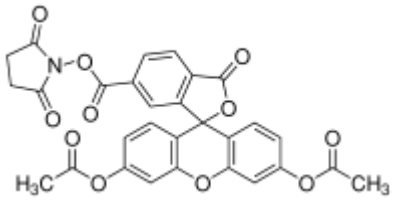


Figure 7: CFDA-SE; http://www.sigmaaldrich.com/catalog/ProductDetail.do?lang=de&N4=08951|FLUKA&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC; accessed April 14th, 2011

Absorption: 492nm
Emission: 517nm

Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) is a cell permeable dye. This dye is inapplicable to TECAN. Because of the highly pH dependence, CFDA-SE is used commonly for detecting damaged cells. CFDA-SE is soluble in DMSO.

To prepare the samples, 500µl of each fluorescent dye were added to 500µl of bacteria suspension; the save lock tubes were covered by aluminium foil, to protect the sample from light. After that, the samples were vortexed for about 10 seconds and incubated at 37°C.
The incubation time was 15 minutes for PI and 1 hour for the other fluorescence dyes. Prior to the measuring, the samples needed to be vortexed again. The samples were measured using fluorimetry as well as flow cytometry.

2.7.3.3 Measurement of fluorescent dyes using fluorimeter and flowcytometer

Principle of fluorimeter[36]

The excitation light (λex) strikes the sample. While a part of the light is absorbed by the sample, the relevant part emits fluorescence. The fluorescent light (λem) is emitted in all directions. A part of the emitted light reaches a detector by passing through a filter which eliminates scattered light. In order to minimize the risk of falsification by reflected light, the detector is usually placed at 90° to the incident light beam.

To measure the fluorescence spectrum, the wavelength of the excitation light is kept constant, preferably at a wavelength of high absorption. In general at low concentrations the fluorescence intensity is proportional to the concentration of the fluorophore.

Main reasons for using fluorescence measurements are higher selectivity and sensitivity compared to absorptiometry.

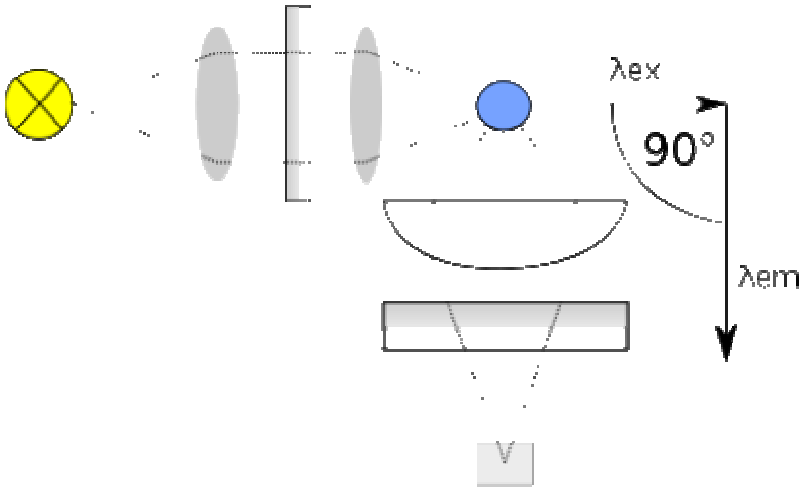


Figure 8: Fluorimeter;
http://de.wikipedia.org/wiki/Fluorimetrie; accessed April 14th, 2011

Principle of flow cytometry[37,38]

For flow cytometric measurements, the sample is injected into a stream of liquid (sheath fluid). The sheath fluid is lead to the point of measurement, so called flow cell. Laser light of single wavelength is focused on the flow cell. Detectors are placed at the cross point where the stream passes through the light beam. The extinction light as well as the scattered light is recorded. Each suspended particle (from 0.2 to 150 micrometers) passing through the beam cuts the light in some characteristic way which is measured.

This technique can be used for different parameters:

- enzymatic activity
- intracellular pH
- cell viability
- membrane integrity
- cell pigments
- DNA
- RNA
- chromosome analysis and sorting
- protein expression and localization
- Protein modifications, phospho-proteins
- transgenic products *in vivo*
- intracellular antigens
- nuclear antigens
- membrane fluidity
- apoptosis

After the incubation of the samples, 50µl were added to 1ml of filtered phosphate buffer and instantly measured.

2.8 Investigation of the impact of spray drying on culturability and cellular properties

2.8.1 Spray drying

Harvested and washed cells were dispensed in 50ml phosphate buffer pH 6,8. Just before spray drying the calculated amount of additives were added to 50ml of cell suspension.
Unprotected cells were also prepared for spray drying by washing and re-suspending them in phosphate buffer pH 6,8.
Four different kinds of additives were used, in concentrations of 0%, 10%, 50% and 100% based on the moist cell mass after harvestation. According to the literature, the concentrations were chosen in order to detect a possible correlation between concentration and beneficial effects. The additives were solved in phosphate buffer pH 6,8.
The applied additives were:

- Gum arabic (carbohydrate)
- Maltodextrin (polysaccharide)
- Gelatine (denaturated protein)
- Skimmed milk (mixture of proteins)

Thermal conductivity and thermal diffusivity vary with the excipients used and so does the physical qualities[19].

To obtain comparable results, it was important to optimize the following parameters in order to have an equivalent stress range.

Inlet Temperature:	115°C
Outlet Temperature:	70°C
Aspirator:	50%
Pump:	7rpm → 1,5g/min
Air pressure:	700 l/h

After processing, the dried cell mass was resuspended in 50ml phosphate buffer and 2ml of the suspension was used to determine the DNA content. While the DNA determination the bacterial suspension was centrifuged and stored at 4°C under anaerobic condition.

2.8.2 DNA determination

During spray-drying there occurs a loss of bacterial mass. In order to put a relation between the bacterial mass before and after the spray-drying, the concentration of DNA must be measured. The concentration of DNA is the only constant term between different treatments. The loss of bacterial mass is adjusted by a factor using the below mentioned formula.
Determination of bacterial mass loss was done by using the protocol of InstaGene® Matrix kit, which is described below:
2ml of the bacteria suspension were filled in a safe lock tube 2ml (Eppendorf) and centrifuged at 8000rpm for 10min. The supernatant was disregarded and the pellet was resuspended in 1ml autoclaved distilled water. After centrifugation at 10500rpm for 1 minute, the supernatant was disregarded and the pellet resuspended in 200µl of InstaGene® Matrix kit by BIO RAD and incubated at 56°C for 30 minutes in a themomixer at 500rpm. The sample was vortexed for 10 seconds and once more incubated at 99°C for 8 minutes. After centrifugating at 10500rpm for 3 minutes, the DNA resided in the supernatant[39].
After following this procedure it was possible to determine, by filling 50µl of the supernatant in a quartz cuvette and adding 450µl autoclaved, distilled water, how much µg DNA could be found in 1ml of sample solution. This was done by measuring the sample in a spectro photometer at 260nm. As blank, autoclaved and filtered, water was used.

The amount of DNA/ml was evaluated using the following formula:

Absorbance x 20 x 50 = µg D.N.A/ml sample

After the adjustment of loss, the bacteria were resuspended in the determined volume of phosphate buffer and prepared for investigations of culturability and cellular properties.

2.8.3 Investigation of culturability

The method is described in chapter 2.7.2.

2.8.4 Investigation of cellular properties

The method is describe in chapter 2.7.3.

2.8.5 Protective agents using for spray drying

Spray drying of unprotected cells results in an increase of stained cell number compared to freshly harvest stained cells.

For further investigations, the following protective agents were used:

Gum arabic is an air hardened, rubber like, natural excretion of *Acacia Senegal* L. Willdenow, or other *Acacia* species[40]. It presents a complex mixture of glycoproteins and polysaccharides[41].

Gelatine is a purified and irreversibly hydrolysed form of collagen[42], gained by partial acidic hydrolysis (Typ A) or partial alkaline hydrolysis (Typ B), it also can be a mixture of both[43].

Maltodextrin is a mixture from glucose, di- and polysaccharide, produced from starch by partial hydrolysis[44,45].

Skimmed milk is made when all the milkfat is separated from the milk[46].

2.9 Statistic analysis

Statistical analyses were performed using GraphPad Prism3 (GraphPad Software, San Diego, USA) and the Microsoft Excel integrated analysis tool.

Data were analyzed using one-way ANOVA with post-hoc Tukey test cross-comparing all study groups. Values of $p<0.05$ were considered as significant.

3 Results

3.1 Heat stress

3.1.1 Effects of heat stress on the culturability of *B. bifidum* Bb12

For the investigation of culturability *B .bifidum* Bb12 was stressed for different time intervals at the following temperatures:

60°C: 2min, 4min, 6min and 10min
70°C: 1min, 2min, 4min and 10min
80°C: 30sec, 1min, 2min and 4min
90°C: 30sec, 1min, 2min and 4min

		60°C			
	Fresh cells	2min	4min	6min	10min
CFU (±sd)	1 (± 0)	0.645 (± 0.08)	0.541 (± 0.09)	0.171 (± 0.06)	0.007 (± 0.01)

Table 1: Fold change of culturability of fresh cells after heat stress at 60°C (fresh cells = 1)

At 60°C the data showed no significant difference of culturability between two and four minutes of stress. Stressing the cells for 4 minutes at 60°C resulted in a decrease of the culturability less than 0.5 log units. After four minutes the CFU decreased dramatically.
At 6 minutes of heat stress the cell damages are significant, and the culturability decreases 1 log unit. The culturability decreases 2 log units after 10 minutes at 60°C.

		70°C			
	Fresh cells	1 min	2 min	4 min	10 min
CFU (±sd)	1 (± 0)	1.186 (± 0.53)	0.999 (± 0.29)	0.001 (± 0.00)	0.001 (± 0.00)

Table 2: Fold change of culturability of fresh cells after heat stress at 70°C (fresh cells = 1)

At 70°C there is no significant difference between 1 and 2 minutes. The first 2 minutes at 70°C does not harm the cell culturability significantly. But after 2 minutes, the CFU decreased dramatically.
After 4 minutes, the culturability decreases 3 log unites.

		80°C			
	Fresh cells	30 sec	1 min	2 min	4 min
CFU (±sd)	1 (± 0)	1.064 (± 0.02)	0.459 (± 0.38)	0.005 (± 0.0)	0.000 (± 0.00)

Table 3: Fold change of culturability of fresh cells after heat stress at 80°C (fresh cells =1)

Exposing the samples 30 seconds at 80°C showed no significant difference in culturability as compared to fresh cells. After 1 minute of heat stress at 80°C, the culturability decreases by 0.5 log units. After more than 2 minutes at 80°C, the culturability of the bacteria is no longer detectable.

		90°C			
	Fresh cells	30 sec	1 min	2 min	4 min
CFU (±sd)	1 (± 0)	0.537 (± 0.17)	0.456 (± 0.29)	0.001 (± 0.00)	0.000 (± 0.00)

Table 4: Fold change of culturability of fresh cells after heat stress at 90°C (fresh cells = 1)

No significant difference in culturability could be found after stressing cells at 90°C for 30 seconds or 1 minute. In both cases culturability decreased by 0.5 log units compared to fresh cells. At 2 minutes of stress the culturability decreases for 3 log units and after this time, there is no more growth detectable.

3.1.2 Effects of heat stress on cellular properties of *B. bifidum* Bb12

The effect of heat stress was investigated on membrane integrity, esterase activity and the production of hydrogen peroxides.

3.1.2.1 Cell membrane integrity

Alteration in membrane integrity was investigated by using PI for staining.

This dye is membrane impermeant and generally excluded from viable cells. PI is commonly used for identifying damaged or dead cells in a population. Once the cell membrane is damaged, PI can bind to the DNA.

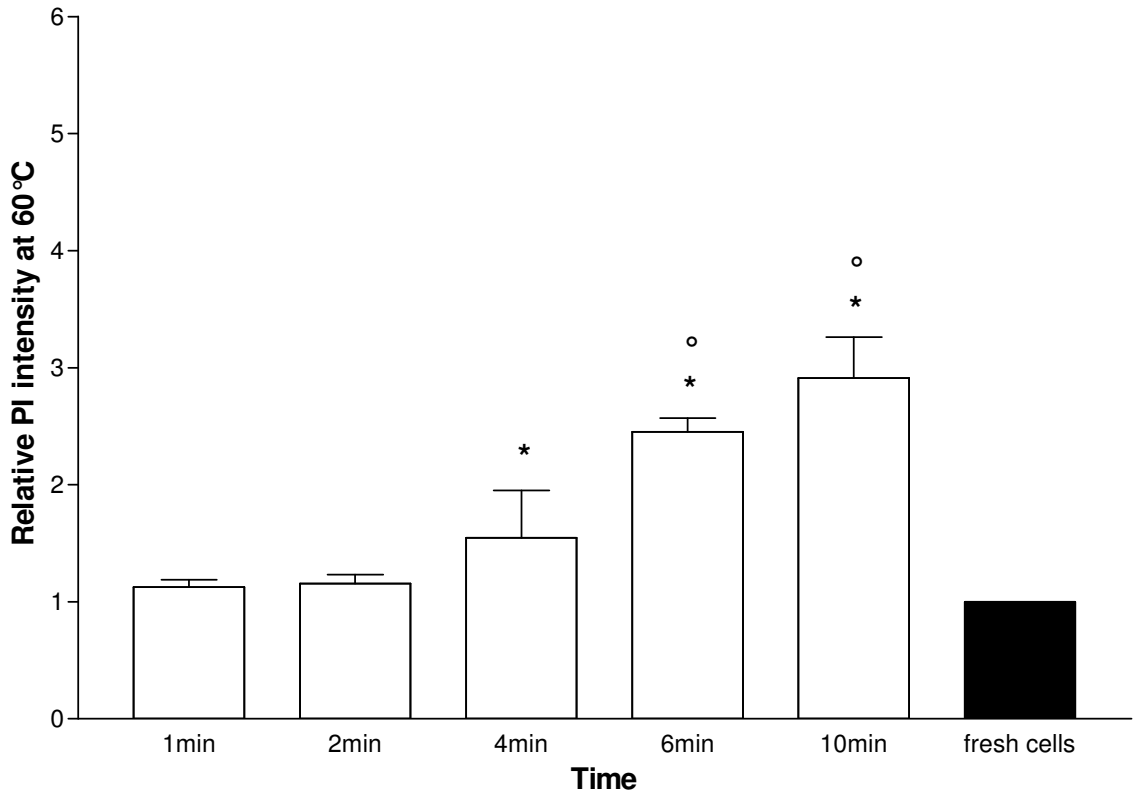


Figure 9: Relative PI intensity of freshly harvested cells and stressed cells at 60°C for different time intervals. * significance between relative PI intensity of stressed cells compared to freshly harvested cells ($p<0.05$). ° significance of changes in PI intensity of stressed cells compared to prior time intervals ($p<0.05$)

Figure 9 shows the impact of exposure of cells to 60°C on the PI intensity.

It can be seen, that the PI intensity of samples stressed for 1 and 2 minutes is comparable with that of fresh cells. After 4 minutes, the PI intensity of samples starts to increase.

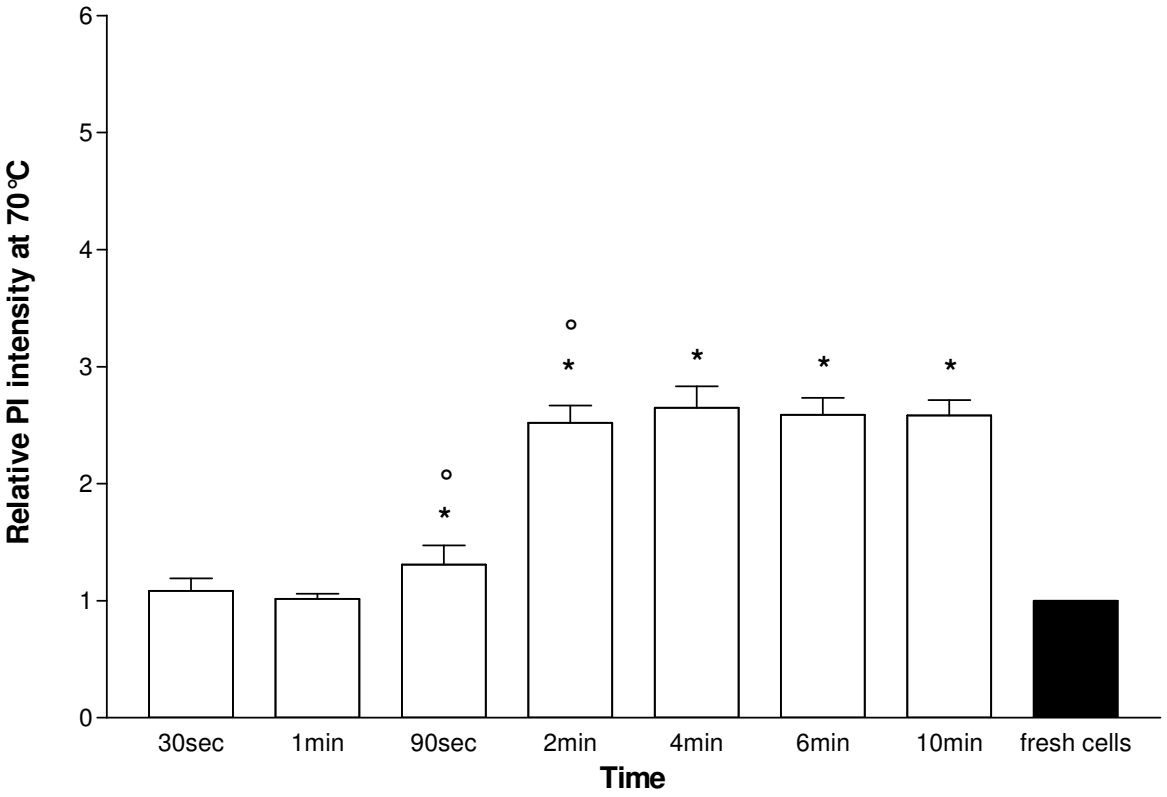


Figure 10: Relative PI intensity of freshly harvested cells and stressed cells at 70°C for different time intervals. * significance between relative PI intensity of stressed cells compared to freshly harvested cells ($p<0.05$). ° significance of change in PI intensity of stressed cells compared to prior time intervals ($p<0.05$).

Alteration in PI intensity as the result of exposure of cells to 70°C is shown in figure 10.

This figure shows that the relative intensity of samples treated at 70°C for a stress time of 30 seconds and one minute is comparable to the relative intensity of freshly harvested cells.

After 90 seconds of heat stress, the damages on the cell membrane integrity was significantly higher than after 1 minute of stress ($p<0.01$).

Increasing the time to 2 minutes resulted in further increasing of the PI intensity ($p< 0.001$).

However, using time intervals longer than 2 minutes, the PI intensity shows no further increase.

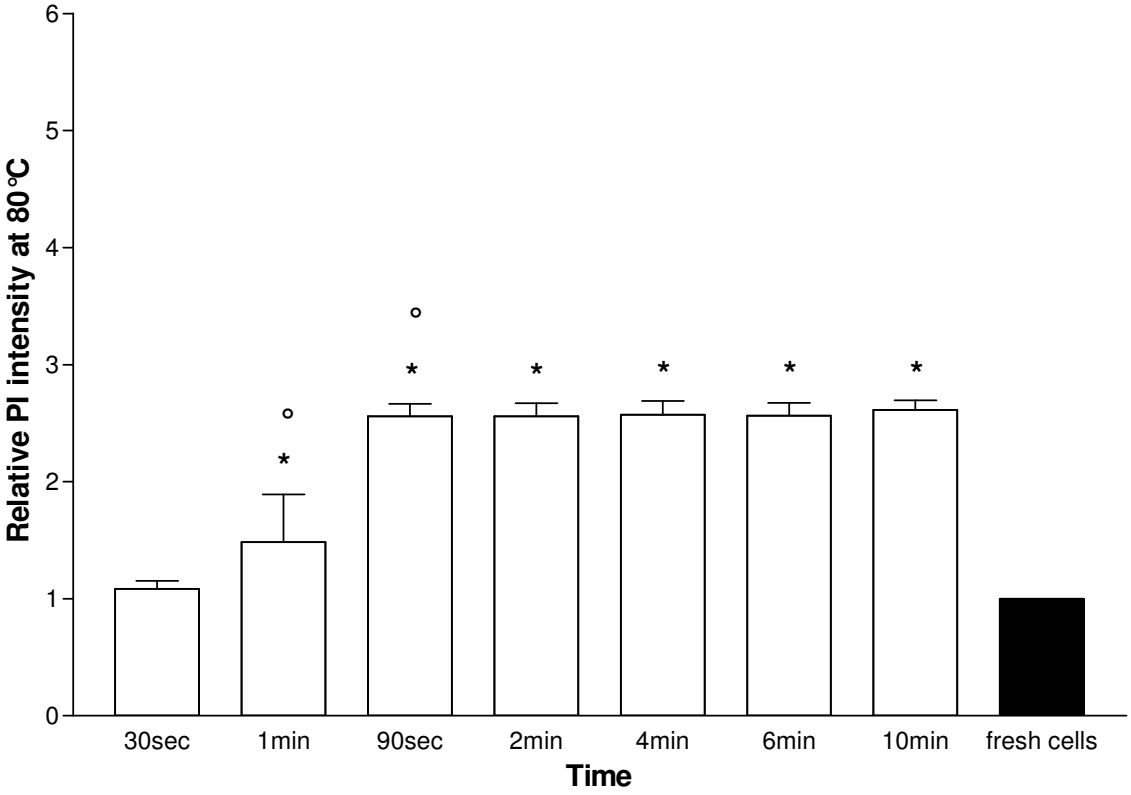


Figure 11: Relative PI intensity of freshly harvested cells and stressed cells at 80°C for different time intervals. * significance between relative PI intensity of stressed cells compared to freshly harvested cells ($p<0.05$). ° significance of change in PI intensity of stressed cells compared to prior time intervals ($p<0.05$).

The impact of 80°C on PI intensity is shown in figure 11. This figure shows that samples stressed for 30 seconds at 80°C are comparable to freshly harvested cells. But with a stress time of 1 minute the relative intensity of PI increase significantly ($p < 0.001$).

Comparing figure 11 with figure 10 shows that increasing the stress temperature from 70°C to 80°C the first significant increase of PI intensity appears sooner. (90 seconds for 80°C versus 2 minutes for 70°C)

Moreover using 80°C, the highest PI intensity was achieved after 90 seconds (versus after 2min using 70°C).

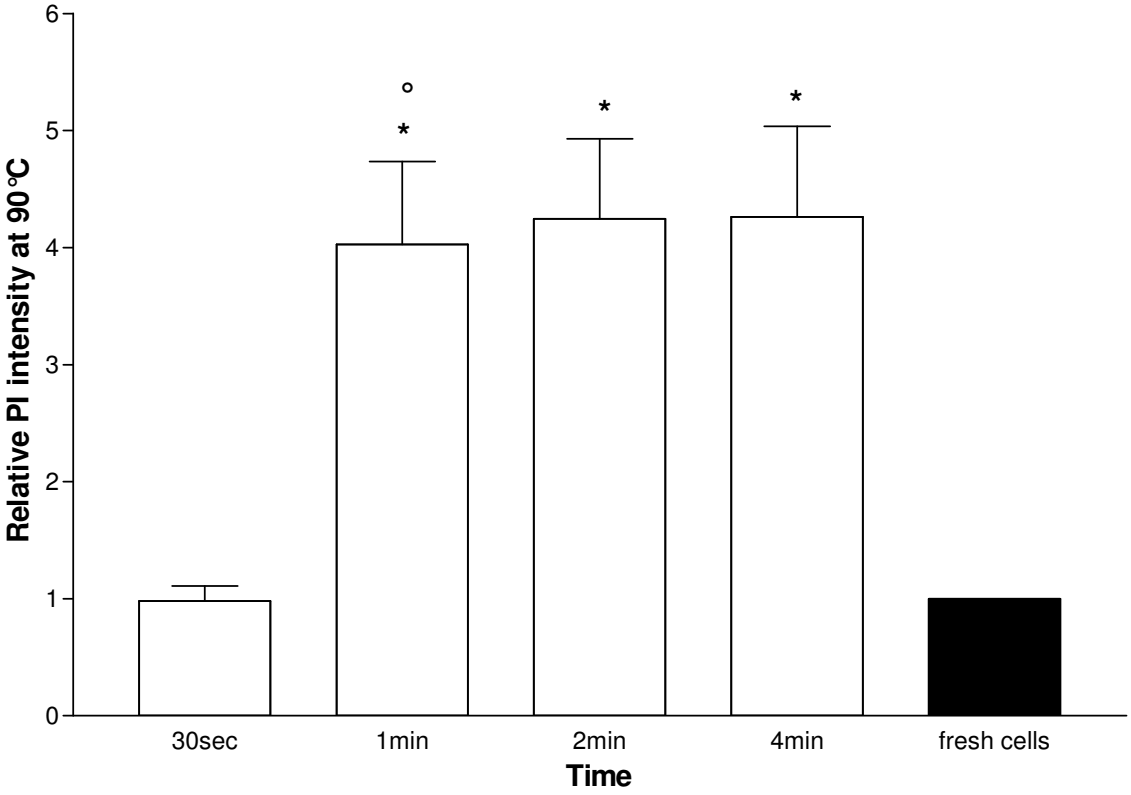


Figure 12: Relative PI intensity of freshly harvested cells and stressed cells at 90°C for different time intervals. * significance between relative PI intensity of stressed cells compared to freshly harvested cells ($p<0.05$). ° significance of changes in PI intensity of stressed cells compared to prior time interval ($p<0.05$).

Figure 12 shows that 30 seconds is the only time interval which does not have a significant difference of fluorescence intensity to fresh cells.

Comparing figure 12 with figure 11 shows that increasing the stress temperature from 80°C to 90°C the first significant increase of PI intensity appears sooner (1 minute for 90°C versus 90 seconds for 80°C).

Moreover using 90°C, the highest PI intensity was achieved after 1 minute (versus after 90 seconds using 80°C).

Following this results, it can be observed that the intensity of PI fluorescence is increasing with exposure to time and temperature until it reaches a maximum.

3.1.2.2 Esterase activity

FDA is per se non fluorescent. It can pass through the cell membrane, where intracellular esterases hydrolyze the diacetate group and can emit fluorescence. Only cells with intact metabolism and esterase activity are able to produce fluorescence.

The higher the level of damage, the lower is the fluorescence intensity.

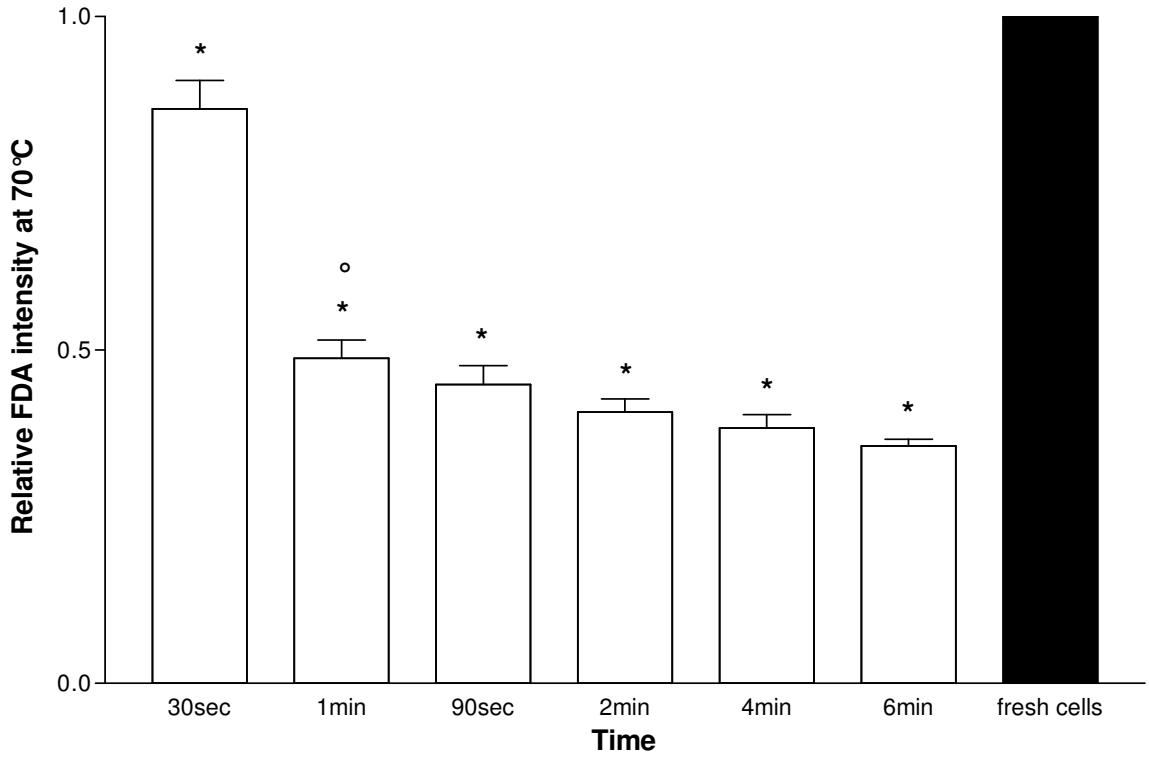


Figure 13: Relative FDA intensity of freshly harvested cells and stressed cells at 70°C for different time intervals. * significance between relative FDA intensity of stressed cells compared to freshly harvested cells ($p<0.05$). ° significance of changes in FDA intensity of stressed cells compared to prior time interval ($p<0.05$).

Figure 13 shows the relative FDA intensity of freshly harvested cells and stressed cells at 70°C. The FDA intensity of fresh cells is significantly higher than that of stressed cells in all time intervals ($p< 0.001$).

In the case of stressing time of 30 seconds at 70°C, the FDA intensity was lower than in freshly harvested cells ($p< 0.001$). However it was significantly higher than the intensity of cells at other time intervals. There is no significant difference of relative FDA intensity between 1 minute and 90 seconds ($p>0.05$) and there is no significant difference of relative FDA intensity between 4 minutes and 6 minutes.

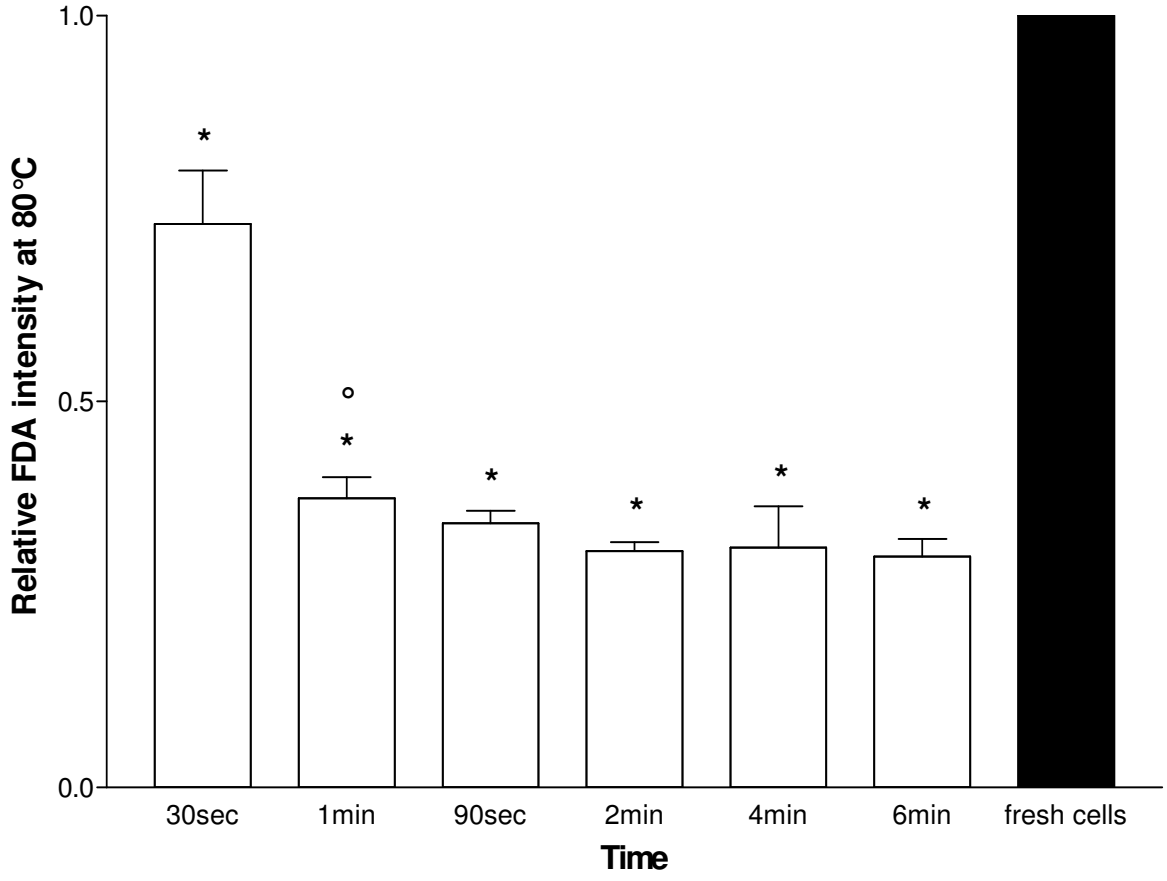


Figure 14: Relative FDA intensity of freshly harvested cells and stressed cells at 80°C for different time intervals. * significance between relative FDA intensity of stressed cells compared to freshly harvested cells ($p<0.05$). ° significance of changes in FDA intensity of stressed cells compared to prior time interval ($p<0.05$).

Figure 14 shows the relative FDA intensity of freshly harvested cells and stressed cells at 80°C. The FDA intensity of freshly harvested cells is significantly higher than the intensity of stressed cells in all time intervals ($p<0.001$).

A lowest stressing time of 30 seconds shows a significant decrease in the fluorescence intensity compared to figure 13 and to all higher stress times ($p< 0.001$). This intensity is however higher than that of other stressed cells.

A stress time of 2 minutes shows no significant difference in the fluorescence intensity to 90 seconds, 4 and 6 minutes ($p> 0.05$).

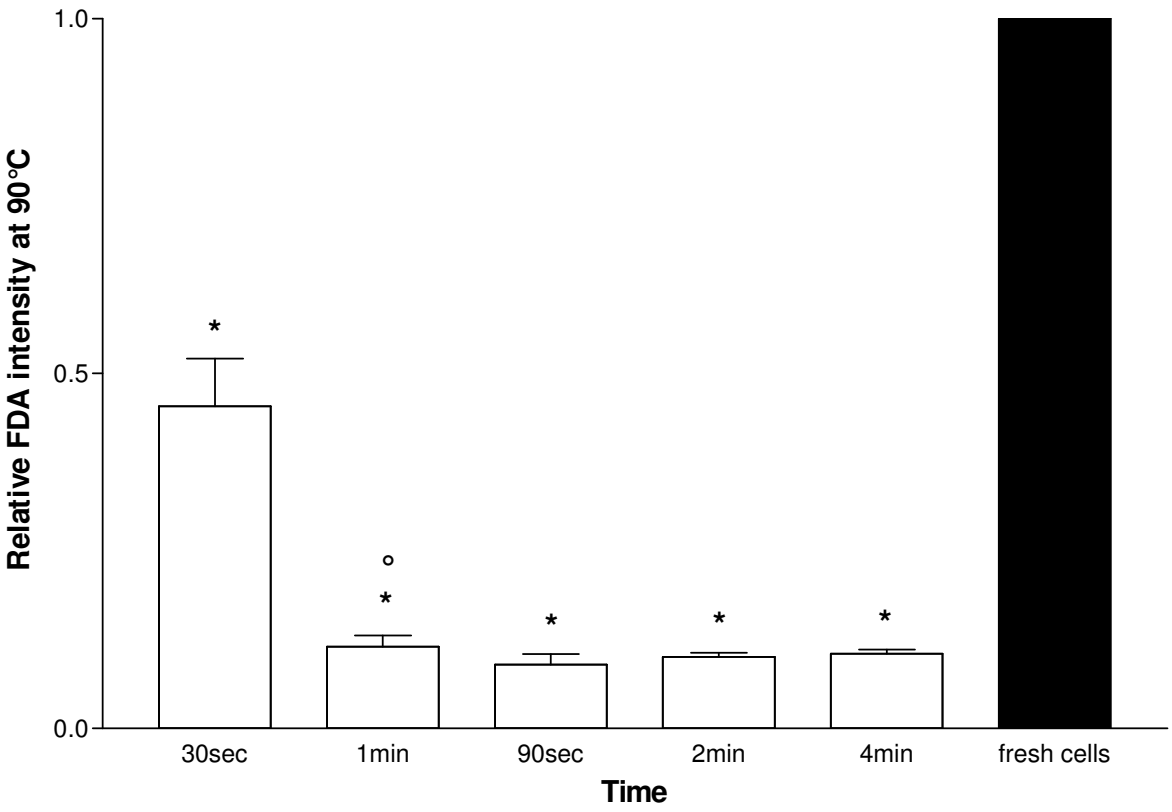


Figure 15: Relative FDA intensity of freshly harvested cells and stressed cells at 90°C for different time intervals. * significance between relative FDA intensity of stressed cells compared to freshly harvested cells ($p<0.05$). ° significance of changes in FDA intensity of stressed cells compared to prior time interval ($p<0.05$).

Figure 15 shows the relative FDA intensity of freshly harvested cells and stressed cells at 90°C. Obviously relative intensity of FDA of the freshly harvested cells is significantly higher than stressed cells.

A heat stress for 30 seconds provokes the decreasing of the value more than 50%, but also showed significantly better results than the other time intervals ($p<0.001$). There is no significant difference between the other time intervals.

The intensity of FDA fluorescence was constantly decreasing with higher temperature and higher exposition times.

3.1.2.3 Production of hydrogenperoxide

DHR 123 does not fluorescent. It enters the cells and is oxidized by the intracellular redox systems and so a fluorescent derivate is produced. Cells have redox systems, which are able to increase the superoxide production for defending against stress factors. During a longer period of stress, the DHR 123 fluorescence first increases (the cell is already in the condition to compensate the stress factors). But when the stress became to strong, the cell is no longer able to produce H_2O_2 and the fluorescence finally decreases.

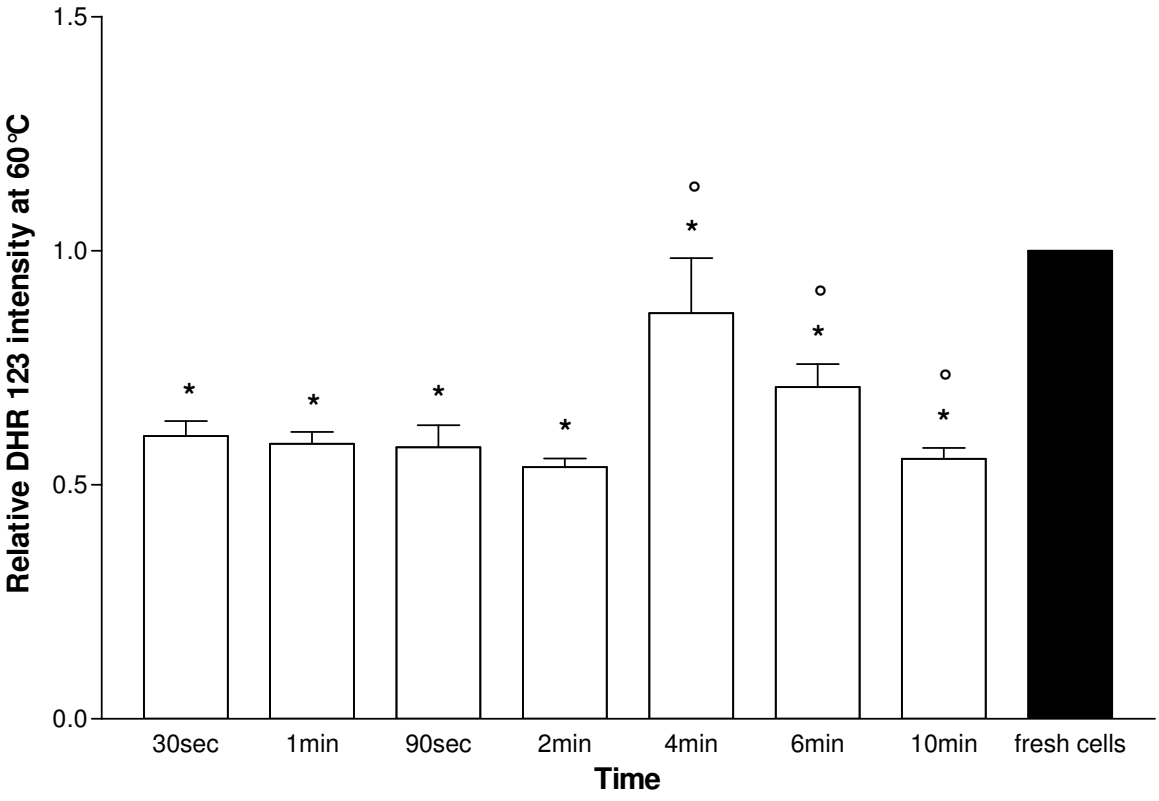


Figure 16: Relative DHR 123 intensity of freshly harvested cells and stressed cells at 60°C for different time intervals. * significance between relative DHR 123 intensity of stressed cells compared to freshly harvested cells ($p<0.05$). ° significance of changes in DHR 123 intensity of stressed cells compared to prior time interval ($p<0.05$).

Figure 16 shows the relative DHR 123 intensity of freshly harvested cells and stressed cells at 60°C. The intensity of fresh cells is significant higher to the stressed cells ($p < 0.01$ for 4 minutes and $p< 0.001$ for the rest)

From 30 seconds until 2 minutes the fluorescence intensity lightly is decreasing:

30 seconds, 1 minute, 90 seconds and 2 minutes all have a significant lower relative DHR 123 intensity than 4 and 6 minutes. After 4 minutes at 60°C, the production of superoxide increased, which marks a higher stress level, but the cells are still able to defend against heat damages. After 6 minutes the relative intensity begins to decrease again. The cells are no longer able to compensate the heat damages.

For this part of the work it can be conclude, that when stressing begins, the production of superoxide increases. By this increased production of superoxide, the cells are able to compensate for stress over a limited period of time. After that, the production of superoxide decreases which marks the collapse of the cell.

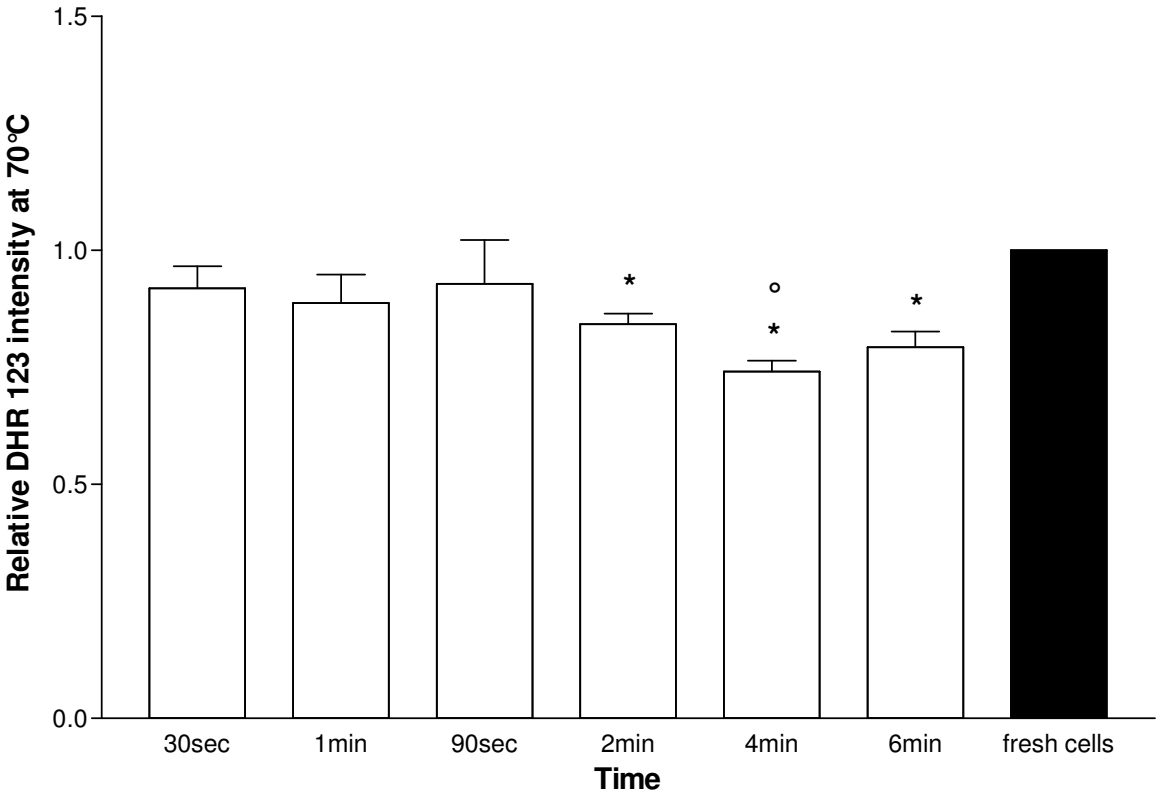


Figure 17: Relative DHR 123 intensity of freshly harvested cells and stressed cells at 70°C for different time intervals. * significance between relative DHR 123 intensity of stressed cells compared to freshly harvested cells ($p<0.05$). ° significance of changes in DHR 123 intensity of stressed cells compared to prior time interval ($p<0.05$).

Figure 17 shows the relative DHR 123 intensity of freshly harvested cells and stressed cells at 70°C. The DHR 123 intensity of freshly harvested cells is significantly higher than the intensity of cells stressed for two minutes or longer.

A stress time up to 2 minutes show not significant decrease in the intensity of fluorescence compared to fresh cells ($p>0.05$), cells are compensating heat damages by producing superoxide.

Between 2, 4 and 6 minutes there is not significant difference in the intensity of fluorescence.

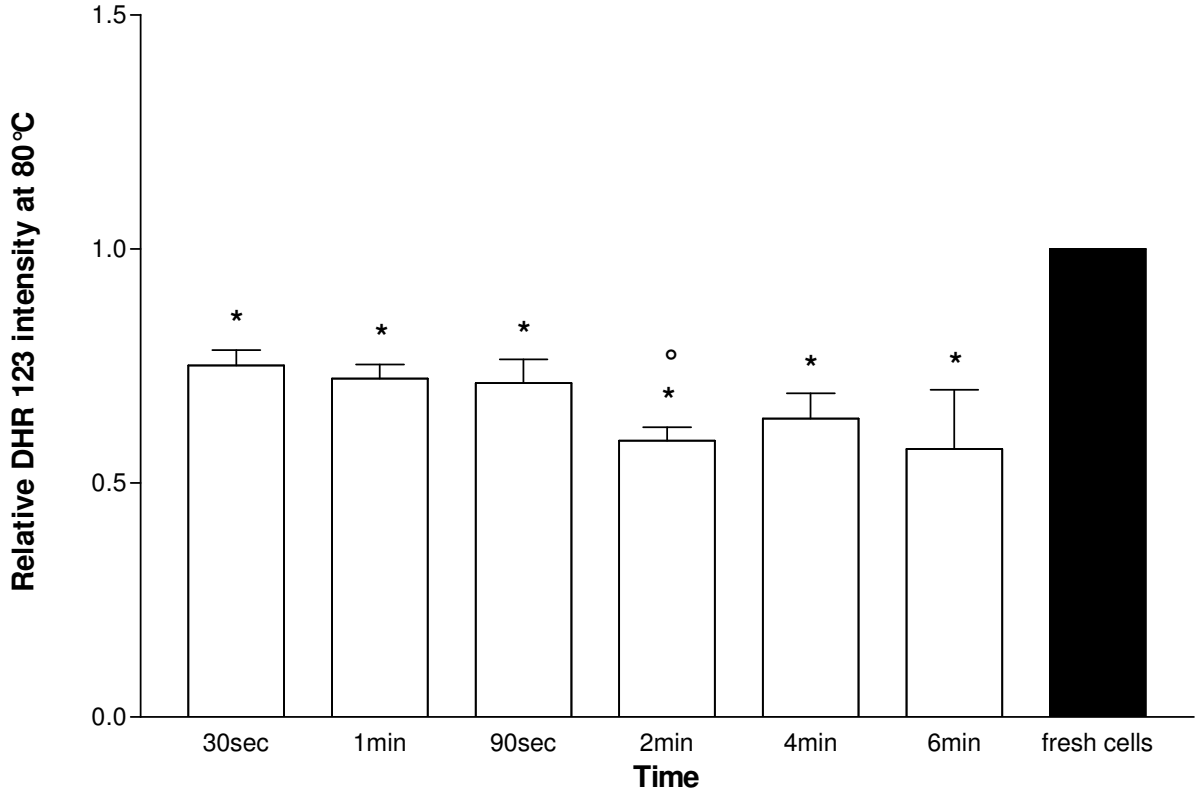


Figure 18: Relative DHR 123 intensity of freshly harvested cells and stressed cells at 80°C for different time intervals. * significance between relative DHR 123 intensity of stressed cells compared to freshly harvested cells ($p<0.05$). ° significance of changes in DHR 123 intensity of stressed cells compared to prior time interval ($p<0.05$).

Figure 18 shows the relative DHR 123 intensity of freshly harvested cells and stressed cells at 80°C. A decreasing of the fluorescence intensity after stress during different time intervals can be observed. The relative intensity of freshly harvested cells was significantly higher than the intensity of stressed cells using all time intervals ($p< 0.001$).

For a stress time interval of 30 seconds the fold changes of fluorescence intensity are significantly higher than the intensity using two minutes or longer stress time.

It can be concluded that by using 80°C, the longer stress times resulted in more decreased levels of DHR 123 intensity.

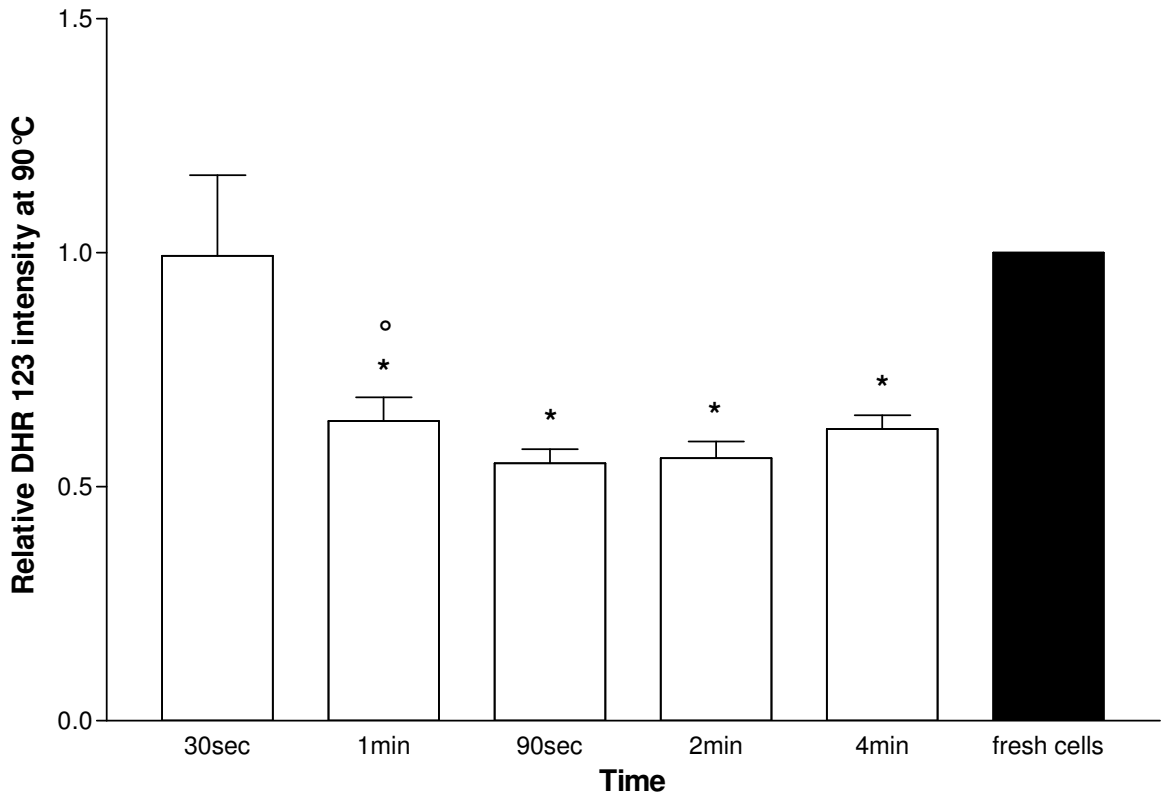


Figure 19: Relative DHR 123 intensity of freshly harvested cells and stressed cells at 90°C for different time intervals. * significance between relative DHR 123 intensity of stressed cells compared to freshly harvested cells ($p<0.05$). ° significance of changes in DHR 123 intensity of stressed cells compared to prior time interval ($p<0.05$).

Figure 19 shows the relative DHR 123 intensity of freshly harvested cells and stressed cells at 90°C.

Compared to 80°C, the decreasing of the relative intensity goes further. The fluorescence intensity of freshly harvested cells is comparable to 30 sec ($p>0.05$), but the fluorescence intensity of 30 seconds is significantly higher to all others time intervals ($p<0.001$)

It can be concluded, that the cell metabolism is no more able to maintain the superoxide production.

3.2 Spray drying

3.2.1.1 Culturability

Parallel to the fluorimetry and flow cytometry measurements, the culturability of *B. bifidum* Bb12 was investigated. Freshly harvested cells showed a colony forming unit (CFU) value of $1.59x10^{10}$CFU/ml sample (± 33,93%). It should be noted, that the growth of *B.bifidum* Bb12 poses severe experimental challenges. Even slightest contamination during spreading the plates, influence the bacteria count during the incubation significantly. This explains the relative high dispersion of the data (standard deviation of 34%).

In the optimized spray drying conditions, we measured a CFU value of $4.58x10^{7}$ (± 32,73%) after the spray drying process for the untreated bacteria. This is equivalent to a loss of 99,7% or almost three log units and evidences the high sensitivity of *B.bifidum* Bb12 to thermal stress.

These values were the baseline for the comparison of the culturability of the cells treated with the studied excipients, i.e gum arabic, gelatine, maltodextrin and skimmed milk.

3.2.1.2 Cellular properties

1) Fluorimetry measurement

Data of the fluorimetry measurements were only used for the determination of the degree of membrane damage after spray drying, using the fluorescence dye PI.

2) Flow cytometry measurements

By flow cytometrie measurements after staining the cells with PI or DHR 123, only the number of stained cells was considered.

For PI and DHR 123 the fluorescence intensity was not taken into account due to the fact that all of the dye binds to the DNA of damaged cells and DHR 123 is oxidised by cellular redox system. Which means that the intensity of the emitted light is much higher by a few damaged cells than it is on a lot of damaged cells.

The results of staining with DHR 123 (superoxide production), does not permit to deduce if the cells are only exposed to.

Reliable results have been deducted from the fluorescence intensity of stained cells with CFDASE and DioC6(3), was taken into account. With these two fluorescence dyes, all cells are stained, not only the damaged ones. The distribution of the dye is homogeneous, because the fluorescence dye binds on ions.

CFDASE-Intensity: The fluorescent dye penetrates in cells and binds intracellular on the ionic structure of amino sources.
The lower the intensity of stained cells with CFDASE is, the better the interstitial condition.

Even fresh cells show a certain level of fluorescence when dyed with CFDASE. When stressed, after drying, fluorescence intensity increases. If the level of stress is further increased or duration of stress is prolonged, a decrease in fluorescence intensity can be noted.

This is also true for *B. bifidum* Bb12, an anaerobic bacterium, which means that it will show increased fluorescence values as soon as getting in contact with oxygen.

DioC6(3) is used to measure the cell membrane potential. For this dye the intensity should be as low as possible, thereby indicating a low level of stress. The dye penetrates in cells and binds on the ions. A high intensity means that DioC6(3) accumulate on a hyperpolarized membrane of stressed cells.

The results with the fluorescence dye Fluorescein diacetate (esterase activity) were not included in the analysis. The obtained results were in no case significant.

3.2.2 Protective impact of excipients on spray dried cells

Concentrations of protectants added to solutions prior to the spray drying are based on the wet cell mass of freshly harvested cells.

3.2.2.1 Gum arabic

1) Culturability

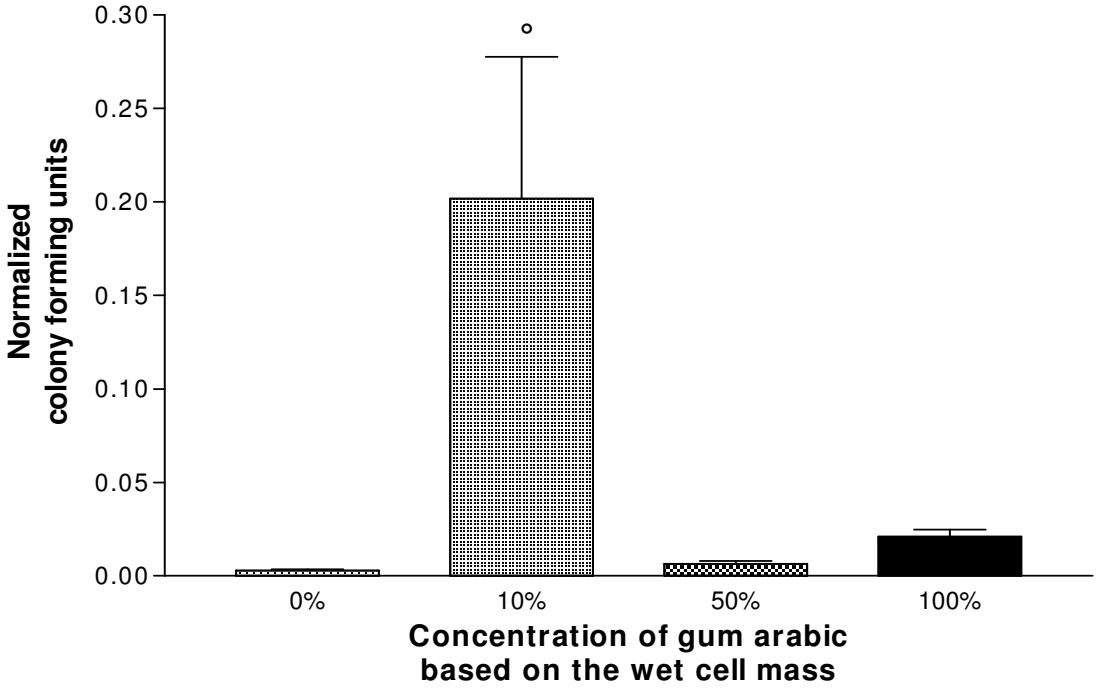


Figure 20: Culturability of B. *bifidum* Bb12 after spray drying with different concentrations of gum arabic. (fold change of the culturability is compared to the culturability of freshly harvested cells, p<0.05; °significance of CFU of protected and spray dried cells compared to CFU of unprotected spray dried cells, p<0.05)

The culturability of spray dried cells was related to the culturability of freshly harvested cells and demonstrated in figure 20.

Using 50% or 100% of gum arabic could not protect the culturability of cells, as the culturability was comparable with that of unprotected cells.

10% gum arabic provided the best protective effect on the culturability of *B. bifidum* Bb12.

The result of culturability of spray dried cells with 10% gum arabic (0,21) is comparable to the result of heat stress results at 60°C of 6 minutes (0,17).

2) Fluorimetry measurements (TECAN)

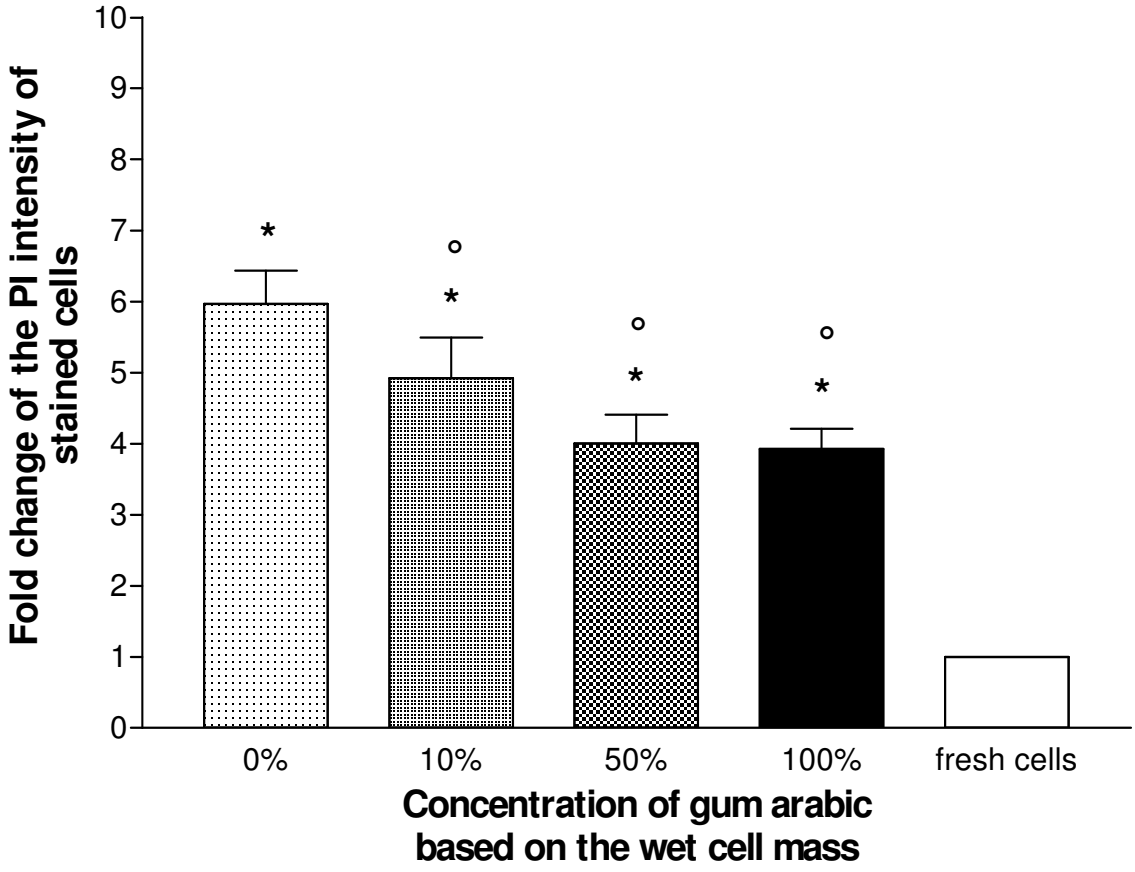


Figure 21: Fold change of the PI intensity of stained cells after spray drying with gum arabic in different concentrations, measured with micro plate reader (*significance of changes in PI intensity of spray dried cells compared to freshly harvested cells, $p<0.05$, ° significance of changes in PI intensity of spray dried cells with Gum Arabic compared to spray dried cells without gum arabic (0% protectant),$p<0.05$)

Figure 21 shows the PI intensity of cells pre-treated with gum arabic and spray dried. Using all concentrations, the PI intensity of protected dried cells was significantly lower than the PI intensity of unprotected dried cells.

The best protective impact was observed by cells protected with 50% or 100% gum arabic, these results comply with 1 minute at 90°C.

3) Flow cytometry measurements

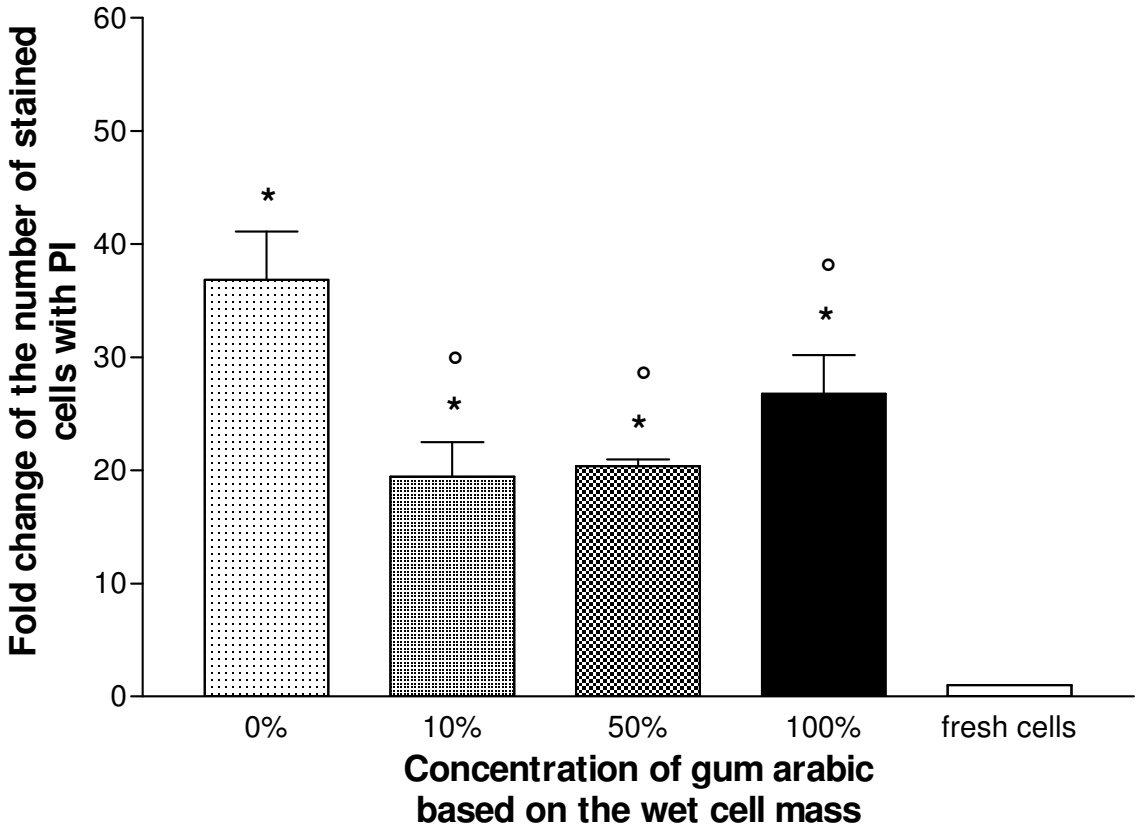


Figure 22: Fold change of the number of stained cells with PI after spray drying with gum arabic in different concentrations. (*significance of changes in the number of spray dried stained cells compared to freshly harvested cells; $p<0.05$, ° significance of changes in the number of protected, spray dried, stained cells compared to unprotected (= 0%), spray dried, stained cells; $p<0.05$)

Figure 22 shows the number of spray dried cells pre-treated with gum arabic and stained with PI. The number of stained fresh cells is also indicated as control. The number of stained unprotected and spray dried cells was significantly higher than spray dried cells protected with gum arabic and freshly harvested cells.

Pre-treatment of cells with gum arabic in all used concentrations before spray drying, protected the cell membrane. The number of stained cells pre-treated with gum arabic and spray dried was significantly less than the number of stained unprotected and dried cells.

All concentration of gum arabic used, presents a protective impact on the membrane integrity. The different concentrations were comparable ($p>0.05$).

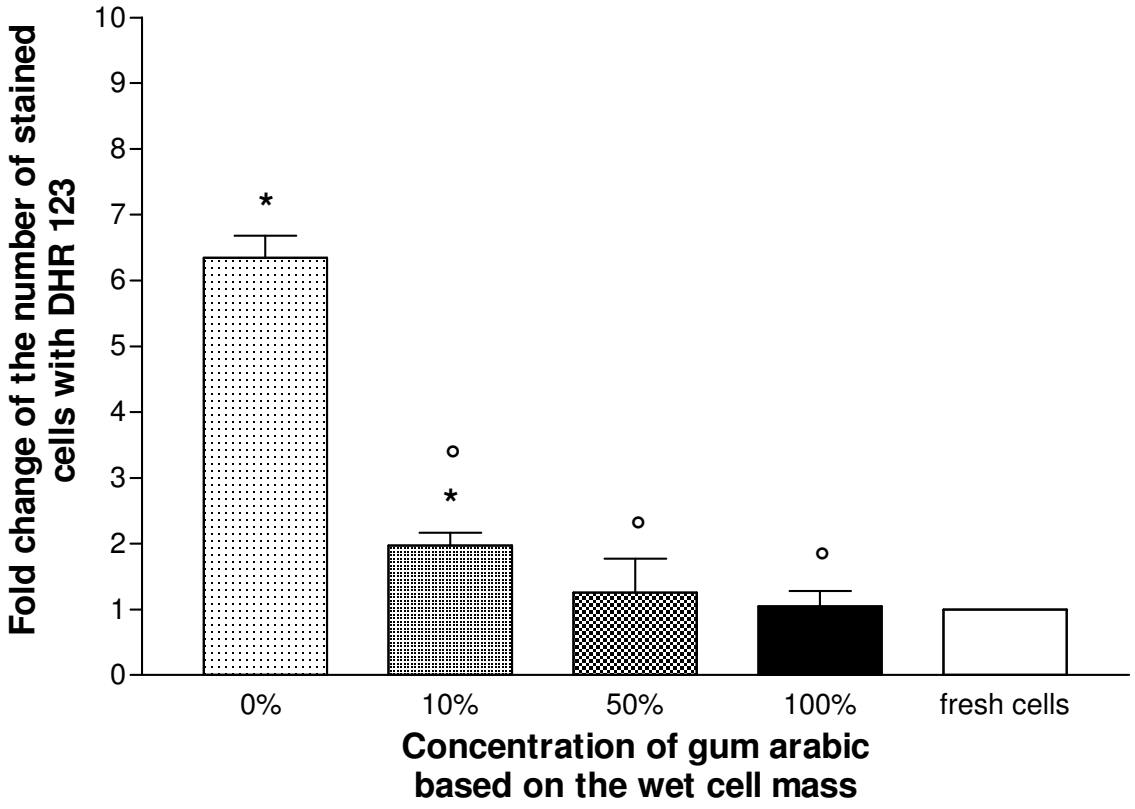


Figure 23: Fold change of the number of stained cells with DHR 123 after spray drying with gum arabic in different concentrations. (*significance of changes in the number of spray dried stained cells compared to freshly harvested cells; $p<0.05$, ° significance of changes in the number of protected, spray dried, stained cells compared to unprotected (= 0%), spray dried, stained cells; $p<0.05$)

Figure 23 shows the number of spray dried cells stained with DHR 123. The number of stained fresh cells is also indicated as control. The number of stained unprotected and spray dried cells was significantly higher than spray dried cells with gum arabic and freshly harvested cells. Pre-treatment of cells with 50% or 100% (based on the wet cell mass) shows that the number of stained cells, after spray drying, was comparable with the number of stained fresh cells.

It was observed, that the best protective effect was obtained by using 50% and 100% gum arabic.

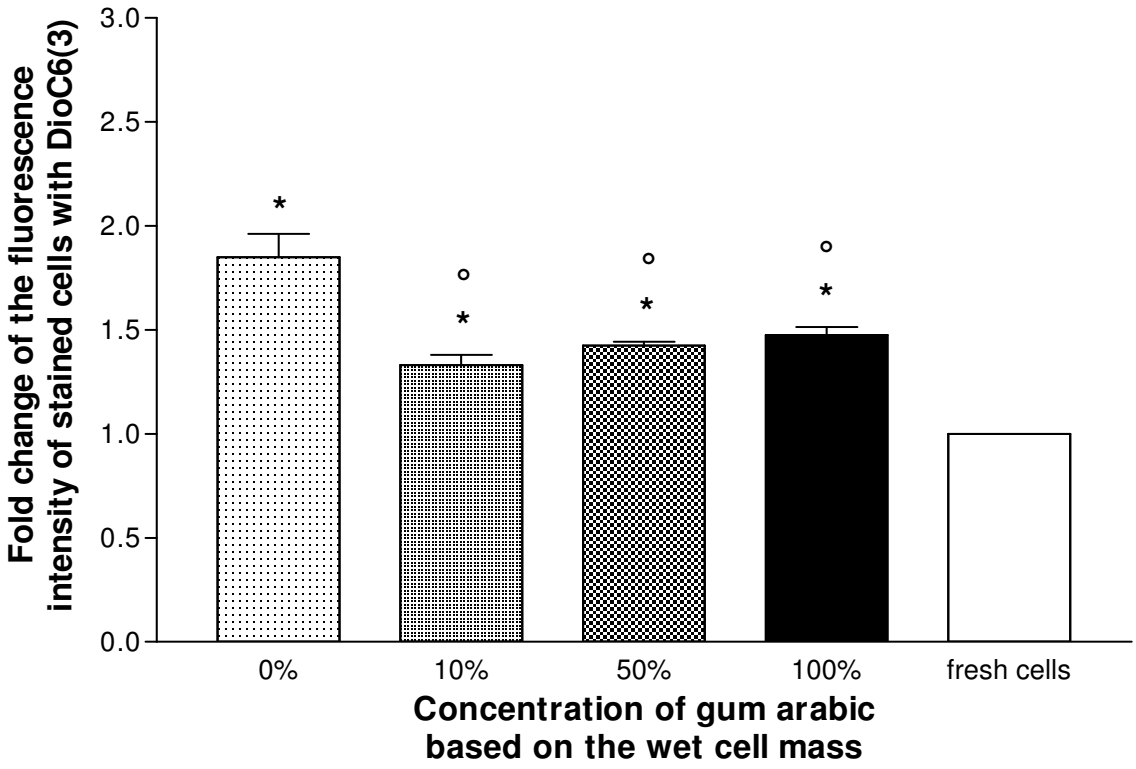


Figure 24: Fold change of the fluorescence intensity of stained cells with DioC6(3) after spray drying with gum arabic in different concentrations. (*significance of changes in the fluorescence intensity of spray dried stained cells compared to freshly harvested cells; $p<0.05$, ° significance of changes in the fluorescence intensity of protected, spray dried, stained cells compared to unprotected (= 0%), spray dried, stained cells; $p<0.05$)

Figure 24 shows the fluorescence intensity of spray dried cells stained with DioC6(3). The number of stained fresh cells is also measured as control. The fluorescence intensity of stained cells pre-treated with gum arabic was significantly lower compared to the intensity of stained unprotected cells. The fluorescence intensities were comparable with each other regardless to the concentration of gum arabic ($p>0.05$). Each concentration of gum arabic shows better results than unprotected spray dried cells. ($p<0.001$)

All in all it was observed that all concentrations of gum arabic show better protective effects than unprotected cells.

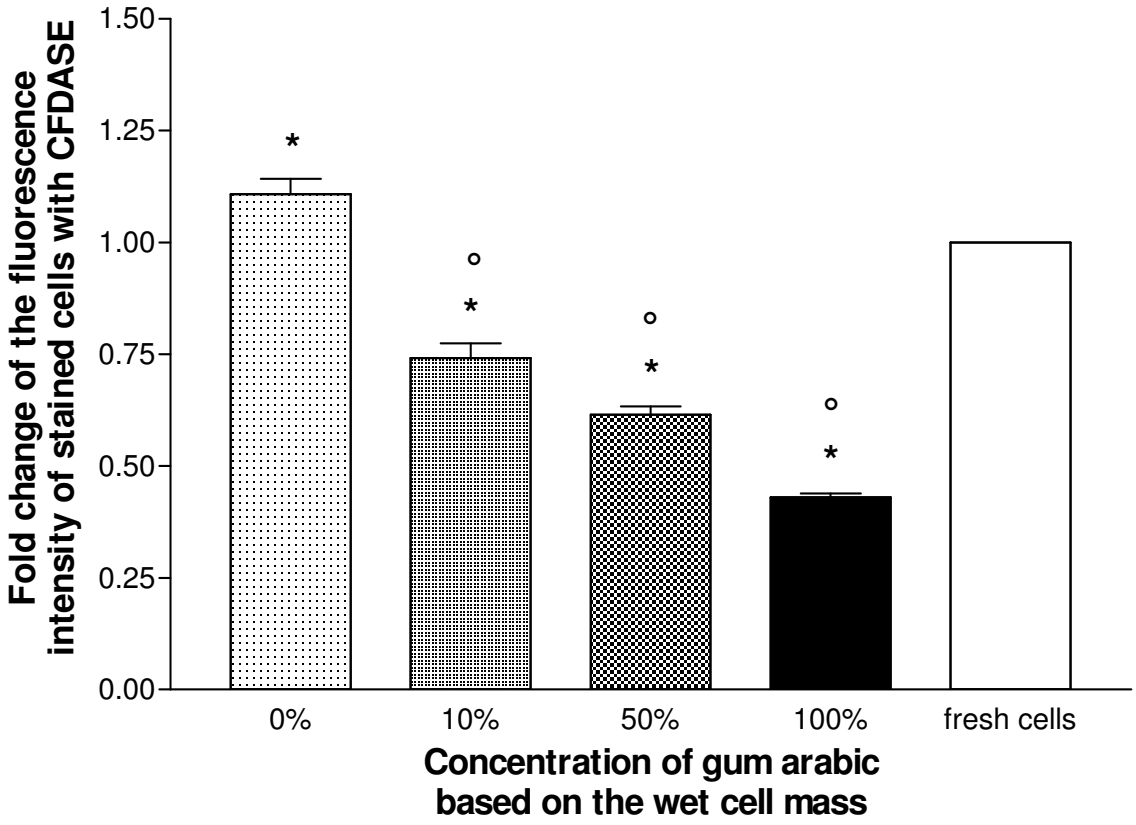


Figure 25: Fold change of the fluorescence intensity of stained cells with CFDASE after spray drying with gum arabic in different concentrations. (*significance of changes in the fluorescence intensity of spray dried stained cells compared to freshly harvested cells; $p<0.05$, ° significance of changes in the fluorescence intensity of protected, spray dried, stained cells compared to unprotected (= 0%), spray dried, stained cells; $p<0.05$)

Figure 25 shows the fluorescence intensity of spray dried cells stained with CFDASE. The number of stained fresh cells is also measured as control. The number of stained unprotected and spray dried cells was significantly higher than spray dried cells with gum arabic and freshly harvested cells.

The fluorescence intensity of stained cells pre-treated with gum arabic was significantly lower compared to the intensity of stained unprotected cells and freshly harvested cells.

The fluorescence intensity of freshly harvested cells compared to pre-treated spray dried cells with gum arabic was significant higher. ($p<0.001$)

In summary it can be said, that spray drying cells with gum arabic presents a protective effect on the reproduction capability and on the cellular properties. This is especially true in terms of membrane integrity. Here gum arabic leads to a significant drop of fluorescence intensity and number of stained cells compared to unprotected cells. In the case of membrane potential all

concentrations used were significantly better than unprotected spray dried cells. Concerning the culturability, 10% gum arabic presented significantly better results than any other concentration of gum arabic.

3.2.2.2 Gelatine

1) Culturability

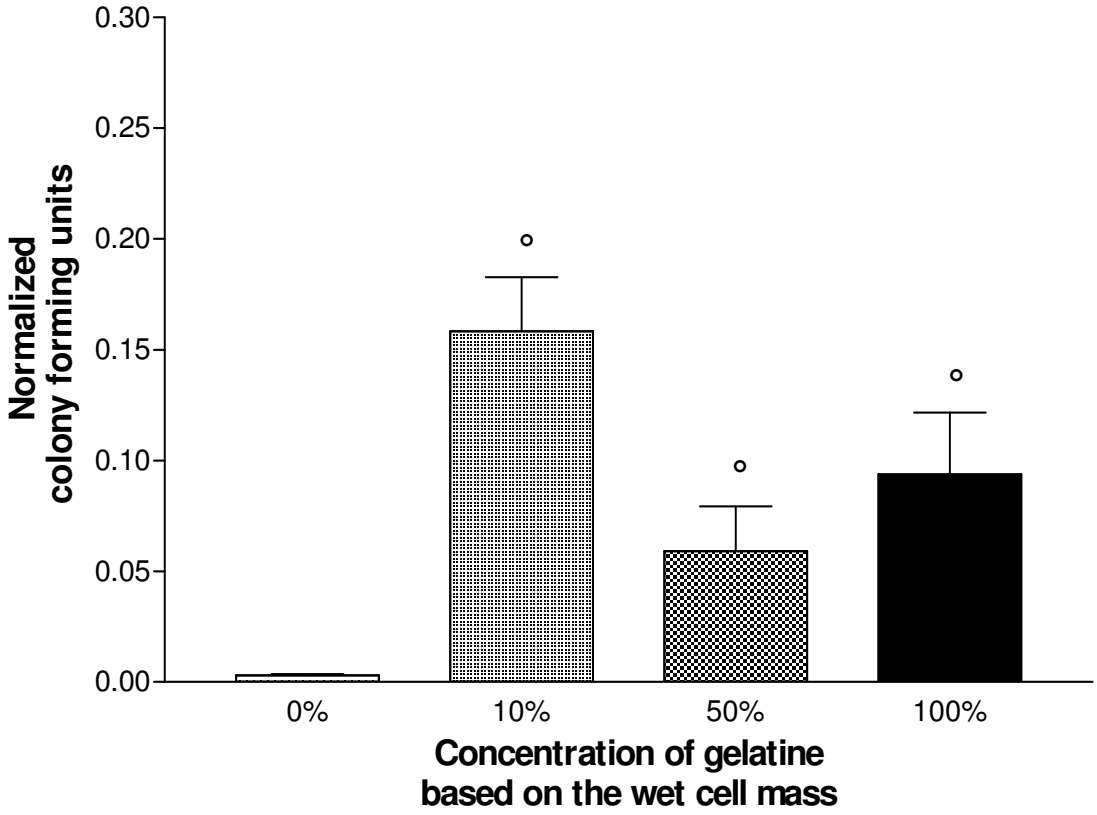


Figure 26: Culturability of *B. bifidum* Bb12 after spray drying with different concentrations of gelatine. (fold change of the culturability is compared to the culturability of freshly harvested cells, $p<0.05$; °significance of CFU of protected and spray dried cells compared to CFU of unprotected spray dried cells, $p<0.05$)

The culturability of spray dried cells was related to the culturability of freshly harvested cells and demonstrated in figure 26. The culturability of spray dried cells is considered as significant different to freshly harvested cells.

The results show that gelatine has in all concentrations used protective attributes on the culturability of *B. bifidum* 12. Whereas the best protective effect was observed using 10% concentration of gelatine, the less protective effect was observed by using 50% concentration of gelatine.

Using 10% of gelatine (0,16) complies to a heat stress of 60°C for 2 minutes (0,17).

2) Fluorimetry measurements (TECAN)

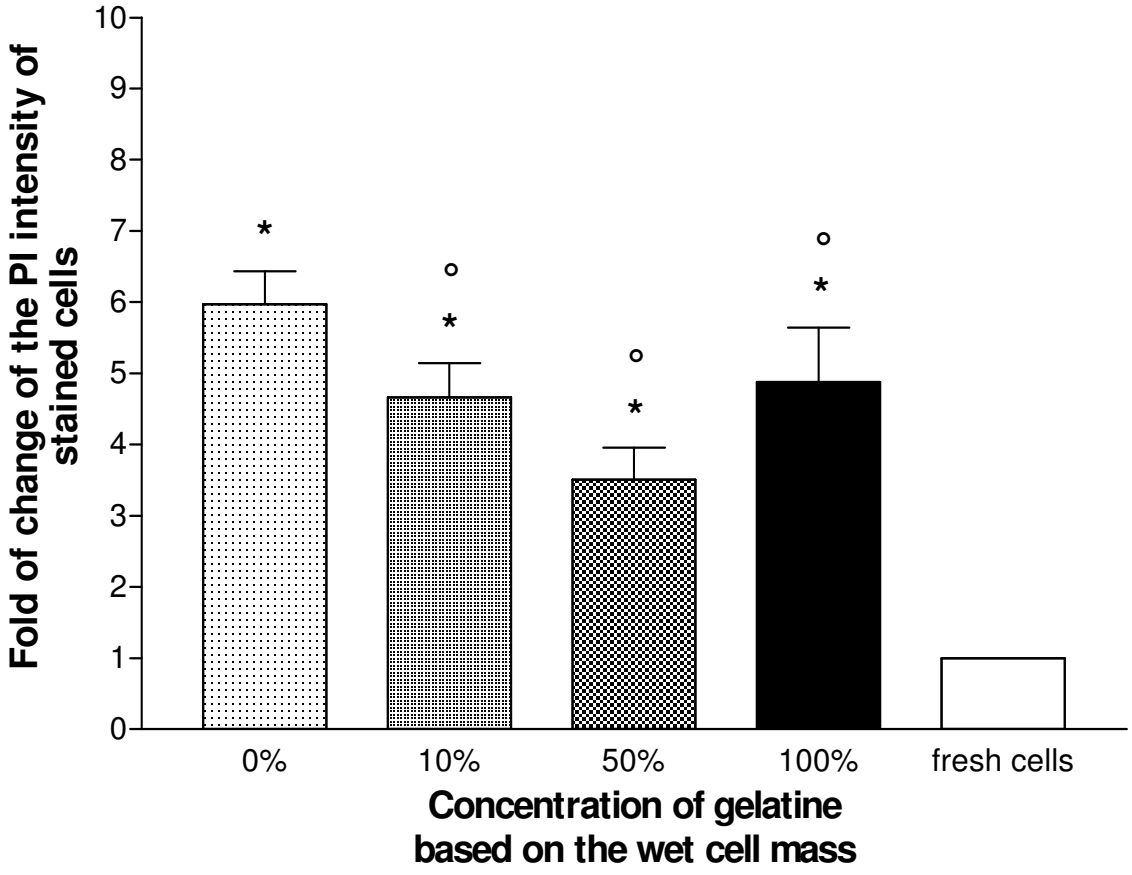


Figure 27: Fold change of the PI intensity of stained cells after spray drying with gelatine in different concentrations, measured with micro plate reader (*significance of changes in PI intensity of spray dried cells compared to freshly harvested cells, p<0.05, ° significance of changes in PI intensity of spray dried cells with Gelatine compared to spray dried cells without gelatine (0% protectant),p<0.05)

Figure 27 shows the fold change of PI intensity of cells pre-treated with gelatine and spray dried. The number of stained fresh cells is also indicated as control. As can be seen the intensity of both protected and unprotected dried cells was significantly higher than the intensity of freshly harvested cells, indicating the damage of cell membrane of drying process. The fluorescence intensity of pre-treated cells with gelatine in all used concentrations was however significantly lower than the intensity of untreated and dried cells, showing the protective impact of gelatine on the cell membrane. Especially a gelatine concentration of 50% (based on the wet cell mass) presented the best protective effect. Whereas the fluorescence intensity of 10% and 100% was comparables, it was significantly higher than using 50% of gelatine.

3) Flow cytometry measurements

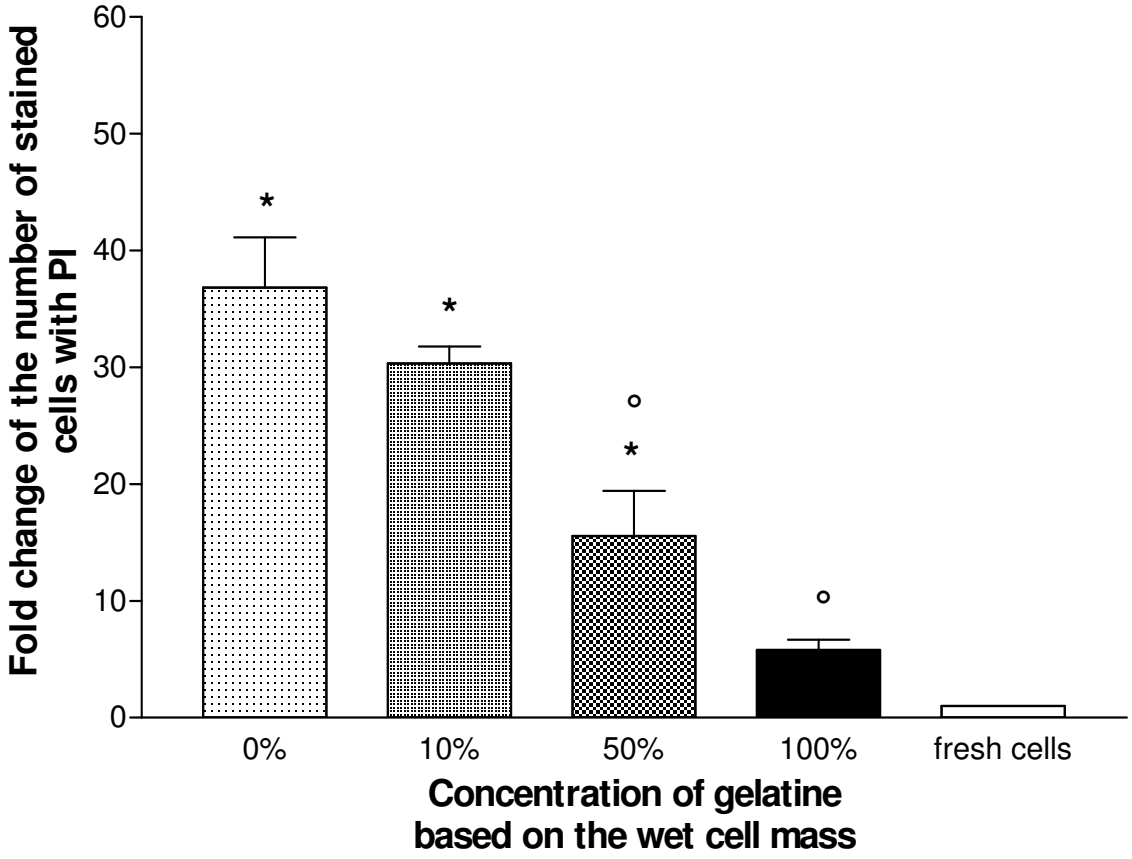


Figure 28: Fold change of the number of stained cells with PI after spray drying with gelatine in different concentrations. (*significance of changes in the number of spray dried stained cells compared to freshly harvested cells; $p<0.05$, ° significance of changes in the number of protected, spray dried, stained cells compared to unprotected (= 0%), spray dried, stained cells; $p<0.05$)

Figure 28 shows the number of spray dried cells stained with PI. The number of stained fresh cells is also indicated as control.

The flow cytometry measurement confirms the protective impact of gelatine on the cell membrane of dried cells observed using PI.

Moreover, it can be observed from figure 28, that 100% of gelatine provided the best protective effect on the cell membrane - at this concentration, the number of stained cells was comparable with the number of stained freshly harvested cells. On the other hand, there was no significant difference between the number of unprotected cells and stained cells and cells which where pre-treated with 10% of gelatine.

It can be concluded that 100% gelatine has the best protective effect on the cell membrane integrity.

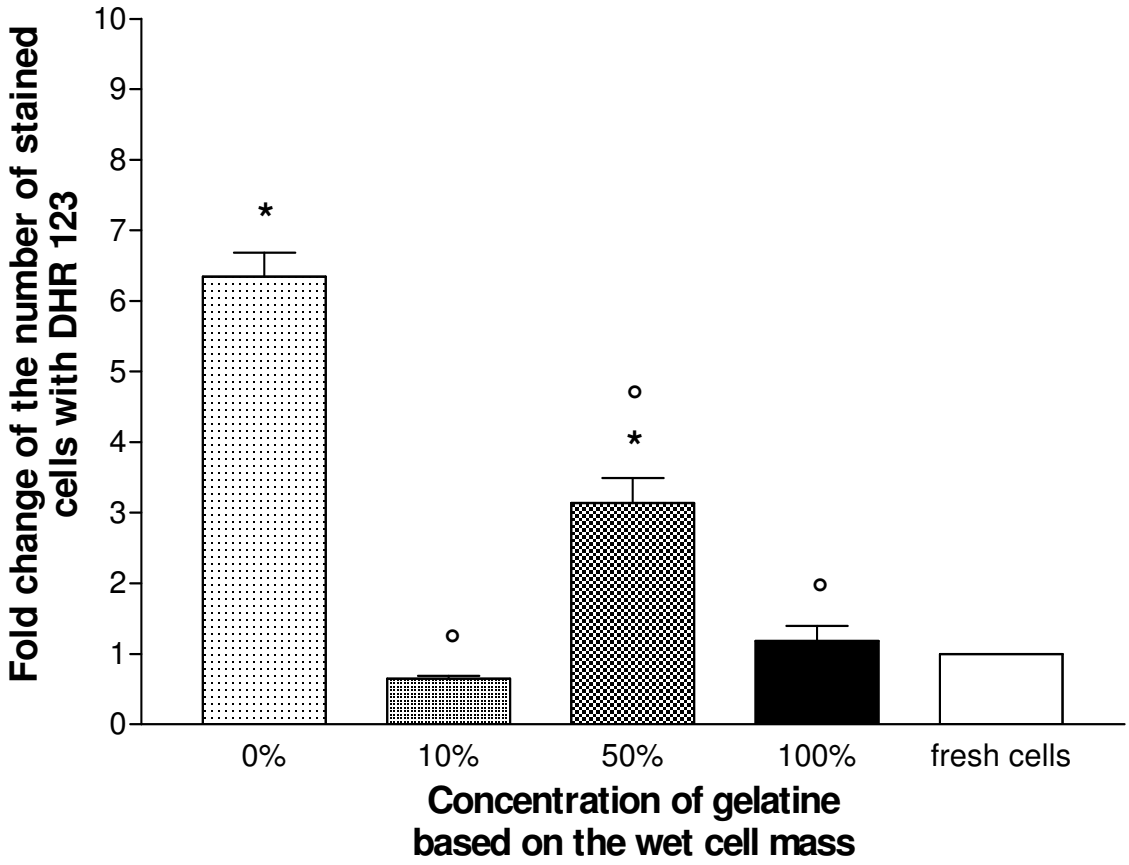


Figure 29: Fold change of the number of stained cells with DHR 123 after spray drying with gelatine in different concentrations. (*significance of changes in the number of spray dried stained cells compared to freshly harvested cells; $p<0.05$, ° significance of changes in the number of protected, spray dried, stained cells compared to unprotected (= 0%), spray dried, stained cells; $p<0.05$)

Figure 29 shows the number of spray dried cells stained with DHR 123. The number of stained fresh cells is also indicated as control.

The number of stained unprotected and spray dried cells was significant higher than the number of pre-treated spray dried cells or freshly harvested cells ($p<0.001$).

The number of stained cells, pre-treated with 10% or 100% was comparable with the number of freshly harvested cells.

The number of spray dried, stained cells pre-treated with 50% of gelatine was comparable to the number of stained cells spray dried without gelatine.

Regarding the production of superoxide, 10 % and 100% of gelatine present the most protective effect on spray dried cells. This result is supported by the investigation measured with PI.

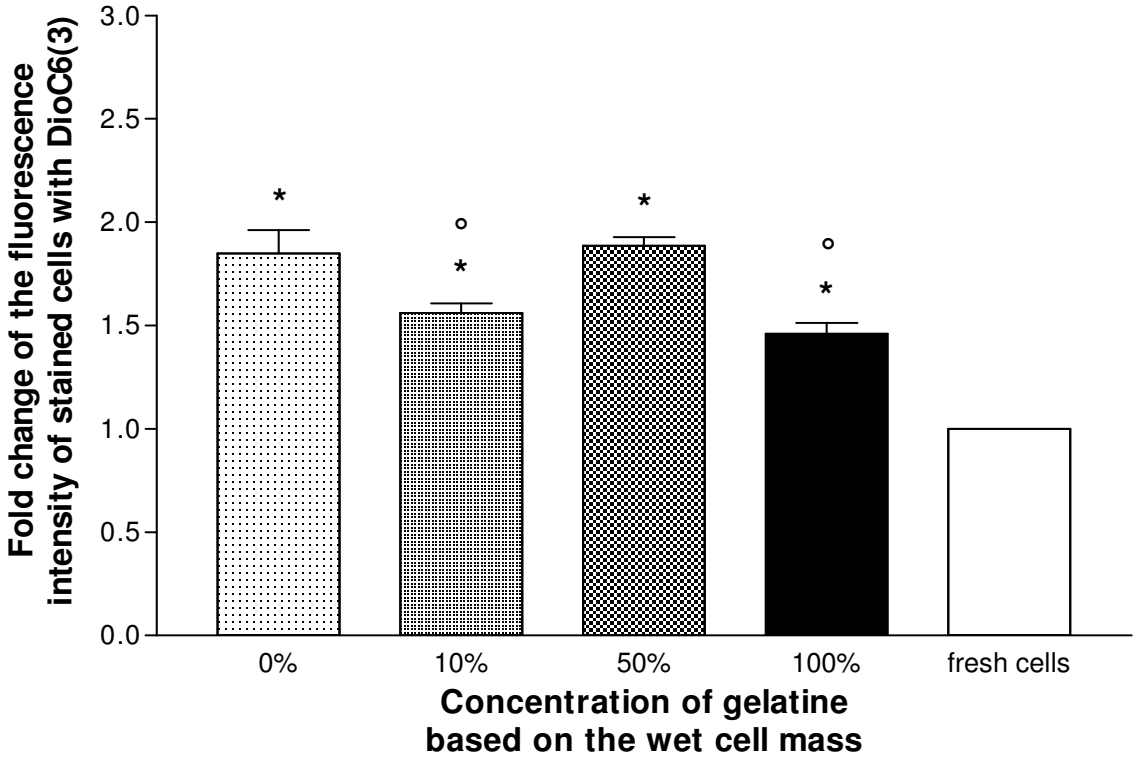


Figure 30: Fold change of the fluorescence intensity of stained cells with DioC6(3) after spray drying with gelatine in different concentrations. (*significance of changes in the fluorescence intensity of spray dried stained cells compared to freshly harvested cells; $p<0.05$, ° significance of changes in the fluorescence intensity of protected, spray dried, stained cells compared to unprotected (= 0%), spray dried, stained cells; $p<0.05$)

Figure 30 shows the intensity of spray dried cells stained with DioC6(3). The number of stained fresh cells is also indicated as control.

The fluorescence intensity of stained spray dried cells pre-treated with gelatine in the concentration of 10% ($p<0.01$) and of 100% ($p<0.001$) was significantly lower compared to the intensity of stained unprotected spray dried cells. Whereas the fluorescence intensity of stained spray dried cells pre-treated with 50% gelatine was comparable to unprotected cells ($p>0.05$).

Regarding the cell membrane potential spray dried cells pre-treated with 10 % or 100% of gelatine present the most protective effect against depolarisation of the cell membrane, because DioC6(3) can accumulate on a hyperpolarized membrane (and increases their fluorescence).

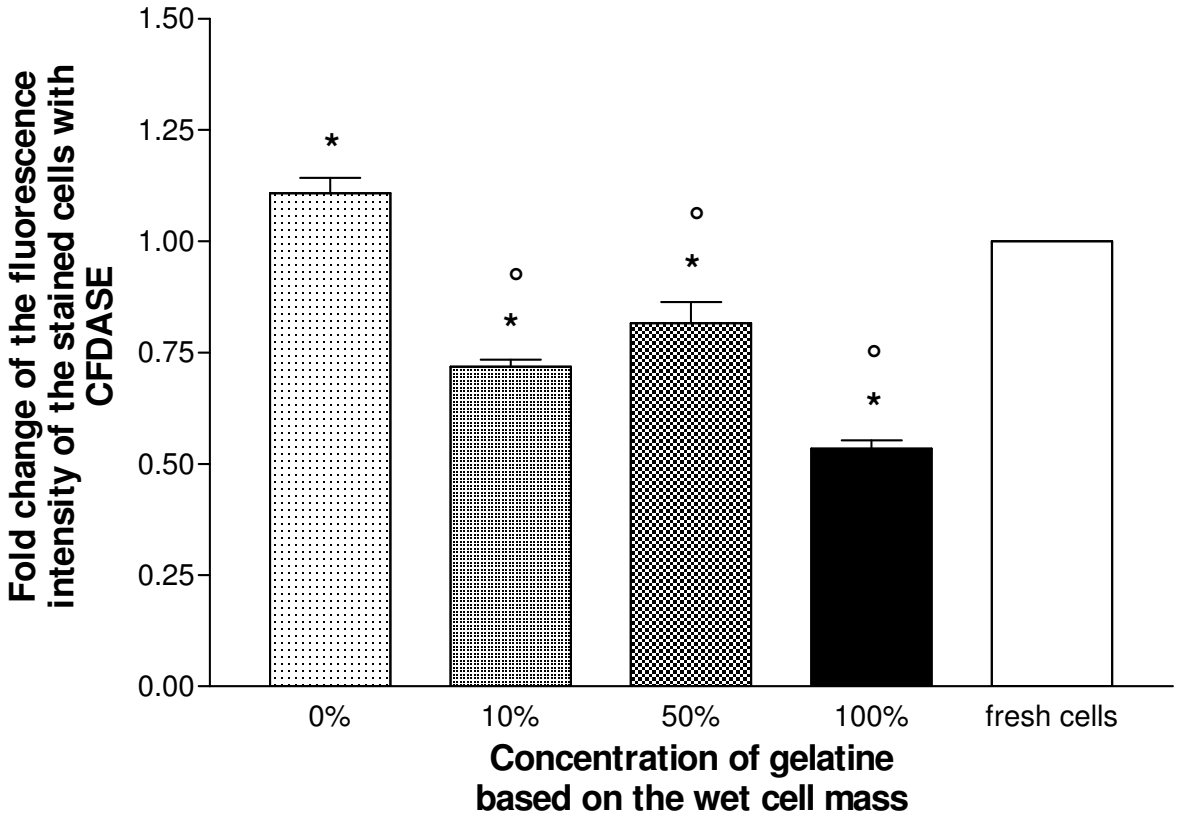


Figure 31: Fold change of the fluorescence intensity of stained cells with CFDASE after spray drying with gelatine in different concentrations. (*significance of changes in the fluorescence intensity of spray dried stained cells compared to freshly harvested cells; $p<0.05$, ° significance of changes in the fluorescence intensity of protected, spray dried, stained cells compared to unprotected (= 0%), spray dried, stained cells; $p<0.05$)

Figure 31 shows the intensity of spray dried cells stained with CFDASE. The number of stained fresh cells is also indicated as control. The fluorescence intensity of stained cells pre-treated with gelatine was significantly lower compared to the intensity of stained unprotected cells and freshly harvested cells ($p<0.01$). For this assay the fluorescence intensity of freshly harvested cells stained with CFDASE was significantly different to the intensity of all spray dried cells pre-treated with gelatine ($p<0.001$).

The intensity of cells pre-treated with 10% or 50% gelatine was comparable. However these values were significantly higher than the intensity of cells pre-treated with 100% gelatine ($p<0.001$).

Therefore, it can be declared that 100% of gelatine presents the most protective effect.

All in all, it can be said, that spray drying cells with gelatine presents a protective effect on the reproduction capability and on the cellular properties in all used concentrations. Compared

to unprotected spray dried cells, the pre-treatment of cells with gelatine resulted in a significant drop of cells stained with PI. In the case of membrane potential concentrations of 10% and 100% of gelatine used, a significant decrease of DioC6(3) intensity was provided, compared to unprotected spray dried cells. Concerning the culturability, 10% of gelatine presented significantly better results than any other concentration of gelatine.

3.2.2.3 Maltodextrin

1) Culturability

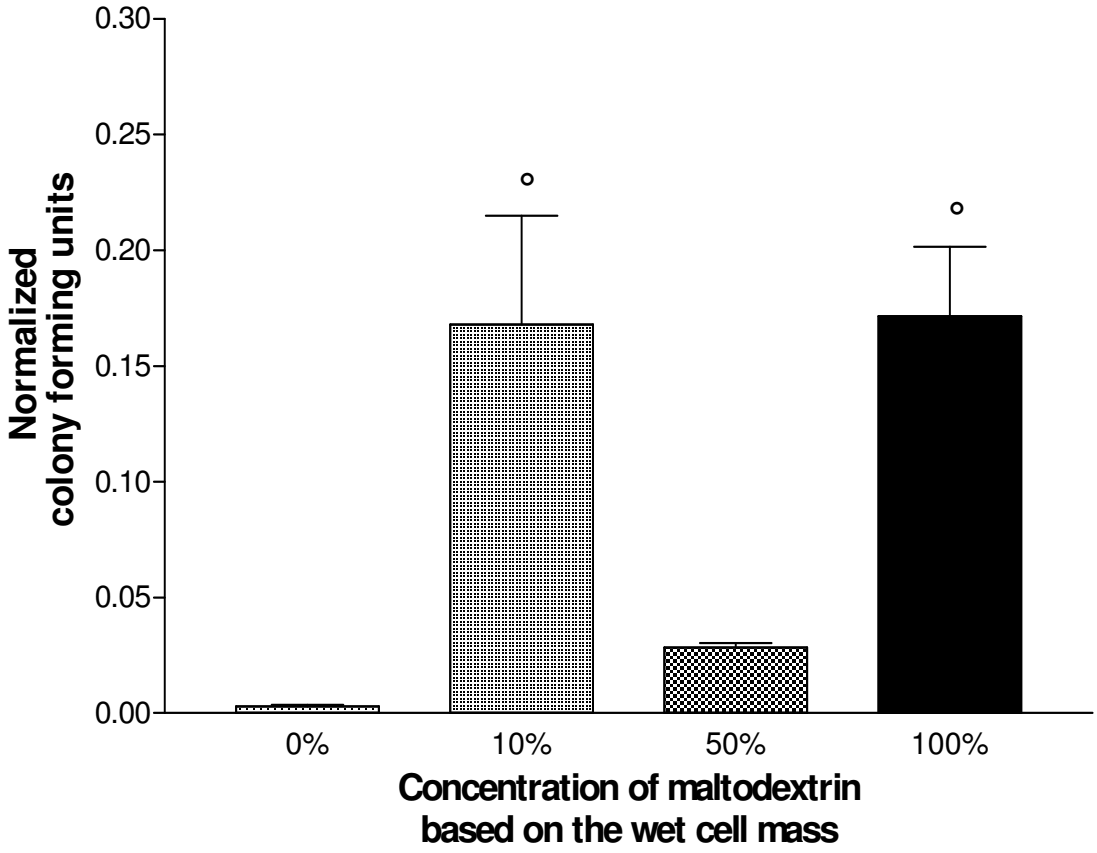


Figure 32: Culturability of *B. bifidum* Bb12 after spray drying with different concentrations of maltodextrin. (fold change of the culturability is compared to the culturability of freshly harvested cells, $p<0.05$; °significance of CFU of protected and spray dried cells compared to CFU of unprotected spray dried cells, $p<0.05$)

The culturability of spray dried cells was related to the culturability of freshly harvested cells and demonstrated in figure 32.

The figure shows, that spray dried cells pre-treated with 10% and 100% of maltodextrin have significant higher culturability, compared to unprotected spray dried cells ($p<0.001$). Using 50% of maltodextrine could not protect the culturability of cells, as the culturability was comparable with that of unprotected cells ($p>0.05$).

Using 10% and 100% of maltodextrin complies with a heat stress at 60°C for 6 minutes.

2) Fluorimetry measurements (TECAN)

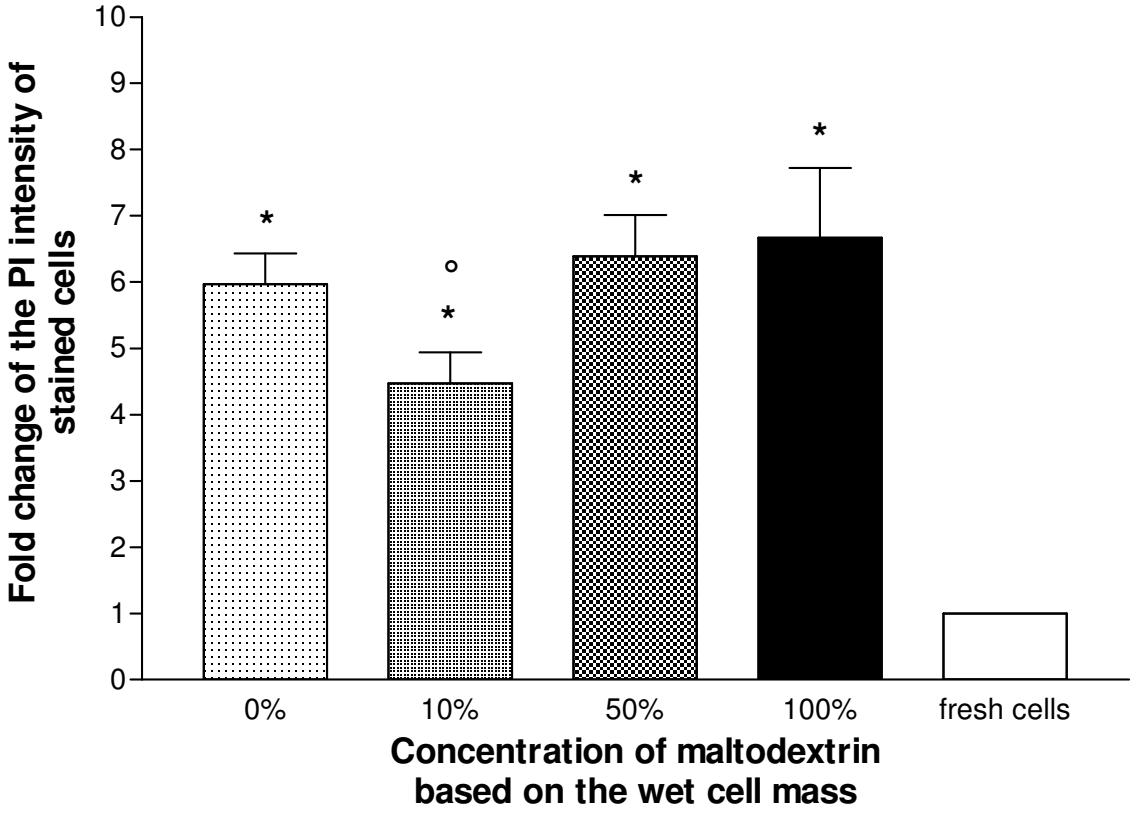


Figure 33: Fold change of the PI intensity of stained cells after spray drying with maltodextrin in different concentrations, measured with micro plate reader (*significance of changes in PI intensity of spray dried cells compared to freshly harvested cells, $p<0.05$, ° significance of changes in PI intensity of spray dried cells with maltodextrin compared to spray dried cells without maltodextrin (0% protectant),$p<0.05$)

Figure 33 shows the relative intensity of spray dried cells stained with PI. The fluorescence intensity of fresh cells stained with PI is also indicated as control. The figure shows, that the fluorescence intensity of freshly harvested cells is significant lower than the intensity of unprotected cell ($p<0.001$).

The fluorescence intensity of spray dried cells pre-treated with 50% and 100% maltodextrin was comparable with the intensity of stained unprotected and spray dried cells ($p>0.05$).

It can be seen, that using 10% of maltodextrin protected the cell membrane better than using other concentrations. This can explain the high culturability of cells using this concentration.

3) Flow cytometry measurements

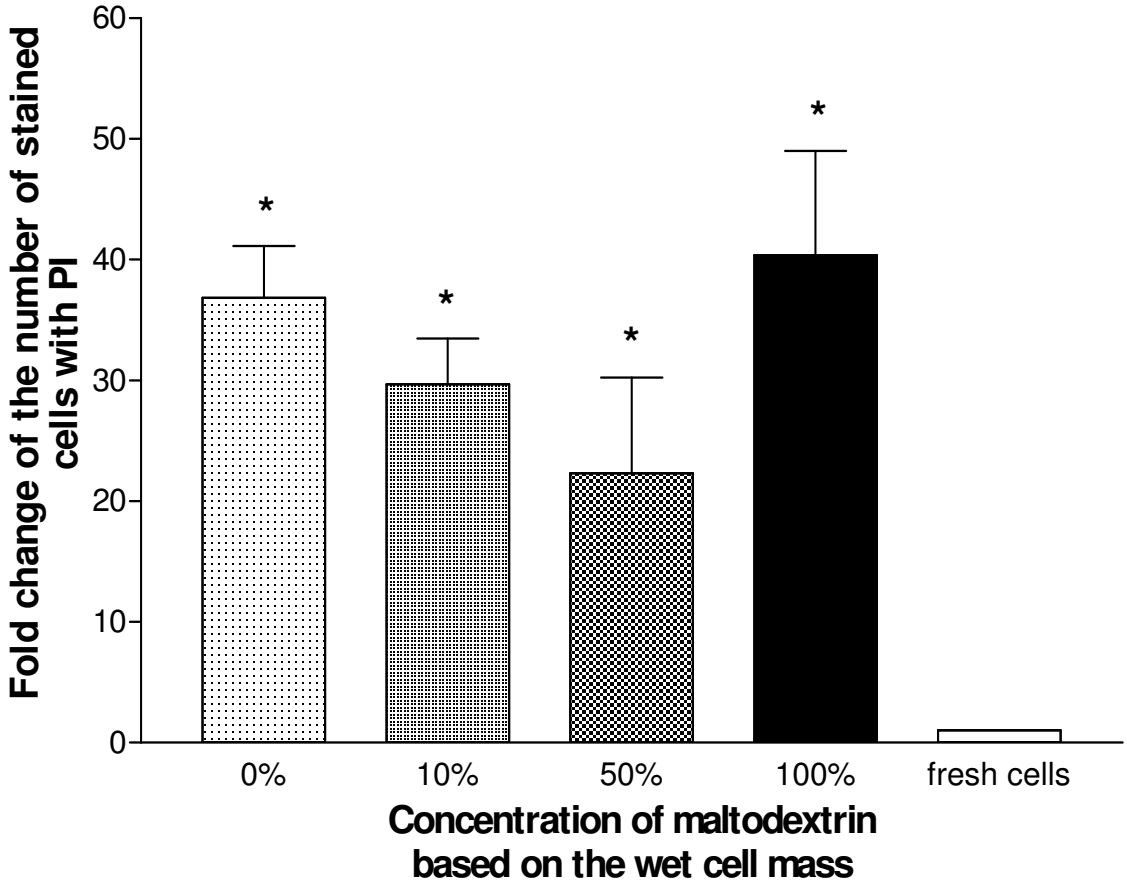


Figure 34: Fold change of the number of stained cells with PI after spray drying with maltodextrin in different concentrations. (*significance of changes in the number of spray dried stained cells compared to freshly harvested cells; $p<0.05$, ° significance of changes in the number of protected, spray dried, stained cells compared to unprotected (= 0%), spray dried, stained cells; $p<0.05$)

Figure 34 shows, the fold change of number of spray dried cells stained with PI. This figure shows that the PI intensity of freshly harvested cells is significant lower compared to the fold change of spray dried stained cells ($p<0.001$).

The PI intensity of protected cells in all concentrations was comparable with the intensity of unprotected and spray dried cells.

The results show that maltodextrin cannot protect the cell membrane.

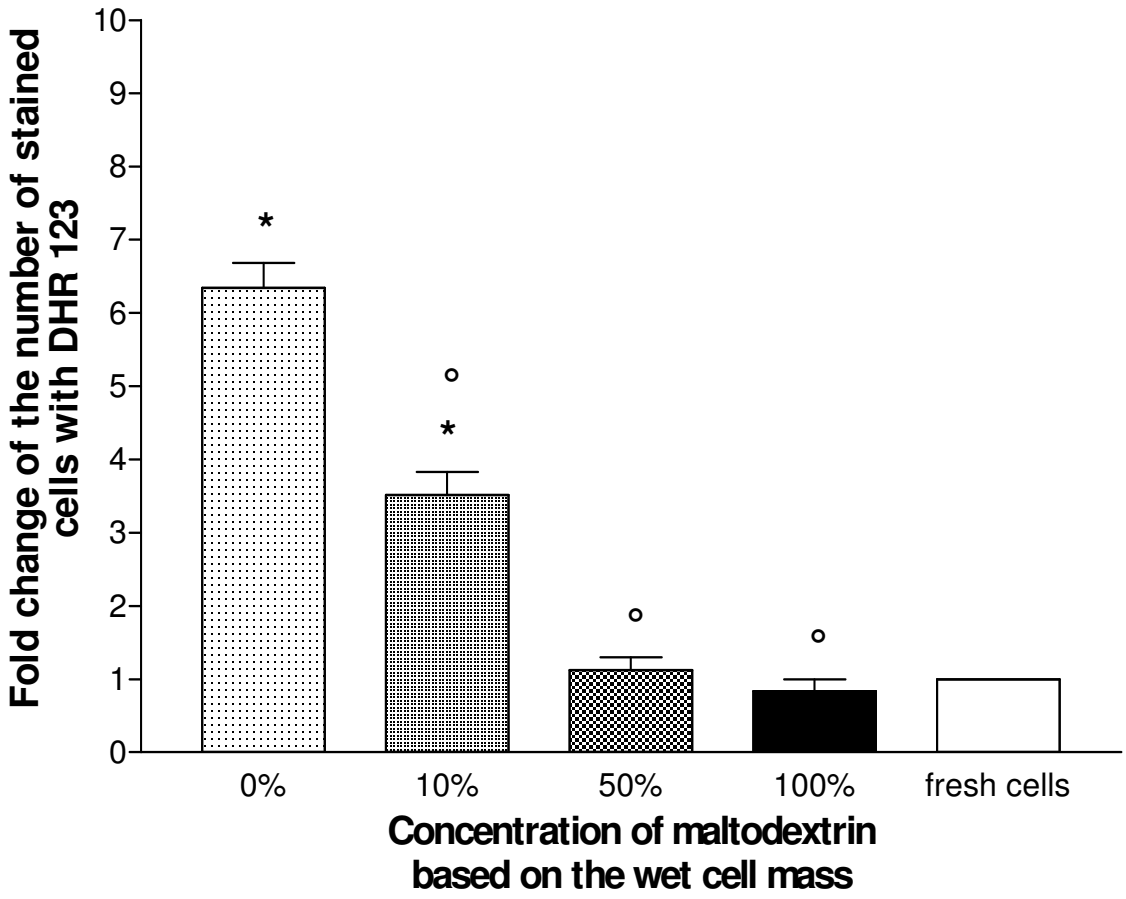


Figure 35: Fold change of the number of stained cells with DHR 123 after spray drying with maltodextrin in different concentrations. (*significance of changes in the number of spray dried stained cells compared to freshly harvested cells; $p<0.05$, ° significance of changes in the number of protected, spray dried, stained cells compared to unprotected (= 0%), spray dried, stained cells; $p<0.05$)

Figure 35 shows the fold change of number of spray dried cells stained with DHR 123. The number of stained fresh cells is also indicated as control. The number of stained unprotected and spray dried cells was significantly higher than spray dried cells with maltodextrin and freshly harvested cells ($p<0.001$). Using maltodextrin with 50% or 100% concentration, the fold change of the number of stained cells was comparable with those of fresh cells. The number of pre-treated cells with 10% maltodextrin and stained with DHR 123 was significant higher than pre-treated cells with 50% and 100% protectant.

In summary it can be said, that concentrations of 50% or 100% showed beneficial effects on the cell capability to maintain the superoxide production.

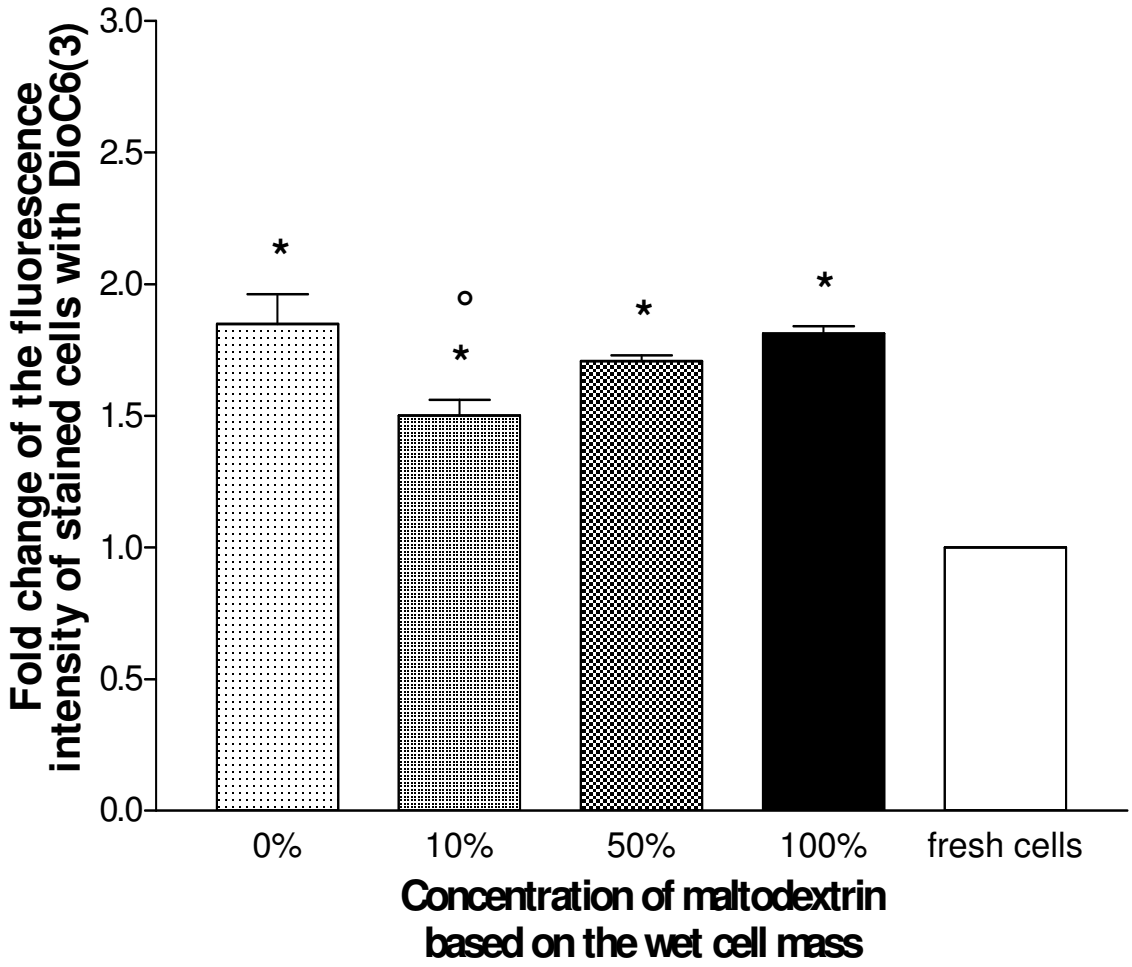


Figure 36: Fold change of the fluorescence intensity of stained cells with DioC6(3) after spray drying with maltodextrin in different concentrations. (*significance of changes in the fluorescence intensity of spray dried stained cells compared to freshly harvested cells, $p<0.05$; ° significance of changes in the fluorescence intensity of protected, spray dried, stained cells compared to unprotected (= 0%), spray dried, stained cells, $p<0.05$)

Figure 36 shows the fold change of fluorescence intensity of spray dried cells stained with DioC6(3). The florescence intensity of stained fresh cells is also indicated as control. The fluorescence intensity of unprotected stained spray dried cells was significantly higher compared to freshly harvested cells ($p<0.001$).

Using maltodextrin with 50% and 100% concentration, the fold change of fluorescence intensity of stained cells was significant higher than those of fresh cells.

The fluorescence intensity of pre-treated cells with 10% of maltodextrin and followed spray drying was significant lower compared to unprotected spray dried cells ($p<0.001$).

The results from staining maltodextrin-protected cells with CFDASE are not presented here, as those results did not show any significance.

3.2.2.4 Skimmed milk

1) Culturability

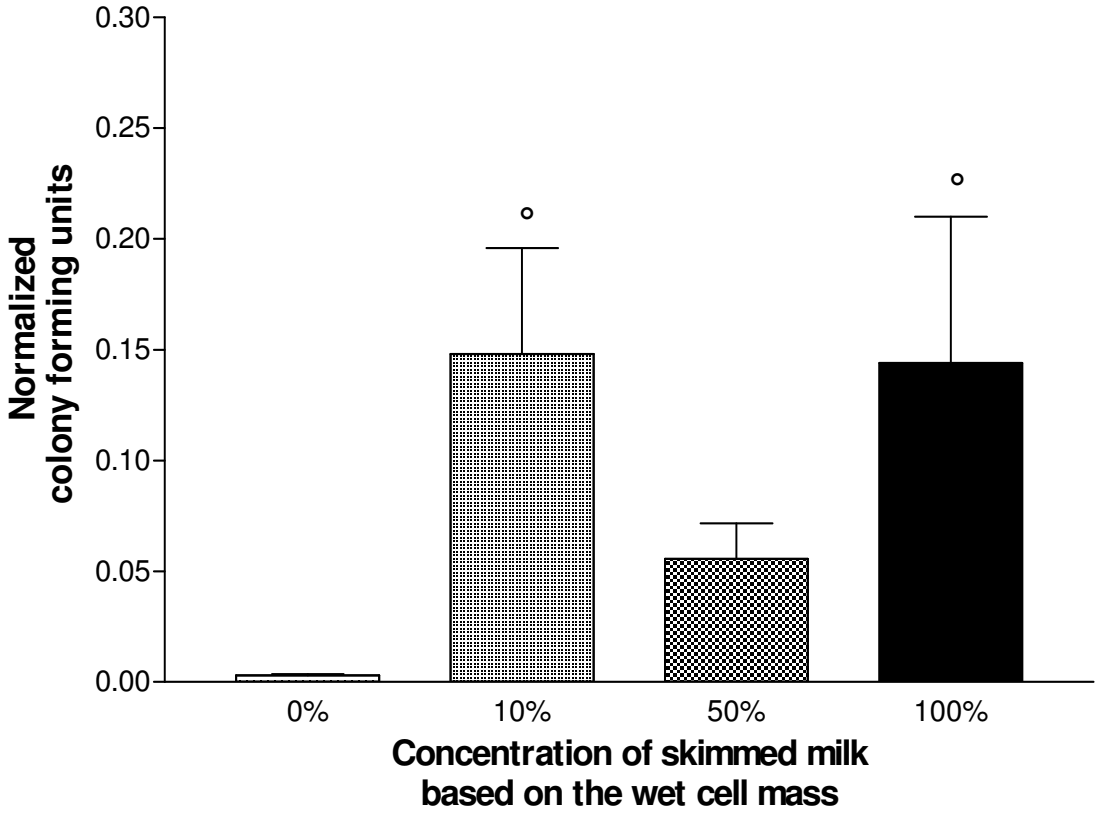


Figure 37: Culturability of *B. bifidum* Bb12 after spray drying with different concentrations of skimmed milk. (fold change of the culturability is compared to the culturability of freshly harvested cells, $p<0.05$; °significance of CFU of protected and spray dried cells compared to CFU of unprotected spray dried cells, $p<0.05$)

The culturability of spray dried cells is related to the culturability of freshly harvested cells and demonstrated in figure 37.

The culturability of freshly harvested cells are considered as significantly higher to spray dried cells without skimmed milk. Using 50% of skimmed milk presents significant better effects on the culturability than spray dried cells without protectant. 10% and 100% of skimmed milk presented the best protective effect on the culturability, even better than 50%.

The result of culturability of spray dried cells with 10% (0,15) and 100% (0,14) skimmed milk is comparable to the result of heat stress results at 60°C of 6 min (0,17).

2) Fluorimetry measurements (TECAN)

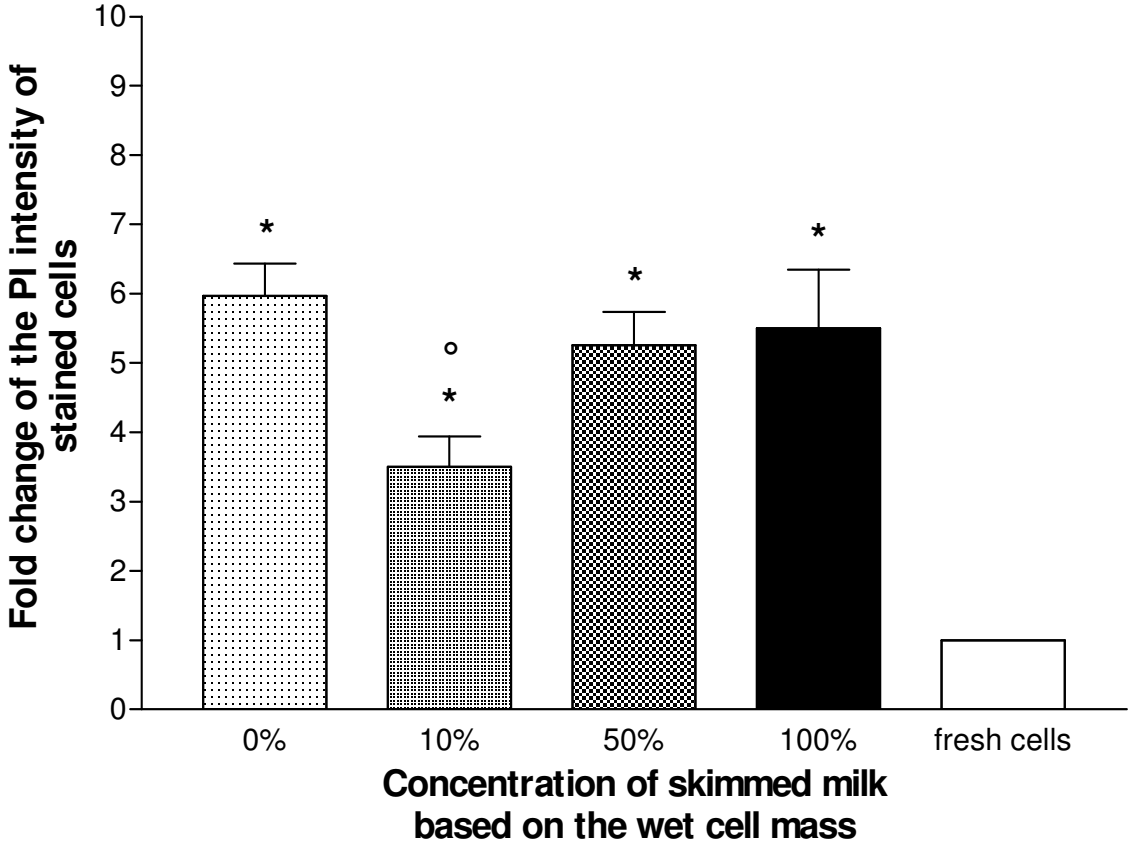


Figure 38: Fold change of the PI intensity of stained cells after spray drying with skimmed milk in different concentrations, measured with micro plate reader (*significance of changes in PI intensity of spray dried cells compared to freshly harvested cells, $p<0.05$, ° significance of changes in PI intensity of spray dried cells with skimmed milk compared to spray dried cells without skimmed milk (0% protectant), $p<0.05$).

Figure 38 shows the fluorescence intensity of spray dried cells stained with PI. The fluorescence intensity of stained fresh cells is also indicated as control. The fluorescence intensity of spray dried cells is significantly higher than the intensity of fluorescence of freshly harvested cells ($p<0.001$).

Spray dried cells pre-treated with 10% of skimmed milk presented a fluorescence intensity much lower than 50% or 100%. In this last two cases, there is no significant fold change of the PI intensity of stained spray dried cells with skimmed milk compared to unprotected spray dried cells ($p>0.05$). There is no significant difference of fluorescence intensity between protected cells with 50% and 100% skimmed milk ($p>0.05$). However, these intensities are significantly higher than using 10% skimmed milk ($p<0.001$).

This part of the study showed, that treatment of cells with 10% skimmed milk protected the cells during spray drying process better than 50% or 100% skimmed milk.

3) Flow cytometry measurements

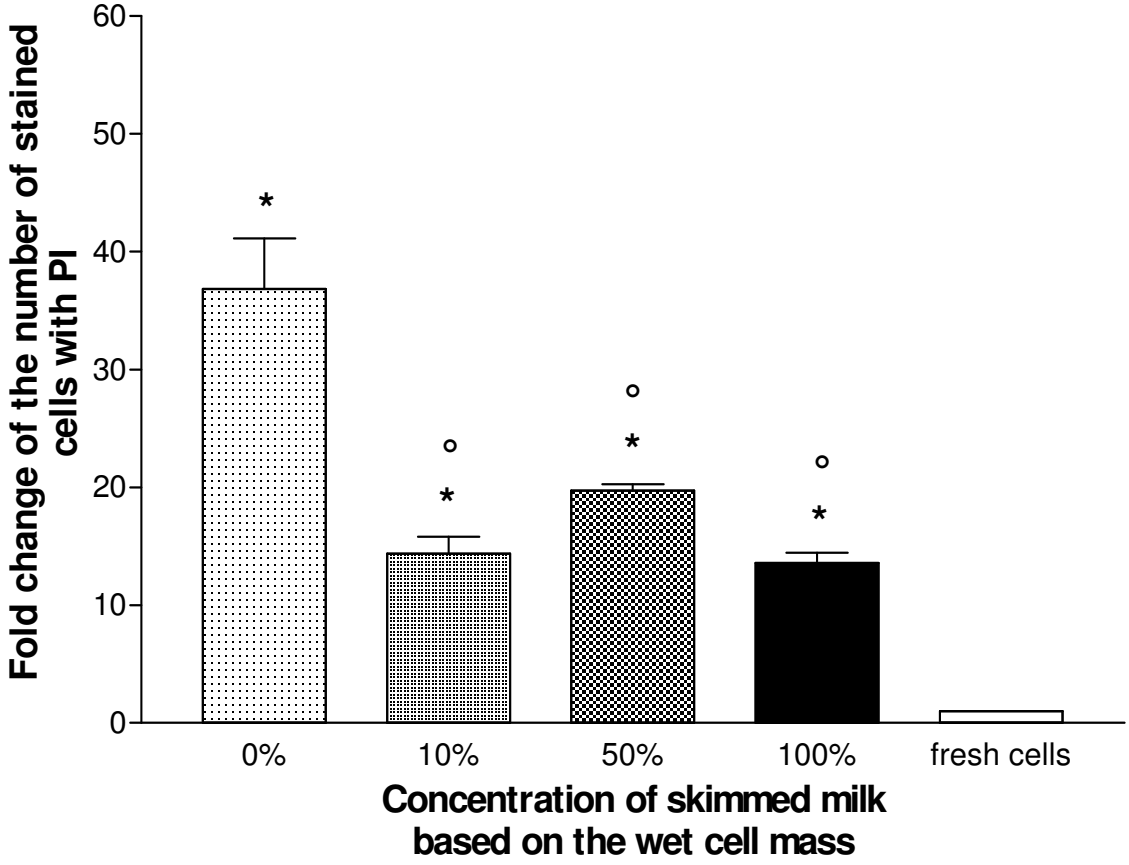


Figure 39: Fold change of the number of stained cells with PI after spray drying with skimmed milk in different concentrations. (*significance of changes in the number of spray dried stained cells compared to freshly harvested cells; $p<0.05$, ° significance of changes in the number of protected, spray dried, stained cells compared to unprotected (= 0%), spray dried, stained cells; $p<0.05$)

Figure 39 shows the number of spray dried cells stained with PI. The number of stained fresh cells is also indicated as control. The flow cytometry measurements confirm the protective impact of skimmed milk on the cell membrane of spray dried cells observed during the fluorimetry measurements. The number of stained unprotected and spray dried cells was significantly higher than spray dried cells protected with skimmed milk and freshly harvested cells ($p<0.001$).The fold change of the number of stained spray dried cells protected with 50% skimmed milk is significantly higher than the number of stained spray dried cells with 10% skimmed milk ($p<0.05$).Using 10% skimmed milk before spray drying, is not significant different to 100% skimmed milk. ($p>0.05$)

The fold change of the number of stained spray dried cells which were protected with 10% skimmed milk was comparable with those protected with 50% or 100% skimmed milk.
Obviously, all concentrations of skimmed milk used, present a protective impact during spray drying. Because of a large standard deviation there is no significance between 10%, 50% and 100%.

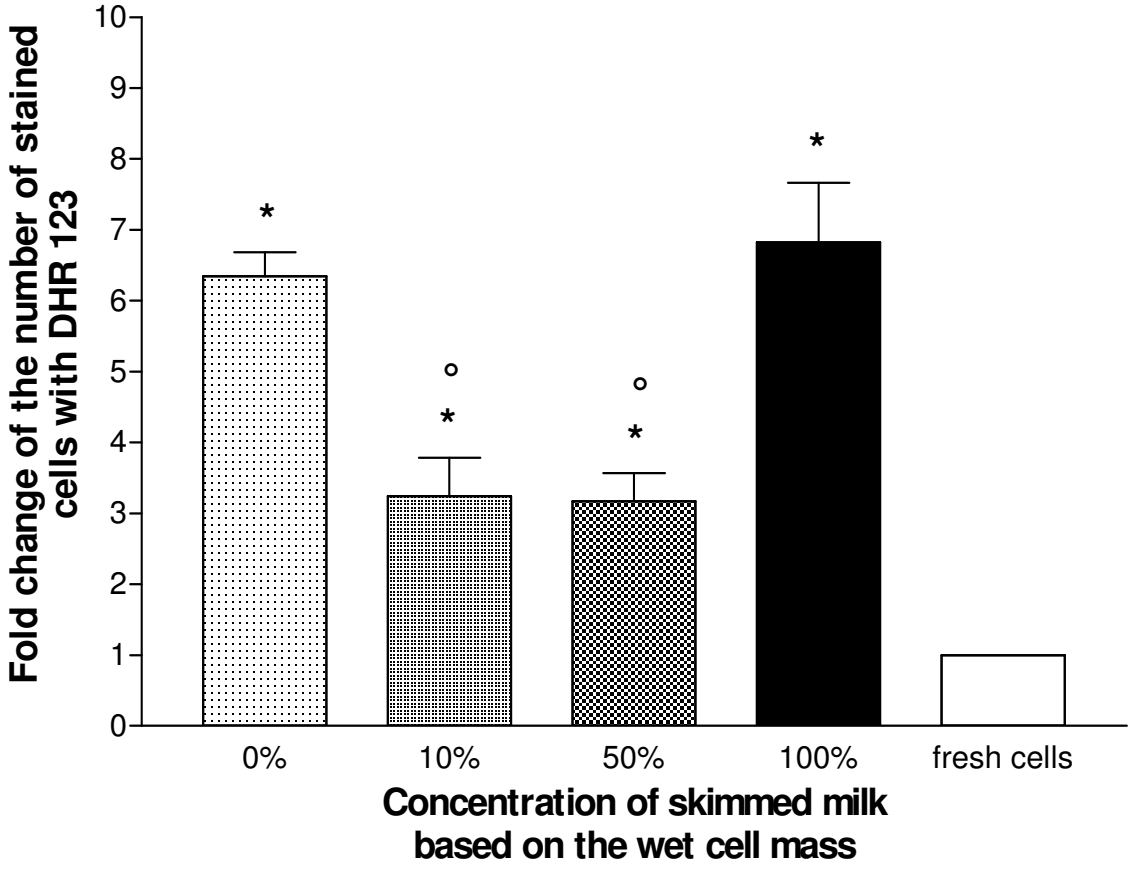


Figure 40: Fold change of the number of stained cells with DHR 123 after spray drying with skimmed milk in different concentrations. (*significance of changes in the number of spray dried stained cells compared to freshly harvested cells; $p<0.05$, ° significance of changes in the number of protected, spray dried, stained cells compared to unprotected (= 0%), spray dried, stained cells; $p<0.05$)

Figure 40 shows the fold change of number of spray dried cells stained with DHR 123 after spray drying. The number of stained fresh cells is also indicated as control. The fluorescence intensity of stained, freshly harvested cells is significantly lower than the one of spray dried cells ($p<0.001$). Using 100% skimmed milk, the fold change of the number of spray dried and stained cells was comparable with those of unprotected and spray dried cells.
The fold change of the number of stained cells protected with 10% or 50% skimmed milk were comparable with each other and significantly lower than those of cells protected with 100% skimmed milk.

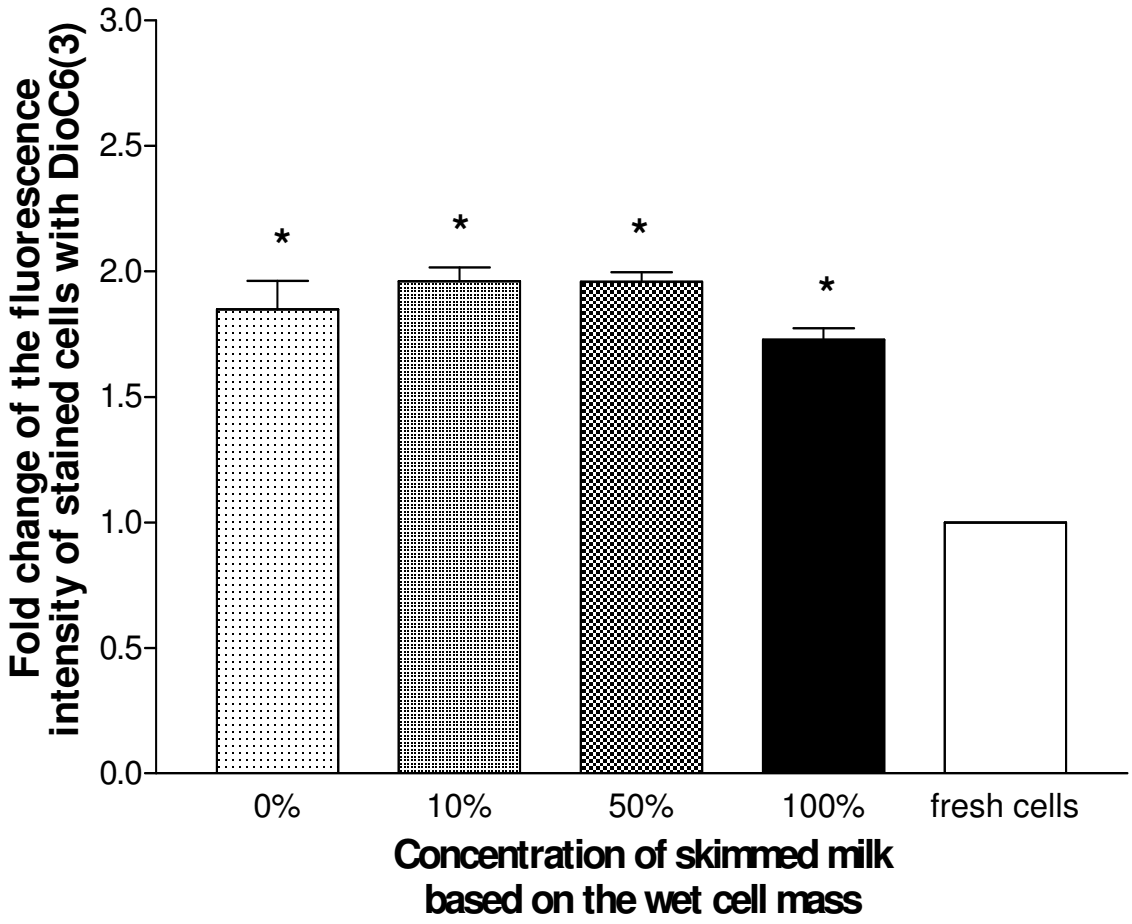


Figure 41: Fold change of the fluorescence intensity of stained cells with DioC6(3) after spray drying with skimmed milk in different concentrations. (*significance of changes in the fluorescence intensity of spray dried stained cells compared to freshly harvested cells, $p<0.05$; ° significance of changes in the fluorescence intensity of protected, spray dried, stained cells compared to unprotected (= 0%), spray dried, stained cells, $p<0.05$)

Figure 41 shows the fluorescence intensity of spray dried cells stained with DioC6(3) after spray drying. The fluorescence intensity of fresh cells is also indicated as control. The DioC6(3) intensity of unprotected spray dried cells was in all concentrations significantly higher than the intensity of freshly harvested cells ($p<0.001$).

The fluorescence intensity of spray dried cells, pre-treated with 10%, 50% or 100%, was comparable with the intensity of unprotected cells ($p>0.05$).

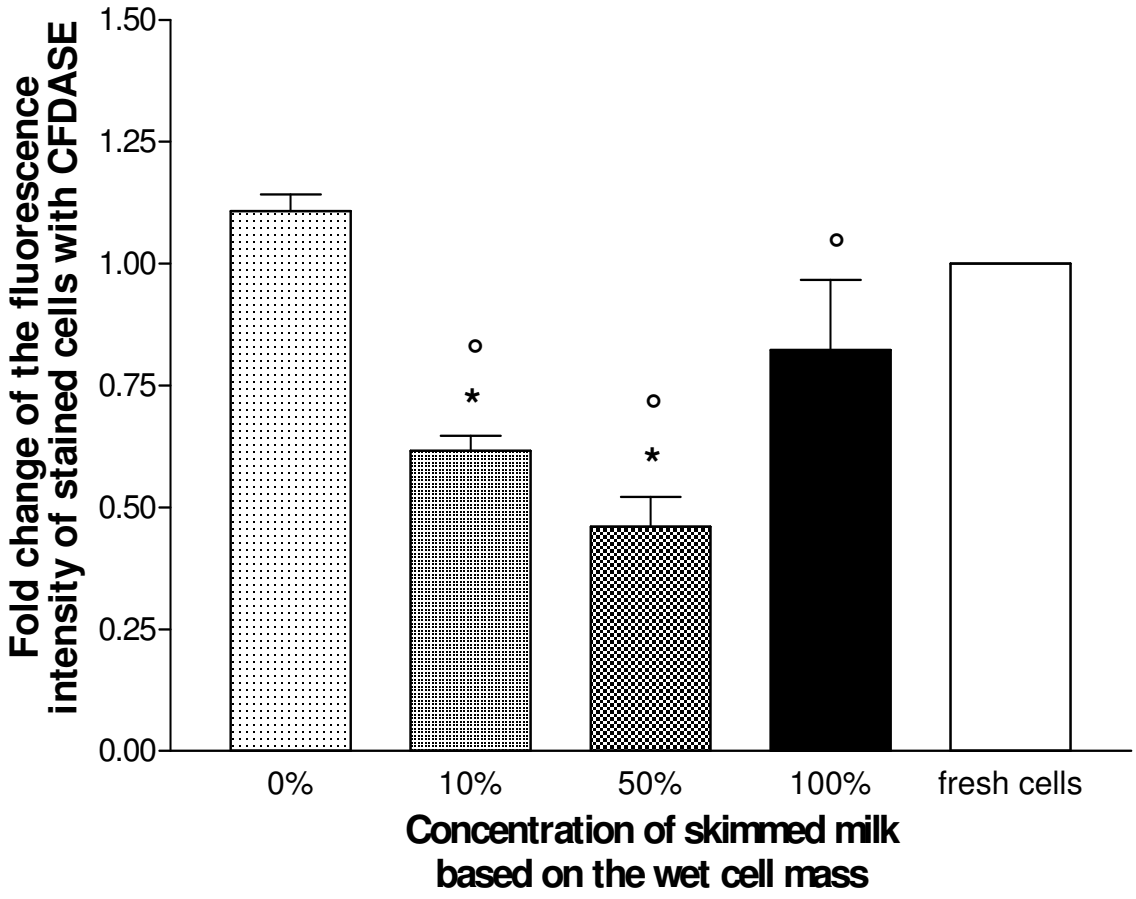


Figure 42: Fold change of the fluorescence intensity of stained cells with CFDASE after spray drying with skimmed milk in different concentrations. (*significance of changes in the fluorescence intensity of spray dried stained cells compared to freshly harvested cells; $p<0.05$, ° significance of changes in the fluorescence intensity of protected, spray dried, stained cells compared to unprotected (= 0%), spray dried, stained cells; $p<0.05$)

Figure 42 shows the fluorescence intensity of spray dried cells stained with CFDASE. The intensity of stained fresh cells is also indicated as control. No significant difference between freshly harvested cells and unprotected spray dried cells was detectable ($p>0.05$). There is also no significant difference between the intensity of freshly harvested cells and spray dried cells pre-treated with 100% skimmed milk ($p>0.05$).

Using a concentration of 10% and 50% skimmed milk presents a significant difference in the intensity compared to 0% skimmed milk ($p<0.001$)

4 Discussion

It is well known that the composition of probiotic formulations causes many challenges in regards to the stabilisation of the microorganisms. There are multiple ways to dry microorganisms, among them spray drying, which is one of the cost-saving predominant methods used in industry[18].

However, "There are obvious challenges associated with using spray drying to produce viable cultures, including the requirement that the microorganisms survive the high temperatures used"[18]. As a consequence spray drying often results in "high loss of cell viability"[20,47].
B.*bifidum* Bb12 is an anaerobic microorganism; Kailasapathy et al noted an incipient drop of viability which could be conferred to an incorporation of air[12].
It is generally admitted, that to assess the viability of microorganisms, not only the capability to reproduce, but also parameters such as intact plasma membrane, enzyme activity, membrane potential and the intracellular pH have to be taken into account[22,23].
For the demonstration of these metabolic activities, the cells can be marked with fluorescence dyes. "Some fluorochromes bind specifically to cell molecules or components (nucleic acids, proteins, lipids) increasing their fluorescence. Others accumulate selectively in cell compartments or modify their properties through specific biochemical reactions in response to changes in the environment such as pH, membrane polarization (cyanines, oxonols) or enzymatic activity (fluorogenic substates)"[24].

4.1 Heat treatment

To obtain a reference of the viability of the *B. bifidum* Bb12 for the spray drying experiments and the subsequent comparison with the different protective agents, we investigated the influence of heat stress under controlled conditions in a first step.
Looking at the evolution of the culturability, a decrease of the CFU with increasing temperature and prolonged exposition can be seen. Whereas at 60°C after 6 min the CFU value has dropped to 17% compared to fresh cells, at 90° we obtained a value of 0,1% after only 2 minutes of heat stress.

Observing the effect of heat on the membrane integrity, PI, a nuclei acid stain, was used[18,31]. When freshly harvested cells are compared with stressed cells, the relative intensity of PI from fresh cells is much lower than from stressed cells. Because of the cell membrane damages, the fluorescence dye could bind on the DNA and is able to emit fluorescence. Exposing *B. bifidum* Bb12 at 60°C went well for two minutes before causing significant damages. At 70°C the time interval, in which B. *bifidum* Bb12 is able to compensate the heat damages, decreased to about one minute. Above a temperature of 80°C it decreased to approximatively 30 seconds.

FDA was used to investigate the esterase activity. Fluorescence is only emitted, when the dye is hydrolysed by intracellular esterases. This means that only an intact metabolism is able to hydrolyse this dye. The higher the level of damage, the lower is the fluorescence intensity. The FDA concentration decreased significantly after 30-60 seconds exposure at 70° and 80°C and far before 30 seconds at 90°C.

DHR 123 was used to measure the hydrogenperoxid production. Hydrogenperoxid protects the cell against stress factors. This dye becomes fluorescence when it oxidized in the intracellular redox system. Unfortunately this mechanism works only for a short time period, which expresses itself in an increase of fluorescence. During a longer period of stress, the system is not longer able to produce H_2O_2 and fluorescence decreases finally. This typical behaviour was only observed clearly at 60°C. For the other temperatures we only observed a decrease of DHR 123 intensity after 90-120 seconds at 70° and 80°C and between 30-60 seconds at 90°C.

Comparing culturability of B.*bifidum* Bb12 after heat stress with other investigated parameters of cell activity, a correlation between loss of membrane intergrity and the decrease of culturability can be observed (between 6-10 minutes at 60°C; 60-120 seconds at 70°C , 30-90 seconds at 80°C and about 30 seconds at 90°C). The decrease of esterase activity is observed somewhat earlier, whereas the decrease of the hydrogenperoxid production correlates again reasonably well with the onset of the culturability decrease.

Overall, processing parameters such as the absolute temperature and time interval of heat treatment can be supposed as essential[23].

4.2 Spray drying

After preliminary investigations in order to test the response of *B. bifidum* Bb12 to heat at different temperatures and over different time intervals, it was possible to start the spray drying. The heat stress results show that temperature and duration have significant effects on the cell activities. Therefore, special attention was paid to the choice of process parameters. Inlet and outlet temperature, spray rate, and the selection and concentration of protectants have great influence on the bacterial survival rate[47,48].

A drop in cell viability[18] was evident with increasing temperature. Otherwise, temperature during the spray drying process was crucial to ensure that the powder was kept dry and did not absorb any moisture[47].
According to available literature, outlet temperature should be within the range of 70°C to 120°C[47,48]. The lowest outlet temperature, where it was still possible to keep the powder dry, was around 70-80°C at an inlet temperature of 115°C. The setting was the same for all experiments in this series.

Temperature ranges used in order to keep the powder dry cause considerable stress on the microorganisms *B. bifidum* Bb12. In order to reduce the stress, protective agents are used.
Literature states that the common used protectants during drying (freeze-drying and also spray-drying) are skimmed milk,[16,18,19,47] sugar derivatives like maltodextrin[18,47] gelatine[19,20,47] and gum arabic[16,19,20]. These substances are used in order to minimize the damaging effects on cells caused by extreme temperatures.

In this series of experiments, the selected protectants were added in an amount of 0%, 10%, 50% and 100% (based on the wet cell mass) in order to investigate a possible correlation between protection and concentration. According to Lian et al, the different chemical composits of the used protectants provide positive effects on culturability and viability. The present study supports this findings as will be shown hereafter.

Focusing on culturability, it is noticeable that the best effect within all protectants used in this study is reached by adding an amount of 10% based on the wet cell mass before spray dryring. These results are comparable with culturability results reached by stressing unprotected cells at 60°C.

Regarding that spray drying takes 60 minutes at a temperature range of 115°C (inlet temperature) to 75°C (outlet temperature), it can be seen, how effectiv the addition of protectants is:
After spray drying, $5x10^7$ CFU/ml was measured for unprotected cells. With protectants we observed a range of CFU values between $10x10^7$ CFU/ml (≈ 0.63%) for 50% gum arabic and $3x10^9$ CFU/ml (≈ 18,62%) for 10% gum arabic. Using 10% gum arabic increased the culturabilty for almost 2 log units during spray drying compared to unprotected cells.
The lowest rate of culturability for all protectants was reached by adding an amount of 50% (based on the wet cell mass). Adding an amount of 100% (based on the wet cell mass) only leaded to good results with maltodextrin.
Therefore, it can be concluded that the concentration of protective additives is crucial for the result. Whereas it is to mention that higher concentrations do not provide better protection. Such a correlation could not be proven.

Membrane integrity proved to be a major indicator in terms of cells viability. Flowcytometry and fluorimetry were used to measure membrane integrity after spray drying. In case of membrane integrity measurements, it was striking that the best results were not achieved by using the same concentration of the investigated protectant.
While in terms of membrane integrity, the overall best performance seemed to be achieved with gum arabic (in all concentrations) and gelatine (where best results were achieved at 50% and less favourable results with 100% and 10%), cell membrane protection can be also obtained by using 10% skimmed milk.
Compared to the beneficial effects offered by the other protectants used, the results of maltodextrin in term of protection can be neglected.

Maltodextrin, gelatine and gum arabic in a concentration of 100% showed very favourable results in terms of superoxide production. Also in a concentration of 50% the use of maltodextrin and gum Arabic can be recommended but not gelatine. Gelatine showed better results at a concentration of 10%.
The fourth used protectant in this study was skimmed milk which in comparison to the other used protectants showed neither positive effect on the superoxide production nor on membrane potential.

In regards to membrane potential diverse results were obtained by using the other protectants. Gum arabic showed significant improvement in all concentrations, wherease this was only true for gelatine in amount of 10% und 100%. In terms of maltodextrin significant results could only be proven by using 10%.

In a final step the intercellular pH was investigated by evaluating the fluorescence intensity of CFDASE. Even in this step maltodextrin proved to be, compared to the other protectants, of less value, as results obtained were statistically not usable. Best results could be reached by using 100% gum arabic. The same is true for gelatine whereas best results with skimmed milk where reached with 50%. Notably is that for all concentrations of protectant lower values were measured than for freshly harvested and unprotected cells. In fact, values between unprotected, spray dried cells and freshly harvested cells don't differ in terms of intercellular pH.

In summary it can be said, that the used protectants show different qualities in protecting cells *B.bifidum Bb12* during spray drying.

Gum arabic showed the best protective results in all concentrations used. Especially, on culturability, but also on membrane integrity, intracellular pH and membrane potential, gum Arabic provided a benefical effect. Interestingly no significant difference in the superoxide production could be found, when comparing freshly harvested cells to protected cells with 50% and 100% gum arabic.

Gelatine also provided positive effects on the investigated cell parameters, particularly when added in an amount of 100% (based on the wet cell mass). An amount of 10% was seen to have notably beneficial effects on the superoxide production.
These findings are in agreement with the results of Lian et al[19]. Which state that gum arabic and gelatine showed the best protective qualities in regards to *B. bifidum* Bb12 in terms of survival after spray drying.

The effects of maltodextrin diverge with the concentrations used. Thus, adding an amount of 10% affects mainly the membrane potential, while 50% affects the membrane integrity and 100% the production of superoxide. The use of maltodextrin proved to be much more efficient upon the production of superoxide than any of the other protectants.

Skimmed milk influenced the membrane integrity and the intercellular pH when added in an amount of 10% and 100% (based on the wet cell mass). Adding an amount of 50% improves the membrane potential whereas an added amount of 10% and 50% improves the production of superoxide.

It can be noted, that the different protectants used in this study, exerts different degrees of beneficial impact during spray drying. Based on obtained results, gum Arabic and gelatine provided best protection in terms of viability. This finding is supported by studies of Lian et al[19]. In their work, the protectetiv impact of gum arabic, gelatine, soluble starch und skimmed milk after spray drying of *B. infantis* und *B.longum* were compared.
In terms of viability, the best protective impact for *B. infantis*, was gum arabic followed by gelatine. In the case of *B. longum* the survival rate with gelatine was higher than with gum arabic.

Ananta et al reported about their investigation, that a high survival is offered by the performance of different protectants during drying[47]. The best results were provided by a mix of protectants.
In this work, the individual protectants in concentrations of 10%, 50% and 100% (based on the wet cell mass), were treated and measured separately from each other. It would therefore be of interest to repeat these tests with a mixture of those protectants that proved, in this study, to be the most effective ones.
In conclusion it can be say, that *B.bifidum* Bb12 is especially heat-sensitive and needs to be protected at all times during the heat treatment in order to prevent cells damages and thereby prevent decrease of the viability.

5 Summary

The aim of the present study was to investigate the impact of spray drying on the cellular activity and culturability of *B. bifidum* Bb12.
Probiotics cover a large field of applications in the human medicine and nutrition supplement industries. Their benefits are documented in many in vivo studies.

Spray drying proposes a low cost approach. But the drying step itself poses a great challenge during the manufacturing, because the microorganisms are submitted to high temperatures and physical forces.
To investigate cell condition, parameters like cell membrane integrity, esterase activity, intracellular pH, and superoxide production must be observed. There we have stained the cells with fluorescence dyes and measured them by fluorimetry or flow cytometry. The culturability was investigated by means of the plate count method.

In a first step, it was important to quantify the heat tolerance on the base of heat stress. Therefore *B. bifidum* Bb12 was incubated for different time intervals at 60°C, 70°C, 80°C and 90°C.
Whereas *B. bifidum* Bb12 can tolerate 60°C for two minutes, the tolerance capability decreases at 70°C to one minute and at 90°C to only 30 seconds.
These results were also supported by measurement of culturability. With increasing temperature, the culturability decreases.
Before *B. bifidum* Bb12 can be spray dried, it was important to define the spray drying parameters. It was found out that a combination of low inlet temperature (115°C), a moderate spray rate (1.5g/min) and a maximal outlet temperature of 70°C, provided the best environment for success. However even in the optimized conditions the spray drying process lead to a decrease of almost 3 log units in the CFU value.

After the setting was completed, different excipients were added in order to investigate their protective activities on *B. bifidum* Bb12 during spray drying. The excipients were selected on the base of literature and previous experience. Gum arabic, gelatine, maltodextrin and skimmed milk were chosen. Cells were treated with these protectants, using concentrations of 10%, 50% and 100%, based on the wet cell mass. After spray drying culturability, membrane

integrity, esterase activities, superoxide production, the membrane potential and intracellular pH after spray drying were measured.

Gum arabic and gelatine provided in all used concentrations the highest protection regarding cellular activities and culturability when compared to unprotected cells.
In comparison 100% maltodextrin showed only beneficial influence for superoxide production and culturability.
Skimmed milk showed promising results concerning membrane integrity and intracellular pH.

The highest culturability obtained in this study was $3x10^9$ CFU/ml using 10% gum arabic. This value compares favourably with the $5x10^7$ CFU/ml of the unprotected cells and shows that an increase of almost 2 log units could be reached.

Overall it can be said, that *B. bifidum* Bb12 is a very heat sensitive microorganism, which has to be protected during the spray drying process, in order to set limits to the grade of damages.

6 Zusammenfassung

Die vorliegende Arbeit befasst sich mit der Sprühtrocknung der probiotisch aktiven Bakterien des Stammes *B. bifidum* Bb12.
In Medizin und Ernährung haben probiotische Zubereitungen einen hohen Stellenwert und ihr Nutzen wird durch zahlreiche Studien belegt. Die entsprechenden Präparate enthalten eine standardisierte Anzahl an ruhenden, aber lebens- und vermehrungsfähigen Keimen, die nach Einnahme im Darm wieder revitalisiert werden. Um die lebenden Mikroorganismen jedoch in lagerungsbeständige Überdauerungsstadien zu überführen, müssen sie zuerst getrocknet werden.

Die Sprühtrocknung ist eine bewährte und kostengünstige Methode zur Herstellung von lagerungsstabilen Probiotika. Allerdings werden die Mikroorganismen bei der Sprühtrocknung diversen Stressfaktoren, wie hohe Temperaturen, Dehydrat und mechanischen Scherkräften ausgesetzt, welche den Zustand der Zellen negativ beeinflussen. Ziel dieser Arbeit ist es, den Effekt der Sprühtrocknung auf die Vermehrungsfähigkeit und die zellulären Aktivitäten von *B. bifidum* Bb12 zu untersuchen, sowie die protektiven Eigenschaften von diversen Hilfsstoffen zu ermitteln. Der tatsächliche Einfluss wird anhand der Fähigkeit zur Koloniebildung auf Agar-Platten sowie einer Fluoreszenzfärbung und anschließender Auswertung unter Verwendung eines Microplatereader beziehungsweise eines Flowcytometers belegt.

In einer ersten Versuchsreihe (Hitzestress) wurden die Bifidobacterien hinsichtlich ihrer Reaktion auf steigende Temperatur getestet, um anschließend sprühgetrocknet zu werden.
In einer zweiten Versuchsreihe (Sprühtrocknung) wurden Arabischer Gummi, Gelatine, Maltodextrin und Magermilchpulver hinsichtlich ihrer protektive Eigenschaften während des Spühvorganges untersucht.

Während der Hitzestress-Untersuchungen wurde vor allem Wert auf den Zustand der Zellmembran, die Wasserstoffproduktion, die Esteraseaktivität sowie auf die Vermehrungsfähigkeit gelegt.
Die Vermehrungsfähigkeit wurde anhand von koloniebildenden Einheiten auf Agar-Platten ermittelt, während die Zellzustände mittels fluoreszierenden Farbstoffen bestimmt wurden.

Allumfassend lässt sich sagen, dass sich mit steigender Temperatur die Zeit in der die Zellen noch in der Lage sind sich zu erholen, erwartungsgemäß sinkt. Während bei 60°C nach 2 Minuten Belastung noch kein Unterschied zu frischen Zellen bemerkbar ist, fällt die Hitzetoleranz bei 90°C auf 30 Sekunden.

In Anbetracht der Erkenntnisse der ersten Versuchsreihe, war es besonders wichtig die optimalen Sprühtrocknungsparameter festzulegen. Die besten Ergebnisse lieferte die Mischung aus möglichst tiefer Einsprühtemperatur (115°C), einer moderaten Sprührate (1,5g/min) sowie einer maximalen Auslasstemperatur von (70°C). Selbst unter diesen optimierten Parametern, führte der Sprühtrocknungsprozess allerdings immer noch zu einer Reduktion der Kolonie bildenden Einheiten (CFU/ml) um nahezu 3 Zehnerpotenzen. Nach Festlegung der Parameter, wurden die gewählten Protektanten zu 10%, 50% und 100% (bezogen auf die Feuchtmasse der Mikroorganismen) vor dem Sprühvorgang der Sprühlösung zugesetzt. Anschließend wurde die Vermehrungsfähigkeit, die Membranintegrität, die Wasserstoffproduktion und die Esteraseaktivität, sowie der intrazelluläre pH Wert und das Membranpotential ermittelt.

Im Vergleich zu den Werten von ungeschützten sprühgetrockneten Zellen, zeigten Arabischer Gummi und Gelatine im Hinblick auf Vermehrungsfähigkeit und zellulärer Aktivitäten, die besten protektiven Effekte. Magermilchpulver zeigte wohl positive Auswirkungen im Bereich der Membranintegrität und des intrazellulären pH-Wertes, nicht jedoch beim Membranpotential. Im Vergleich dazu zeigt der Einsatz von 100% Maltodextrin, nur bei der Superoxidproduktion und der Vermehrungsfähigkeit, deutliche Vorteile zur ungeschützten Zellen.
Die beste Vermehrungsfähigkeit die in dieser Studie mit einer CFU von $3x10^9$ CFU/ml nach der Sprühtrocknung erreicht wurde, konnte bei Zusatz von 10% arabisches Gummi erzielt werden. Dieser Wert hebt sich deutlich von dem Vergleichswert der ungeschützten Zellen mit $5x10^7$ CFU/ml ab, und zeigt, dass eine Verbesserung der Vermehrungsfähigkeit um fast 2 Zehnerpotenzen erreicht werden konnte.

Zusammenfassend lässt sich sagen, dass *B. bifidum* Bb12 einen besonders hitzeempfindlichen Mikroorganismus darstellt. Um die Schäden während der Sprühtrocknung gering zu halten, sollte *B. bifidum* Bb12 daher unbedingt geschützt werden.

7 Abbreviations

B. *bifidum* 12 (Bb12)	Bifidobacterium bifidum 12
°C	degree celsius
µl	microliter
CFDA-SE	carboxy fluorescein diacetate succinimidyl ester
cfu	colony forming units
DHR 123	dihydrorhodamin 123
DioC6(3)	3,3´dihexyloxacarbocyanine iodide
DMSO	dimethyl sulfoxide
FDA	Fluorescein diacetate
g/l	gram per liter
h	hour
l	liter
min	minute
ml	milliliter
mw	molecular weight
nm	nanometer
PI	propidium iodide
RCA	reinforced clostridium agar
RCM	reinforced clostridium medium
rpm	rotations per minute
sec	seconds

8 Figures

9 Tables

10 References

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