FLOW CYTOMETRY, FLUORESCENT PROBES, AND FLASHING BACTERIA

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FLOW CYTOMETRY, FLUORESCENT PROBES, AND FLASHING BACTERIA

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Abstract

In food industry there is a perceived need for rapid methods for detection and viability assessment of microbes. Fluorescent staining and flow cytometry provide excellent tools for microbial analysis. This thesis describes fluorescent techniques for assessment of the physiological state of lactic acid bacteria.

Lysis of lactic acid bacteria plays a crucial role in cheese manufacturing. It is generally considered that lysis results in leakage of intracellular enzymes in the cheese curd and, thus, plays an important role in ripening and flavor formation. *Bac*Light (Molecular Probes) was applied for monitoring the lysis process of *Lactococcus lactis* MG1363 in a buffered suspension with high osmolarity to mimic cheese conditions. The *Bac*Light kit combines the nucleic acid dyes propidium iodide (PI) and SYTO 9. PI is commonly used to determine membrane integrity based on dye exclusion. When used in combination with the permeant SYTO 9, membrane-damaged cells are stained by PI (red) while the intact cells are stained by SYTO 9 (green). Lysis was induced with mutanolysin and followed in time using fluorescence microscopy and flow cytometry. Also, enzyme assays and plate counts were performed. The results demonstrated a transient permeable cell status that has a significant role in the lysis process. Furthermore, permeable cells were demonstrated in ripening cheese with confocal scanning laser microcopy and *Bac*Light.

Viability assessment by conventional plate counting requires long incubation times and provides limited information. Flow cytometric assessment of the viability of lactic acid bacteria was investigated and compared with plate counts. The esterase substrate carboxyfluorescein diacetate (cFDA) and the impermeant nucleic acid dyes PI and TOTO-1 were tested using exponential phase at 70°C heat-killed cultures of a *Lactococcus*, a *Streptococcus*, three *Lactobacillus*, two *Leuconostoc*, an *Enterococcus*, and a *Pediococcus* species. The combination of cFDA and TOTO-1 gave the best results. The intact and membrane-damaged subpopulations were distinguished well. Sorting and plating showed that cFDA stained the culturable and TOTO-1 the nonculturable cells. The assay was applied to cultures exposed to deconjugated bile salts or to hydrochloric acid and results corresponded well with plate counts.

Subsequently, flow cytometry with cFDA and TOTO-1 staining was applied to *Lactobacillus plantarum* WCFS 1 suspended in milk. To facilitate flow cytometry clearing of the milk was required. A procedure based on a milk clearing solution was optimized to increase the signal-to-noise-ratio and flow cytometry enumerations were accurate to a lower limit of 10^5 cells/ml.

Finally, the novel assay was applied to starter cultures for cheese and yogurt and to the probiotic products Yakult, Mona Vifit, and Orthiflorplus. Flow cytometry in combination with plate counts revealed three populations: culturable cells, cells that are intact and metabolically active but not culturable, and permeabilized cells. The proportions of the populations differed between the tested products.

In conclusion, the development of flow cytometry for bacteria is an important asset for microbiological research. The rapid novel methods described in this thesis provide possibilities for examination of fermentation processes and food products.

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General Introduction

SCOPE

Job applicants get a physical examination before they are taken on. Thereby the employer is assured that the worker is in good physical health and is likely to perform the tasks of the job well. Many industrial companies rely, however, not only on the performance of human workers. The activity of microbes is also crucial in many industrial processes! Therefore, great effort is spent in the selection of suitable microbes and optimal process conditions, and in fundamental studies of how microbes function. This thesis describes the use of fluorescent dyes and flow cytometry for detection and physiological examination of microbes. Moreover, it describes the development of novel methods for assessment of functional physiology and the applications to lactic acid bacteria in pure culture, fermentation processes, and in food products.

Lactic acid bacteria play an important role in many food and feed fermentations, such as the production of cheeses, yogurt, creams, wine, sauerkraut, pickles and silage. Lactic acid bacteria acidify the food by conversion of sugars to lactic acid. This inhibits growth of spoilage microbes and thus conserves the product. In addition lactic acid bacteria give specific flavor and texture characteristics to fermented products. Some lactic acid bacteria are supposed to provide certain health effects beyond inherent basic nutrition (probiotics). A number of food products and food supplements that contain probiotic bacteria can be found on the market place.

The development of flow cytometry for microbes has been an important asset for microbiological research. With flow cytometry multiple parameters of thousands of individual (fluorescently stained) cells can be measured within a few minutes while the cells pass a focussed light beam in a fluid stream. Flow cytometry can be regarded as a tool to perform population studies on microbial communities. The population statistics show not only the average values but also the heterogeneity, and it can reveal the presence of subpopulations. Flow cytometry provides information that can not be obtained by conventional plate counts and biochemical assays.

The conventional methods that are commonly applied to assess fermentation capacity of dairy starters are the plate count technique and acidification assays. However, these methods require long incubation times and provide limited information. In food industry there is need for rapid novel methods. Fluorescent staining combined with flow cytometry provides an excellent tool for microbial analysis. This should be applicable in dairy research to examine starter cultures and dairy products, as well as fermentation processes. The aim of the PhD project was to develop fluorescent methods to assess viability of lactic acid bacteria.

OUTLINE OF THE THESIS

This chapter, the general introduction, is written for everybody with a scientific interest. It introduces the wonderful world of microbes and the fundamental problem inherent with the study of microbes: heterogeneity amongst individuals of the population. It focuses on flow cytometry, an important tool for the analysis of individual cells. This chapter narrates some historical aspects of the development of flow cytometry, gives theoretical background of fluorescence, and explains how a flow cytometer works.

Chapter 2 is a review of current and future applications of fluorescent staining and flow cytometry in food microbiology. The concepts of microbial viability and physiological states are discussed and an overview is given of physiological functions that can be assessed with fluorescent probes. Next, approaches for detection of specific microbes are described. Furthermore, Chapter 2 summarizes reported applications of fluorescence staining of microbes in drinks and foodstuffs, and presents an outlook to the future.

Chapters 3 to 6 describe the experimental research of the PhD project. Chapter 3 describes the application of the probes carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) as indicators for culture viability and activity. The subject of Chapter 4 is the monitoring of permeabilization and lysis of cheese starter bacteria with fluorescent methods. Chapter 5 deals with the flow cytometric viability assessment of individual cells and describes a novel combination of viability probes, cFDA and TOTO-1 iodide, which makes an excellent live/dead assay. Chapter 6 shows the application of the viability assay with cFDA and TOTO to milk samples, involving a milk clearing procedure, and presents flow cytometry results of dairy starters and probiotic products obtained with this assay.

Chapter 7 summarizes the research and discusses the potential of the developed methods for application in microbiological research and in food industry practice. Lastly, in Chapter 8 the background, aims, and results of this PhD research are summarized in Dutch.

THE WORLD OF MICROBES

Microbes are everywhere

Microbes or microorganisms are, literally, small organisms. Most microorganisms are visible only with a microscope. The major groups of microorganisms are protozoa, algae, fungi, and bacteria. Protozoa, algae, and fungi are eukaryotes. Protozoa are unicellular, algae and fungi may be unicellular or multicellular, and some can be actually very big. Unlike the other groups, bacteria are prokaryotes, lacking nuclear membrane and cell organelles. Bacteria are unicellular, though may appear in pairs, chains or clusters. Bacteria are very small; their cell size is usually between 0.5 and 5 μm.

Microbes are virtually omnipresent. There may be billions of them in just a teaspoonful of fertile soil. Microbes inhabit all aquatic environments. Air currents carry them to the upper atmosphere, and to other continents. Animals carry large populations of microbes on their body surfaces, in the intestinal tract, and in their body openings. The human body contains approximately 100 trillion (10^{14}) bacteria, thereby outnumbering 10 times our own body cells.

There is a huge genetic variety among microbes and they are diverse in their nutritional requirements and in the biochemical reactions they perform.

Good and bad

Plant and animal life depend on microbes. First of all because microbes play a key role in recycling of elements in nature. Degradation or decomposition of organic waste brings the chemical elements back in a form that can be used by plants. Furthermore, in aquatic ecosystems algae and cyanobacteria are the principal photosynthetic organisms. These microbes (and not the rainforest) are the main producers of oxygen worldwide. Also, microbes are at the bottom of the food chain (plankton). A number of microbes live in symbiosis with plants, or animals or each other. Well known is the symbiotic nitrogen fixation by *Rhizobium* bacteria that grow in nodules attached to the roots of legumes. Bacteria in the intestines of herbivores help in the digestion of plant material. Lichens are composite microorganisms consisting of an alga or cyanobacterium growing in intimate association with a fungus.

Some microbial species are of direct use to us, humans. These good guys include microbes used in food fermentation, production of pharmaceuticals, and other industrial processes. The industrial use of microbes will be described in more detail in the next paragraphs. Other microbes are attractive because we can eat them. These include fungi that give edible fruiting bodies, such as *Marasmius oreades* (champignon) and *Cantharellus cibarius* (chanterelle), and edible algae, such as seaweed (nori) used to make maki sushi.

Of the thousands of species of microbes there are relatively few which are pathogenic to humans. These bad bugs take various ways of transport. Some are foodborne, such as *Salmonella* Typhimurium, *Staphylococcus aureus* and *Bacillus cereus*, which can cause food infection or food poisoning. Others are arthropod-borne, such as *Yersinia pestis,* a bacterium that can be transmitted by infected fleas and that caused immense pandemics of the plague in the Middle Ages. Others again are transmitted by direct contact, such as *Bacillus anthracis*. It can infect people through breaks in the skin

or when inhaled as dust and causes the highly mortal anthrax. Either in a good or bad way, microbes have a key role in the complex world of life.

Industrial use of microorganisms

Many species of microorganisms are used commercially because they are able to transform substrates into valuable substances or because they can transform wastes into harmless substances. Important industrial products from microbial dissimilation are alcohol, lactic acid, and acetic acid. The metabolic productions are integral processes in production of alcoholic beverages, fermented dairy products, and vinegar. Products from biosynthetic activities of microorganisms include antibiotics, enzymes, peptides, and amino acids. Pharmaceuticals such as insulin, human growth hormone and interferons are now produced by genetically engineered microorganisms. The conversion of waste material by microorganisms is applied to purify wastewater, to clean up oil spills, and to remove pollutants from soil. These processes depend on specific characteristics of the microorganisms involved.

Lactic acid bacteria

Lactic acid bacteria are characterized by the metabolic capacity to convert sugars to lactic acid. Various lactococci, lactobacilli, streptococci, leuconostocs, and pediococci play an essential role in diverse food fermentations. Think of the wide range of fermented cheeses, milks, breads, wines, pickles and meats to recognize the valuable contribution of lactic acid bacteria to our daily diet. In addition, some lactic acid bacteria are supposed to provide certain health affects beyond inherent basic nutrition (probiotics). This has lead to the introduction of new functional foods with lactic acid bacteria as the functional ingredient. These features explain the major economic importance of lactic acid bacteria for the food industry, and for the dairy industry in particular.

MICROBIAL HETEROGENEITY ASKS FOR SINGLE-CELL ANALYSIS

The size of a sample

For industrial applications of microorganisms a great deal of effort is spent in selection of species and strains, as well as in optimization of the growth and production conditions. Furthermore, with molecular biological tools microbes can be genetically modified for better performance or for introduction of new properties. Performance testing involves measurements of enzyme activities and metabolite concentrations of the microorganism under study. Let us compare this with a physiological measurement of, for example, a human. A biochemical assay needs a certain amount of material for the measurement. For example, to measure blood sugar content one drop of blood is used. The result gives information about the condition of the sampled individual. Microbes are simply too small to be sampled, so a part of the population is taken to be the sample. For example, to measure ATP content in cheese starter bacteria a sample is used that contains millions of cells. The result gives the total or average ATP content. By this approach, the physiological condition of microorganisms is measured as a group phenomenon, with no information given about the individuals.

Population versus individual

The measurement of the group as one entity is sometimes valid from a practical point of view. An example from dairy practice is the monitoring of a fermentation process by measurement of the pH decrease in the product. By sampling the fermentation culture this is measured as a group phenomenon. Another example is the spoilage of food by putrefaction. When a food product is spoiled by proteolytic degradation, the concentration of protein degradation products, such as ammonia and hydrogen sulfide, has become too high. The spoilage is established as a state of the food product, as a consequence of the total activity of the microbes.

However, a microbial community is not one entity, nor a homogeneous group of cells all doing the same thing at the same time. It is a heterogeneous collection of individual organisms. This may not be surprising for a natural microbial community since that is an ecosystem formed by various species. But it is also true for a laboratory culture of one strain. In the examples mentioned above microbial processes are studied just by measuring their over-all effect on the environment. But if one wants to get insight into the microbial physiology, one should be aware of the heterogeneity. Consider a physiological study in which one wants to establish the relationship between a metabolic property of interest and the viability (judged by the capacity to form a colony). Take, for example, a study of lactase activity of *Lactococcus* under acid stress. If in a culture half of the activity is lost under stress, this can be because all cells have lost half of their activity, or because half of the cells have lost all of their activity, or a combination in between these two extremes. With the enzyme activity being measured as a property of the culture and survival as a property of individual cells one can not define the relation. However, if both properties were measured on individual cells it would be possible to observe whether the two are related and what would be the minimum activity for maintaining viability.

Causes of heterogeneity

Next to the obvious heterogeneity in a mixed microbial community there are three other sources of heterogeneity, which are also relevant for axenic laboratory cultures. Firstly, heterogeneity can be genotypic through mutations. A pure culture is derived form one cell and with each cell division there is a certain chance on mutations. Bacteria can multiply fast and with each generation the genetic variability in the culture may increase. Secondly, heterogeneity can be phenotypic by progression through the cell cycle. The expression of particular proteins depends on the phase of the cell cycle and microbial performance depends on the distribution of cells throughout the cell cycle. Thirdly, heterogeneity can be phenotypic by changes in place and time. Microbial activities are modulated by the local environment of a cell and its history.

Statistics of heterogeneity

Besides giving mechanistic explanations, we can also look at heterogeneity from a statistical point of view. A quantitative characteristic of a (micro-) biological population, such as size, DNA content, or enzyme activity, is often observed to have a normal distribution. In a normally distributed population only 0.9545 (95.45%) of the population falls, by definition, within the range of the mean +/- 2 times the standard deviation and the rest has a value higher or lower than that. The central 95% are often taken in clinical and other research as the reference or normal value range. Thus, for a number of n independent quantitative attributes 0.9545^n individuals will fall within the reference range of standard biochemical criteria. The more quantitative attributes are taken into account, the fewer individuals will be regarded as "normal".

Single-cell analysis

Given the considerable degree of heterogeneity in a microbial culture, it is clear that we can not understand the physiology by treating a culture as one entity or as an ensemble of identical cells. Measuring only the mean value does not define a physiological property of a culture very well. One should define the distribution of a physiological property throughout the population. Evidently, this asks for measurement of many individual cells. This will give fuller and more accurate information about the physiology of a culture.

Flow cytometry is the obvious tool for single-cell analysis of large numbers of cells. Flow cytometry measures thousands of cells in a few minutes. It is an optical, purely quantitative, technique, which measures scattered light and fluorescence properties of individual cells. By using appropriate fluorescent probes specific assets of cell physiology can be studied. Flow cytometry is inherently a population statistical approach and it allows for multiparametrical analysis.

It is important to realize that the term 'single-cell analysis', as used in this thesis, does not indicate that it concerns the analysis of one single cell. Single-cell analysis, as by flow cytometry, is actually the analysis of many individual cells as a random sample survey for a population study. And a population should be seen as a heterogeneous collection of individuals rather than one entity.

HISTORIC HIGHLIGHTS

This part gives some historic background on developments towards the flow cytometry technology we have today. The starting point is the observation of bacteria, followed by the development of microscopes, in particular the fluorescence microscope. The next subject is the history of staining and of fluorescence. Then, the building of flow cytometry instruments is covered and finally the progress in the use of flow cytometry, with special reference to microbiological applications.

Microbes

Microbiology as a science started with the Dutchman Antonie van Leeuwenhoek (1632-1723), who worked in Delft where he inspected weaving in cloth with the use of magnifying glasses. As a hobby he ground glass lenses and made simple microscopes (Fig. 1) that he used to observe rainwater, pepper infusions, saliva, etc. He saw tiny living creatures not visible to the naked eye, which he called animalcules. Van Leeuwenhoek was not the first person to make such observations, but his detailed descriptions have made him famous as the discoverer of microorganisms. He carefully recorded his observations in a series of letters to the British Royal Society and we owe to him the first recorded drawing of microorganisms.

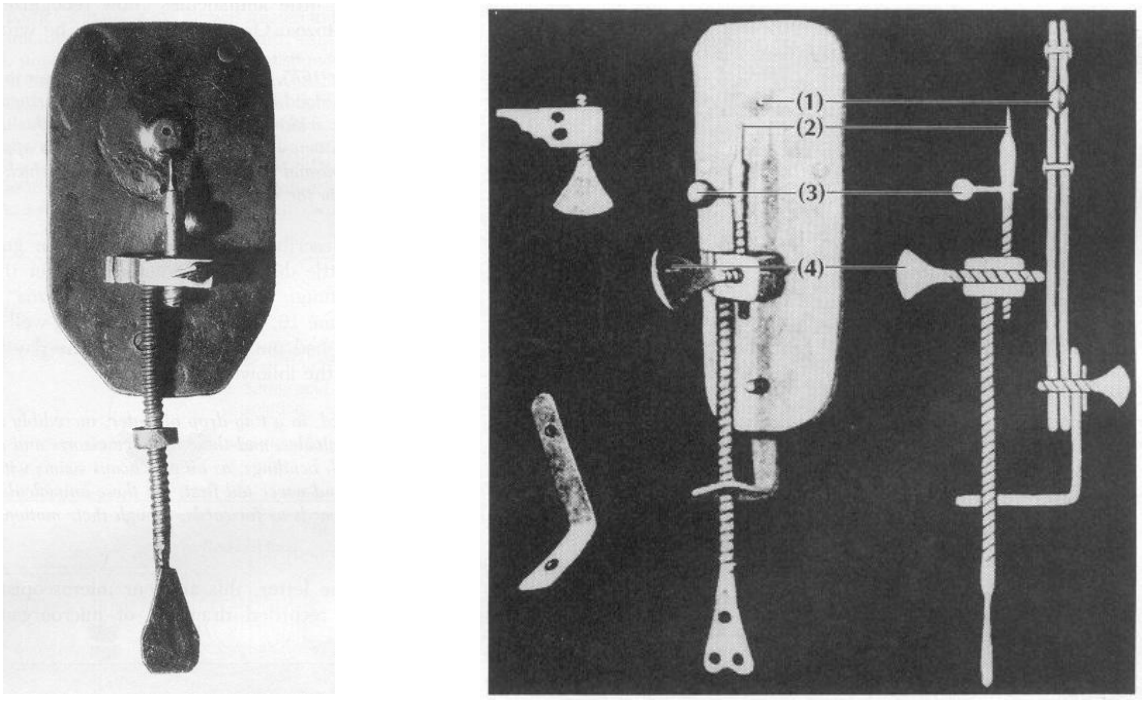

Fig. 1. The Van Leeuwenhoek microscope. Left: replica of a microscope made in 1673 by Van Leeuwenhoek. Right: construction of the Van Leeuwenhoek microscope: (1) lens; (2) pin for placement of specimen; (3, 4) focusing screws (left).

Microscopes

The lenses of Van Leeuwenhoek were of very good quality, the best ones able to magnify an object 200 to 300 times. His simple microscopes consisted of a lens in a holder with a pin for the specimen that could be focussed with screws (Fig. 1). Another design that also already existed at that time is the compound microscope, first built in 1590. A compound microscope has an objective lens that gives a magnified image of the specimen, and a second lens, the ocular, which gives further magnification of the image. Compound microscopes were easier to produce than single-lens microscopes.

Around 1900 Carl Zeiss Works in Germany introduced many refinements of optical design and technique now associated with modern microscopes. The theory of microscopy of Ernst Abbe teaches us that resolution improves at shorter wavelength (1). In an effort to resolve finer detail, transmitted light microscopes were built with ultraviolet light sources and quartz optics. These microscopes required photography rather than direct observation, since the human eye can not perceive ultraviolet light. August Köhler of Zeiss constructed the first UV microscope (32). When he examined biological tissues he saw fluorescence, and he recognized immediately that it would be possible to make practical use of this. Thus, fluorescence microscopy had arisen, as a by-product of the search for increasing resolution.

The construction of specialized fluorescence microscopes started first with use of a cadmium spark as light source, soon to be abandoned in favor of the carbon arc, which gives more intense light. The carbon arc was applied in combination with a set of three filters that transmitted only light in the range of 280-400 nm. Around 1911 the companies Carl Zeiss and Reichert had started production of commercial fluorescence microscopes, soon joined by Leitz. A carbon arc lamp is not the most ideal light source. It requires a high current (10-20 ampere), gives insufficient intensity, needs continual adjustment of the electrodes because they wear away, and produces unpleasant fumes. Other lamps were tried including the high-pressure mercury arc, which was first used in 1937. This light source has become the most common lamp in fluorescence microscopes.

Staining

Dyes

A stain or dye is a compound that imparts a color to the substance to which it is applied. People have used dyes since ancient times to paint their bodies and to color their clothes. Dyes were obtained as crude extracts from plants or animals. A well-known example is indigo, a deep violet blue dyestuff extracted from indigo plants, especially the tropical shrub *Indigofera tinctoria*. Two other long-known natural dyes are cochineal and logwood. These dyes also found application in histology in the eighteenth and nineteenth century (7). Cochineal consists of dried bodies of the females of the insect *Dactylopius coccus* that are cultivated on the Mexican cactus *Opuntia ficus-indica*. It contains the rich purplish red colorant carmine. Logwood (*Haematoxylon campechianum*) is a Central American tree. Its heavy reddish wood contains colorless hematoxylin that oxidizes to the red-brownish hematein (not structurally similar to hematin of red blood cells!). From the 1850's on dye synthesis by organic

chemistry developed. This has increased the number of available compounds and applications, and has replaced uses of scarce and expensive dyes from nature to compounds produced by chemical industry.

Early applications in histology

The pioneers in histological staining only had natural dyes to their disposal. Antonie van Leeuwenhoek was probably the first person who deliberately stained a biological object for microscopic study (7). In 1719 he reported the use of a solution of stigmas of *Crocus sativa* (saffron) to elucidate microscopic structure of almost transparent muscle tissue. For his observations of bacteria, however, he did not use stains. Staining of bacteria was not reported until after 1870 (7). By that time various staining solutions, mostly made from cochineal or logwood, had been applied with more or less success on (tissues of) animals and plants. Carmine had been applied, for example, to observe the division of the cell nucleus in onion. Also, it had led to the discovery of intracellular granules in algae, now known as chlorophyll. Another application was the study of transport streams, by injection of dye into blood vessels of animals or vessels of plants and observation of what parts became colored.

Staining of bacteria

When systematic study of bacteria started, around 1870, staining was soon implied in the studies (7). By then the researchers could use not only the natural dyes, but also some newly synthesized dyes. Aniline dyes, for example, were first extracted from indigo, but could be manufactured now cheaply and in new varieties from coal tar. Early successes of staining of bacteria were that of the pathologist Weigert. With carmine he could differentiate bacteria in tissue, for example the bacteria in smallpox crusts, and the anthrax bacteria in organ sections of dogs with anthrax. Bacteria in liquids could also be recognized by staining but their trembling or independent motility and low refractivity made examination difficult. Koch found that these difficulties could be avoided by drying a bacterial suspension in a very thin layer on the cover glass. After fixation on the glass upon the cells could be stained, washed and observed (31).

In 1881 Ehrlich, who worked with Koch, was the first to employ methylene blue as a bacterial stain (16). At that time Koch was working on the tubercle organisms and he applied alkaline methylene blue to stain this organism in tissues. In 1882 Ehrlich introduced the principle of adding aniline water to a solution of a basic dye. He observed that when a preparation stained with methyl violet in aniline water is washed with strong hydrochloric acid, the tissue and other bacteria decolorized while the tubercle organisms kept the staining color. Ehrlich was the first to realize the important difference between acid and basic dyes for staining purposes and his work has been very important to staining technology (7, 48).

The discovery of fluorescence

Fluorescence, as a macroscopic phenomenon, appears to have been known already in the sixteenth century. Wood from the tree *Eysenhardtia polystachya*, exported from the New World to Europe, attracted attention because it had apparent medical virtues, it had an unusual yellow color, and it gave

blue light by infusion in water. The report of this phenomenon by the Spanish physician and botanist Nicolas Monardes in 1577 is probably the first record of fluorescence (36).

In the 1830's this phenomenon was described for fluorite (CaF_2), quinine and chlorophyll. It was Stokes in 1852 who gave this phenomenon the name 'fluorescence', after fluorite in analogy to opalescence (the typical play of colors as produced by an opal). He did that in a publication that described his observation of 'refrangible radiations' from biological materials, such as paper, bones, horn, cork and cotton, for which he had used sunlight as a source of UV, a liquid excitation filter of cuprammonium and a barrier filter of potassium dichromate (55).

Fluorescent dyes

Organic chemistry brought many new compounds, including fluorescent ones. In 1971 Adolf von Baeyer synthesized a new compound from phthalic anhydride that was found to be very fluorescent, and it was called fluorescein (48). Ehrlich employed this newly synthesized compound shortly after to study the dynamics of ocular fluids.

After Ehrlich's lifetime, fluorescence microscopy set the stage for great advantages in analytical cytology, as well as in microbiology. At first it was used to study autofluorescence of various plant tissues, animal tissues, and microbiological material. Later, people searched for fluorescent stains (fluorochromes) suitable for histological staining, analogous to colored stains (diachromes), but visible with fluorescence microscopy (7). Particularly acridines were found to be very useful. Like fluoresceines, acridines were suitable for vital staining. This is staining which does not require fixation of the cells and which is not lethally toxic to the cells.

Acridines

Acridine was first found as a side product of the production of magenta out of coal tar in 1870 (47). A few years after, several orange and yellow acridines were produced for the dye market. There was also medical interest in these substances. Antimicrobial action of proflavine and acriflavine was discovered in 1913 and quinacrine, the first synthetic antimalarial drug that was as good as quinine, was discovered in 1930. Histologists found acridines to stain cell nuclei and after 1940 especially acridine orange became widely used for this purpose. Acridine orange is a so-called metachromic fluorochrome. It fluoresces green when bound to DNA in living cells, but it fluoresces red when bound to denatured DNA or RNA. The metachromasia of acridine orange is explained by polymerization *in situ* when two dye molecules are very close together, which occurs in RNA and denatured DNA (47). This vital stain has many applications, for example in clinical cytology where acridine orange has been used to distinguish leukocytes and erythrocytes. Also, acridines have been used for chromosome banding.

Further developments

Besides fluoresceines and acridines there are now many more fluorochromes available for staining of cells or cell components such as nucleic acid, mitochondria and cell wall. Also, fluorescent probes have been developed for cell functions such as enzyme activities, transporter activities, intracellular

pH, calcium concentration, and membrane potential (23, 35). The next phase after fluorochromy was the development of immunofluorescence, from the 1940's on (48). In immunofluorescence, antibodies tagged with a dye are used to measure the presence of antigens. Another application is to use a fluorescent dye as a tag to oligonucleotide probes (3). A recent development is the use of genetic engineering to make fluorescent mutants. This was initialized by the cloning of the gene for the Green Fluorescent Protein (GFP) from the jellyfish *Aequorea victoria* in 1992, and subsequent expression in other organisms, and it has proven to be an excellent tool for studies of gene expression, protein localization, and many other purposes (56, 58). The number of dyes and applications still increases. Molecular Probes, the world's leading supplier of fluorescent probes, sells now more than 2,500 different products (23).

Flow cytometry instruments

Flow cytometer ancestors

Flow cytometry technology started in the 1920's and 1930's when the ancestors of modern flow cytometers were built for analysis of flowing colloidal suspensions and for detection, counting, and sizing of particles in aerosols such as mine dust. In the 1940's, during World War II, the detection of bacterial cells in aerosols was accomplished by Gucker and coworkers (17, 21, 22). It was the first flow cytometric detection of a biological specimen. The work was sponsored by the U.S. Army with the aim of rapid identification of airborne bacteria and spores used as biological warfare agents. The instrument used a sheath of filtered air to confine an air sample stream to the center of the flow chamber. The air sample stream was subjected to dark-field illumination by a Ford headlight, which was the most powerful light source then known. A photomultiplier tube was introduced as a detector.

Early developments

The principle of flow cytometry was taken up for development of a clinical application: the counting of blood cells. Until the 1950's the counting of blood cells could only be done by visual observations. Countings of erythrocytes (red cells), leukocytes (white cells) and thrombocytes (platelets) were done with a microscope using a counting chamber called a hemocytometer. The hemocytometer counts were rather imprecise, which made accurate diagnosis of anemias difficult. Thus, there was a perceived need for instruments that could count with a higher precision. Based on the sheath flow principle of the earlier aerosol counter, Crosland-Taylor developed a flow system for counting blood cells detected with light scattering and dark-field illumination (10). Several companies in Germany, England, and the United States attempted to build such apparatus in the 1950's.

Wallace Coulter also developed a flow instrument for blood cells, but he explored another means of detection (9). He developed a blood cell counter based on the fact that the electrical conductivity of cells is lower than that of saline solutions. Thus, cells could be counted when passing one at a time through a small orifice. The cell size is also measured because it relates to the amplitude of the signal. Coulter Counters are still widely used for simple enumeration of cells in a suspension, including bacteria. However, flow cytometry with optical techniques exceeds the limits of the electrical

technique of the Coulter Counter. The orifice of a Coulter Counter is easily blocked and limits the dynamic range in terms of size. In optical flow cytometers cell clumping and blocking are much less of a problem, the light scatter is largely independent of cell orientation and the dynamic range is much larger. Moreover, fluorescent probes can be used for actual analytical single-cell analysis, which is the power of flow cytometry.

The first real flow cytometer was built by Kamentsky at IBM, using spectrophotometric techniques to measure absorption of cells in a flow stream (30). To verify the instrument's performance he introduced a cell sorter based on a syringe pump (29). Around the same time Fulwyler developed a cell sorter that used electrostatic deflection of charged droplets (19), based on the principle of the ink jet printer developed at that time.

Commercial flow cytometers

From the 1970's on commercial flow cytometers have been produced (48). The first machines were the Impulscytophotometer (ICP) of Phywe AG in Göttingen, which was built around a Zeiss fluorescence microscope, the Cytograf and Cytofluorograf of Kamentsky's newly founded company Bio/Physics Systems, the Two Parameter Sorter of Coulter Electronics, and the Fluorescence Activated Cell Sorter (FACS) of Becton-Dickinson.

A microscope-based flow cytometer for bacteria

A revolutionary new design was introduced by Steen and Lindmo in 1979 (54). Their basic principle was that "a flow cytometer is essentially a fluorescence microscope with cells flowing through the focus" (51). Starting with a fluorescence microscope, their main problem was to run the cells in a narrow stream through the focus. The solution that they came up with was to use hydrodynamic focussing in stead of a stream in a capillary flow cell or in air. A pressurized water jet around the outlet of the sample tube forms a laminar stream with the sample stream confined to a narrow and stable section in the center of the flow. In their flow cytometer design the stream hits a glass coverslip at a low angle and passes through a focus of exciting light from a mercury arc lamp through microscope optics epi-illumination. With this system the measurement of fluorescence and light scattering at small and large angle was sufficiently sensitive to characterize bacteria (53). The system was commercialized as the Skatron Argus and later the Bio-Rad Bryte HS, but has now disappeared from the market place. The principle of hydrodynamic focussing, though, has been adopted for laser-based flow cytometers designed with horizontal flow cells.

Later developments

Other developments in the 1970's were the incorporation of mini-computers, the addition of a second fluorescent parameter, dual-wavelength excitation, and the use of powerful lasers, which needed to be water-cooled. These systems took a lot of space for all the equipment and were operated in darkened rooms. Better optical design made it possible to use smaller, air-cooled, lasers or arc lamps. In the 1980's the bench-top flow cytometers came up, such as Beckton-Dickinson's FACS Analyzer and Coulter's EPICS C. The flow cytometers of this new generation were smaller in size and price, and

became user-friendly because instrument control and data acquisition and analysis were computerized. Technical improvements made mainstream instruments also useful for measurements on bacteria.

Flow cytometry is generally a research or routine laboratory technique, but not necessarily. For microbiological measurements 'in the field' flow cytometers in cars or on-board of ships are used. For even more flexibility, the Microcyte flow cytometer, a portable instrument for microorganisms, was developed (see (13)). This is makes flow cytometry a tool which, in principle, can be used anytime and anywhere. Furthermore, there are automated flow cytometers for routine laboratory analysis in specific applications, such as the UF 100 for clinical testing of particles, including bacteria, in urine samples (14), the Bactoscan FC (Foss Electric, Hillerød, Denmark) for total bacterial counts in milk, and the Chemflow (Chemunex, Maisons-Alfort, France) which has various applications in food and pharmaceutical industry.

Flow cytometry applications

The clinical drive

The driving force of flow cytometry development was the belief in its clinical use. From the 1970's on flow cytometry has been used for the measurement of DNA content for diagnosis of cancer and leukemia, and for determination of effects of drugs on tumor cell proliferation kinetics. Furthermore, flow cytometry has been applied for differential leukocyte counts. And since monoclonal antibodies became available by the 1980's flow cytometry has been applied for the detection of CD (Cluster of Differentiation) Antigens on T-cells and other cell types. Antigen detection has been used for applications such as the evaluation of autoimmune diseases, matching transplantation donor and acceptor, and predicting the course of HIV infection.

Early applications to microorganism

Gucker already recognized the potential of flow cytometry for microbiology in 1947 as he wrote "The principle (of flow cytometry) should have wide application in ... bacteriology" (21). However, the applications in microbiology were somewhat limited by the small size and low amounts of DNA and protein of microorganisms compared to mammalian cells. A *Saccharomyces cerevisiae* cell has about 200 times less DNA than a normal diploid human cell, and an *Escherichia coli* cell about 1,400 times less (50). In the 1970's, after improvement of optics technology and development of better fluorescent stains, the first reports on flow cytometry of microorganisms appeared. These early studies concerned the cell cycle of bacteria and yeast, DNA and protein content in microorganisms, autofluorescence of algae, purity of cultures, and even detection of viruses (4, 24, 25, 40).

The breakthrough for bacteria

The real breakthrough in flow cytometry of bacteria came with the flow cytometer of Steen and Lindmo which used hydrodynamic focussing (54). With this system the DNA content of individual bacteria could be measured with an accuracy of a few percent (5, 52). The high signal-to-noise ratio of

this flow cytometer design and the improvements of laser-based instruments made flow cytometry a technique very suitable for measurements on microorganisms.

Increasing range of applications in microbiology

Steen's microscope-based flow cytometer and modifications and optical improvements of the traditional laser-based instruments have led to many different applications in microbiology. These include identification of phytoplankton by DNA content and autofluorescence (57), detection and characterization of bacteria in urine of infected patients by DNA content and base composition (GC percentage) (61), and detection of bacteria in blood by ethidium bromide staining (34). Another line of applications involve immunofluorescence probes for specific detection, for example of *Bacillus anthracis* spores (41), *Legionella pneumophila* (26, 59) and *Listeria monocytogenes* (15). Furthermore, flow cytometry has been used with physiological probes. Examples include the investigation of bacterial cell damage provoked by antibiotics and surfactants (8), study of the sequence of processes during starvation (28), and study of physiological changes during biofilm formation (64). In addition, the role of quorum sensing in bacterial growth has been investigated by flow cytometry (65). Yet another application is the selection of mutants, which was for example used to isolate acid-inducible promoters of *Salmonella* from a promoter library using random gene fusion to a GFP reporter gene (60).

The examples mentioned are just a grasp out of many more. The increasing number of studies over the last two decades has provided a wealth of information. Some information has been brought together in review papers about flow cytometry of bacteria in general (13, 18, 62) and in review papers focussing towards various research fields such as environmental microbiology (42, 43), aquatic microbiology (63), microbial ecology (3), clinical detection of pathogens (2), susceptibility testing (2), viability assays (6, 27, 37, 38), and biotechnology (44). Certainly the developments in fluorescent probe technology, electronic hardware performance, computing power, and methods of artificial intelligence will further increase the potential of flow cytometry, and a continuing rise of applications is anticipated.

Growth in literature

The development of flow cytometry applications is also shown by an analysis of the number of papers (Fig. 2). The first papers on flow cytometry appeared in the late 1970's, including a few on microbes, and each year more papers were published than the year before. In 1980 the International Society for Analytical Chemistry (ISAC) started the publication of *Cytometry*; a journal dedicated to analytical cytology with many articles about flow applications. At the end of the 1980's the PUBMED database contained approximately a hundred papers on flow cytometry of microbes, out of approximately 2000 papers on flow cytometry in total. During the last decade the literature has grown steadily and now, December 2001, the total number of flow cytometry papers in the database has exceeded the 50,000 of which approximately 4,000 (8%) deal with bacteria and fungi.

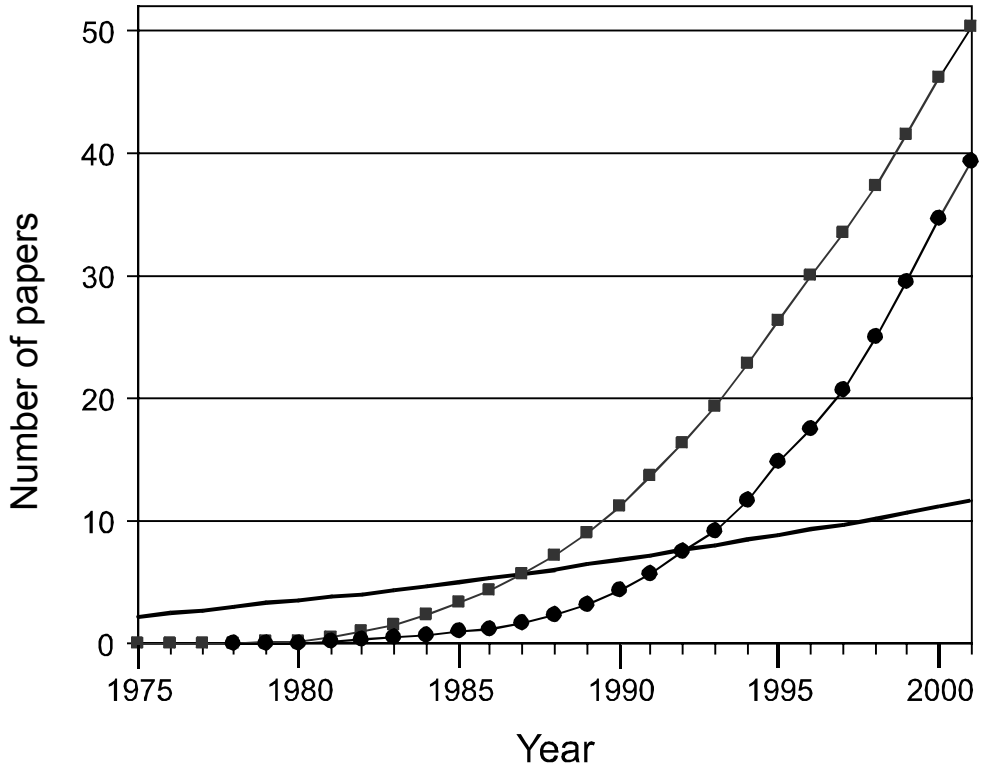

Fig. 2. Bibliometric analysis. The PUBMED database was searched for papers on flow cytometry and its subset on bacteria and fungi. The search terms used were 'flow cytometry' and 'bacteria OR fungi'. The cumulative numbers of the papers on flow cytometry are shown in thousands (squares), and of the subset on microbes in hundreds (circles). The black line shows the total size of the database in millions.

FLUORESCENT PROBES

The physics of fluorescence

Light

Light is a small part of the electromagnetic spectrum (Fig. 3). Electromagnetic radiation has a wave nature as well as a particle nature. It can be pictured to propagate through space in the form of corpuscular radiation, consisting of small packets of energy termed quanta. In the case of light these quanta are named photons. In other words, light is a stream of particles, called photons, each carrying a definite amount of energy but no mass. Wavelength (λ) and frequency (ν) of electromagnetic radiation are inversely proportional, the relation given by the formula $\nu = c / \lambda$, with c being the speed of light (3.0×10^8 m/s). The amount of energy of a photon is $h\nu$, with h being Planc's constant. Photons of a higher energy are perceived as light of a color more towards the UV. More photons are perceived as a higher light intensity.

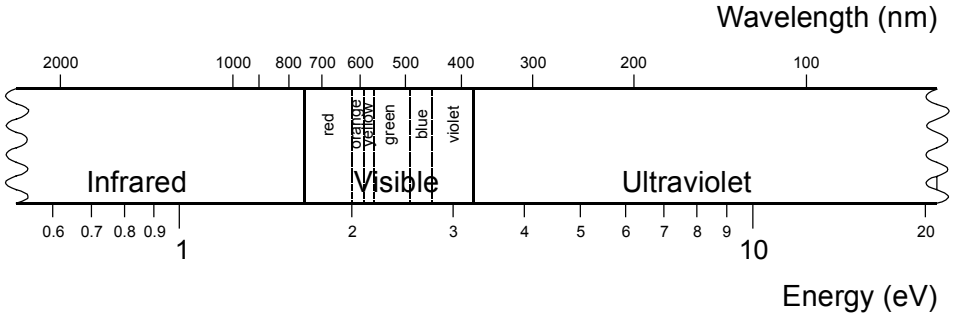

Fig. 3. Small part of the electromagnetic spectrum showing energy and wavelengths of visible light.

Matter

The principle of fluorescence is best explained on the molecular level. Therefore, we start with the nature of the molecule as explained by quantum mechanics. The quantum mechanic theory says that an atom or molecule can only exist in discrete energetic states specific for that atomic element or molecule species. To change from one state into another, and atom or molecule needs to absorb or emit a discrete quantum of energy exactly matching the energy difference between the states. Bohr's model of the atom, which pictures electrons in discrete orbits around the nucleus, is an approximation towards the phenomenon of discretely quantized energy states. In fact, electrons and other subatomic particles exhibit both wave nature and particle nature, and we can not know exact position and momentum at the same time (Heisenberg uncertainty principle). This leads to an atomic model in which the position of an electron is given by a probability distribution that can be depicted as a cloud surrounding the nucleus.

Each electron of an atom has another electronic state. Each spatial orbit can occupy a pair of electrons, provided that they have opposite spin. Molecules consist of two or more atomic nuclei,

surrounded by more or less shared electron orbitals. The electronic state of an atom or molecule is the overall pattern of electrons. In the ground state electrons neatly fill up the electronic energy levels. In an excited state there is at least one unoccupied electronic level below the highest level that is occupied, thus, the molecular electronic energy level is higher than in ground state.

The multiplicity of an electronic state is defined as 2S + 1, with the molecule spin quantum number S being the absolute value of the sum of the electron spin quantum numbers. If there are as many electrons with positive spin as with negative spin, S = 0 and the molecule is in a singlet state. With one unpaired electron it is in a doublet state, and with two unpaired electrons it is in a triplet state. A molecule with an even number of electrons has in ground state the orbitals filled up from the lowest level on and thus all electrons are paired. This state is called S_0. If one electron jumps to the next orbital and keeps the direction of spin, the molecule reaches the first singlet excited state (S_1). If the electron jumps to the next orbital and changes the direction of spin, the molecule reaches the first triplet excited state (T_1).

The total energy level of a molecule is defined by the electronic state, and by the vibrational state of the nuclei, the bond rotation state, and the bond flexion state. All are quantized but the steps are of different scale. Electronic transitions require most energy. Vibrational transitions are smaller, and rotation and flexion transitions are again smaller. To bring a molecule in a higher electronic state an amount of energy between 2 and 20 eV is needed, depending on the molecule and the transition. Higher energy may lead to ionization.

When light and matter meet

When a beam of light meets matter, e.g. a cell, various kinds of interactions take place, as shown in Fig. 4. Part of the incoming light is reflected by the surface (point A). The angle of reflection equals the angle of incidence. Therefore, the rougher the surface is, the less specular (mirrorlike) and the more diffuse reflection will occur. The rest of the incoming light enters the material. Some of the light is scattered inside the material and may eventually exit the material. Some of the light, directly or after being scattered, is absorbed. Absorption of light means that the molecules take up the light energy, and by doing that they transform into a higher energy level. Excited molecules loose the energy again by various processes, including the emission of light. This new light is either fluorescence or phosphorescence (explained later) and it exits the material in all directions, like the scattered light. Some of the light is reflected internally at the other surface (point B), and may then be scattered and absorbed in the material. The residual beam exits the material. The beam of light is usually deflected by some degree when it passes the surface between two different media. This is because light travels at different velocities in different media, which is measured as a difference in refraction index. All these interactions take place to a greater or smaller extent, depending on the characteristics of the material and the wavelength of the light.

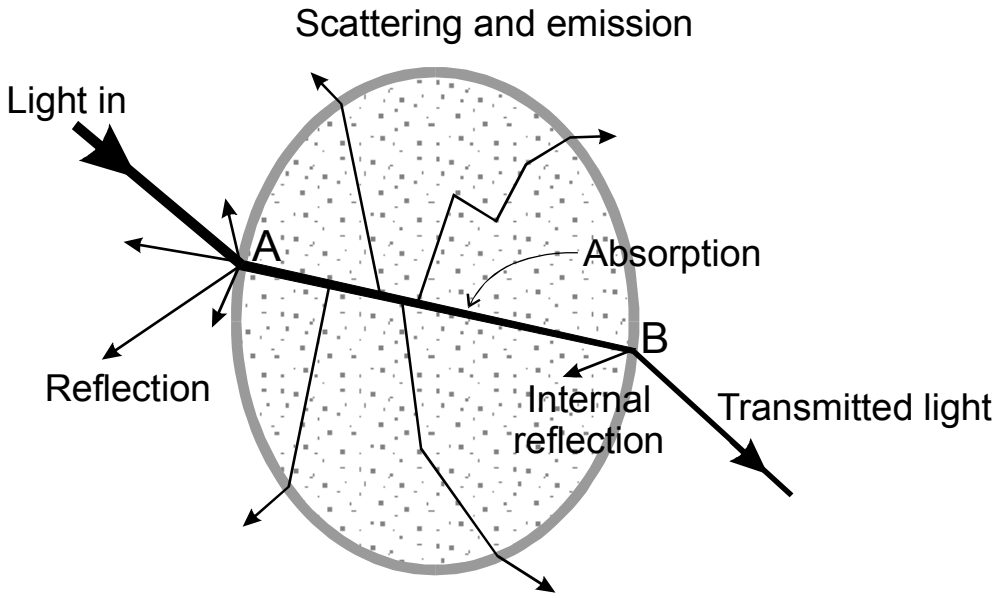

Fig. 4. Interactions of light with matter.

Excitation and emission

A photon of sufficient energy can be absorbed and bring a molecule into an excited state (Fig. 5). The molecule is then in some vibrational level of a higher electronic energy level. This transition is an instantaneous process and the energy of the photon is equal to the difference between the energy level of the excited state and the state before transition (usually the lowest vibrational level of the ground state). The absorption spectrum is the measure of probability that photons of given wavelength, i.e. quantum energy, are being absorbed by the molecule (Fig. 6). An absorption spectrum consists of bands rather than lines because of the possible vibrational energy levels on top of the electronic states.

After excitation the molecule usually undergoes fast (within 10^{-11} s) relaxation to the lowest vibrational energy level of the electric level to which it was brought. The energy is lost by conversion into heat upon collision with other molecules. From this energy level various processes can take place to return the molecule back into the ground state (Fig. 5). Molecules can fall to a lower electronic energy level by internal radiationless conversion. This generates heat and it happens in most molecules in most cases. Alternatively, molecules can loose energy by luminescence (fluorescence or phosphorescence). Excited molecules can also perform a chemical reaction.

Fluorescence is the transition from the first excited singlet state to some vibrational level of the ground state in which the difference in energy is lost by generation of a photon. Fluorescence takes place after a decay time of 10^{-9} to 10^{-8} s. To return finally to the lowest level of the ground state the surplus vibrational energy is converted into heat. The transition of S_2 to S_1 has a much higher probability to occur by radiationless conversion then by fluorescence because the difference in energy level is much smaller than between S_1 to S_0 and conversion is fast (less than 10^{-11} s). Thus fluorescence practically always occurs from S_1, and therefore the emission spectrum is independent of the excitation

energy. The shape of the emission spectrum is usually a mirror image of the lowest energy band of the excitation spectrum and it also consists of a band rather than a line because of the possible vibrational energy levels of the ground state (Fig. 6). The difference between the excitation maximum and emission maximum is called the Stokes shift.

From the lowest level of S_1 an 'alternative route' may be taken to return to the ground state. Energy transition may take place to some vibrational level of the first excited triplet state T_1 (intersystem crossing). It is immediately followed by vibrational relaxation. Transition from the lowest vibrational level of T_1 to some vibrational level of S_0 occurs by emission of a photon and is called phosphorescence. Phosphorescence takes place after a long delay time, 10^{-4} to 1 s, therefore it is less probable to occur than fluorescence.

It should be clear that an energy transition in an excitation or emission process is not a predisposed fate, but that it is determined by a probability distribution of occurrence. The ratio of the number of quanta emitted to the number absorbed is called the quantum efficiency (ϕ). It is the probability that an excited molecule will luminesce, which is determined by the statistical competition between the various processes involved. The fluorescence intensity in its turn is determined by the amount of light absorbed and the quantum efficiency.

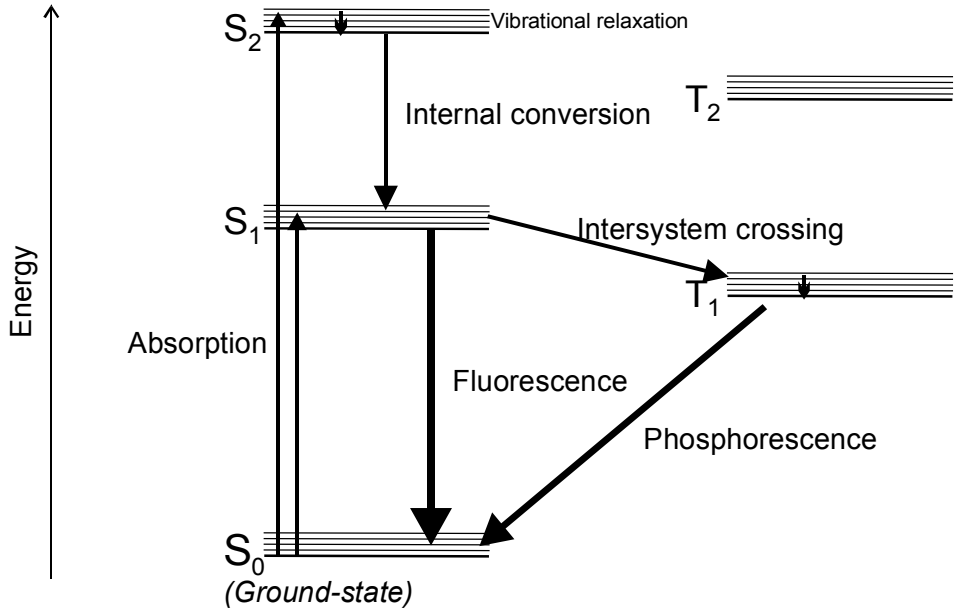

Fig. 5. Jablonski diagram showing transitions between molecular energy levels during absorption of light, fluorescence and phosphorescence. Only electronic and vibrational energy levels are shown.

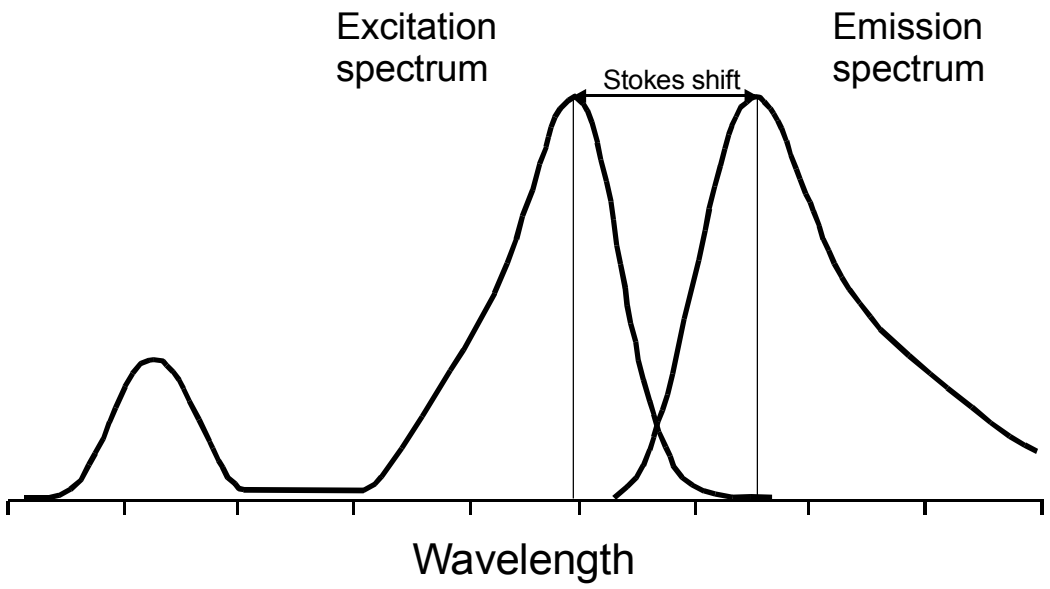

Fig. 6. Typical fluorescence excitation and emission spectrum.

The chemistry of fluorescence

Molecule structure and environment

The structure of a molecule determines whether it can fluoresce or not. Many organic and some inorganic molecules are fluorescent. Most of the useful fluorescent organic compounds are aromatic or heterocyclic structures and possess a fairly large conjugated system (see figs. 7, 8 and 9). Such a molecule is promoted from ground state to first excited state by the transition of an electron from the π bonding orbital to the π^* antibonding orbital. This does not excessively disrupt the bonding. The bigger the conjugated system the less energy this transition requires and the higher the quantum yield is. Strongly fluorescent aromatic compounds usually have a rigid planar system of aromatic rings, which make a large conjugated system.

Fluorescence is strongly dependent on environmental factors such as pH, ionic strength, temperature, rigidity of the medium, and binding to macromolecules. The pH determines the ionic dissociation of acid groups. The protonation or deprotonation of a molecule can markedly change the structure and therefore the absorbance of light and the quantum efficiency. In some compounds, e.g. fluorescein, the pH effect is very strong. A lower temperature and a more viscous or rigid medium decrease motion of the molecules and therefore the chance of collision. This decreases the occurrence of radiationless conversion and increases the occurrence of fluorescence at the returns from excited state to the ground state. Binding to a macromolecule may change fluorescent properties of the dye, including excitation and emission spectra and quantum efficiency.

Photobleaching and quenching

Molecules in excited state are more reactive than molecules in ground state. Chemical reactions that can take place are decomposition, polymerization, or a reaction with another molecule. Molecules can keep on cycling between ground state and excited state by absorbing and emitting photons until it undergoes a chemical reaction by which it is transformed into other molecule(s). The occurrence of a chemical reaction is a matter of chance and the probability depends on the structure of the molecule and the energy used for excitation. The irreversible destruction of excited fluorophores, called photobleaching, leads to a decrease in fluorescence intensity of a fluorescent preparation. This is mainly due to photochemical reactions induced by the excitation light. Especially chemical reactions with oxygen are believed to cause photobleaching. Photobleaching can be a problem for quantitative studies and photomicrography.

Quenching is the reduction of fluorescence by a competing deactivating process resulting from the presence of other molecules in the system. Four common types of quenching are recognized: temperature, oxygen, impurity and concentration quenching. Temperature quenching is caused by increased molecular motion and collisions as temperature increases. Oxygen quenching is caused by chemical reactivity between compounds in excited state and oxygen. Impurity quenching is caused by energy transfer to less-fluorescent compounds. Energy transfer can be very efficient and take place over distances as great as one micrometer. Concentration quenching, finally, is caused by a decrease in intensity of excitation light deeper inside the specimen because of light absorption by molecules closer by the light source.

Probes used in this research

Carboxyfluorescein diacetate

5(6)-Carboxyfluorescein diacetate (cFDA) is a hydrophobic non-fluorescent substrate that yields the fluorochrome 5(6)-carboxyfluorescein (cF) upon hydrolysis (fig. 7). The molecular weight of cFDA is 460, that of cF is 376. cFDA is moderately permeant to the cell membrane. Once in the cell, the acetate groups of cFDA are cleaved by esterases releasing the highly fluorescent cF, which is well retained because of its negative charge. cFDA is a vital stain, which means that it has been found to stain living cells. It is widely used for cell tracing, assessment of viability, and measurement of intracellular pH (23, 35, 47). Carboxyfluorescein has four protolytic groups and thus five protolytic forms: cation, neutral, monoanion, dianion, and trianion. The species differ in their fluorescence and since the distribution among the protolytic forms is determined by the pH, the fluorescence is pH sensitive. The trianion is the most fluorescent. The equilibrium between dianion and trianion has a pK_a of approximately 6.4. The excitation peak is at 492 nm and the emission peak at 516 nm for both 5-cF and 6-cF (pH 8). The extinction coefficient at the excitation maximum is 78,000 cm^{-1} M^{-1} (pH 9), and the quantum yield is 0.92 (in 0.1 M NaOH) (23). Carboxyfluorescein belongs to the class of the fluorones (47). This class includes fluorescein, which is the simplest fluorone, and derivatives thereof.

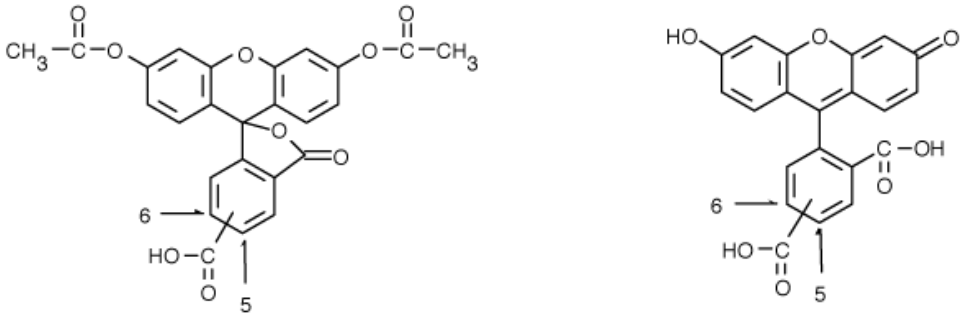

Fig. 7. Chemical structures of 5(6)-carboxyfluorescein diacetate (left) and 5(6)-carboxyfluorescein (right).

Propidium iodide

Propidium iodide (PI) is an impermeant dye that binds to nucleic acids forming a red-fluorescent complex (Fig. 8). The molecular weight is 668. Unbound PI has low fluorescence intensity. Upon binding to nucleic acids the fluorescence is enhanced 20- to 30-fold. PI intercalates with double stranded nucleic acids, has little or no sequence preference and binds with a stoichiometry of one dye molecule per 4-5 base pairs of DNA. The absorption maximum of the PI-DNA complex in water is at 535 nm and the emission maximum at 617 nm. The molar absorptivity is 5,400 $cm^{-1}M^{-1}$. This is not high, however the large Stokes makes PI a useful probe anyhow. The large Stokes shift facilitates simultaneous detection of PI with other probes, such as fluorescein, provided that proper optical filters are used. PI has significant anti-tumor activity and is a potent mutagenic as well. PI is widely used as viability test, based on exclusion by cells with intact membranes. PI is a phenanthridine dyes and an analogue to ethidium bromide (23).

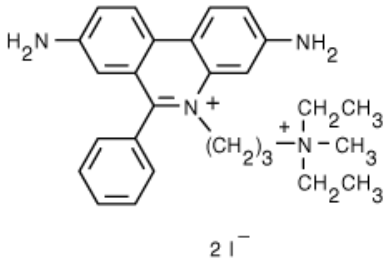

Fig. 8. Chemical structure of propidium iodide.

TOTO-1 iodide

TOTO-1 iodide is an impermeant dye that binds to nucleic acids forming a green-fluorescent complex (Fig. 9). The molecular weight is 1303. The affinity for DNA is high, and the complex of TOTO-1 with DNA is extraordinary stable. It acts as a bis-intercalator with interactions in the minor groove of DNA. The fluorescence enhancement upon binding is very high, approximately 1400-fold. The TOTO-1-DNA complex has an excitation maximum at 514 nm and an emission maximum at 533 nm. TOTO is used mainly in DNA gel electrophoresis and chromosome analysis, but can also be used to assess cell viability since it enters damaged cells only. It is one of the cyanine dimer nucleic acid dyes developed by Molecular Probes (23).

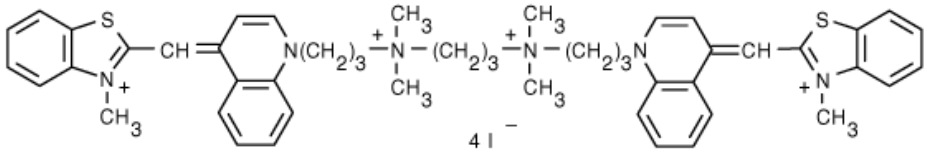

Fig. 9. Chemical structure of TOTO-1 iodide

SYTO 9

SYTO 9 is a permeant dye that binds to nucleic acids forming a green-fluorescent complex. The structure is proprietary information of Molecular Probes. SYTO 9 is one of the series of SYTO dyes. These dyes are permeant to cell membranes and bind to nucleic acids but their binding affinity is relatively low. They have a high molar absorptivity of more than 50,000 cm^{-1} M^{-1} at visible wavelengths. Free SYTO compounds have low intrinsic fluorescence with quantum yield < 0.1 while in complex with nucleic acids the quantum yield is typically > 0.4. The members of the SYTO series differ from each other with respect to permeability, fluorescence enhancement and spectra. The excitation maximum of SYTO 9 in complex with nucleic acids is at 480 nm and the emission maximum at 500 nm. SYTO 9 generally stains all bacteria: those with damaged membranes and those with intact membranes of both Gram-positive and Gram-negative species. The LIVE/DEAD *Bac*Light Bacterial Viability Kit of Molecular Probes consists of SYTO 9 and PI. By using an appropriate mixture of this permeant and impermeant nucleic acid dye the intact cells are stained green-fluorescent and the cells with damaged membranes are stained red-fluorescent.

FLUORESCENCE MICROSCOPY

A fluorescence microscope is an optical microscope. Like other light-microscopes it contains lenses to produce an optically magnified image, which allows observation of small objects and a high resolution of details. A fluorescence microscope contains furthermore filters that select the wavelength for illumination and for observation. These filters facilitate the observation of fluorescent compounds.

The optical system of a fluorescence microscope consists of an illumination system and an observation system (Fig. 10). The illumination system is composed of the light source, the lenses, and the filters in between the light source and the specimen. Each light source has its own specific spectrum. Mercury arc lamps, which are widely used in fluorescence microscopy, have major bands at about 365, 405, 436 and 546 nm. With an appropriate excitation filter one of those excitation bands can be selected. For a uniform illumination of the specimen a field lens and field diaphragm can be placed between source and specimen (Köhler illumination). A specimen may be illuminated either from below (dia-illumination) or from above (epi-illumination). For epi-illumination the objective can act as a condenser. One advantage of epi-illumination is that non-transparent specimens can be examined, which is not possible with dia-illumination. Secondly, the transmitted-light pathway can be used independently for alternative or simultaneous examination in phase-contrast, darkfield or brightfield. A fluorescence microscope usually has a semitransparent mirror, also called dichromatic or dichroic mirror. This mirror reflects short-wavelength light and is transparent to long-wavelength light. Also, there is usually a barrier filter that filters out the light of the wavelength band used for excitation. The entire filter system (excitation filter, dichromatic mirror, and barrier filter) is usually contained in one unit called the filter block. A fluorescence microscope can be equipped with an apparatus for photography, photometry or imaging.

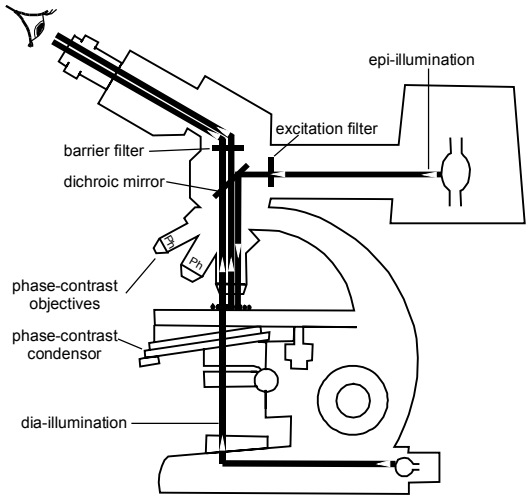

Fig. 10. Fluorescence microscope equipped with epi-illumination system, filters, phase contrast condensor and phase contrast objectives.

FLOW CYTOMETRY

What is flow cytometry?

Flow cytometry provides the means to investigate bacteria as being a population of individuals. The flow cytometer is the tool that measures several parameters quantitatively of many individual cells within a few minutes. Subsequent automated data analysis provides the population statistics of the sample and subpopulations may be distinguished. In addition, these subpopulations can be physically sorted for further study.

The anatomy of a flow cytometer consists of a flow cell, a light source, optics, detectors, electronics and a computer. In addition, a flow cytometer can be equipped with a cell-sorting device. How the different parts work is explained below. For more information the texts from Ormerod, Givan, and Shapiro are recommended (20, 39, 48).

The flow cytometer instrument

Flow cell

The purpose of the flow cell is to deliver cells in a single file (one after another) pass a focussed light beam. This is achieved by hydrodynamic focussing using a so-called sheath fluid, normally a saline solution. The sheath fluid and the sample can be delivered to the flow cell using air pressure or pumps. The sample is injected into the center of a stream of sheath fluid. This focuses the sample stream, so that the cells are delivered at a constant velocity to the point of detection with an accuracy of 1 μm or better. There, in the illumination zone, the particles interact with light.

Light source

The light source is normally either a laser or an arc lamp. Lasers are generally preferred because they produce monochromatic light and have small 'spot' size. Argon-ion lasers are used most often. The major line at 488 nm gives a convenient source of blue light for excitation of many probes, including fluorescein, phycoerythrin, and propidium iodide.

Optics

A flow cytometer contains a number of lenses and filters to select and direct excitation and emission light. Figure 11 shows a scheme of flow cytometer optics. In case the light source is an arc lamp, a filter is needed to select the wavelength of excitation. In case of a laser this is not needed because laser light is monochromatic. The excitation light beam is focussed on the sample stream by a lens.

The scattered and emitted light from the flow cell is collected by two lenses. One collection lens placed in forward direction (head on along the direction of the laser beam) and the other is placed orthogonal to the illumination beam. In front of the forward lens an obscuration bar is placed to block the light from the laser beam itself. Only light that is refracted or scattered by particles in the sample stream will avoid the obscuration bar. Light bent to small angles will hit the forward-positioned lens,

which focuses it onto a photodiode. The amount of this forward angle light scatter (FALS), for short forward scatter (FSC), depends on the size of the cells, but also on the refractive index of the cell constituents with which the light interacts. The lens that is placed orthogonal to the illumination beam has a high numerical aperture to collect as much of the fluorescence as possible. In between that collection lens and the detectors dichroic mirrors and bandpass filters are placed to select a part of the spectrum for each detector. There is one detector for the light scattered to wide angles, called right angle light scatter (RALS), for short side scatter (SSC). The amount of SSC depends on the 'granularity' of the cells. The FSC and SSC provide information about the physical characteristics of a cell. The other photodetectors collect light of different colors produced by fluorescent molecules in the cell upon interaction with the source light. These can be endogenous compounds, such as chlorophyll, or fluorescent probes. Commonly, red light (> 650 nm), orange light (585 nm) and green light (530 nm) are collected.

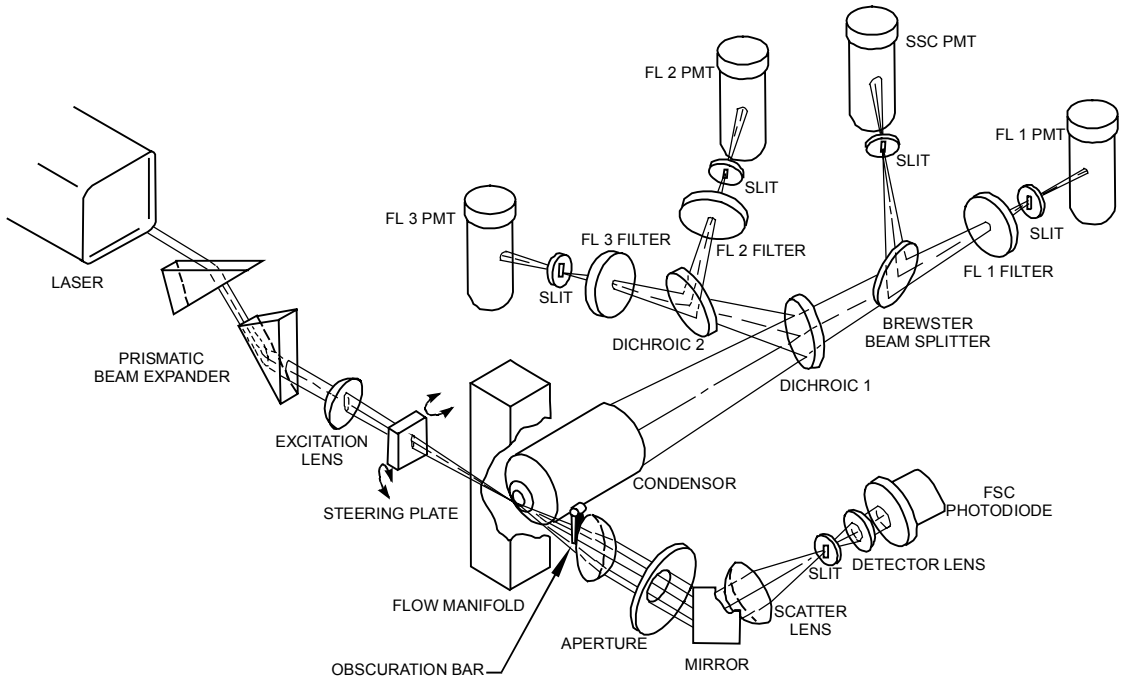

Fig 11. Schematic diagram of the optics of a flow cytometer. The laser beam is focussed on the sample stream passing through the flow cell. Forward angle light scatter is measured at low angle with a photodiode. Right angle light scatter and fluorescence are collected at 90°, split by a series of dichroic mirrors and filters in different colors, and measured by photomultiplier tubes.

Detectors

The number of photons collected from a cell varies over a wide range, depending upon what is measured. The detected FSC consists of much more photons than the detected SSC. For the detected fluorescence signal it depends on the fluorescent characteristics of the dye and the amount of dye in the cell. Small cells give less signal than big cells. Two types of photodetectors are used: forward

scattered light is usually detected by a photodiode, while side scattered light and fluorescence are detected by photomultiplier tubes (PMTs). Photodiodes as well as photomultiplier tubes produce a current at their anodes when photons impinge upon their light-sensitive photocathodes. Basically, photons go in and electrons come out. A photodiode does not require external power and has a responsivity of maximally 7 electrons out for 10 photons in. A PMT has a series of dynodes over which a voltage is applied. Emitted electrons are accelerated, so they acquire energy, and at each stage more electrons are dislodged. This gives a PMT current gain, which can be quite high (10^7 electrons out for each photon in).

Signal processing

The signal processing ultimately converts the detected light signals into a data file. The current produced by a detector is fed into a preamplifier, which produces a voltage pulse proportional to the number of photons that originally reached the detector. Debris and electronic noise also produce signal. To ignore this, a threshold level must be set on one of the parameters. The electronics are only triggered if the signal rises above the threshold level.

The processing electronics may apply compensation subtraction or ratio calculation. Compensation may be used to subtract spectral overlap of different fluorochromes. Ratio circuits may be used for normalization in cases when not the amount of dye in the cell is relevant but only the change in the spectrum of the dye, for example in calcium flux measurements or pH measurements. In stead of already during data acquisition, compensation and ratio calculations can also be made afterwards, using the raw data in the saved file.

The next step in signal processing is to amplify the signal, either linearly or logarithmically. With linear amplification the output voltage is directly proportional to the input voltage from the preamplifier. This is used for example for cell-cycle measurements with DNA stains. With logarithmic amplification the output voltage is proportional to the logarithm of the input. Logarithmic amplification increases the dynamic range so that both weak and strong signals can be recorded on the same scale. This is used for example for immunofluorescence and functional probes.

The last step is the analog-to-digital conversion. Most flow cytometers use 10 bits, so there are 210 or 1024 bins or channels. Thus, each light signal is converted into a number on a scale of 0 to 1023. These are put in list mode in a data file. The list contains one row for each measured cell and one column for each measured parameter.

Data analysis

To get information out of a list file it comes to proper statistics and data display methods. The main statistical parameters that are calculated in flow cytometry data analysis software are the total counts, counts of defined regions, mean and median channels, standard deviation and coefficient of variation. Various data display methods can be used. A histogram displays the distribution of the values of one parameter for all the cells in the data file (see for example figs. 2 and 3 in Chapter 5). Bivariate histograms or cytograms display two parameters. One type of cytogram is the dot plot, with each dot

representing a single event (see for example fig. 1 in Chapter 6). Other forms of bivariate displays are density plots, contour plots, and pseudo three-dimensional isometric plots.

Flow cytometry data analysis can involve 'gating', which means that events that meet certain criteria are designated. To gate the data one or more parameters are displayed and regions of interest are defined. Thus, certain populations are selected for analysis of the final parameters. Gating can be helpful in analysis of microbiological samples to distinguish cells from background.

Sorting

To sort cells with a flow cytometer two types of sorting devices may be used. The first type works by deflection of charged droplets. The flow cell is vibrated along the axis of the fluid stream so that a uniform stream of droplets emerges from the exit of the flow cell. The cell concentration has to be sufficiently low, preferably so that, on average, every tenth drop will contain a cell. The flow cell is charged positively or negatively for a short interval of time when a cell of interest is enclosed in the drop formed at the break-off point. The stream of droplets passes through a pair of charged plates so that charged droplets are deflected and collected. The second type of sorting uses a piezo-electric device that deflects a collector flow channel into the sample stream to gather the cells of interest. These collectors are very stable, but are limited in sorting rate, cells are diluted, and only one population can be sorted. Sorted cells can, for example, be examined for culturability by plating.

Pro's and con's of flow cytometry

Flow cytometry enjoys the benefit of the ability to analyze single cells. Furthermore, appropriate combinations of stains, light source(s) and detectors permits the estimation of multiple determinants on each individual cell. With multivariate data analysis various cell types in a mixed population can be determined, or relationship between different cellular variables can be analyzed. The speed of analysis and therefore the number of cells analyzed is high. Usually, some 100 to 1,000 cells per second are analyzed, so the acquisition of 10,000 events may be effected within 10 to 100 seconds. The high-speed analysis allows the detection of rare events. The data can be analyzed rapid enough to allow electronic sorting of a selected population of cells.

Flow cytometry also has limitations. One is the sample preparation needed. For proper analysis of each individual cell the sample has to be a clean suspension of single cells. Cells have to be separated from each other; else cells that stick together will be measured as one event. The suspension has to be clean; it should not contain particles that interfere with the detection of cells, or particles that are falsely interpreted as cells. Cell-sticking, interfering particles and background noise limit the accuracy of a measurement. Furthermore, specialist knowledge is required, in particular to set up new applications. The last disadvantage of flow cytometry is the price. A flow cytometer is an expensive piece of laboratory equipment. However, the analysis does not require much of the chemicals nor much time per sample. Advantages and disadvantages of the flow cytometry technique are summed up in Table 1.

Flow cytometry can be compared with fluorescence microscopy. Like flow cytometry, fluorescence microscopy analyzes single cells. However, with standard microscopy a qualitative, detailed, assessment of a few cells is made by eye, while flow cytometry allows quantitative measurements on many individual cells. Clear advantages of fluorescence microscopy are that the instrument itself is not that expensive and that it can be applied on tissue, so without the need of preparing a cell suspension. However, analyzing many cells with microscopy is laborious. Automating with image analysis techniques can meet this drawback to some extent and those methods are improving, but flow cytometry is superior in rapid acquisition and analysis of multiple parameters.

Flow cytometry can also be compared with biochemical measurements. The main differences are the analysis of individual cells versus a cell suspension and multivariate analysis versus univariate analysis. A biochemical measurement gives an average value of one parameter, such as enzyme activity, DNA content, or protein content, for all the cells in the sample volume needed for the assay. Flow cytometry measures multiple parameters of each cell individually, and therefor allows the detection of subpopulations based on one or more parameters.

Table 1. Advantages and disadvantages of flow cytometry

Advantages	Disadvantages
Individual cell analysis	Need for suspension of single cells
Multiparameter data acquisition	Expensive equipment
Multivariate data analysis	Need for skilled operators
High speed analysis	
Ability to sort cells	

Sources of information

For people interested in using fluorescent probes and flow cytometry, key sources that give introduction, background and overview of flow cytometry, with special interest to microbiology are listed here.

A good introduction in fluorescence microscopy is given in Rost's two-volume *Fluorescence Microscopy* (46, 47). Fluorescence probes are the subject of Slavik's *Fluorescent probes in cellular and molecular biology* (49), and *Fluorescent and Luminescent probes for biological activity*, edited by Mason (35). A must-have is the *Handbook of Fluorescent Probes and Research Chemicals*, edited by Haugland, which is also available on CD-ROM and Internet (www.probes.com) (23). This is not just the catalog of the Molecular Probes company, it also contains a wealth of information on several thousands fluorescent dyes, and a literature database including more than 40,000 references.

There are several recent books about flow cytometry. Definitely the most recommendable is Shapiro's *Practical Flow Cytometry* (48). There is a lot to learn from this book, especially for a beginner, and it is entertaining. General introductory information is given in *Flow cytometry*, a slim book by Ormerod (39). Basic principles and many applications, including measurements on microorganisms are given in *Flow cytometry* edited by Darzynkiewicz, Crissman and Robinson. This

is the third edition of flow cytometry volumes in the *Methods in Cell Biology* series and appeared as volumes 63 and 64 in 2001 (11, 12). Another book with some chapters relevant for microbiologists is *Current protocols in Cytometry*, edited by Robinson et al. and published in affiliation with the International Society for Analytical Cytology (ISAC). This is frequently revised and published as loose-leaf and CD-ROM (45). A specialized book for microbiologists is *Flow cytometry in microbiology*, edited by Lloyd (33).

A recommendable review article for microbiologists is the1996 paper of Davey and Kell on flow cytometry and cell sorting of microbial populations (13). Recently, reviews were published about applications of flow cytometry in aquatic microbiology by Vives-Rego et al. (63), about applications in clinical microbiology by Álvarez-Barrientos et al. (2), and about use of fluorescent probes for physiological assessment of bacteria by Joux and Lebaron (27).

A lot of information about flow cytometry can be found on the Internet, including information on courses and a discussion group. A good starting point is the site of Purdue Flow Cytometry Labs (www.cyto.purdue.edu).

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2

Review:
Monitoring of Microbes with Fluorescent Probes and its Applications in Food Microbiology

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A modified version of this chapter is submitted for publication

INTRODUCTION

Detection of foodborne pathogens

Food safety control is of crucial importance in the food production chain: from raw materials, processing and retailing, to the moment of consumption. Microorganisms are a principal cause of food spoilage and food poisoning. Foodborne diseases are a major problem for public health worldwide (147, 222). For example, it is estimated that in The Netherlands alone several hundreds of thousands of people are affected with foodborne diseases or food poisoning each year. Though the symptoms are usually mild and mortality is low, the social and financial impact on society is enormous.

Nowadays consumers want fresh, wholesome, and easy-to-prepare foods. Traditional ways to control microbial spoilage, such as freezing, sterilization, blanching, and use of preservatives are being replaced by innovative techniques including mild heating, modified atmosphere and vacuum packaging, natural antibiotics, and use of high hydrostatic pressure. These minimal processing techniques have led to new risk factors and changed the spectrum of pathogens that may cause food spoilage. In addition, the last years national and international food authorities have introduced more stringent microbiological safety regulations. To ensure the safety of food, good detection methods for pathogenic and spoilage microbes are of critical importance (162).

Industrial controls are needed not only for detecting pathogens in finished products, but also for a rapid analysis of process samples to detect problems at the earliest possible stage of the production. Classical methods that rely on culturing and colony counting are customarily applied in food industry and food authorities have developed standardized protocols, for example for testing milk hygiene (99). Culture methods have a low detection limit and do not need expensive equipment. However, these conventional methods require several days to give results, are labor-intensive, and provide limited information. Therefore, the food industry is in need of faster and more reliable methods for detection of foodborne pathogens in raw materials and food products.

Food biotechnology

Fungi, yeasts and fermentative bacteria are widely used in food production. For example, alcoholic fermentation by *Saccharomyces cerevisiae* and other yeasts is used in production of beer and wine. *Saccharomyces cerevisiae* is also used in bakeries, for the rising of bread dough. Lactic acid fermentation is applied in the manufacturing of fermented dairy products, such as yogurts, sour creams, and cheeses. The main bacterial species used in the dairy industry are lactococci, lactobacilli, and leuconostocs. The bacterial cultures added to the milk to start lactic acid fermentation (so-called starter cultures) produce lactic acid and volatile compounds like diacetyl and acetaldehyde. Factors such as antibiotics, bacteriophage attack and low viability of the starter culture can reduce or stop the normal process of acidification and, thus, lead to defective products and require costly clean-up procedures. Plate counts on solid medium can be used to test the viability of starter cultures. However, with the plate count technique the delay between sampling and the result is a couple of days. Also, culturability of the starter culture does not yet guarantee a good fermentation process. Storage conditions can significantly affect bacterial viability. Another control test commonly used in dairy

industry is the acidification capacity assay. This test may be a more appropriate control for a starter culture, however it takes a couple of hours of incubation (100). Thus, in addition to rapid methods for safety control in food industry, there is also need for rapid methods for control of the viability of the cultures of microorganisms that are used in food fermentations, for process control during manufacturing, and for control of the products. These rapid methods are needed in dairy industry, as well as in breweries, wineries, and in production processes of fermented meats and fermented vegetables.

Rapid automated methods

During the last two decades a number of rapid microbiological methods have been developed (52, 81, 114, 237). Examples are the ATP bioluminescence assay, impedimetry, and enzyme-linked immuno-absorbent assays (ELISA). Also, there are genetically based methods for detection and characterization (52). Most of these methods require lysis of the microbial cells and often also the isolation of DNA. Target sequences may be detected in isolated DNA that is immobilized on a membrane, directly from solution, or after separation in an electrophoresis gel using radioactive or fluorescent labeled oligonucleotide probes. Alternatively, amplification by PCR with specific primers may be used to enable detection of target sequences. Furthermore, molecular subtyping of different strains within a species is possible with DNA fingerprinting methods. All the methods mentioned above measure microbes on the population level.

On the other hand there are rapid methods that detect microorganisms on the single-cell level. These include *in situ* detection (in the food itself) using a microscopic technique (light microscopy, epifluorescent microscopy, or scanning microscopy), the direct epifluorescent filter technique (DEFT), and flow cytometry. The principle of DEFT is that microbes are collected on a membrane by filtration of the sample, in this case (stomachered) foodstuff, stained with a fluorescent dye, and counted by microscopy. Flow cytometry is an automated optical technique that measures light scatter and fluorescence of (stained) particles in a liquid stream that passes a focussed laser beam. Samples can be fluorescently stained with dyes that directly stain certain cell components, such as DNA or protein, or with fluorochromic substrates. Alternatively, fluorescently labeled antibodies or fluorescently labeled nucleic acid probes can be used with flow cytometry.

Scope of this review

This review focuses on fluorescent methods for direct detection and examination of microbial cells. For a description of fluorescence microscopy and flow cytometry techniques see Chapter 1 of this thesis. Here, the issue of microbial viability is discussed and a classification of physiological states is described. Next, an overview is given of fluorescent dyes used for microbial analyses, with examples mainly about food-related microorganisms and flow cytometry. Subsequently, application of fluorescent techniques for the detection of (specific) microbes in drinks and food stuffs as well as in food production processes are reviewed. Finally, challenges and limitations are discussed and a future outlook is presented.

MICROBIAL VIABILITY

'Viable' and 'nonviable' cells

A basic question in microbiology is whether microorganisms are dead or alive. This simple question is however not always easily answered. During the last two decades a debate is going on about microbial viability, in philosophical as well as in experimental sense (8, 9, 115, 128, 158). At the simplest level, bacteria may be classified as either viable or non-viable. In microbiological practice this is usually tested by culture and enumeration of 'viable counts' (180). Thus, *viability* is equated with *culturability*. However, bacterial physiology is much more complex than the simple two-valued logic system of live or dead. This section will describe different physiological states of bacteria. The concept presented here should provide a basis for better insight into bacterial physiology in laboratory, natural, and industrial environments.

Life and death in macrobes and microbes

The concept of life and death of microbes is different from that of macrobes, as was clearly described by Postgate in 1976 (181). Amongst macrobes the ability to multiply at the level of the organism (fertility) is not synonymous with being alive. Also, cell death and death of the organism are two very different events in macrobes. Normal somatic cells are 'committed' to death: they are capable of only a limited number of divisions. Natural death of a macroorganism is the result of loss of effective interaction between cells. One can say that macroorganisms and their component cells are both intrinsically mortal. In multicellular microbes, such as filamentous moulds and algae, death of individuals or parts can also be observed. Among unicellular microbes there are also some in which aging and death are an intrinsic part of the life cycle of individual cells, for example in budding yeasts. These microbes can be used to study processes of aging and cell death.

Bacteria (and other fissile, haploid, vegetative microbes) live and die in a way of their own. Bacteria do not produce progeny and then grow old and die; they vanish as they are replaced by two new cells. A multiplying cell doubles its DNA and other cell components and then divides into two individuals. The DNA of the new cell consists half of the DNA that the parent cell originally had, and half of the DNA that the parent cell synthesized before division. Therefore, the two daughter cells can be considered as equally young. Thus, bacteria reproduce by an equal division of the cell in two. This implies that if viability is equated with the ability to multiply, we can never state that a given cell *is* alive, only that it *was* alive. In bacteria, no processes can be observed of a commitment to death. Yet, bacteria do die if cultures are left to age, or are otherwise lethally stressed. In the two-valued system death is discovered only retrospectively by the lack of forming progeny up to the limit of detection in liquid or on solid medium.

Uncultured microbial species

The discrepancy between the number of bacterial cells that can be detected by microscopy and the number of viable plate counts, especially in environmental samples, has been recognized for many years (8). Phylogenetic research, mainly by 16S ribosomal RNA (rRNA) sequence data (255), has

established a practical foundation for the ‘great plate count anomaly’. We know now that there are many species that are not detected by culture, but can be identified by molecular genetic techniques. These techniques are either based on PCR of 16S rRNA genes or detection with labeled rRNA-directed oligonucleotide probes (2, 262). In aquatic systems 0.1 to 10% of the total bacterioplankton has been found culturable, meaning that the majority of the population requires other methods to be detected (14, 146, 243). Also, the microbiota community in the gastro-intestinal tract of animals, including humans, is a very rich ecosystem of which many species are not yet known. The culturability of gut microbiota detected by plate counts has been estimated to range from 10 to 50% of the total present (240). Other examples of ecosystems containing many unknown species are artisan fermentation starters, communities used in wastewater treatment, and biofilm communities in industrial and hospital environments.

It is important to realize that species that have not yet been cultured are not necessarily unculturable (8). Attempts to recover bacteria from environmental samples often leads to new species (261). This might require powerful selective culture methods, which might not be available yet but need to be developed. Furthermore, there are many cultured organisms in collections that remain to be identified. However, it should also be realized that the cultivation of part of the as-yet-uncultured species might be difficult or even unachievable. This might be caused by slow or cell density-limited growth, or by highly specific nutritional or environmental requirements. One example is *Mycobacterium leprae*, discovered in 1880 and despite all attempts still not culturable in axenic culture, so its viability has to be investigated using test animals.

Survival

One point of confusion that may arise by using the term 'viability' has clearly been explained by Barer and Harwood (8). In their paper on bacterial viability and culturability they wrote: “In common parlance, use of the terms ‘viable’ and ‘viability’ reflects our expectation that an entity, which may be animate, inanimate or abstract, will survive over a generally accepted (but rarely stated) period of time”. In microbiology the considered time frame often extends for several generations. This rationalizes that reproduction is considered inherent to bacterial viability, as it is in long-term survival of any species. It also explains the possible confusion since bacterial lifetime is highly variable (8). To unravel this point of confusion we need to state clearly whether we are considering the individual or the community in the experiment.

An individual bacterium usually exists only for a limited time. What happens depends on the (fluctuating) condition of the cell and on the external circumstances. At some time a cell may go into the process of division resulting into two new cells. Contrarily, a cell may loose physiological fitness below a certain physiological threshold level leading eventually to cell death. Under some circumstances the lifetime of a cell may be extraordinary long as shown by the discovery of million years old bacteria in amber crystal that were still culturable (246).

On the level of a bacterial community cell reproduction has to be at least in balance with cell death to guarantee survival of the community.

Recognition of other physiological states

One of the most important consequences of the increasing biotechnological exploitation of bacteria is the recognition that their performance depends on their physiological status. Microbial physiology of readily culturable species is usually studied using exponentially growing cultures, where enzyme activities are expected to be high. However, in biotechnological processes organisms often encounter suboptimal conditions and extended periods of nutrient deprivation. Thus, bacterial cells in industrial processes are likely to be in physiological states such as 'stationary phase', 'resting cell suspension', or 'non-proliferating phase' (128). The conditions that microbes have to cope with in natural environments may be even worse. As the understanding of bacterial physiology improved it has become clear that the two-valued system of either viable or non-viable is inadequate (115). Cells may be not culturable in one point in time, but regain this capacity later. Microbes can adopt so-called 'cryptobiotic', 'dormant' (111), 'moribund' (180), or 'latent' states. The survival in a natural environment can depend on the ability to persist in such a state for some time (193). Resuscitation, i.e. the transition back to a culturable state, may require medium free of a 'lethal' substrate, a temperature shift or other factors. In the case of *Micrococcus luteus* it was found that resuscitation required the presence of culturable cells or a proteineous pheromonal factor of such cells (113, 154, 245). Also, cells may be injured because of damaging factors such as freezing, antibiotics, or electroporation treatment, but may recover from that after some time. All these states of temporal unculturability can, in retrospect, be identified as 'not immediately culturable' (NIC) (115). Furthermore, cells can be in a state in which they exhibit certain activities, but no cell division. Such cells are often called 'viable but nonculturable' (VBNC) (41, 190, 221). However with the common definition that viability means culturability this is a contradiction in terms, and therefore such a state should better be referred to as 'active but nonculturable' (ABNC) (115). The existence of these phenomena requires a more extended concept of microbial viability than the either-viable-or-nonviable classification (7, 50, 111, 156).

Heterodynamic stability

A living organism is much more complex than inorganic matter, however it is all the same subjected to the laws of physics (196). From a physical approach a living system performs generally not far from equilibrium. It does rarely operate in steady state, but there are oscillations and rhythms in the states and rates of the system. The living cell is a blend of chaotic determinism and random processes (48, 128). There is periodic turnover: energization and biosynthesis alternate with de-energization and breakdown. Mechanisms of control are to be expected distributed (bottom up) rather then centralized (top-down). The oscillatory performance enhances the sensitivity to perturbations from within (free radical damage, erroneous transcription and mutations) or from the environment (nutrient supply, temperature, oxygen). Small perturbations may be tolerated, but extreme disturbance may lead to cell death. Such a physical approach also holds true for a community of different species. It has been recognized that long-term survival of a species in mixed communities is very difficult to predict. For example, the ecosystem of mixed phytoplankton appeared very sensitive for small variations and behaved as a chaotic process (94). The recognition of heterodynamic stability explains, in a physical way, fluctuations in what could be called the 'degree of viability'.

Classification of cell functionality

Fluorescent indicators and flow cytometry facilitate demonstration of activity, integrity and composition of bacterial cells. This allows for a differentiation of the status of an individual bacterium far beyond the classical viability system solely based on culturability. New terminology, definitions, and classifications have been given in a number of recent papers (8, 9, 109, 115, 158). In recent literature the term 'viability' is often not used as a pure synonym for culturability, but rather to indicate the general physiological condition. Such broad use of a term may lead to confusion. Thus, when terms such as 'viable' or 'alive' or 'active' are used to indicate the cell status this needs to be clarified with regards to measured property and the interpretation.

Fig. 1 shows a diagram of physiological states based on measurable cell characteristics. On top there is the state of reproductive growth. This requires both metabolic activity and membrane integrity, and it may be regarded as the highest level of physiological fitness. A cell may loose the capacity to reproduce due to irreversible DNA damage or other factors.

Metabolic activity can be detected on different 'levels'. Biosynthesis, the level just below reproduction, requires the activity of a number of systems (active enzymes, generation of energy, availability of building blocks). Pump activity and generation of membrane potential are less demanding and can still take place in situations where growth is not longer possible. Enzymatic conversions, which do not require energy but just the presence of the enzyme, can be regarded as the

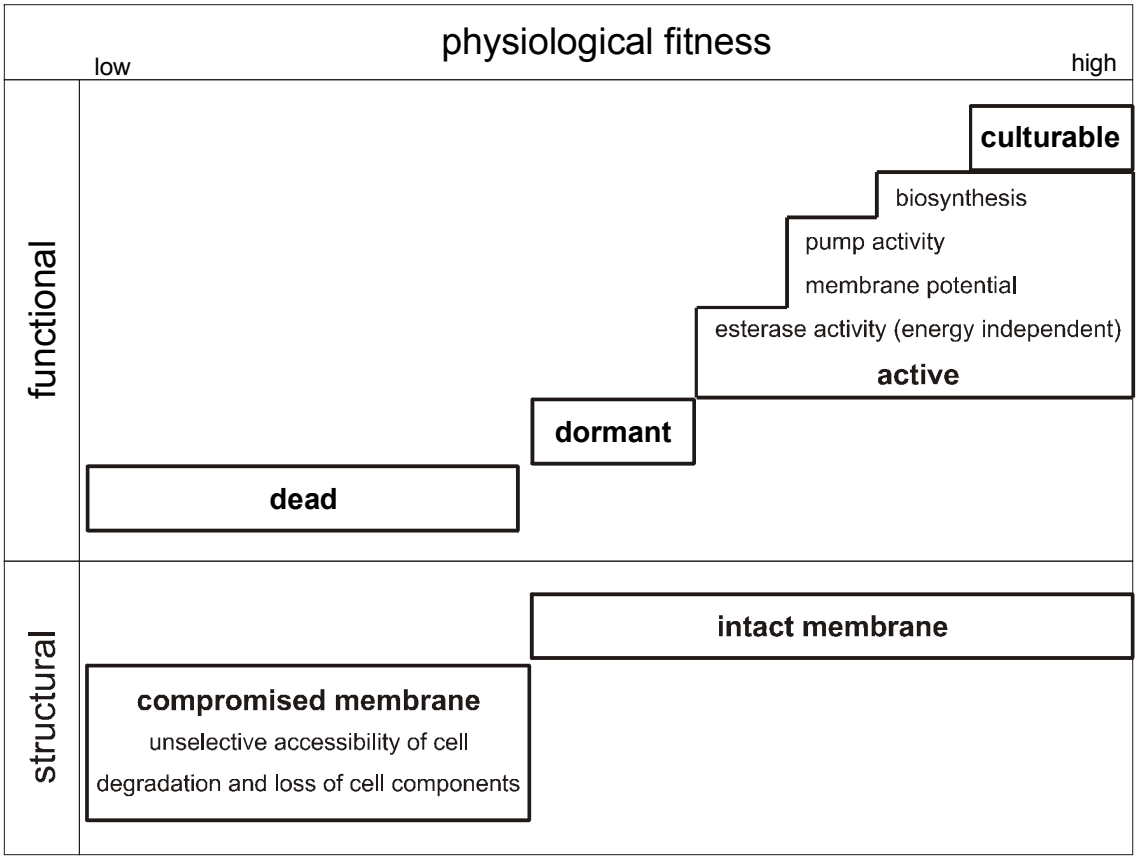

Fig 1. Classification of physiological cell states

lowest level of metabolic activity. Even in membrane-damaged cells or cells that are de-energized, such enzyme activity can remain for some time. Dormant, injured, and extremely starved cells may have no detectable activity, but may in time reverse to an active state.

Metabolic activity, also in the absence of growth, plays an important role in biotechnological processes, for example in dairy fermentations. Likewise, food pathogens may still cause food spoilage or accumulation of toxins when they are in an active but nonculturable status (7). If the loss of activity is irreversible cells may be considered dead.

An intact membrane is required for maintaining the capability of metabolic activity. Cells can recover from a transient permeabilization, but if the membrane is irreversibly compromised the cell is doomed to die. Without an intact membrane a cell can not maintain electrochemical gradients so it will loose its membrane potential and pH gradient. As the intracellular compartment is no longer separated from the environment, components leak out of the cell and potentially toxic chemicals from the environment diffuse freely into the cell. This will lead to breakdown of cell components and finally the degradation of the whole cell.

FLUORESCENT ASSESSMENT OF PHYSIOLOGICAL FUNCTIONS

A large number of fluorescent dyes has been described as probes for assessment of cell viability (86). These so-called 'viability probes' actually measure specific physiological attributes and it is a matter of debate, and a subject of study, how the various properties relate to each other and to culturability (as measured by viable plate count). Here an overview is given of the various microbial assets that can be measured with fluorescent probes and flow cytometry and that are used to study the physiological state of microbes. Table 1 lists dyes that are used to assess those physiological characteristics and gives references to applications of those dyes in flow cytometry studies of microbes.

Culture growth

An individual bacterium only lives for a limited time. At a certain time that particular cell ceases to exist. Either the cell divides into two new cells, or the cell is degraded. If the rate of reproduction is higher than the rate of cell death, the culture grows. If reproduction is too low the bacterial community will eventually extinct. On the level of the cell, fission can be deduced in cell tracking experiments. In each cell division the fluorescence is divided, so this allows for tracing the generation of a cell (231).

On the level of the culture, growth and decrease can be monitored by the number of cells. An increase of the number of cells in time indicates the capacity of reproduction. A decrease indicates that cells have lysed and are broken down. Counting and monitoring of cell numbers has many applications, for example for investigating bacteria in soil (56), in compost (57), and in water (126, 142). Counting of cell numbers is often combined with assessment of membrane integrity or metabolic activity, for example in studies of lactic acid fermentation (31, 121).

Biosynthesis

Biosynthesis is the build-up of cell compounds and cell structures. Cells need to perform biosynthesis to remain in good condition and stay functional. They need to synthesize enzymes, nucleic acids, polysaccharides, phospholipids and other chemical components and perform repair of damage in order to keep the cell machinery going. Moreover, to grow and multiply cells need to produce chemical components and construct structural cell parts, such as the ribosomes, cell wall, and membrane. When cells are growing and dividing there is net biosynthesis. During starvation there is net degradation of cell components and this can eventually lead to cell death.

The measurement of the contents of cellular components such as DNA and protein is used to assess biosynthesis or degradation. The cells may need to be fixated to allow access of the probe applied. Light scattering is also used as a parameter to indicate cell growth or cell starvation. These three parameters (light scatter, DNA content and protein content) were used in the earliest applications of flow cytometry to microorganisms (5, 166, 207). These parameters are still commonly used in viability assessment, for example for testing toxicity and microbial susceptibility for antibiotics, including surfactants (see reviews (1, 249)). Measurement of DNA content has also been used to

assess cell cycle parameters and to study the mechanism of chromosome replication (18, 205, 206). Steen et al. (207) started with testing ethidium bromide and propidium iodide for measuring DNA content of microbes, since these probes were also used for mammalian cells. However, these probes proved less suitable for bacteria, probably because they contain relatively greater amounts of RNA and a combination of mithramycin and ethidium bromide was found much more DNA specific (206, 208). DNA amount has also been measured using Hoechst, DAPI and novel nucleic acid dyes such as SYBR Green, SYTO 13 and TOTO-1 (74, 83, 141).

Protein is commonly measured using fluorescein isothiocyanate (FITC). Also, scattered light has been found a good measure for protein content (19). A novel probe for proteins is SYPRO (86), which has been applied to measure protein content of bacteria in seawater (263).

Another approach for assessment of biosynthesis is the measurement of the rRNA content. A high concentration indicates a high level of cell activity. This was used for example in a starvation study of *Salmonella* (229).

The increase of cell size is used as a direct proof of biosynthesis in the direct viable count (DVC) assay developed by Kogure et al. (119). In this assay cells are incubated with nutrients so that they can grow, but the presence of an antibiotic inhibits cell division. Thus, the number of cells is kept constant, and the cells that have the potential to proliferate are revealed by their elongation. The DVC assay was developed with microscopy, but it can also be used with flow cytometry. It has been applied for example for flow cytometry analysis of natural waters (106).

Membrane integrity

An intact membrane protects the cell constituents and is a prerequisite to generate gradients between the cytoplasm and the environment. Cells need electrochemical gradients to remain functional. The selective permeability of the membrane ensures that the cytoplasm is a separate entity from the environment and therefore the cell has the potential of metabolism and proliferation. Keeping the membrane intact hardly requires cell activity (only for maintenance) Thus, cells can survive starvation or other harsh conditions by turning down their metabolism and staying in a 'dormant' state until better times.

Membrane integrity is tested by the capacity of cells to exclude impermeant dyes. These do not enter an intact cell because they are virtually insoluble in the hydrophobic membrane phase. Nucleic acid dyes are ideal to test membrane integrity because the high concentration of nucleic acids provides for many target sides and binding usually leads to large fluorescent enhancement. This leads to a clear distinction between cells with intact membranes and cells with compromised membranes. Several impermeant nucleic acid dyes are available, of which PI has been used most frequently (43, 129, 160, 232). In the last decade a series of cyanine dyes with superior fluorescence characteristics has been developed for nucleic acid staining, including the impermeable SYTOX Green, monomeric TO-PRO series and dimeric TOTO series compounds, and the permeant SYBR Green and SYTO dyes. These new dyes are increasingly used (122, 126, 141, 153, 194). The permeant dyes can be combined with impermeant dyes to facilitate easy identification of all cells and total counting.

*Bac*Light, Molecular Probes' kit for bacterial viability testing, is such a combination (86). It combines propidium iodide with SYTO 9 and thus it stains membrane-compromised cells red (propidium iodide) and intact cells green (SYTO 9). *Bac*Light has been applied to a number of bacterial species using flow cytometry (109, 159, 215, 242).

Metabolic activity

Esterase activity

Esterases are present in all living organisms and assays that measure esterase activity are commonly used to assess metabolic activity (86, 107, 241). Though synthesis of the enzyme requires energy, the enzyme-substrate reaction itself is energy-independent and esterase functionality is found to be quite persistent. It was shown that H_2O_2-killed cells, as well as γ-radiated cells, maintained detectable esterase activity for over a week, and even 70°C-heat killed cells had considerable remaining esterase functionality (30, 243). The detection of cells with esterase activity requires not only the enzymatic conversion, but also membrane integrity. Esterase activity assays use lipophylic non-fluorescent fluorogenic substrates that can diffuse across the membrane. Once within the cell the substrate is cleaved by non-specific esterases releasing a more charged and polar product that is well retained by cells that have an intact membrane, but that leaks out of the cell in case the membrane is permeabilized. This happens for example when cells have been killed by 70°C treatment (30). A number of fluorescein derivatives have been used and compared for various applications and species, including the widely used fluorescein diacetate (FDA) and carboxyfluorescein diacetate (cFDA), as well as calcein-AM, Chemchrome B and Chemchrome V6 (commercial preparations of Chemunex), and 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxy methyl ester (BCECF) (29, 44, 59, 129). Reported limitations of fluorogenic esterase substrates are poor dye uptake, low labeling efficiency of some species, and active extrusion (30, 150). Furthermore, the outer membrane of Gram-negative bacteria is generally impermeable to the lipophylic probes, and permeabilization of the outer membrane is required (29, 86, 150).

Dehydrogenase activity

Respiration is a process central to cellular energy metabolisms in most organisms other than fermentative bacteria. Tetrazolium salt reduction is often used as an indicator of respiratory activity. A tetrazolium salt can be reduced by dehydrogenases. Therefore, it can act as electron acceptor in the electron transport system. Reduction converts the colorless soluble tetrazolium salts to fluorescent formazan precipitates. Tetrazolium salt reduction is used as a direct measure of the cells actively engaged in respiration. The compound 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) is often used in studies of bacterial viability (55, 86), (129). For example to study starvation and resuscitation of *Micrococcus luteus* (111), and study of antibiotic induced damage of *Staphylococcus aureus* (215). In both cases substantial heterogeneity in dehydrogenase activity was detected among the population. A drawback of CTC is that it is rather toxic (107).

Membrane potential

Cells build up potential energy in metabolic processes by the unequal distribution of protons and of electric charges (outside more H^+ ions and positively charged ions than inside). The proton distribution determines the pH-gradient and the charge distribution determines the membrane potential. Together they form the proton motive force, which plays a critical role in bacterial physiology. Proton motive force is needed for various energy-requiring processes, such as uptake of amino acids and sugars and rotation of flagella. In addition, respiring bacteria use proton motive force, which they generate by the electron transfer chain, for synthesis of ATP. Fermentative bacteria on the other hand may need to maintain proton motive force at the expense of ATP by extrusion of H^+ ions by H^+ATPase. The membrane potential of active bacteria is typically in the order of -150 mV. Membrane potential can be measured by the uneven distribution of ionic probe molecules. Either positively charged or negatively charged dyes may be used. Positively charged dyes will concentrate in cells with a membrane potential. Rhodamine 123 is frequently used for viability assessment (110, 112, 129, 229). It has also been applied for mitochondrial membrane potential measurements in yeasts (135). Other cationic dyes applied to microbes are the carbocyanine dyes $DiOC_2(3)$ and $DiOC_6(3)$ (60, 151, 163, 187). Negatively charged dyes on the other hand are excluded from cells that maintain a membrane potential. These compounds can only accumulate in cells of which the membrane potential is dissipated, for example by the action of uncouplers. The anionic membrane potential dye that is used most often is the lipophylic bis-(1,3-dibutylbarbituric acid)-trimethine oxonol ($DiBAC_4(3)$) (43, 54, 103, 129, 144, 215). Novo et al. (163)suggested that for accurate bacterial membrane potential measurements the fluorescence should be corrected for cell size.

Intracellular pH

Microorganisms can be classified as acidophiles, neutrophiles or alkalophiles, and they can exhibit a wide range of intracellular pH values, from 5.6 to 9. Under normal growth conditions, the intracellular pH of acidophiles and neutrophiles is, in general, higher than the extracellular pH. Thus the pH gradient contributes to the proton motive force as explained above. The intracellular pH can be measured using probes of which the fluorescence is dependent of the pH, such as fluorescein, BCECF, and cSNARF-1 (20). Various studies suggest that the intracellular pH or maintenance of a pH-gradient provide good indications of cell viability (22, 24, 37, 97). In many studies fluorescence microscopy is used to assess the intracellular pH of individual cells (22, 27, 97, 202), but flow cytometry has been applied as well (37, 125, 137).

Pump activity

Assays of pump activity by fluorescent probes are not (yet) commonly used in microbiology. However, there are several reports of extrusion of fluorescent probes from loaded cells upon energization. For example, rhodamine 123 is extruded by *Listeria* (231) and several yeast species (183), ethidium bromide by *E. coli* (104) and *Salmonella enterica* serovar Typhimurium (90, 91, 157, 158), and various fluorescein derivatives by *Lactococcus lactis* (23, 30, 150) and by *Saccharomyces cerevisiae* and other yeast species (23, 183). Transporter proteins have been identified in *Lactococcus*

lactis (15, 77) and in *Saccharomyces cerevisiae* (93). Cells that extrude a viability probe during incubation give false negative results. This can give underestimations, for example in viability assessment of environmental samples.

However, the probe efflux can also be used as an additional measure of cell viability. Chapter 3 of this thesis describes a novel two step assay using cFDA to assess viability (30). The first step is the measurement of the fraction of intact cells by counting the total number of cells and the number of cF-stained cells. Before the incubation with cFDA the cells need to be de-energized to prevent false negatives because of active extrusion. The population of intact cells may consist of two subpopulations: cells that have the capacity to pump cF out of the cell and cells that lack this capacity. This is tested in the second step of the assay by energizing with a carbon source and measuring the extruded cF by spectrofluorimetry. The two-step assay proved useful for predicting fermentation capacity of *Lactococcus lactis* cultures, even when exposed to various stress conditions. The extrusion of cF by lactic acid bacteria can also be measured using flow cytometry (unpublished results). This has the advantage that heterogeneity among cells can be detected while with spectrofluorimetry only the average of culture is determined. Da Silveira et al. applied flow cytometry to measure cF-efflux of *Oenococcus oeni*. This bacterium is responsible for malolactic fermentation in wine. The cF-efflux was used to assess the metabolic activity of *O. oeni* under ethanol stress and after adaptation. Efflux indicated the capacity of the cells to generate energy both by phosphorylation at substrate level (during glucose consumption) and strictly dependent on the proton motive force (during malolactic activity) (Da Silveira, personal communication). Nebe-von Caron and co-workers used ethidium bromide extrusion in viability assessment. They combined ethidium bromide with propidium iodide and bis-oxonol in one assay for functional assessment of starved *Salmonella* Typhimurium (90, 158). With this combination of probes subpopulations of permeabilized, depolarized, de-energized, and actively pumping cells could be distinguished.

Furthermore, efflux may be used to investigate drug efflux transport. Fluorescent probe efflux is commonly used to test the drug efflux capacity of mammalian carcinoma cells (86). The last decade fluorescent probes have also been identified as substrates of bacterial drug efflux pumps (184, 239). Elucidating the mechanisms of bacterial multidrug resistance has become of great relevance since pathogenic microorganisms have evolved resistance against an increasing range of antibiotics. Pump activity tests using fluorescent probes may be a valuable tool in the investigation of drug resistance.

Thiols

Glutathione (γ-glutamylcysteinylglycine) and other thiols have important functions in cellular metabolism. Thiols have a reactive sulfhydryl (-SH) group and act as reductor in several metabolic processes. Flow cytometric procedures to measure thiols were first discussed in 1983 (63). One of the fluorescent probes that has been used to determine the total level of thiols is monobromobimane, which reacts with sulfhydryl groups (198). Another is monochlorobimane, which is most specific for glutathione. The reaction is catalyzed by the enzyme glutathione-S-transferase. Rice et al. described the use of this probe for glutathione measurement in mammalian cells by flow cytometry (188). The importance of glutathione in eukaryotes has been well established (148, 195, 203). Its main functions

are the maintenance of redox balance, detoxification of reactive oxygen species and multidrug efflux. Some studies have been performed to determine the occurrence of thiols in bacteria. Bacteria seem to be more versatile in the thiols they use than eukaryotes. Glutathione has been detected in some bacterial species, though not in all (68), and recently some novel thiols were detected in prokaryotes (67). The role of thiols in bacterial oxidative stress response, multidrug resistance and redox balance may be studied using fluorescent techniques (33, 184).

Table 1. Functional cell parameters amendable to flow cytometric analyses and examples.

Functional parameter	dye[a]	Applications
Culture growth		
(i) Cell counting	EB	bacteria in body fluids (40)
	PI	bacteria in soil (56)
	Hoechst 33342	*E. coli* (142), bacteria in seawater (141, 142)
	SYTO-13	*E. coli* (43, 44), *Staphylococcus aureus* (42, 44), bacteria in seawater (123, 130)
	SYTO BC	bacteria in milk (84)
	SYBR Green	*E. coli* (6), *Bacillus subtilis* (6), bacteria in seawater (14, 123, 141)
	TOTO / TO-PRO	*E. coli* (142), bacteria in seawater (126, 142)
(ii) Cell tracking	cFDA-SE	*Lactobacillus plantarum* (232)
Biosynthesis		
(i) Morphology	Light scatter	*E. coli* (64, 72, 80, 208, 244, 247, 248), *Pseudomonas aeruginosa* (80), *Klebsiella pneumoniae* (247)
(ii) Nucleic acid content	EB	various bacteria (177)
	Mithramycin and EB	*E. coli* (13, 19, 205, 247, 248), *Klebsiella pneumoniae* (247)
	PI	*E. coli* (13, 64, 80), *Salmonella* Typhimurium (229), *Bacillus subtilus* (5), *Pseudomonas aeruginosa* (80)
	Hoechst 33342	*E. coli* (142, 151), *Salmonella* Typhimurium (109), *Staphylococcus aureus* (58), *Deleya aquamarina* (108), bacteria in seawater (141, 142)
	SYBR Green	bacteria in seawater (74, 141)
	SYTO-13	*E. coli* (83), bacteria in seawater (74, 83, 227)
	TOTO / TO-PRO	*E. coli* (83), bacteria in seawater (83, 126)
(iii) Protein content	Light scatter	*E. coli* (19)
	FITC	*E. coli* (64, 208), *Salmonella* Typhimurium (229), *Staphylococcus aureus* (58), *Bacillus subtilus* (5)
	SYPRO Red	bacteria in seawater (263)
(iv) DVC	Light scatter	bacteria in natural waters (14, 106)
	DAPI	*Salmonella* Typhimurium (109)
Membrane integrity	PI	*E. coli* (6, 43, 72, 129, 131, 153), *Salmonella* Typhimurium (129, 131, 157), *Staphylococcus aureus* (42), *Bacillus subtilus* (6), *Lactococcus lactis* (29, 31, 160), Various lactic acid bacteria (29), Lactobacillus spp (232, 256), *Saccharomyces cerevisiae* (3, 26, 54, 182), *Zygosaccharomyces balii* (182), *Bifidobacterium* spp (12), *Oenococcus oeni* (47), *Trichomonas vaginalis* (95), *Pasteurella piscicida* (138)
	SYTOX Green	*E. coli* (194) (122, 153, 215), *Salmonella* Typhimurium (122), *Staphylococcus* spp (120, 194, 215), *Pseudomonas* spp (120, 215), *Bacillus cereus* (194)
	TOTO / TO-PRO	*E. coli* (153), *Staphylococcus aureus* (164), *Listeria monocytogenes* (137), *Lactobacillus plantarum* (28), various lactic acid bacteria (29), *Micrococcus luteus* (51, 164, 245)
	*Bac*Light	*E. coli* (215, 242), *Salmonella* Typhimurium (109), *Staphylococcus* spp (120, 215), *Pseudomonas* spp (120, 215), *Bacillus subtilis*(242), *Listeria monocytogenes* (159, 217), *Lactococcus lactis* (31), *Deleya aquamarina* (109)

Table 1. (continued)

Functional parameter	dye[a]	Applications
Metabolic activity		
(i) Esterase activity	FDA	various bacteria (60), bacteria in freshwater (38), *Trichomonas vaginalis* (95), *Saccharomyces cerevisiae* (182)
	cFDA	*Listeria monocytogenes* (159, 191), *Bacillus subtilis* (57), various bacteria (59), bacteria in water (178), various lactic acid bacteria (29), *Lactobacillus plantarum* (28), *Bifidobacterium* spp (12), *Oenococcus oeni* (47), *Klebsiella pneumoniae* (59), *Saccharomyces cerevisiae* (3)
	calcein AM	*E. coli* (44), *Staphylococcus aureus* (42, 44), various bacteria (59)
	cDFDA	filamentous fungi (25)
	BCECF-AM	*Listeria monocytogenes* (159), various bacteria (59)
	Chemchrome	*Salmonella* Typhimurium (39) (229), *Bacillus subtilis* (57), various bacteria (59), *Listeria monocytogenes* (159), *Lactobacillus acidophilus* (121), bacteria in waters (167, 178), *Deleya aquamarina* (108), *Saccharomyces cerevisiae* (54)
(iii) Membrane potential	Rh123 (cationic)	*E. coli* (43, 60, 129, 131), *Salmonella* Typhimurium (129, 131, 229), *Staphylococcus aureus* (42, 58), *Staphylococcus* spp (120), *Bacillus subtilis* (57, 60), *Pseudomonas* spp (60, 120), *Listeria monocytogenes* (159), *Micrococcus luteus* (110-112, 245), *Pasteurella piscicida* (138), *Saccharomyces cerevisiae* (54, 135), *Zygosaccharomyces balii* (135)
	$DiOC_2(3)$ (cationic)	*E. coli* (151, 163), *Staphylococcus aureus* (163, 164), *Pseudomonas aeruginosa* (163), *Micrococcus luteus* (164), *Listeria* species (187)
	$DiOC_6(3)$ (cationic)	various bacteria (60)
	$DiBAC_4(3)$ (anionic)	*E. coli* (43, 54, 103, 129, 131, 132, 144, 215), *Salmonella* Typhimurium (129, 131, 144, 157, 229), *Staphylococcus aureus* (42, 54, 144, 214, 215), *Pseudomonas aeruginosa* (215), *Bacillus* spp. (54), *Bifidobacterium* spp (12), *Trichomonas vaginalis* (95), *Saccharomyces cerevisiae* (3, 54)
	Oxonol VI (anionic)	*E. coli* (132)
(iv) Intracellular pH	cFDA-SE	*Listeria monocytogenes* (137), *Clavibacter michiganensis* (37)
	c-SNARF1-AM	*Corynebacterium glutamicum* (125)
(v) Pump activity	EB	*E. coli* (104), *Salmonella* Typhimurium (157)
	cF	*Saccharomyces cerevisiae* (23),
(vi) Thiols	monobromobimane	various bacteria (67, 68)

[a] abbreviations: EB, ethidium bromide; PI, propidium iodide; SYTO BC, SYTO® BC Bacterial Stain; SYBR Green, SYBR® Green I nucleic acid gel stain; TOTO / TO-PRO, compounds of the dimeric TOTO or monomeric TO-PRO nucleic acid stain series; cFDA-SE, carboxyfluorescein diacetate succinimidyl ester; FITC, fluorescein isothiocyanate; SYPRO Red, SYPRO® Red protein gel stain; DAPI, 4',6-diamidino-2-phenylindole, dihydrochloride; SYTOX Green, SYTOX® Green nucleic acid stain; *Bac*Light, LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (consists of SYTO 9 and PI); FDA, fluorescein diacetate; cFDA, carboxyfluorescein diacetate; calcein-AM, calcein acetoxymethylester; cDFDA, carboxy-dichloro-fluorescein-diacetate; BCECF, 2',7'-bis-(2-carboxyethyl) -5-(and-6)-carboxyfluorescein; CTC, 5-cyano-2,3-ditolyl tetrazolium chloride; Rh123, rhodamine123; $DiOC_2(3)$, 3,3-diethyloxacarbocyanine iodide; $DiOC_6(3)$, 3,3'-dihexyloxacarbocyanine iodide; $DiBAC_4(3)$, bis-(1,3-dibutylbarbituric acid)-trimethine oxonol; Oxonol VI, bis-(3-propyl-5-oxoisoxazol-4-yl)pentamethine oxonol; c-SNARF1-AM, carboxy SNARF®-1 acetoxymethyl ester; cF, carboxyfluorescein.

DETECTION AND ENUMERATION

Flow cytometry can be applied for total or specific detection and counting of microbes. In addition, flow cytometry can be used for cell tracking. Various approaches for detection of microbes will be described in the following paragraphs.

Total detection and enumeration

Flow cytometry has been used for counting microbes in pure cultures and in diverse environments such as water, air, blood, feces, compost, soil, food or industrial production lines (50, 57, 123, 140, 177). Counting is often performed using a dye that stains nucleic acid. Impermeant stains such as ethidium bromide, propidium iodide and SYTOX Green require fixation to permeabilize the membrane. Permeant dyes such as acridine orange, Hoechst 33342, DAPI and SYTO probes can be used directly, although fixation may increase the labeling. Fluorescent staining facilitates the detection of the microorganisms. In samples from environments such as foods, soil, and feces, the microbes would not be detectable without staining. However, in buffer or in an environment with low background, such as seawater, bacteria can also be detected just by their light scatter.

The concentration of the cells in the sample can be calculated directly from the number of cells counted, provided that the flow rate is known. For a known flow rate, a constant-volume sample delivery system needs to be incorporated, such as a syringe pump. However, most currently used flow cytometers have not incorporated either constant volume sample feed or flow monitoring. Flow rate can also be calculated from a test sample that is weighted before and after a run with a set time. However, the flow rate often varies between the samples and is affected by the composition of the cell suspension. For example, a sample of bacteria in buffer has a significantly different flow rate than a sample of bacteria in cleared milk (unpublished results).

A better approach is to calculate the concentration of cells proportional to an included standard. Fluorescent polystyrene beads added to the sample in an exactly known concentration are commonly used. The beads have to be detectable with the same settings as the cells, but should not overlap with the cell population, at least not for all parameters. After running the sample, the cells of interest and the beads are counted. Since the bead concentration is known, the concentration of cells can be calculated.

Specific detection by intrinsic parameters

A number of intrinsic cell parameters may be used for detection of specific (groups of) microorganism or for discrimination between microorganisms in a mixture. Autofluorescence is commonly used to identify autotrophic marine microorganisms such as algae and cyanobacteria. They contain pigments such as chlorophylls, cartenoids, phycoerythrin, phycocyanin, allophycocyanin, etc. The type and amount of pigments differ among the species. Therefore, each autotrophe has a distinctive autofluorescence dependent on its photosynthetic pigment signature. This has been used to classify

unicellular phototrophes in marine samples (126, 226). DNA content and cell size are also used to differentiate species, for example to in bacterioplankton (74, 141, 227). Flow cytometry of intrinsic parameters has led to discovery of new species, such as the marine bacterium *Prochlorococcus* (35) and the green alga *Ostreococcus tauri*, which is less than 1 µm and was reported as the smallest known eukaryote (45). Another intrinsic parameter is the DNA base composition (GC content). This has been used to detect various species in urine (238). Finally, Gram-positive and Gram-negative organisms can be detected specifically by a fluorescent Gram stain (65, 86).

Specific detection by antibodies

The growing range of monoclonal antibodies has greatly extended the utility and discriminating power of flow cytometry. Antibodies may be labeled with fluorescent tags, thus enabling detection of a given cell type in a mixed population as well as quantification of surface antigens on the target cells. A particular benefit of the approach is that cells need not be in a culturable status; dormant cells and active but nonculturable cells are detected as well. One example of the use of fluorescently stained antibodies is the screening for the presence of *Legionella* subsp. in cooling-tower water (98, 230). The speed of flow cytometry analysis is a big advantage over the conventional plating and animal experiments, as well as the fact that cells are detected that are not culturable at the moment of sampling, but that may recover and cause illness when the water is drunken. Another example is the detection of the foodborne pathogen *Listeria monocytogenes* (61) and *Salmonella* Typhimurium (145) in milk by FITC-labeled antibodies. Careful antibody preparation is required if cross reactivity is to be avoided. In flow cytometric-immunofluorescence studies on detection of *Bacillus* subsp. the antibodies used were specific enough to discriminate between *B. anthracis* and *B. subtilis*. However, the antigenic overlap between the moderately pathogenic food poisoning *B. cereus* and the mortal, infectious *B. anthracis* was a problem in these assays (175, 176). This may be explained by their close genetic relationship (87). The last example here is the detection of *Escherichia coli* O157:H7. This serotype of *E. coli* is one of the most virulent foodborne pathogens causing the so-called Hamburger disease outbreaks. *E. coli* O157:H7 can be lethal, which convinced authorities as well as food manufacturers of the value of rapid detection methods. Research has shown that this is possible by flow cytometry using antibodies. By using enrichment followed by separation with immunomagnetic beads as few as four *E. coli* O157:H7 cells per gram of ground beef could be detected (199).

Specific detection by oligonucleotide probes

Ribosomal RNA contains more and less variable regions and this allows for phylogenetic typing on various levels of specificity (255). Highly variable regions may be specific to the species level, while more conserved regions give a higher, more general, hierarchy classification. Ribosomal RNA-targeted oligonucleotide probes have been used for more than a decade for various microbial applications (2, 127, 179). The probes are about 20 nucleotides long and usually tagged with a single dye molecule. The most frequently used dyes are fluorescein, tetramethylrodamine and the

indocarbocyanine dyes CY3, CY5 and CY7. One dye molecule per probe molecule is enough because a cell contains multiple copies of rRNA molecules, so there are enough targets to provide sufficient labeling. The labeled probe is quite small and can penetrate fixed cells. Under the right conditions, the probe hybridizes specifically to the rRNA target sequence. Phylogenetic staining can be used to study microbial consortia *in situ*. For example, Wallner and co-workers used flow cytometry and rRNA-targeted probes for identification of bacteria in sewage sludge (250). They could unambiguously identify 70 to 80% of Hoechst-stained cells in samples from a wastewater treatment plant with probes specific for a variety of bacterial domains. Another example is a study of Zoetendal et al. of bacterial communities in the human intestine by 16S rRNA-targeted probes using fluorescent *in situ* hybridization and flow cytometry on fecal samples (261). Various groups of bacteria were quantified and it was found that the uncultured *Ruminococcus obeum*-like bacteria are a subgroup of the *Clostridium coccoides - Eubacterium rectale* group, and that *R. obeum*-like bacteria are predominantly present in human feces.

Cell tracking

Cell tracking can be used to study bacterial transport and the fate of cells in an environment over a prolonged period of time. It requires long-term fluorescent staining which does not affect viability, adhesion and transport characteristics. Various vital stains have been tested for cell tracking, including DAPI and cFDA-SE. Cell tracking has been used to investigate where underground water streams end up and to monitor bacterial transport through soil. The latter application was used in bioremediation of contaminated soil and groundwater (71). Cell tracking dyes have also been used for rapid isolation of novel yeasts hybrids by flow cytometric sorting (11). By using one parent strain with a fluorescent green stain and another with a fluorescent orange stain, hybrids were selected by their dual green and orange fluorescence. Even with rare mating a high enrichment is obtained by this technique. Thus, only few colonies need to be screened to identify novel strains that have desirable industrial traits. The 'conventional' method for selection uses introduction of antibiotic resistance markers. This is an effective but undesirable method for selecting industrial hybrid yeasts, since the products are released live into the environment. Therefore, flow sorting of hybrids based on cell tracking dyes provides a very attractive alternative.

Detection using the Green Fluorescent Protein

In the past five years the Green Fluorescent Protein (GFP) of the bioluminescent jellyfish *Aequorea victoria* has attracted considerable attention as a marker and reporter protein (216, 228). The gene for this fluorescent protein was cloned in 1992 and was subsequently expressed in various eukaryotes as well as prokaryotes. GFP is a protein of 238 amino acids that spontaneously emits light at 508 nm when excited with blue light at 395 nm. GFP can easily be detected with epi-fluorescent microscopy, as well as with flow cytometry. The protein requires post-translational oxidation to become fluorescent. It is stable, photobleaches very slowly, and does not require energy to fluoresce. Full

activity is achieved at pH values around 7. Genetically engineered mutant GFPs with shifted excitation and emission spectra or changed stability have increased the range of applications. These fluorescent proteins are attractive markers to monitor microbial cells in the environment and reporters for the analysis of gene expression (66, 192, 216, 229). Furthermore, some mutants of GFP can be used as internal pH indicator (118).

GFP has been used as a reporter for gene expression in various bacteria. One example is the study of *E. coli* stress response using gfp_{uv} fused to heat shock stress protein promoter elements (34). Another application is the study of virulence factors of bacterial pathogens. GFP was used as a transcriptional reporter and genes could be identified that were up-regulated in response to engulfment of *Salmonella* Typhimurium by host macrophages (235). Furthermore, GFP was applied to follow expression of genes involved in sporulation in *Bacillus subtilis* (124). Recently, GFP was expressed in *Lactococcus lactis* and it was shown that GFP-mediated fluorescence could be detected in milk background even at a pH of around 5 (53).

A number of bacterial species have been marked with GFP for environmental detection and monitoring in various environments, including soil, water, activated sludge, rhizospere, and biofilms (66, 233). Examples include the monitoring of cell numbers of GFP-tagged *E. coli* and *Pseudomonas fluorescence* in soil samples during 30 days by flow cytometry (234), and the localization and viability assessment of *Pseudomonas fluorescence* in barley rhizospere by CSLM and counting of fluorescent microcolonies (161). Also, *gfp*-tagging was used for tracing and survival studies of lactic acid bacteria in the gastrointestinal tract, which is of relevance in the development of bacteria as live vaccine vectors (75, 197).

The effect of various physiological cell states on GFP fluorescence has been investigated using *gfp*-tagged *Pseudomonas fluorescence* (133). It was shown that starved cells and viable but nonculturable cells remained fluorescent, however fluorescence was lost by treatment with UV light or incubation at 39°C or higher. Results indicated that loss of membrane integrity resulted in loss of fluorescence. In a viability study of the fungus *Aureobasidium pullulans* exposed to biocides GFP fluorescence correlated with the number of viable cells, indicating the potential of this technique for susceptibility testing (253).

GFP and its mutants provide elegant tools for the study of gene expression, permeability, intracellular pH, localization, and survival. The range of applications will surely further expand the coming years.

COMBINATION OF FLUORESCENT PROBES

Multiple physiological probes

Cell physiology is often assessed using multiple probes. The aim can be to obtain a more complete impression of the physiological state by measuring various cell properties, or to obtain a more confident assessment of one physiological property by using various probes. One can use multiple probes in multiple assays (each assay with one probe), or one can use a combination of probes simultaneously in a multi-staining assay. For multi-staining assays probes have to be selected with excitation and emission wavelengths which allow distinction of each probe in the presence of other. Some multi-staining combinations have just one discriminatory probe combined with a 'counterstain' that stains all cells. For example, the commonly applied *Bac*Light LIVE/DEAD Kit combines PI with SYTO 9 (86). The same principle has been used by multi-staining with PI combined with other SYTO stains (43), or with SYBR Green I (6). The advantage of this kind of multi-staining in stead of the discriminatory probe solely is that all cells are labeled and therefor easily detected and counted.

Other multi-staining assays combine two probes that both discriminate populations based on physiological properties. Examples are the combination of PI (red fluorescence, compromised membrane) with cFDA (green fluorescence, esterase activity and intact membrane) (29, 30, 36, 258), PI with rhodamine 123 (green fluorescence, polarized membrane), PI with bis-oxonol (green fluorescence, depolarized membrane) (43, 129, 131), CTC with rhodamine 123, and CTC with bis-oxonol (129).

Simultaneous incubation with multiple probes may result in double staining. This has for example been observed with the combination of cFDA and PI applied to ethanol-stressed *Oenococcus oeni* (47) and to bile-stressed *Bifidobacterium bifidum* (12). In both studies staining with cFDA and PI resulted in three populations: one population stained only by cF indicating the intact cells, a second population stained only by PI indicating membrane-compromised, dead cells, and a third population stained with both cF and PI. This population is just minorly damaged; the membrane has permeabilized, but to a low extent. Thus, the cells do not exclude PI any more, but still the membrane has enough integrity to maintain cF in the cell. This physiological status is a transient phase in the progressive change towards cell dying, however, death is not yet irreversible and cells may still recover. Sorting of the double stained population of stressed *Bifidobacterium bifidum* showed that a significant part could resume growth on plates after resuscitation (12).

A physiological multi-staining assay can even use a combination of three probes, as demonstrated by Nebe-von Caron and co-workers (157). They applied PI for membrane integrity, bis-oxonol for membrane potential, and ethidium bromide for pump activity in one assay. With the use of cocktails of fluorescent stains, good multivariate data analysis methods are important for the extraction of information from the data obtained. Davey et al. showed the value of artificial neural networks for such analysis (49).

In many viability studies two or more physiological properties are examined in separate assays. For example, Comas and Vives-Rego (43) studied effects of antibiotics on membrane integrity and

membrane potential of *E. coli*. Another example is a study by Suller and Lloyd (215) who monitored antibiotic induced damage of clinical isolates of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *E. coli* with membrane integrity probes, the respiration probe CTC and a membrane potential probe.

Also, multi-assay analysis has been used in starvation studies. Kaprelyants et al. studied starvation, dormancy, and resuscitation of *Micrococcus luteus* using a variety of approaches including nucleic acid dyes PI and TO-PRO3 for membrane integrity, CTC for respiration, and rhodamine 123 for membrane potential (51, 111, 112). The results showed a detailed picture of the heterogeneity in starved cultures. Similar heterogeneity has been detected in long-term batch cultures of *Salmonella* Typhimurium (229). Furthermore, Joux et al. measured DNA content, DVC, respiration, membrane integrity and total cell count to provide insight into the succession of cellular states of *Salmonella* Typhimurium and *Deleya aquamarina* during starvation in seawater (108, 109).

Luppens et al. (136, 137) used multiple probes to study the effect of the disinfectants benzalkonium chloride and hydrogen peroxide on viability of *Listeria monocytogenes* exponential- and stationary phase cells. Comparison of plate counting with CTC staining, TOTO-1 exclusion, and maintenance of the pH-gradient (determined with cFDA-SE) gave more insight into sub-lethal and lethal damage caused by disinfectants. The last example is the study of physiological changes in the early stages of surface-attached growth in a biofilm by Williams et al. who used FITC for total protein content, CTC for respiratory activity, and bis-oxonol and rhodamine 123 for membrane potential (254). These examples show the value of using multiple probes in providing single-cell based information on the physiological condition of bacteria and the use for monitoring changes and investigating heterogeneity.

Species-specific and physiological probes

When specific detection and physiological assessment are combined this allows for the study of one species within a complex community. This may be applied for example to measure the activity of specific pathogens in food or water, to detect which bacteria are involved in waste water treatment, or to monitor the fermentation activity of the various species of a mixed starter culture. Specific detection with rRNA-targeted probes requires permeabilization of the cells, so this limits their use in combination with physiological probes. Contrarily, immunofluorescence probes can be targeted against surface antigens so labeling can be performed without cell fixation. Immunofluorescent probes can conveniently be combined with physiological probe, provided that a fluorescent label is chosen with suitable excitation and emission wavelength.

Nebe-von Caron et al. (158) applied immunostaining with physiological probes for specific isolation of intact *S. sanguis* cells from dental plaque with FITC-tagged antibodies. Intact cells (PI negative and EB positive) were sorted and their viability was tested by cultivation. Also, monoclonal antibodies have been combined with rhodamine to study survival of *Francisella tularensis* during aerosolation (88), and with Chemchrome B for specific detection of viable *Salmonella* Typhimurium cells (39). Furthermore, the combination of an antibody against wall lipopolysaccharide detecting

Gram-negative bacteria with PI and SYBR-I has been suggested as a novel method for viable bacteria immunodetection (6).

Van der Waaij et al. analyzed noncultured anaerobic bacteria in human feces using PI to discriminate bacteria from other compounds and FITC-labeled antibodies against human IgA to detect the IgA-coated bacteria (236). A final example of a combination of viability and taxonomic probes is the research of Ben Amor et al. who investigate gastro-intestinal microbiota using fecal samples (Ben Amor, personal communication). Their approach is first to sort subpopulations with the viability probes PI and cFDA and then to examine these subpopulations with rRNA-targeted probes for the presence of specific species. This is applied to monitor bifidobacteria, which are considered as good colon bacteria and are often used in probiotics. With the combined approach more insight can be gained about survival and activity of bifidobacteria in such a complex ecosystem as the colon.

APPLICATIONS IN FOOD MICROBIOLOGY

In situ microscopic detection

Microbes can be detected in foods *in situ* by microscopic methods. A long established approach is light- or fluorescence microscopic analysis of smears of the food specimen onto a microscopic slide, stained with an appropriate dye. A dye reduction test using methylene blue is commonly employed to estimate the number of viable organisms. This is used for example in dairy industry for microbial quality control of raw milk and dairy products according to a method originally developed by Breed (143).

For the microscopic analysis of solid foods cryosectioning is applied. Yiu (259) tested various viability stains in a fluorescence microscopic study of cheese and found acridine orange suitable for simultaneous detection of various cheese structures, including the microflora. Microscopy can also be applied using antibodies for specific detection. The antibodies may be tagged either with a fluorescent or a diachromatic dye (204, 211, 257). For example, Stringer *et al*. (209) applied cryosectioning and light microscopy to detect colonies of the spoilage bacterium *Brochothrix thermopshacta* in sausage using a specific antibody against this species. Furthermore, nisin producing *L. lactis* colonies were detected using an anti-nisin antibody (210). Beimfohr et al. applied fluorescence microscopy for *in situ* detection of specific bacteria in milk (10). After extraction of the bacteria the cells were fixed to coated glass slides and lactococci, enterococci and streptococci were identified using fluorescent-labeled specific rRNA-targeted oligonucleotide probes.

Microscopic examination of food sections is labor-intensive, because it often requires the examination of many fields of view. However, detection and quantification can be (semi-)automated by image analysis techniques. Fernandes et al. (69) developed a light microscope-based system that could scan heterogeneous foodstuff for bacteria using Gram-staining and select those fields that contained bacteria to be examined by a microscopist.

A second method for *in situ* detection of microbes in food is with confocal scanning laser microscopy (CSLM). This technique has the advantage that it can be used with non-smooth surfaces and does not require fixation. CSLM provides an excellent tool to study localization and behavior of food pathogens as well as food fermentation bacteria. For example, Kim et al. applied CSLM for the three-dimensional visualization of *Salmonella* attachment to chicken skin by staining with Pyronin-Y (117). Seo and Frank (201) used CSLM to study attachment of *E. coli* O157:H7 to lettuce. With FITC-labeled specific antibodies they were able to show that this pathogen preferentially attached to cut edges on lettuce leafs, and not to a *Pseudomonas* biofilm which grew mainly on intact surface. Also, they showed that many cells survived chlorine treatment using PI staining. Recently, *E. coli* O157:H7 tagged with GFP was used in CSLM studies of food, which has the obvious advantage that no staining procedure is needed. This was applied to detect attachment on green leaf lettuce, cauliflower, and tomato (218), and to surfaces and internal structures of apples (32).

CSLM has also been used in food fermentation studies. Starter bacteria have been observed with CSLM in Gouda cheese (31), and in fermented milk, cheddar cheese, and spray-dried probiotic milk

powder (4, 165). The permeabilization and lysis of the starter bacteria are of crucial importance in the maturation of cheese. With propidium iodide and SYTO 9 (the *Bac*Light LIVE/DEAD bacterial viability kit) both intact cells and permeabilized cells could be detected in young Gouda cheese, which confirmed the hypothesis of persistence of permeabilized cells in the cheese matrix (31). O'Sullivan et al. (165) used *Bac*Light to compare cheese curd made with a lytic or a non-lytic *L. lactis* strain and Auty et al. (4) used the same staining to assess viability of probiotic bacteria in dairy products. These studies demonstrate the potential of CSLM to study the status of the bacterial cells *in situ* during dairy fermentation processes.

The direct microscopy techniques enable the study of preferential attachment, growth, and behavior of cells and colonies on the food product. Thus, *in situ* microscopy is extremely useful for understanding the interaction of microbes with foodstuff. However, it is not very suitable for quantitative measurement of the bacterial concentration. The presence of clumps and colonies and background staining of food structures may result in inaccurate counting.

Direct epifluorescence filter technique (DEFT)

Bacteria in liquids can be concentrated on membrane filters, stained with fluorochromes, and analyzed with epifluorescence microscopy. This is widely used for direct counting of aquatic bacteria (21, 92). Direct filtration of milk is impractical because somatic cells and fat globules cause blockage of the pores of the filter. However, this can be solved with a suitable surfactant or protease. Pettipher and colleagues developed a DEFT method for raw milk (171, 172). In this method milk is pretreated with Triton X-100 and trypsin, filtered through a 0.6 μm-pore-size polycarbonate membrane filter, stained with acridine orange, and examined visually with an epifluorescent microscope. Further development automated the microscopic examination by using an image analyzer (169, 173). The DEFT method was successfully applied to a range of food products including ice cream, butter, meat, fish, and vegetables (169, 173). DEFT has also been used to enumerate bacterial spores in broth and in milk (116, 152). Bacterial staining with acridine orange is usually very efficient and, in principle, it should differentiate between live and dead microorganisms. Stained RNA fluoresces orange while stained DNA fluoresces green, so, as the ratio of RNA/DNA is higher in actively growing cells, these should fluoresce orange whereas dead cells should fluoresce green. However, several observations question the reliability of acridine orange as culturability indicator (116, 169, 186).

DEFT has also been applied with other dyes. For example, *Bac*Light was used to count total and viable bacteria in drinking water (17), processed meats (62), and wine (46). Fluorescent antibodies have been used as well with DEFT, for example to detect *E. coli* O157:H7 in milk and juice (223).

In the last decade several commercial DEFT instruments have been developed, such as COBRA (BIOCOM, France), Autotrak (A. M. Systems, UK), Bio-Foss Automated Microbiology System (Foss Electric, Denmark) and Scan RDI (Chemunex, France) (114). The COBRA instrument was described and evaluated by Pettipher (174). This instrument fully automated the sample filtration, staining, rinsing, drying and counting stages of DEFT. It could enumerate bacteria in cultures, raw milk, meat and fish. One operator could process more than 100 samples per hour, and results were available

within one hour (174). Scan RDI is based on detection of fluorescently stained bacteria on a membrane filter by scanning with a laser (149, 251).

Flow cytometry

Flow cytometry is extremely useful for counting and analyzing cells suspended in liquid at concentrations of at least 10^3 cells/ml. Applications of flow cytometry to food products are summarized in Table 2.

Yeasts

In the brewing industry, flow cytometry has been used for process monitoring and optimization, and for quality control. For example, wild yeast infections could be detected with flow cytometry using selective enrichment (105). Furthermore, the changes in DNA, β-hydroxysterol, and neutral lipids during fermentation and storage were investigated to determine their correlation to beer quality (155). The lower limit for direct detection of yeasts in beer is approximately 10^3 cells/ml (105). Pettipher (170) used the Chemflow system to detect the presence of spoilage yeasts in soft drinks. Depending on the sample type the sensitivity ranged from 50 to 14,000 cells/ml. Flow cytometric detection of yeast in beer or soft drinks is relatively simple. Yeasts are much bigger than bacteria, and beer and soft drinks have a relatively low concentration of particles that may give background signal. The detection of bacteria in food products such as milk or meat with flow cytometry proved to be a much bigger challenge and proper sample preparation is an important aspect of the analyses.

Bacteria in milk

Direct enumeration

Patchett et al. (168) tried direct enumeration of total bacteria in milk, as well as in meat and pâté, using light scattering and ethidium bromide and mithramycin staining. The results of the meat and pâté are described later. Application to milk was severely restricted by the presence of high levels of suspended material. *E. coli* inoculated at 6×10^7 CFU/ml could not be detected. It was clear that further development was needed to reduce background signal from the food matrix.

Enrichment

Specific enrichment can be used when the target microbe is present in concentrations too low for direct detection. It is often applied to detect pathogens in foods by plating. Donnelly et al. (61) used specific enrichment prior to flow cytometry analysis to detect low-level contamination of milk with the pathogen *Listeria monocytogenes*. An initial cell population as low as 8 CFU/ml grew to 1.7×10^6 CFU/ml in 24 h. At this level *Listeria monocytogenes* could be detected, using light scatter and DNA fluorescence profiles in addition to immunofluorescence for gating of the targeted population. Specific enrichment may be suitable for detection of the presence of a specific microbe, however it is not a method for direct and accurate counting.

Milk clearing

McClelland and Pinder (145) adopted a milk clearing procedure that was commercially available in a milk assay kit for total viable organisms by means of an ATP bioluminescence assay (Promega, Southampton, UK). In this procedure the milk it is mixed with a reagent containing the detergent Triton X-100 and a chelating agent. This results in lysis of somatic cells, flocculation, and coalescence of the micelles. By centrifuging the milk separates in a clear liquid with a cream pad on top and a bacterial pellet on the bottom. The reagent also contains polystyrene beads that sediment slightly slower than cells. The beads visualize the location of the pellet, also if the amount of cells is too small to form a visible pellet by itself. After careful aspiration of the fat and liquid the pellet is resuspended in buffer. McClelland and Pinder proved that that it was possible to enumerate *Salmonella* Typhimurium in milk or eggs with this clearing procedure and fluorescently labeled monoclonal antibodies with a detection limit of 10^3 cells/ml within an assay time of 40 minutes. In addition, with nonselective enrichment *Salmonella* could be detected at a concentration of at least 10 cells/ml in milk and 1 cell/ml in egg.

Gunasekera et al. (84) also applied milk clearing. They used proteases and Triton X-100 and stained milk samples with SYTO BC to determine total bacteria. The sensitivity of this method was 10^4 cells/ml, which is below the level of detection required for milk hygiene assurance in many countries. Furthermore, flow cytometric counts of bacteria in raw milk correlated well with plate counts and direct microscopy.

Immunomagnetic separation

Another useful tool is immunomagnetic separation (IMS). IMS is a capture method that uses paramagnetic beads coated with a monoclonal antibody specific to a target organism. A magnetic field is used to isolate the bead-organism complex away from contaminating particles. IMS acts both as a separation and concentration step, and is extremely suitable to facilitate detection of bacteria in environments such as foodstuff. Seo et al. (199, 200) applied IMS with flow cytometry for rapid detection of *E. coli* O157:H7 in milk, ground beef and apple juice. The foods were artificially contaminated with very low numbers (2 - 6 CFU/g). Provided that long enough enrichment was used the contaminated samples could be positively identified using IMS to capture the cells and a second, FITC-labeled antibody against *E. coli* O157:H7 for staining prior to flow cytometry. Goodridge et al. (78) developed another approach for the detection of *E. coli* O157:H7. They combined IMS with fluorescently stained highly specific bacteriophages to label the target cells and by using enrichment, low contamination numbers in milk and ground beef were detectable (79).

Bactoscan

In the beginning of the 1980's the Bactoscan method (Foss Electric, Hillerød, Denmark) was developed as a routine method for determination of the bacteriological quality of raw ex-farm milk (212). After the version Bactoscan 8000, which has been used in many dairy companies, a new generation instrument has been introduced recently coined Bactoscan FC. This is a fully automated instrument based on a real flow cytometric technique. First the milk sample is filtered through a 100-

µm filter. Then bacteria are stained with ethidium bromide and disturbing milk components are reduced by chemical/heat treatment. After this incubation, which takes 8.5 minutes, part of the sample is injected into the flow cell where sheath fluid carries it past a laser beam. Stained particles emit light pulses, which are counted if they are higher than a fixed threshold (213).

The predecessor, Bactoscan 8000 counted cells with a fluorescence microscope in stead of a cytometer with laminar flow (212). Acridine orange was applied with Bactoscan 8000 in stead of ethidium bromide, and a different pretreatment of the milk sample, which included gradient centrifugation to separate bacteria from other milk constituents. The stained bacterial solution was transferred in the form of a thin, standardized film onto the surface of a rotating disc and the stained particles were registered by a fluorescence microscope. Events were counted as cells when the pulse height is above threshold.

The Bactoscan methods have been evaluated for their accuracy, and have been compared with each other and with the standard macrocolony method (16, 186, 212, 213, 219). The Bactoscan methods proved suitable for qualitative analysis of milk and may be used in stead of standard plate counts. Such evaluation is required for new or modified routine methods for microbiological examination of milk to become accredited according to international standards (101, 102, 220).

Microcyte

The Microcyte, a relatively cheap and portable (10 kg) flow cytometer, was introduced in 1994 (76). It was designed primarily for analysis of microorganisms (50) but it has also been applied successfully to mammalian cell cultures, for example to study apoptosis (85). The instrument employs a 635-nm laser diode as light source and has two solid-state photodiode detectors, one for light scatter and one for fluorescence. It can detect and provide accurate counts of particles as small as 0.4 µm and the lower limit of detection is below 10^3 cells/ml. Total counts and viable counts of bacteria and yeasts could be performed readily in samples with complex background such as pasteurized whole milk (50). Total cell counts were performed with the nucleic acid probe TO-PRO-3 after permeabilization of the cells, and viability was tested using carboxynaphtofluorescein diacetate, which works in a similar way as fluorescein diacetate. These probes were shown to be suitable for the 635-nm excitation in the Microcyte.

Assessment of starter bacteria

Besides for the bacterial hygiene control of milk, flow cytometry can be used to study fermentation starter bacteria in dairy productions. Laplace-Builhé reported flow cytometric enumeration during production of a dairy starter. After 4 hours of fermentation of milk with *Lactobacillus acidophilus*, when the bacteria had reached a level of 10^7 cells/ml, viable cells could be enumerated using Chemchrome staining (121).

In Chapter 6 of this thesis the application of flow cytometry for the assessment of total counts and viability of (probiotic) fermentation starter bacteria is described. An optimized procedure using Promega's milk clearing solution was developed for better clearing and flow cytometry performance. The samples were stained with cFDA and TOTO-1 to discriminate intact and membrane-damaged

Table 2. Flow cytometric detection of microbes in foods and drinks

target	food product	detection method	pretreatment	detection limit[a]	references
yeasts	soft drinks	Chemchrome (Chemflow)		10^2-10^4	(170)
	beer	Chemchrome (Chemflow)		10^2-10^3	(105)
	wine	lights scatter or viability stain[b]		10^3	(139)
total bacteria	skim milk medium	Chemchrome (Chemflow)		N.D.[c]	(121)
	milk	mithramycin and ethidium bromide		$> 10^7$	(168)
	milk	SYTO BC	clearing	$< 10^4$	(84)
	milk	acridine orange (Bactoscan 8000)	clearing	$< 10^4$	(212)
	milk	ethidium bromide (Bactoscan FC)	clearing	$< 10^4$	(213)
	milk	cFDA + TOTO-1	clearing	$< 10^4$	(28)
	dairy starters	cFDA + TOTO-1	clearing	N.D.[c]	(28)
	probiotic drinks	cFDA + TOTO-1		N.D.[c]	(28)
	wine	lights scatter or viability stain[b]		10^4	(139)
	meat	mithramycin and ethidium bromide	stomachering	10^5	(168)
	pâté	mithramycin and ethidium bromide	stomachering and filtration	10^6	(168)
	apple juice	immunofluorescence	enrichment (24 h)	0.1	(224)
Listeria monocytogenes	milk	immunofluorescence[d]	enrichment	8	(61)

cells. The enumeration was very accurate and the sensitivity was at least 10^5 cells/ml. Results showed that dairy starters and various probiotic products contained considerable numbers of permeabilized cells as well as intact but nonculturable cells. The relatively high numbers of intact but nonculturable cells indicate that the fermentation or probiotic potential of a culture may be considerably higher than expected based on plate counts.

Bacteria in other beverages

Malacrinò et al. (139) used flow cytometry for direct detection of yeasts and malolactic bacteria in wine with viability probes. The detection limit for yeast (10^3 cells/ml) was the same as for pure culture showing no significant interference due to wine debris. However, the natural debris of wine did interfere with detection of bacteria. Therefore the sample preparation procedure was optimized by centrifuging and washing.

Seo et al. (199) applied IMS-FCM for specific detection of *E. coli* O157:H7 using antibodies to milk, as described above, and also to apple juice. Furthermore, Tortorelli et al. (224) compared

Table 2. (continued)

target	food product	detection method	pretreatment	detection limit[a]	references
Salmonella Typhimurium	milk	immunofluorescence	clearing	10^3	(145)
	milk	immunofluorescence	clearing and enrichment	10	(145)
	eggs	immunofluorescence	clearing	10^3	(145)
	eggs	immunofluorescence	clearing and enrichment	1	(145)
	chicken carcass washes	immunofluorescence	IMS	10^4	(252)
	chicken carcass washes	immunofluorescence	enrichment (18 h) and IMS	0.2	(252)
E. coli O157:H7	ground beef	immunofluorescence	IMS	$< 10^3$	(200)
	ground beef	immunofluorescence	enrichment (9 h) and IMS	5	(199)
	ground beef	specific bacteriophages stained with YOYO-1	enrichment (6 h)	2.2	(79)
	ground beef	immunofluorescence	enrichment and clearing	20	(225)
	raw milk	immunofluorescence	enrichment (6 h) and IMS	5	(199)
	raw milk	specific bacteriophages stained with YOYO-1	enrichment (10 h)	10^2-10^3 [e]	(79)
	apple cider	immunofluorescence	enrichment (6 h) and IMS	2-6	(199)
	apple juice	immunofluorescence	enrichment (24 h)	0.1	(224)

[a]Lower detection limit in cells/ml or cells/g
[b]The applied viability stains were rhodamine 123, calcein-AM, BCECF-AM, or FDA.
[c]The detection limit was not determined. The concentration in the samples was at least 10^7 CFU/ml.
[d]In all studies applying immunofluorescence FITC-tagged antibodies were used
[e]Concentration after enrichment

methods to detect *E. coli* O157:H7 in apple juice. IMS-FCM required a longer enrichment (24 h), than DEFT (10 h) and PCR (8 h), but was favorable overall, because of the extremely rapid assay and the possibility of cell sorting to recover the pathogen on conventional media. In total (enrichment and incubation) selective plating required more time than the PCR and the fluorescent methods.

Bacteria in meats

The first trials of FCM for detection of bacteria in meat were performed by Patchett et al. (168). They performed direct enumeration using light scatter and ethidium bromide and mithramycin staining of stomached pâté and steak. Such direct analyses of the meats was found restricted by the presence of high levels of suspended material, but the detection was more sensitive than that of milk. With meat samples the lower limit was 10^5 CFU/g and with samples of pâté it was 10^6 CFU/g.

A number of recent papers concern detection of *E. coli* O157:H7 using FCM combined with IMS, including detection in ground beef, the main vehicle responsible for outbreaks (200, 225). Some

studies included detection in milk and apple juice (79, 199, 224). The detection limit reported for FCM using fluorescently stained antibodies was 10^3-10^4 CFU/ml in enrichment broth of ground beef, and FCM results were found similar to Ab-DEFT counts and plate counts (200, 225). An original contamination of 2-6 CFU/gram ground beef was detectable after 6 h-enrichment (199). Detection with fluorescently stained specific bacteriophages could also be used, to detect a contamination as low as 2.2 CFU/g ground beef (79). Another alternative approach used enumeration by scanning microscopy and included CTC staining to determine respiratory activity of the IMS-captured *E. coli* O157:H7 (185).

Wang and Slavik applied IMS-FCM to detect *Salmonella* in chicken washes. (252). The detectable level was $2.3 \cdot 10^4$, and the detection of wash samples inoculated with 0.2 CFU/ml after 18-h enrichment correlated well with selective plate counts. These studies show that IMS-FCM is a sensitive and specific technique valuable for screening foodstuffs for pathogenic contamination.

fermentation monitoring

Control of the fermentative capacity of the inoculum, process control, and process optimization are essential in industrial microbial fermentations. Accurate measurements are important for making decisions on process control because the process performance depends largely on cell numbers and individual cell physiological states. In the next paragraphs examples are given of flow cytometry studies that concern the activity assessment of starters, and of studies on the fermentation process.

Chapter 3 of this thesis describes the use of fluorescence staining to predict lactic acid fermentation activity of starter bacteria (30). A novel assay based on membrane integrity (measured by cF labeling or by PI exclusion) and active metabolism (measured by cF extrusion upon energizing) was developed. *Lactococcus lactis* was used as test strain and it was shown that the assay could predict fermentation capacity after various conditions such as low pH and storage at -20°C. In that study staining and extrusion were examined by microscopy and fluorescence spectroscopy, however, the assessment of efflux activity can also be done with flow cytometry (Bunthof, unpublished results; Da Silveira, unpublished results). Further development and validation testing are needed to make this assay applicable in dairy industry as a rapid single-cell based assay for predicting fermentation activity.

Deere et al. (54) and Attfield et al. (3) investigated fluorescent staining and flow cytometry to test dried yeast, which is used as inoculum in fermentations in baking and wine industry. These studies showed that membrane integrity staining (propidium iodide) combined with membrane potential staining (bis-oxonol) could be used to predict fermentation activity and to examine cell damage by process factors such as vigorous mixing of dried yeast in water, rehydration conditions and temperature.

Chapter 4 of this thesis describes a flow cytometry study of the starter lysis process, which occurs during ripening of cheese (31). A fluid model system was used in which the cheese conditions were mimicked by a buffer with high protein and high salt concentrations that stabilizes the cells. Lysis was induced by the cell-wall hydrolyzing enzyme mutanolysin. With the *Bac*Light kit (SYTO 9 and PI) the total cell number was monitored and intact and permeable cells were distinguished. It was shown that

permeabilized cells remained present for a prolonged time under stabilizing, cheese mimicking conditions. Peptidase activity assays proved that permeabilization caused an increased accessibility of enzymes, which supports a higher rate of substrate conversion. This study suggests that permeabilized cells play an important role in the cheese ripening processes.

Most of the fermentation problems are caused by the induction of bacteriophages. Hutter et al. measured the DNA content of various *Lactococcus* species upon induction of phages. Cells were fixated with alcohol and stained with propidium iodide followed by flow cytometry analysis (96). Results for one of the species showed that approximately 2.5 hours after inoculation with various phages the lysis took place and almost all cells died. Later on, the culture recovered again. Apparently, some cells were phage-resistant and had survived and grown. This research showed that phage induction by starter cultures as well as resistance of starters against phages can be measured by flow cytometry.

Besides for food fermentations, microbes are cultivated in bioreactors to produce for example antibiotics, enzymes, and amino acids. Flow cytometry has been used to monitor production efficiency of various processes. For example, the production of polyhydroxybutyrate, a carbon and energy source that can be used as a biologically degradable thermoplastic polymer, has been tested in various bacterial species using Nile Red staining (189). Another example is the monitoring of viability of *Xanthomonas campestris* with DNA and protein probes during the production process of Xanthum gum (73). This production was shown to be related to bacterial viability.

Hewitt et al. (91) examined cell physiology of *E. coli* under various fermentation conditions using multi-staining with propidium iodide and oxonol. It was found that small-scale, well-mixed fed-batch cultures produced a higher biomass, but lower cell viability than large scale fermentation, which could be explained by the ever-increasing glucose limitation in the well-mixed case (89, 91).

Production of heterologous proteins by genetically modified organisms may be hampered by plasmid instability, i.e. the loss of the plasmid that harbors the gene for the protein of interest. This is a major problem in heterologous protein production. By flow cytometry Lú Chau *et al*. (134) could correlate the fraction of plasmid-containing *Saccharomyces cerevisiae* cells with cellular exo-β-glucanase activity (a marker enzyme encoded by the plasmid) and with total cellular protein content. Also, recombinant cells were shown to have a larger size and a more complex internal structure.

CONCLUSIONS AND PERSPECTIVES

Food microbiology is becoming more challenging, with novel risk factors for food infection and more stringent safety regulations. Scaling-up in agriculture and cattle-raising and global transport of animal feed, food ingredients, and food products are two significant factors that may lead to an increased risk in food safety. If somewhere pathogens arise, they may spread on a much larger scale than in the traditional situation of local production of food. Also, the ongoing change from traditional food preservation methods to novel minimal processing techniques increases the risk of food spoilage. Minimally processed foods are usually not long sustainable, and, especially when not sufficiently cooled, the risk of occurrence of pathogens may increase fast. Moreover, careless handling of food in the kitchen is an important risk factor for foodborne illnesses. Not only the risk factors have increased by modern food handling, but also the range of pathogens being recognized. The last years national and international food authorities have introduced more stringent microbiological safety regulations. This requires good methods for detecting pathogens in food production lines and food products.

In many cases flow cytometry may be the best alternative for detection of pathogens in food. It is well established that conventional culture-based methods have major disadvantages. Many pathogens are either slow or impossible to detect or may be in a nonculturable state. A flow cytometric measurement can be done within 15 minutes. If the concentration of bacteria in a food product is low, enrichment is needed to bring the cells to a detectable number, because the lower detection limit of flow cytometry is in practice approximately 10^3 cells/ml. However, for plate counting a similar enrichment is needed. Other novel rapid methods may also need extra time for enrichment. For example, specific detection of low concentrations of pathogens in food by PCR and gel electrophoresis is often preceded by an enrichment step. Furthermore, PCR and electrophoresis take some hours of incubation. Thus, considering the criterion of time, flow cytometry is very attractive, even if enrichment is needed.

Another important point of consideration is the need for pretreatment. To facilitate flow cytometry the samples need to have a low background-level. Some drinks such as wine, beer, and apple juice may be used directly, or after a simple filtration step. However, foodstuffs such as milk or ground beef require procedures to reduce particle background. In recent years methods have been developed like clearing procedures for milk and specific capture by immunomagnetic separation. These methods facilitate the detection of bacteria in food, with a detection limit as low as that for bacteria in buffer.

Flow cytometry is the superior method when it comes to assessment of physiological states. This makes it a valuable method for detection of bacteria in food. For example, when a food product is infected by *Listeria monocytogenes* many of these bacteria may be in a nonculturable but still virulent state. Plate counts would underestimate the risk, but flow cytometry would be able to detect all the cells and recognize the active-but-nonculturable state.

Finally, flow cytometry can easily be automated for simple and fast routine measurement in food industry. For example, the Bactoscan FC has been developed for automated routine detection of

bacteria and somatic cells in raw milk. It provides an accurate and rapid method for quality and safety control and it has been validated according to international standards.

Flow cytometry of bacteria started as a technique for detection of airborne bacteria and spores used as biological warfare (82). Now, flow cytometry is a well-established method in the armed forces (243). The exposure of frontline soldiers or civilian populations to biological warfare agents needs extremely rapid detection. Flow cytometry is used because it can differentiate between biological and non-biological particles. Moreover, it can determine whether or not a microorganism is potentially virulent.

Though applications to monitor fermentation processes by flow cytometry are still limited, this is definitely a promising prospect. The best approach for process control and optimization is by online measurements. This provides the opportunity to regulate the process without any delay if necessary. Given the speed of analysis and the diversity of cell characteristics that can be measured, flow cytometry can be a valuable tool for online process control. Zhao et al. (260) described a flow-injection system with an integrated microchamber for sample processing (dilution, fixation, staining, and washing). They demonstrated the system performance by monitoring the distribution of DNA content in *Saccharomyces cerevisiae* and GFP production in *E. coli*. Online-monitoring will be valuable to provide detailed knowledge about fermentation processes and it is a sophisticated tool that allows direct regulation of process conditions in order to optimize the production.

Research has shown the potential of flow cytometry to detect pathogens in food and to monitor fermentation processes. Some of the methods are applied in food industry and this is bound to increase further. Anticipated developments of the technique and of fluorescent probes will increase the range of applications. One of the attractive perspectives is the combination of viability probes and probes for detection of specific species. This will facilitate detailed studies on physiological behavior of bacteria in a complex community. Another promising perspective is the combination of flow sorting to isolate a specific population and subsequent analysis using techniques such as PCR amplification, image cytometry, biological activity, electron microscopy, and culturability testing, for identification and characterization of the population of interest.

In the past, reasons often cited for not using flow cytometry more extensively in applied microbiology are the costs for the instrument and need for specialized cytometrists. The development of low-cost light sources such as solid-state lasers and opto-electronics such as diode array detectors that will supersede PMTs will dramatically decrease the costs, complexity and size of cytometers. The Microcyte, introduced in 1994, is a relatively cheap flow cytometer. It is portable and has been found useful for analysis of microorganisms as well as mammalian cells. The potential of the Microcyte flow cytometer for routine analysis in food industry has been demonstrated (50).

An exciting development towards miniaturization of cytometry is the microfabricated fluorescence activated cell sorter (μFACS), in which the fluidics system is replaced by a silicone elastomer chip. External optics and detectors are used to read the chip (70). The μFACS has demonstrated to be able to separate *E. coli* cells expressing green fluorescent protein from a background of nonfluorescent *E. coli* cells and the bacteria were still viable after extraction from the

sorting device. Further development could be the fabrication of detectors and optical filters directly on the chip, which will decrease the costs considerably (70).

In summary, further improvements and applications of the methods for direct analysis of bacteria on the single-cell level will increase our understanding of microbial populations, their heterogeneity and complexity. Furthermore, the developments will lead to cost effective, simple and small instrumentation ideally suited for microbiological applications, including routine analysis in food industry.

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Rapid Fluorescence Assessment of the Viability of Stressed *Lactococcus lactis*

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Rapid Fluorescence Assessment of the Viability of Stressed *Lactococcus lactis*

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The aim of this study was to establish the use of the fluorescent probes carboxyfluorescein (cF) and propidium iodide (PI) for rapid assessment of viability, using *Lactococcus lactis* subsp. *lactis* ML3 exposed to different stress treatments. The cF labeling indicated the reproductive capacity of mixtures of nontreated cells and cells killed at 70°C very well. However, after treatment up to 60°C the fraction of cF-labeled cells remained high, whereas the survival decreased for cells treated at above 50°C and was completely lost for those treated at 60°C. In an extended series of experiments, cell suspensions were exposed to heating, freezing, low pH, or bile salts, after which the colony counts, acidification capacity, glycolytic activity, PI exclusion, cF labeling, and cF efflux were measured and compared. The acidification capacity corresponded with the number of CFU. The glycolytic activity, which is an indicator of vitality, was more sensitive to the stress conditions than the reproduction, acidification, and fluorescence parameters. The cF labeling depended on membrane integrity, as was confirmed by PI exclusion. The fraction of cF-labeled cells was not a general indicator of reproduction or acidification, nor was PI exclusion or cF labeling capacity (the internal cF concentration). When the cells were labeled by cF, a subsequent lactose-energized efflux assay was needed for decisive viability assessment. This novel assay proved to be a good and rapid indicator of the reproduction and acidification capacities of stressed *L. lactis* and has potential for physiological research and dairy applications related to lactic acid bacteria.

Lactic acid bacteria (LAB) are the most important group of bacteria encountered in the food industry. They are used as starter cultures for fermentation of milk, vegetables, and meat. In addition, LAB are used as probiotics and as silage inoculants. The reproduction of LAB and the activities of (starter) cultures containing LAB are important for the success of these fermentations. The production, storage, and use of LAB impose environmental stresses on the bacterial cells, such as freezing and drying of starter cultures, low pH during fermentations, and low temperatures and high salt concentrations during cheese ripening (38). Bacteria that are used as probiotics have to survive the low pH of the stomach and the high bile salt concentrations in the intestine to be effective in the gastrointestinal tract (22). Development of rapid and reliable methods for measuring viability is of the utmost importance for studies on bacterial physiology. Other important criteria for the use of new techniques in the dairy industry are the degree of accordance with established methods and the applicability for starter cultures subjected to different stresses.

The defined aspects of microbial viability are reproduction, vitality, and membrane integrity (27). Reproductive (living) cells are able to proliferate, whereas nonreproductive (dead) cells are not. This status is conventionally assayed by colony counting. Vital cells are metabolically active. Criteria that are used for vitality assessment include nucleic acid synthesis, rate of fermentation, heat production, ATP content, dye extrusion, dye reduction, and maintenance of membrane potential and pH gradients (21, 24). Intact cells have a cytoplasmic membrane with selective permeability. This membrane integrity can be determined by dye exclusion (14, 36). The membrane integrity, the degree of vitality, and the ability to reproduce depend on environmental conditions and the physiological status of the cell at the moment of investigation. Microorganisms can adopt different states, such as a dormant or a latent state, from which they may be resuscitated, i.e., induced to return to a physiologically active state (16).

Techniques that are used in the dairy industry to evaluate culture viability are colony counts and acidification tests (12, 13). The colony count method is the standard method for assessment of viability, but its disadvantages are the long incubation times needed to form countable colonies and underestimation of the viable cell count caused by cell clumping and chain formation. Acidification assays, for example like that described by Pearce (29), are often applied in the dairy industry in addition to plate counts. These assays are empirical ways of testing the acidification capacity of cultures and are supposed to be generally applicable, but they require long incubation times. The acidification test that is commonly used in the Dutch dairy industry takes an entire day, including 6 h of incubation.

A number of fluorescence techniques have been introduced over the past decades for assessment of viability and vitality of microorganisms (7, 21, 24, 32, 44). However, their use in applied food microbiology is still limited. Few fluorescence methods have been made applicable for practical food research and food industry situations. The described applications mainly concern detection and complete enumeration of microorganisms in food samples, such as milk, fixed cheese slides, and cryosectioned sausages (5, 6, 15, 18, 30, 39, 43, 47).

We investigated if the fluorescent probes carboxyfluorescein (cF) and propidium iodide (PI) are good indicators for viability of *Lactococcus lactis* subsp. *lactis* ML3. *L. lactis* is an important organism in dairy fermentations and a model organism for genetic and physiological studies (9, 17, 28, 34, 45). PI is a nucleotide-binding probe, supposed to enter only cells with

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damaged membranes. Therefore, it is used frequently to label dead cells (10, 11, 14, 27). Fluorescein and fluorescein derivatives have been used as viability probes for a wide range of microorganisms (1, 4, 10, 32).

To facilitate passive diffusion into the cell, nonfluorescent fluorescein (derivative) esters, such as 5(6)-cF diacetate (cFDA), are used for fluorescence labeling. The ester bonds are hydrolyzed by enzymes with esterase activity, yielding the green fluorescent dye molecules. Because enzyme activity is needed for hydrolysis, and membrane integrity is needed for the retention inside cells, it is supposed that viable cells accumulate fluorescein (derivatives) but dead cells are not able to do so (37). However, it was shown that the fluorescein derivatives cF and 2',7'-bis(2-carboxyethyl)-5(6)-cF (BCECF) are actively extruded by *L. lactis* cells upon energizing with lactose (2, 17). We hypothesized that this probe efflux could be put to use as an additional measure of cell viability. cF labeling and, subsequently, the efflux could be measured to assess multiple aspects of cell viability: enzyme activity, membrane integrity, and metabolic activity upon energizing. These combined methods could give more information about the physiological condition than cF labeling alone does.

The approach of our study was to measure fluorescence-related parameters and glycolytic activity by fast assays, and also by the traditional but time-consuming plate count and acidification capacity test methods, and to compare these with each other. In dairy industry practice LAB are exposed to different types of environmental stress. Therefore, the applicability of the fluorescence-based methods was tested after exposure to a range of different stress conditions, comprising heating to 60 or 70°C, freezing at -20°C with or without glycerol, exposure to low pH, exposure to conjugated bile salts (CBS), and exposure to deconjugated bile salts (DBS). The results indicated shortcomings of cF labeling and PI exclusion for viability assessment. Combining cF labeling with subsequent cF efflux resulted in a novel assay, which proved to be a good and rapid indicator for the reproductive and acidifying capacities of *L. lactis*.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *L. lactis* subsp. *lactis* ML3 (NCDO 763) was grown at 30°C in M17 broth (Unipath Oxoid, Basingstoke, United Kingdom) supplemented with 0.5% (wt/vol) lactose (42). Overnight cultures were diluted 10-fold in fresh medium, incubated for approximately 2 h, and harvested in mid-exponential phase after they had reached an optical density at 620 nm (OD_{620}) of approximately 0.7 by centrifugation at 4,000 × g for 10 min at 10°C. Cell suspensions were then centrifuged with an Eppendorf centrifuge (Biofuge Fresco; Heraeus Instruments, Ostrode, Germany) at 10,000 rpm for 2 min at 10°C. Harvested cells were washed twice with 50 mM potassium phosphate (KPi) buffer (pH 7.0) and concentrated in 50 mM KPi buffer (pH 7.0) to an OD_{620} of 20.

Treatments. The concentrated cell suspensions were exposed to different types of stress: heat, freezing, low pH, and bile salts. Nontreated cell suspensions served as a positive control. All treatments were done with 400 μl portions of concentrated cell suspension (OD_{620} = 20). Exposure to heat was done at 70°C for 10 min or at 60°C for 90 s. Exposure to freezing was done at -20°C for 24 h with or without 30% glycerol. Exposure to low pH was done by incubation in 10 mM KPi , adjusted to pH 2.0 or 5.0 with hydrochloric acid, at 30°C for 60 min. Exposure to bile salts was done by incubation either with CBS, 0.2 or 1.0% (wt/wt) in 50 mM KPi (pH 6.0), or with DBS, 0.02, 0.06, or 1.0% (wt/wt) in 50 mM KPi (pH 7.0) at 30°C for 60 min. The CBS (Unipath Oxoid) contained mainly sodium glycocholate and sodium taurocholate. The DBS (Sigma-Aldrich, Steinheim, Germany) contained 50% sodium cholate and 50% sodium deoxycholate. For comparison, cell suspensions incubated in KPi buffer (pH 7.0) at 30°C for 60 min were used. After the treatments the cells were spun down, resuspended in KPi buffer (pH 7.0), and put on ice until use.

Plate counts. The reproductive capacity was determined by plate counting. Cell suspensions were serially diluted with 50 mM KPi buffer (pH 7.0), and 100-μl portions of the appropriate dilution were spread out in triplicate on M17 plates containing 0.5% (wt/vol) lactose and 1.5% agar. After incubation for 3 days at 30°C the colonies from plates containing 40 to 300 colonies were counted.

Acidification capacity. The acidification capacity was determined by the standard assay that is used in the dairy industry in The Netherlands. This assay resembles the Pearce test (29). The milk medium, a 10% (wt/wt) suspension of Nilac milk powder (NIZO, Ede, The Netherlands) in sterile water, was equilibrated at 30°C and inoculated to a final microbial protein concentration of 3.6 mg/ml. This is equal to 10 7 CFU/ml for a nontreated cell suspension. Then, 50 ml of the inoculated milk medium was incubated at exactly 30°C for 6 h in the dark. After incubation, the pH was measured and the acidification capacity was determined by titrating 15 ml of milk with 100 mM NaOH to pH 7.0 (determinations were made in triplicate).

Glycolytic activity. The glycolytic activity was assessed by measuring the initial rate of acidification. The applied method was adapted from Gatto et al. (8). Cells were spun down and resuspended in 9.4 ml of 0.5 mM potassium MES (morpholineethanesulfonic acid)–50 mM KCl buffer at pH 6.5 to a final protein concentration of 0.15 mg/ml. After equilibration at 30°C, 200 μl of lactose was added to a final concentration of 6 mM. Acidification of the medium was monitored with a thin (diameter, 5 mm) Schott pH electrode and a pH meter connected to a recorder. Changes in pH values were converted into nanomoles of H^+ by calibration of the cell suspension with 10-μl portions of 100 mM NaOH. The glycolytic activity was expressed in nanomoles of H^+ per minute per milligram of protein.

Fluorescence labeling with cF. Stock solutions of cF were prepared by dissolving cF (Molecular Probes, Eugene, Oreg.) in acetone (4.6 mg/ml) and immediately diluting 10- or 100-fold with 50 mM KPi buffer (pH 7.0). These solutions of 1.0 mM concentration and 100 μM concentration were divided into 0.5-ml aliquots to avoid repeated thawing and freezing and stored at 220°C in the dark. Cell suspensions (OD_{620} = 20) were diluted 1:1 with 100 μM cF and incubated at 30°C for exactly 10 min. Immediately after this labeling, the cells were spun down, washed once, and resuspended in 50 mM KPi (pH 7.0) to an OD620 of 4.0 for microscopic analyses or to an OD620 of 2.0 for fluorimetrical analyses.

Cell suspensions were microscopically analyzed with an Axioskop epifluorescence microscope equipped with a 50-W mercury arc lamp, a fluorescein isothiocyanate filter set (excitation wavelength, 450 to 490 nm; emission wavelength, >520 nm), an ×100 1.3 numerical-aperture Plan-Neofluar objective lens, and a camera (Carl Zeiss, Oberkochen, Germany). Photomicrographs were made with simultaneous light and epifluorescence microscopy, a low light intensity, a magnification of ×1,000, and an exposure time of 15 s, on Kodak 400 ASA color films. In these photomicrographs both the cF-labeled cells and the nonlabeled cells can be counted. In each experiment four photomicrographs were made, each depicting 100 to 400 cells.

To measure the cF labeling capacity, i.e., the average internal cF concentration (cF_{in}), labeled cells were lysed by incubation at 70°C for 15 min and the debris was removed with a Biofuge (13,000 rpm, 2 min at 10°C) (Heraeus Instruments GmbH, Hanau, Germany). The fluorescence of the supernatant was measured fluorimetrically (excitation at 490 ± 5 nm and emission at 515 ± 5 nm), with a Perkin-Elmer LS 50B luminescence spectrometer equipped with a plate reader by using computer-controlled data acquisition. The intracellular cF concentration was calculated by using a calibration curve for cF (concentration range, 0 to 1.5 μM) in 50 mM KPi buffer (pH 7.0).

Fluorescence labeling with PI. To test whether a treatment caused membrane damage, cells were incubated with the impermeant nucleotide binding probe PI. Stock solutions of 1.0 mg of PI (Molecular Probes) per ml were prepared in distilled water, stored in the refrigerator, and kept in the dark. PI was added to a concentration of 44 μM to a cell suspension with an OD_{620} of 2 and incubated at 30°C for 10 min. Photomicrographs were made with the same settings as used for the cFDA-labeled cell suspensions, and the red-labeled and nonlabeled cells were counted.

cFDA hydrolysis activity of cell extract. Cell extracts were prepared by disrupting 600-μl portions of cell suspension (OD_{620} = 40) by sonication (10 times for 15 s with 45-s intervals; amplitude intensity of 15 μm). The cell debris was removed by centrifugation. The cFDA hydrolysis activity of cell extract was determined by incubation of 100 μl of 1.0 mM cFDA and 250 ml of the cell extract in 50 mM KPi buffer (pH 7.0) in a total volume of 1.0 ml at 30°C. The increase of cF concentration over time was monitored by measuring A_{490} every 5 min for 20 min. The measurements were corrected for the chemical hydrolysis of cFDA.

cF efflux activity. Cells were labeled by cF as described above. The labeled cell suspensions (OD_{620} of 2.0) were incubated at 30°C with or without lactose (final concentration, 20 mM). Samples (200 μl) were withdrawn at specific time points and immediately centrifuged to remove the cells. From the fluorimetrically measured fluorescence of the supernatants and the total labeling capacity, the intracellular concentrations of cF at the sampling time points were calculated.

ATP concentration. ATP concentration measurements were made under the same conditions as cF efflux measurements, and samples were withdrawn at the same time points. For measurement of total ATP concentration, a 20-μl sample was mixed with 80 μl of dimethyl sulfoxide and diluted with 5 ml of deionized water. For measurement of external ATP concentration, 20 μl of supernatant from a 80-μl, immediately centrifuged sample was used in the same way. Internal ATP concentration was calculated by subtracting the external ATP concentration from the total ATP concentration. The ATP concentrations were measured in an M 2500 biocounter (Lumac, Landgraaf, The Netherlands), with the Lumac luciferin/luciferase assay.

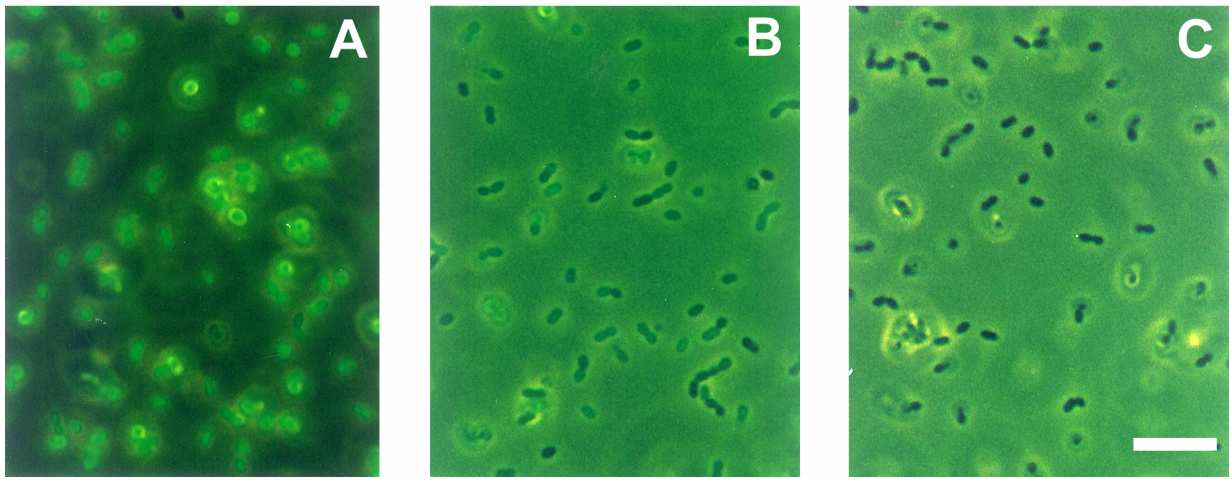

FIG. 1. Labeling of *L. lactis* by cF and subsequent active extrusion. A nontreated cell suspension was labeled with cF, by incubation with 50 μM cFDA at 30°C and pH 7.0 for 10 min, and washed once (A). This labeled cell suspension was incubated with 20 mM lactose at 30°C for 2 min (B) and for 15 min (C). Cell suspensions were photographed with simultaneous light and epifluorescence microscopy (excitation wavelength, 450 to 490 nm; emission wavelength, .>520 nm) to visualize both stained and nonstained cells. Bar represents 10 μm for all micrographs.

Estimation of cell protein content. Protein concentrations were assayed by the method of Lowry et al. (20). Cellular volumes were calculated from the protein concentrations, assuming a ratio of 2.8 ml per mg of cell protein (31).

Experimental design and statistical analyses. The experimental discrimination of viable and nonviable bacteria in mixtures of nontreated and heat (70°C)-treated cell suspensions by plate counts and that by cF labeling were performed with two batches of cells. The correlation between the methods was tested at a significance level of 0.05. In the series of experiments testing various physiological indicators after 13 different treatments, nearly all experiments were performed with at least three batches of cells. The effect of each stress treatment was tested for significance with the Student's *t* test at the levels of 0.01 and 0.10. Furthermore, comparison of each pair of indicators was made for each treatment by calculating the probability values associated with the Student's *t* test (P values). These P values, and the plot of the indicators against each other, were taken into consideration to evaluate the general correspondence between the two indicators.

RESULTS

Accumulation and retention of cF. *L. lactis* can easily be labeled with cF and retains the probe well when not energized, but it extrudes cF rapidly upon lactose addition. Nearly all *L. lactis* cells of a nontreated cell suspension were labeled within a few minutes of incubation with cFDA (Fig. 1A). When labeled cells were stored on ice for up to 2 h, a gradual decrease of intracellular cF at the rate of 8% per hour was observed. When the cells were kept at 30°C the rate of leakage was 18% per hour. The dissipation of the proton motive force (PMF) caused by addition of the ionophores valinomycin and nigericin to nonenergized cells resulted in an additional and immediate loss of 10%, after which the rate of leakage was the same as that for the other cells kept at 30°C (Fig. 2A). These rates of cF leakage were negligible compared to the immediate and rapid extrusion upon energizing by lactose (Figs. 1B and 1C). The time needed to extrude 50% of accumulated cF ($t_{1/2}$) was less than 2 min (Fig. 2B). Also, when the PMF was dissipated there was an immediate and rapid extrusion upon energizing (Fig. 2B). Since cells treated with valinomycin and nigericin also produce ATP upon addition of lactose, this indicates that the extrusion is most likely mediated by an ATP-driven transport system.

Correspondence between cF labeling and reproduction after temperature treatments. Living bacteria could easily be distinguished from dead bacteria in mixtures of nontreated and

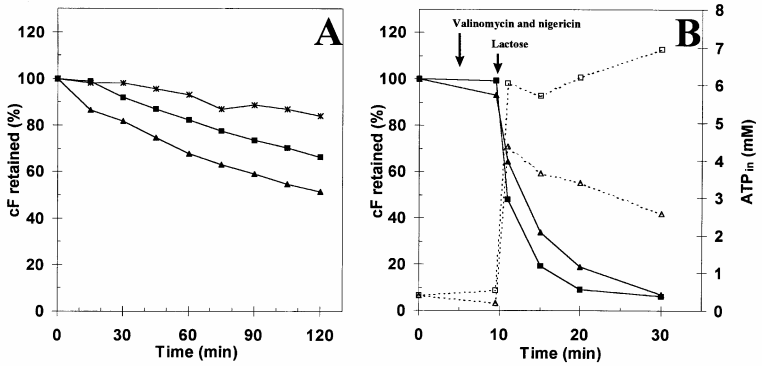

FIG. 2. Retention of cF by *L. lactis*. Cells were loaded with cF and resuspended in 50 mM KPi buffer (pH 7.0). (A) Retention of cF in the absence of an energy source when cells were kept on ice (*), at 30°C (■), and at 30°C with the addition of 2 μM valinomycin and nigericin (▲). (B) Retention of cF in cells with the addition of 20 mM lactose (■) and with the addition of 20 mM lactose after dissipation of the PMF by adding 2 μM valinomycin and nigericin (▲). The internal ATP levels in cells with the addition of lactose (□) and with the addition of lactose after dissipation of the PMF (Δ) were also measured.

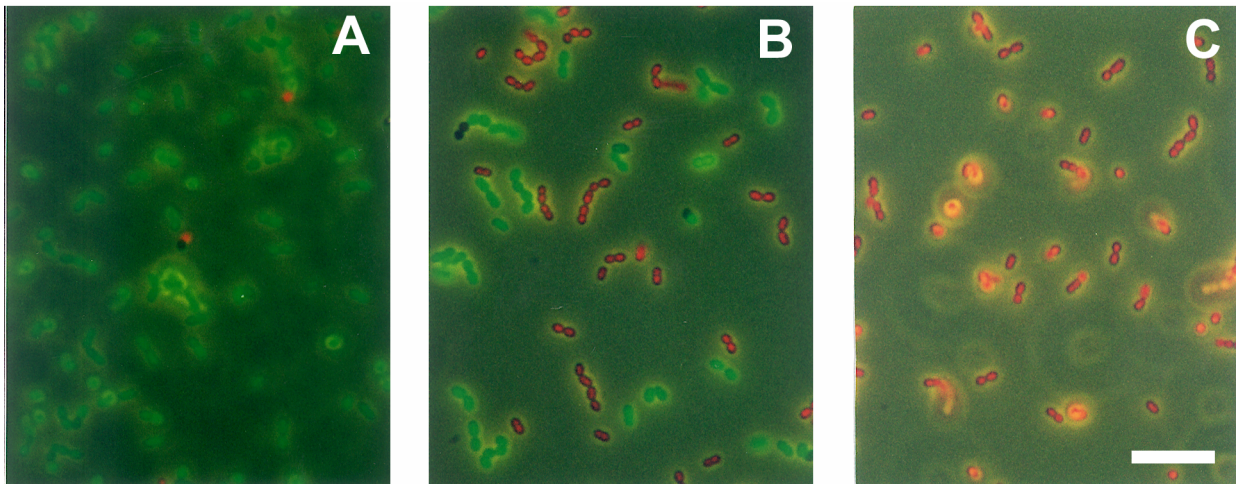

FIG. 3. Fluorescence microscopy of *L. lactis* cells double labeled with cF and PI. Suspensions containing 100% nontreated cells (A), 50% nontreated cells mixed with 50% heat-killed cells (B), and 100% heat-killed cells (C) were incubated with 50 μM cFDA and 44 mM PI. Cell suspensions were photographed with simultaneous light and epifluorescence microscopy (excitation wavelength, 450 to 490 nm; emission wavelength, >520 nm) to visualize both stained and nonstained cells. Bar represents 10 μm for all micrographs.

heat (70°C)-treated cell suspensions by staining with cFDA and analyzing with fluorescence microscopy. This was confirmed by counterstaining the cell suspensions with the membrane-impermeant DNA stain PI. When a nontreated cell suspension was incubated with cFDA and PI, nearly all (97%) of the cells showed bright green fluorescence and very few (3%) showed red fluorescence (Fig. 3A). The small fraction that was not labeled by cF but was labeled by PI is presumably the fraction dead cells present in the cell culture at harvest. Treatment of a cell suspension at 70°C for 10 min resulted in a total loss of reproduction, as determined with standard plate counts (no colonies were detected after plating of $\geq 10^9$ cells). When such a heat-treated cell suspension was incubated with cFDA and PI, not a single green fluorescent cell could be detected but all cells were brightly labeled red (Fig. 3C). This PI labeling indicated that the cells were killed because of major membrane damage. In mixtures of nontreated and heat (70°C)-treated cell suspensions, live and dead cells could be distinguished by double labeling with cF and PI (Fig. 3B). This double labeling gave the same fractions of green- and red-labeled cells as the cF labeling and PI labeling performed separately. This reflects that these probes act in a complementary manner.

viability assessment. The number of reproductive cells determined by plate counts was linearly related to the proportion of nontreated cells (Fig. 4A). The fraction cF-labeled cells and the labeling capacity (the average intracellu-lar cF concentration of the mixed cell suspensions) were also linearly related to the proportion of nontreated cells (Fig. 4B and C). The fluorescence microscopy method has an especially high precision as indicated by the value of R^2. The coefficients of correlation between the microscopic counts and the plate counts and between the labeling capacity and the plate counts were both 0.96 ($P < 0.005$). Thus, cF labeling provides a valid alternative for determining the viability of these mixed cell suspensions, whether it is examined by fluorescence microscopy or by spectrofluorimetry.

However, after the suspensions were exposed to temperatures of 50 to 65°C the cF labeling results were discordant with the plate count results (Fig. 5). No colonies were detected after 90-s exposure to 65°C or higher, less than 0.1% of the cells survived heating to 60°C, 46% survived heating to 55°C, and 69% survived heating to 50°C. Nevertheless, after exposure to 60°C or lower, more than 95% of the cells were labeled by cF. The cF labeling capacity (cF_{in}) decreased with increasing tem-

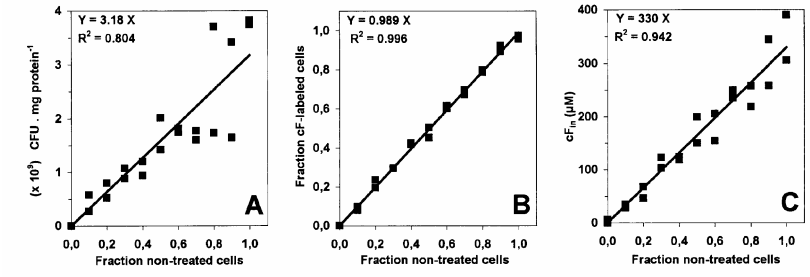

FIG. 4. Experimental discrimination of viable and nonviable bacteria. An *L. lactis* cell suspension (10^{10} CFU/ml) was divided into two portions. One was not treated and the other was exposed for 10 min to 70°C. The nontreated and the heat-treated portions were mixed in various proportions and plated on M17 agar supplemented with 0.5% (wt/vol) lactose or labeled for 10 min with 50 μM cFDA, washed, and analyzed by fluorescence microscopy and spectrofluorimetry. The experiment was performed with two batches of cells. Plate counts (A), fraction cF-labeled cells (B), and average intracellular cF concentration (C) are all plotted against the known fraction of nontreated cells.

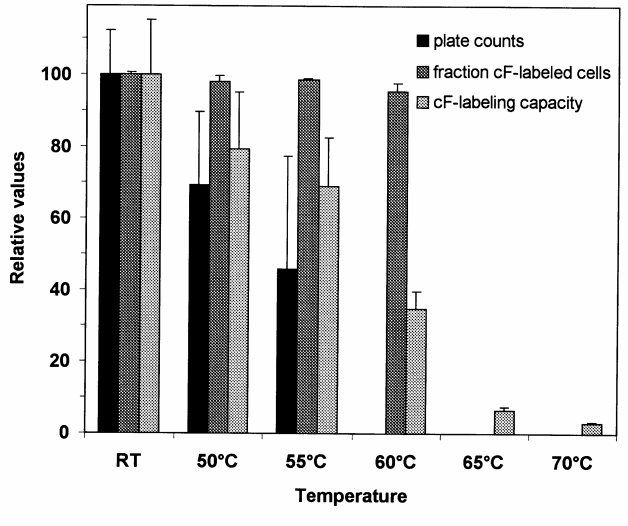

FIG. 5. Effects of temperature treatment on *L. lactis* survival and cF labeling. *L. lactis* cell suspensions (10^{10} CFU/ml) were subjected to elevated temperatures for 90 s. After the treatment the suspensions were plated on M17 agar supple-mented with 0.5% (wt/vol) lactose and tested for cF labeling. Both the fraction cF-labeled cells and the cF labeling capacity were determined. Data are the means and standard deviations of three experiments and are expressed as values relative to that for nontreated cells (taken as 100). RT, room temperature.

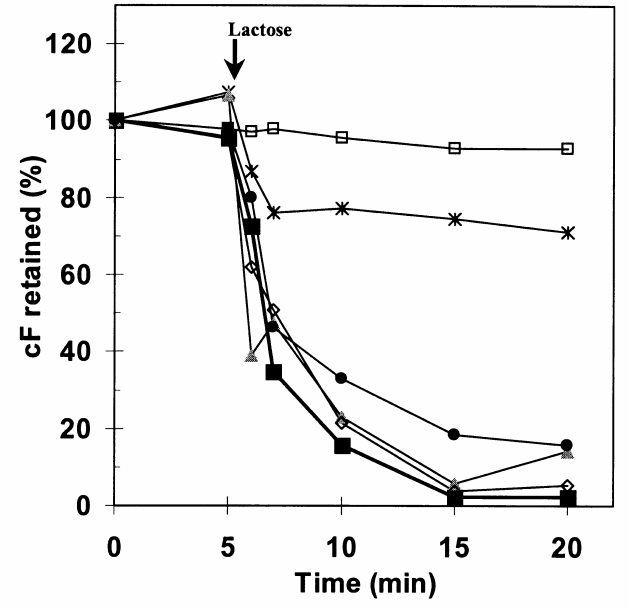

FIG. 6. Effect of stresses on the cF efflux by lactose-energized *L. lactis*. Cell suspensions were pretreated under different stress conditions, loaded with cF, and resuspended in 50 mM KPi buffer (pH 7.0). Five minutes after the start of the incubation at 30°C, lactose was added to a final concentration of 20 mM. Results of representative experiments with a non-treated-cell suspension and five of the stressed cell suspensions are given. No treatment, 100% = 364 μM (■); exposure to 60°C for 90 s, 100% = 149 μM (□); exposure to -20°C for 24 h, 100% = 36 μM (*); exposure to pH 5.0, 100% = 137 mM (▲); exposure to 1.0% CBS, 100% = 105 μM (●); and exposure to 0.02% DBS, 100% = 136 μM (◇).

perature, but the results for this parameter as well were not in accord with those for reproduction. However, when cell suspensions exposed to 60°C were tested for cF efflux, no active transport was detected (Fig. 6). Thus, in this case, the lack of efflux is a good indicator for the lack of reproduction while cF labeling is not. This supports our hypothesis of the additional value of cF efflux for assessment of viability.

Comparison of various physiological indicators after stress. *L. lactis* cell suspensions were exposed to 13 different treatments, including heat, freezing, low pH, and addition of bile salts. Nontreated cell suspensions served as the control. The effects of the stress exposures on the reproduction (plate counts), acidification capacity, glycolytic activity, and various fluorescence-related parameters including the cF efflux are presented in Table 1.

The stress conditions were designated as severe, intermediate, or mild based on their effects on the survival (plate counts). Exposure to 70°C for 10 min resulted in a total loss of reproduction, and exposure to 60°C for 90 s or to 1.0% DBS concentration resulted in a loss of more than 99% of the population. Therefore, these three conditions were classified as severe stress conditions. Exposure to freezing with or without glycerol, to pH 2.0, to 1% CBS, or to 0.06% DBS caused loss of reproduction of part of the population, but more than 5% survived. These were classified as intermediate stress conditions. The other treatments did not decrease the plate counts significantly and were therefore classified as mild treatments. The fraction of cF-labeled cells showed some important deviations from the (in most cases) reasonable to good correspondence (Fig. 7A). After treatment at 60°C, 90% of the cells were able to accumulate cF, but there was hardly any reproduction. Also, after exposure to 1% CBS, the labeling was significantly ($P < 0.05$) higher than plate counts. The cF labeling of cells exposed to low pH (pH 2) had variable results, while the plate counts were reasonably consistent. These results indicate that cF labeling is not a general indicator for reproduction. After exposure to the mild stress conditions most cells were labeled by cF, but the labeling capacity (Fig. 7B), i.e., the cFin , was lower than that of nontreated cell suspensions. The other stress conditions also decreased the labeling capacity more than they decreased the fraction cF-labeled cells. Under all conditions tested there was at least some cFDA hydrolysis activity (Fig. 7C), but it did not correspond with the cF labeling capacity. Neither the hydrolysis activity nor the cF labeling capacity corresponded with the plate counts. The fractions of PI-excluding cells corresponded with the fractions of cF-labeled cells, indicating that the cF labeling depended on the integrity of the membrane. Under most stress conditions the PI exclusion corresponded with the plate counts (Fig. 7D). However, the PI exclusion by cell suspensions treated at 60°C shows that cells can lose their reproductive capacity but still have an intact membrane.

The two stress conditions that caused complete loss of cF labeling (70°C and 1.0% DBS) also caused complete loss of reproductive capacity. For these conditions no further investigation was needed; absence of cF labeling indicates absence of reproduction. Cell suspensions of which (part of) the cells were labeled were investigated further by efflux assays. After exposure to pH 7.0, pH 5.0, and bile salts, cell suspensions showed efflux behavior similar to that of nontreated cell suspensions, although the cF_{in} (the labeling capacity) was lower. After exposure to pH 2.0 and to freezing the efflux was not complete, and after exposure to 60°C there was hardly any efflux (Fig. 6). In all cases the extrusion or leakage of cF without energizing was negligible for the duration of the experiment, so no corrections were necessary. Labeling and efflux were combined into one parameter by multiplying the fraction of cells labeled after incubation with cFDA by the fraction of cells showing efflux up to 15 min after lactose addition. The consequent

TABLE 1. Effects of stress on various physiological parameters[a]

Treatment	Plate counts, CFU • mg of protein^{-1} (%)	Acidification capacity, mmol H^+ • 6 h^{-1} • mg of protein^{-1} (%)	Glycolytic activity, μmol H^+ • min^{-1} • mg of protein^{-1} (%)	Fraction, cF-labeled cells (%)
No treatment	$5.35 \times 10^9 \pm 9.71 \times 10^8$ (100)	20.72 ± 1.55 (100)	1.02 ± 0.08 (100)	0.97 ± 0.02 (100)
70°C, 10 min	0.00 ± 0.00 (0)	0.53 ± 0.46 (3)**	0.00 ± 0.00 (0)**	0.00 ± 0.00 (0)**
60°C, 90 s	$1.24 \times 10^6 \pm 2.20 \times 10^6$ (0)	0.61 ± 0.16 (3)**	0.00 ± 0.00 (0)**	0.90 ± 0.06 (92)**
-20°C, 24 hr, 0% glycerol	$8.11 \times 10^8 \pm 6.83 \times 10^8$ (15)	5.40 ± 0.79 (26)**	0.01 ± 0.01 (1)**	0.31 ± 0.09 (32)**
-20°C, 24 hr, 30% glycerol	$4.19 \times 10^9 \pm 6.99 \times 10^8$ (78)	11.79 ± 2.34 (57)**	0.28 ± 0.17 (27)**	0.86 ± 0.08 (89)
30°C, 60 min, pH 7.0	$4.98 \times 10^9 \pm 1.23 \times 10^9$ (93)	18.21 ± 2.60 (88)	0.69 ± 0.21 (67)	0.97 ± 0.01 (100)
30°C, 60 min, pH 5.0	$4.89 \times 10^9 \pm 1.11 \times 10^9$ (91)	16.91 ± 3.12 (82)	0.54 ± 0.07 (53)**	0.95 ± 0.03 (98)
30°C, 60 min, pH 2.0	$4.40 \times 10^8 \pm 7.26 \times 10^7$ (8)**	5.24 ± 2.29 (25)**	0.00 ± 0.00 (0)**	0.47 ± 0.44 (48)
30°C, 60 min, CBS 0.2%	$4.40 \times 10^9 \pm 1.58 \times 10^9$ (82)	17.00 ± 3.54 (82)	0.56 ± 0.01 (55)**	0.95 ± 0.03 (97)
30°C, 60 min, CBS 1.0%	$2.21 \times 10^9 \pm 9.97 \times 10^8$ (41)**	16.26 ± 2.65 (78)*	0.47 ± 0.05 (46)**	0.86 ± 0.10 (89)
30°C, 60 min, DBS 0.02%	$4.12 \times 10^9 \pm 1.12 \times 10^9$ (77)	18.00 ± 2.62 (87)	0.45 ± 0.08 (44)**	0.85 ± 0.06 (88)**
30°C, 60 min, DBS 0.06%	$2.61 \times 10^9 \pm 1.32 \times 10^9$ (49)*	17.35 ± 1.99 (84)*	0.36 ± 0.06 (35)**	0.40 ± 0.31 (41)**
30°C, 60 min, DBS 1.0%	$2.20 \times 10^3 \pm 1.93 \times 10^3$ (0)**	0.58 ± 0.09 (3)**	0.00 ± 0.00 (0)**	0.00 ± 0.00 (0)**

[a] Results given are the average values ± the standard deviations, and the percentages relative to that for cells no treatment given (set at 100) are shown in parentheses. In addition, the asterisks mark the stress conditions that have significant effects as determined by comparison with the value for cells given no treatment (Student's *t* test; **, $P < 0.01$; *, $P < 0.1$) for all parameters except for the product of cF labeling and efflux. For this combined parameter, Student's *t* test could not be performed because the fraction of cF-labeled cells and the fraction of cF efflux were measured with different cell batches. All experiments were repeated at least two times. In some efflux experiments the labeling was too low (<25 μM) to enable measurements. No efflux could be measured after exposure to 70°C or to 1% DBS, only one experiment could be performed after exposure to 0.06% DBS (therefore, no standard deviation is given), and only two experiments could be performed after exposure to pH 2.

efflux assay improved the estimation of reproductive capacity given by cF labeling (Fig. 7A and E). In general, the results for the combined cF labeling and efflux parameter corresponded well with the plate counts.

The effects of the stresses on acidification corresponded with the effects on plate counts, and the outcome of the comparisons of fluorescence-related parameters with acidification resembled that obtained with plate counts. The acidification capacity is the viability parameter that is used in the dairy industry to estimate the success of fermentations. The acidification capacity corresponded reasonably well with the plate counts (Fig. 7F). Apparently, the results of the industrial acidification tests were largely dependent on the reproductive capacity. Comparison of the combined cF labeling and efflux parameter with the acidification assay revealed a good correspondence (Fig. 7G), whereas none of the other fluorescence-related parameters did. This combined parameter is the best fluorescence indicator for the industrially relevant characteristic of fermentation capacity.

The effects of stress on vitality were assessed by the glycolytic activity assay, which measures the physiological condition of the cell suspensions immediately after the treatments. The glycolytic activity is generally more sensitive to the stress conditions than the reproduction and acidification capacity are (Fig. 7H). Therefore, glycolytic activity is not a good indicator for acidification capacity. None of the fluorescence-related parameters corresponded with the glycolytic activity.

DISCUSSION

In this study we investigated the use of the fluorochromes cF and PI for viability assessment of *L. lactis* subsp. *lactis* ML3. The aim was to develop a rapid assay that provides a generally valid indicator for reproduction. Therefore, cellsuspensions were exposed to different stressconditions. Furthermore, fluorescence-related parameters were compared not only with plate counts but also with acidification capacity and with glycolytic activity.

In mixtures of heat (70°C)-treated cells and nontreated cells, live and dead cells could be distinguished clearly by cF or PI labeling. For cF, this was reflected by the high coefficient of correlation between the number of CFU and the labeling, determined either by fluorescence microscopy or by spectrofluorimetry. Given the precision of the experimental results, fluorescence microscopy combined with photography is the preferred method. For a rapid judgment of viability, the fractions of living and dead cells can be estimated directly with fluorescence microscopy (without photography). When many samples are to be assayed, spectrofluorimetry is preferred. Because these experiments take an hour or less, they are appealing and time-saving alternatives to the classic plate count method. However, cF labeling is not a general indicator for viability. For cells exposed to temperatures from 50 to 60°C or to high concentrations of CBS, the fractions for cF labeling were higher than the reproductive capacity. In addition, the results for PI exclusion by cells treated at 60°C were discordant with reproductive capacity. A disagreement between viability labeling with a fluorescein derivative and plate counts when the cells were incubated at low temperatures (10 and 4°C) for up to 30 days was also reported for *Escherichia coli* (33). This stress induced a so-called viable but nonculturable status, as shown by reduced ability to form colonies even though the cells remained intact and showed intracellular enzyme activity. PI staining is very dependent on the incubation conditions. Under suboptimal conditions staining of fresh and heat (80°C)-killed cells can already give false-positive results, especially when faintly red-labeled cells are interpreted as being dead (46). When they are exposed to milder stress conditions correct distinction of live and dead cells might be even more difficult. For mammalian cells cF fluorescence and PI exclusion were also found to be unreliable indices of viability under stress (23). Because of the complexity of the physiological status and heterogeneity of bacterial cells in a culture, especially after stress, multiparameter analysis is preferable (3, 27). Examples

TABLE 1—*Continued*

Fraction, PI-excluding cells (%)	cF labeling capacity,μmM (%)	cFDA hydrolysis activity, nM • min^{-1} • mg of $protein^{-1}$ (%)	Fraction cF efflux (%)	Product of cF labeling capacity and efflux fraction (%)
0.97 ± 0.02 (100)	293 ± 48 (100)	67.85 ± 8.82 (100)	0.93 ± 0.05 (100)	0.90 ± 0.05 (100)
0.01 ± 0.02 (1)**	7 ± 6 (3)**	10.51 ± 2.57 (15)**		0.00 (0)
0.67 ± 0.29 (69)*	134 ± 46 (46)**	33.76 ± 6.25 (50)**	0.07 ± 0.03 (7)**	0.06 ± 0.02 (6)
0.27 ± 0.11 (28)**	37 ± 15 (13)**	55.38 ± 12.33 (82)	0.32 ± 0.04 (34)**	0.10 ± 0.02 (11)
0.84 ± 0.10 (87)	133 ± 28 (45)**	47.56 ± 13.40 (70)	0.50 ± 0.15 (54)**	0.43 ± 0.11 (48)
0.97 ± 0.01 (101)	243 ± 51 (83)*	49.38 ± 7.84 (73)*	0.91 ± 0.04 (98)	0.88 ± 0.04 (98)
0.95 ± 0.01 (98)	126 ± 39 (43)**	48.12 ± 3.72 (71)*	0.94 ± 0.07 (101)	0.89 ± 0.07 (99)
0.19 ± 0.20 (19)**	32 ± 33 (11)**	20.49 ± 10.10 (30)**	0.30 ± 0.02 (32)**	0.14 ± 0.11 (15)
0.95 ± 0.01 (98)	96 ± 27 (33)**	38.37 ± 5.18 (57)*	0.82 ± 0.07 (89)	0.78 ± 0.07 (86)
0.70 ± 0.26 (73)	103 ± 36 (35)**	40.83 ± 4.54 (60)*	0.68 ± 0.20 (73)	0.59 ± 0.18 (65)
0.88 ± 0.11 (91)	149 ± 29 (51)**	42.39 ± 3.69 (62)*	0.90 ± 0.06 (97)	0.77 ± 0.07 (85)
0.46 ± 0.35 (48)*	52 ± 49 (18)**	48.35 ± 7.85 (71)*	0.92 (99)	0.36 (41)
0.00 ± 0.00 (0)**	7 ± 6 (2)**	36.71 ± 8.09 (54)*		0.00 (0)

indicate that after exposure to stress, cultures may contain dormant and injured subpopulations. Dormant cells may regain growth by resuscitation, while damaged cells may recover from injury and regain growth (16, 19). Study of growth, recovery, dormancy, and adaptation is important for understanding bacterial physiology. Consistent terminology and logical concepts are indeed needed to avoid confusion. Furthermore, we agree with Kell et al. (16) that the validity of a cytological assay can be confirmed only by correlation with culture assays for a specific mechanism of cell death. Therefore, it was our approach to evaluate the validity of the fluorescence assays as indicators for particular practical aspects of viability by comparing the fluorescence-related parameters with plate counts, acidification capacity, and glycolytic activity after different types of stress treatment.

The glycolytic activity assay does not appear to be a good indicator for the acidification capacity. The assay was adapted from the acidification power (AP) test, developed by Gatto et al. (8), which measures the pH decrease during 10 min of spontaneous acidification followed by 10 min of substrate-induced acidification. They suggested that there is a linear correlation between the AP test and the Pearce test (29) for *Lactobacillus delbrueckii* subsp. *bulgaricus*. The results reported by Riis et al. (35) do not indicate a linear correlation. Nevertheless, they evaluated the AP test as promising for use in the dairy industry because it detects minor differences in starter cultures, it is rapid, and it can be automated and standardized. Both the AP test and our glycolytic activity assay measure the ability to utilize exogenous carbohydrates within a short time. From the comparison with the industrial acidification test it appears that glycolytic activity is not a valid quantitative indicator for fermentation capacity. During a fermentation, such as that mimicked by the industrial acidification test, recovery and damage repair processes might result in a higher fermentation capacity than one would predict based on the vitality assessed directly after exposure to stress.

It has been suggested that the rate of FDA hydrolysis can be used to determine bacterial numbers (40) and to monitor microbial activity (41). However, our results show that the effects of the stresses on cFDA hydrolysis do not accord with the effects on plate counts, acidification, or glycolytic activity. The cFDA hydrolysis activity was decreased by all treatments, but it never became limiting for labeling; even dead cell populations still showed hydrolysis activity. Therefore, we conclude that cFDA or FDA hydrolysis is not a valid viability indicator for dairy applications.

Labeled *L. lactis* cells actively extrude the accumulated cF upon energizing, as they do after dissipation of the PMF by valinomycin and nigericin. Under these conditions the internal ATP levels remained high. This suggests that the cF efflux takes place via a primary transport system, which is most probably ATP dependent. The same was found for BCECF efflux (26). However, the rate of cF efflux is much higher than the rate of BCECF efflux. For cF efflux at pH 7 we found a $t_{1/2}$ of less than 2 min, while for BCECF efflux $t_{1/2}$ values of 6 min (pH 6) and 11 min (pH 8) were reported (25). Of the known extrusion systems only multidrug resistance transport systems have demonstrated broad substrate ranges, and since BCECF does not resemble any naturally occurring compound, it was suggested that BCECF is extruded by such an extrusion system (26). cF might be transported by the same or a similar extrusion system. The rapid efflux indicates a high affinity of the extrusion system for cF.

Our experiments showed that cells that had no cF labeling capacity also had no reproductive capacity or acidification capacity, but that the opposite was not generally true. When cF-labeled cell suspensions were subsequently tested for efflux, nonreproductive but labeled cell suspensions proved to be noneffluxing. Cell suspensions with a high labeled fraction and a high rate of efflux also had high relative reproductive capacity and acidification. The heat (60°C)-treated cell suspensions had a high fraction of labeling but hardly any efflux. They also had hardly any reproduction or acidification. The loss of the ability to fully efflux the cF might reflect the inactivation of the transport system or the loss of glycolytic energy generation when cells are killed. The labeling and the efflux ability of a culture were combined into one parameter by multiplying the fraction of cells labeled after incubation with cFDA by the fraction of cF efflux after 15 min of incubation with lactose, with cFin after labeling set at 1.0. This combined cF labeling and efflux parameter gave the best indication of reproduction and acidification capacities. It proved applicable for *L. lactis* after exposure to the tested stress conditions (heat, freezing, low pH, addition of CBS, and addition of DBS) and appears to be a

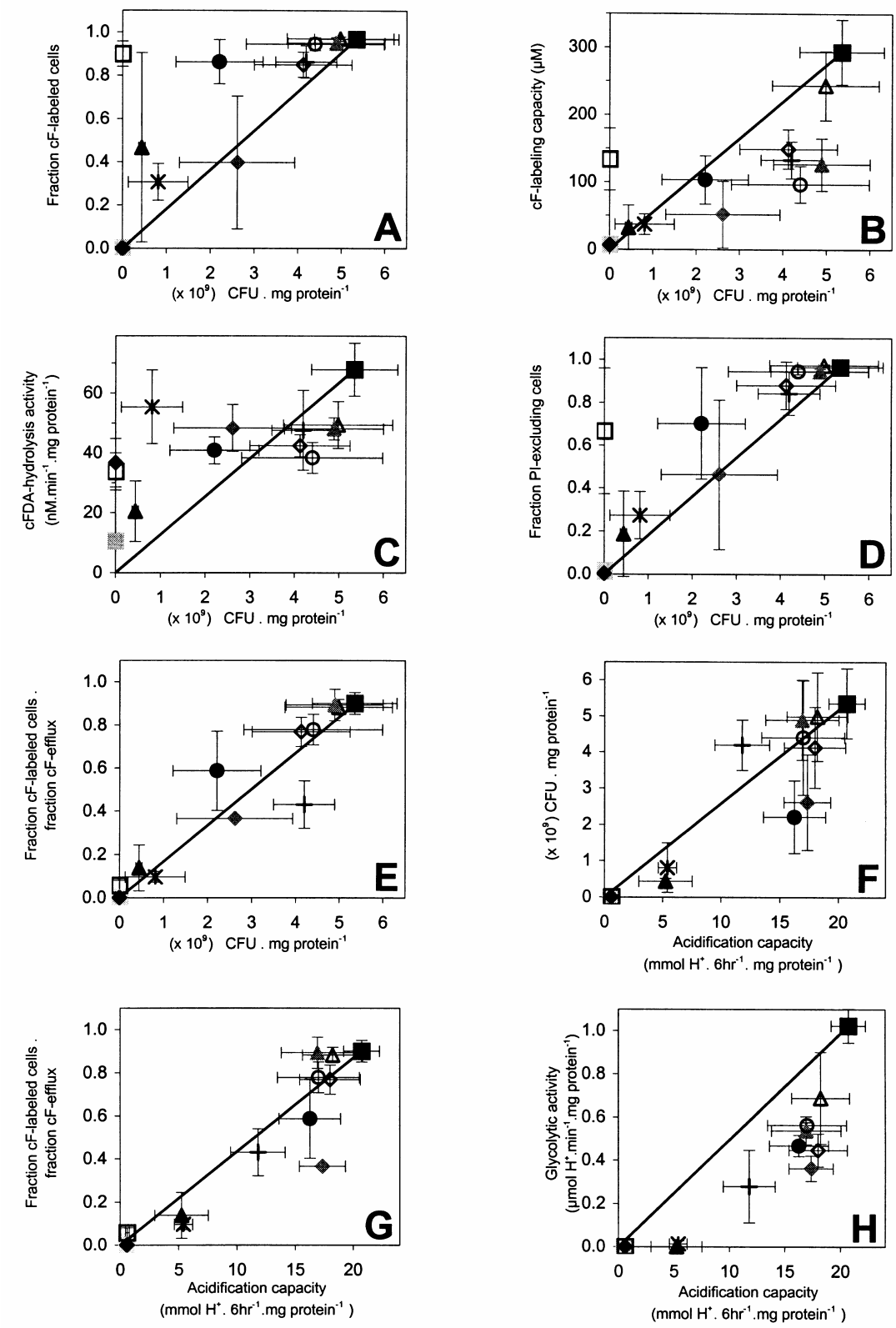

FIG. 7. Comparisons of fluorescence parameters, plate counts, acidification capacity, and glycolytic activity of *L. lactis* after exposure to different types of stress. Comparisons are shown for the fraction cF-labeled cells with plate counts (A), the cF labeling capacity with plate counts (B), the cFDA hydrolysis activity with plate counts (C), the fraction PI-excluding cells with plate counts (D), the product of cF labeling and efflux with plate counts (E), the plate counts with acidification capacity (F), the product of cF labeling and efflux with acidification capacity (G), and the glycolytic activity with acidification capacity (H). For each panel the data are extracted from Table 1. The symbols indicate no treatment (■), exposure to 70°C for 10 min (■), exposure to 60°C for 90 s (□), exposure to -20°C for 24 h (*), exposure to -20°C and 30% glycerol for 24 h (+), exposure to pH 7.0 (△), exposure to pH 5.0 (▲), exposure to pH 2.0 (▲), exposure to 0.2% CBS (○), exposure to 1.0% CBS (●), exposure to 0.02% DBS (◇), exposure to 0.06% DBS (◆), and exposure to 1.0% DBS (◆). The error bars indicate the standard deviations. The bold lines indicate the optimal situation, that is, linear regression through the origin and the point for the non-treated-cell suspensions.

general indicator of *L. lactis* reproduction and fermentation capacities.

This novel assay has potential for physiological research on LAB and for applications in the dairy industry. One application might be the assessment of the acidification capacity of cheese starters by fast fluorescence microscopic examination of cF labeling and efflux prior to the start of the fermentation. In addition, combined cF labeling and efflux assays could be used in the selection of strains of LAB to test, for example, the effect of freezing and storage on cheese starters or the effect of low pH and high bile salt concentrations on probiotics. Furthermore, combination of a cF labeling and efflux assay with strain-specific fluorescent markers could be used to study the viability and population dynamics of (stressed) mixed cultures of LAB. Finally, combining the fluorescence assays with flow cytometry may enable fast measurement of the physiological status of LAB present in cultures and of subpopulations and individual cells of LAB in dairy industry research and applications.

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Fluorescent Method for Monitoring Cheese Starter Permeabilization and Lysis

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Fluorescent Method for Monitoring Cheese Starter Permeabilization and Lysis

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A fluorescence method to monitor lysis of cheese starter bacteria using dual staining with the LIVE/DEAD *Bac*Light bacterial viability kit is described. This kit combines membrane-permeant green fluorescent nucleic acid dye SYTO 9 and membrane-impermeant red fluorescent nucleic acid dye propidium iodide (PI), staining damaged membrane cells fluorescent red and intact cells fluorescent green. For evaluation of the fluorescence method, cells of *Lactococcus lactis* MG1363 were incubated under different conditions and subsequently labeled with SYTO 9 and PI and analyzed by flow cytometry and epifluorescence microscopy. Lysis was induced by treatment with cell wall-hydrolyzing enzyme mutanolysin. Cheese conditions were mimicked by incubating cells in a buffer with high protein, potassium, and magnesium, which stabilizes the cells. Under nonstabilizing conditions a high concentration of mutanolysin caused complete disruption of the cells. This resulted in a decrease in the total number of cells and release of cytoplasmic enzyme lactate dehydrogenase. In the stabilizing buffer, mutanolysin caused membrane damage as well but the cells disintegrated at a much lower rate. Stabilizing buffer supported permeabilized cells, as indicated by a high number of PI-labeled cells. In addition, permeable cells did not release intracellular aminopeptidase N, but increased enzyme activity was observed with the externally added and nonpermeable peptide substrate lysyl-*p*-nitroanilide. Finally, with these stains and confocal scanning laser microscopy the permeabilization of starter cells in cheese could be analyzed.

Lactic acid bacteria in cheese starters have a dual role during cheese manufacture. Initially, they are responsible for the rapid acidification of the milk through efficient conversion of lactose into lactic acid. In a later stage of the process the proteolytic, peptidolytic, and amino acid-converting enzymes of the starter bacteria play a crucial role in the generation of flavor components. Most of these enzymes are located in the cytoplasm, while their substrates are mostly present outside the cells in the cheese matrix. Lysis results in leakage of intracellular enzymes. Therefore, lysis of the starter lactic acid bacteria is generally considered an essential part of the ripening process (2, 12, 20, 30).

Lysis in cheese depends on the choice of the strain and is strongly influenced by cheese-processing conditions such as pH, temperature, and salt concentration (2, 7, 30). By selecting rapidly lysing strains and process conditions that favor lysis, flavor development may be enhanced during ripening (9, 15). A major drawback in this selection is the difficulty in demonstrating lysis, especially in cheese.

Lysis is mostly studied in aqueous systems. In clear growth medium lysis can be observed by the decrease of turbidity. Other markers for monitoring lysis are decrease of viable counts, release of DNA, and release of intracellular enzymes (2, 19, 30). In cheese, however, these methods cannot be applied directly. Usually, an elaborate extraction procedure, which can be so rigorous that major cell damage or cell death is induced, is required. This makes an evaluation of the original cell integrity in the cheese almost impossible (8, 30). Another complicating factor is the occurrence, in cheese, of starter cells in different stages of cell disintegration, such as spheroplast cells (9, 29). Electron microscopy studies have shown that this spheroplast stage, when the cells seemed to leak proteins and ribosomes, was followed by major disruption of the cell membrane and release of the intracellular content. After complete lysis only residual material could be identified in cheese (e.g., ribosomes) (7). The osmotically fragile spheroplasts may be prevented from disruption by an osmostabilizing effect of the cheese environment (27). Alternative methodologies for extraction such as using a cheese press and using hypertonic buffers have been tried (30). However, to date no really good method is available, and an understanding of the role of lysis in cheese ripening is still lacking (8, 22).

Fluorescent probes provide alternative methods for assessment of bacterial physiology (4, 10; P. Haugland, Handbook of fluorescent probes and research chemicals, 7th ed., Molecular Probes, Eugene, Oreg.). With flow cytometry (FCM) individual cells in solution can rapidly be measured with high sensitivity and accuracy (24). With confocal scanning laser microscopy (CSLM) individual cells can be observed in solid matrices without the need for extraction.

The aim of this study was to develop an accurate and rapid method to measure lysis of cheese bacteria. In this work we demonstrate and quantify cell permeabilization and disruption of *Lactococcus lactis* in a buffer that stabilizes the cells, simulating cheese conditions. We applied the stains of the commercially available LIVE/DEAD *Bac*Light bacterial viability kit of Molecular Probes, SYTO 9, and propidium iodide (PI) (P. Haugland, Handbook of fluorescent probes and research

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chemicals, 7th ed., Molecular Probes). We show that the lysis process can be monitored with these stains by counting the number of intact and permeable cells at different time points. We also show the practical relevance of permeable cells in the lysis process by measurement of the accessibility of the intracellular peptidolytic enzyme aminopeptidase N (PepN). Finally, the direct application of the stains in combination with CSLM for cheese studies is described.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *L. lactis* subsp. *lactis* MG1363 was routinely stored in M17 broth (Oxoid, Haarlem, The Netherlands) with 0.5% (wt/vol) glucose and 15% (wt/vol) glycerol at 280°C. A culture was grown overnight in M17 supplemented with 0.5% (wt/vol) glucose (GM17) at 30°C without aeration. The overnight culture was diluted 50-fold with fresh GM17 and further incubated at 30°C until the culture had reached an optical density at 600 nm of approximately 1.0 (exponential growth phase). After harvest the cells were washed once with 50 mM sodium phosphate (NaPi) buffer, pH 6.5, and resuspended in the same buffer.

Induction of lysis and stabilization of permeabilized cells. The cell suspension was divided into two equal portions. One portion was resuspended in NaPi buffer, pH 6.5, referred to as control buffer. The other portion was resuspended in buffer consisting of 50 mM NaPi, 400 mM KCl, 20 mM MgCl2, and 5% (wt/vol) bovine serum albumin (BSA), pH 6.5, referred to as stabilizing buffer. For each cell suspension three 10-ml portions were transferred to new tubes and 0, 10, and 100 U of mutanolysin (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands)/ ml were added, respectively. The cells were incubated at 30°C, and the decrease of turbidity was monitored by measurements of optical density at 600 nm. At various time points samples were taken for analyses.

Plate counts. Samples were serially diluted in peptone physiological salt solution (Tritium Microbiologie B.V., Veldhoven, The Netherlands), and 1-ml portions of the appropriate dilution were spread out on plastic petri disks in duplicate. Twenty milliliters of molten GM17 agar (GM17 with 1.5% [wt/vol] agar, 46°C) was poured out on the plates. After incubation for 2 days at 30°C the colonies were counted.

Fluorescence labeling. The LIVE/DEAD *Bac*Light bacterial viability kit with separate solutions of SYTO 9 and PI (Molecular Probes) contains high-concentration stock solutions in dimethyl sulfoxide: 3.34 mM SYTO 9 and 20 mM PI. Fresh dilutions of 0.5 mM SYTO 9 and 1.5 mM PI in distilled water were prepared daily and kept in the dark at 4°C. Fifty-microliter portions of the samples were incubated with SYTO 9, with PI, or with both or without dye for 10 min at 30°C. The final dye concentrations were 10 μM SYTO 9 and 30 μM PI.

Epifluorescence microscopic counting. Countings were performed with an image analysis system connected to a Dialux microscope (Ernst Leitz, Wetzlar, Germany) that was equipped with a 50-W mercury arc lamp and a Leitz fluorescein isothiocyanate filter (excitation wavelength, 450 to 490 nm; emission wavelength, >515 nm). The emitted light was directed to a charge-coupled device (CCD) camera with C-mount at × 0.63 (COHU high-performance CCD camera; Leica, Rijswijk, The Netherlands). Images were recorded using the Q-Fluoro software package (Leica). Samples labeled only with PI were used in these experiments. The total number of cells was determined from images recorded with phase-contrast illumination. The number of PI-labeled cells was determined from images recorded with epifluorescence illumination. The averages of three image fields were calculated.

FCM. FCM was performed with cell suspensions labeled with SYTO 9 and PI, with SYTO 9 only, or with PI only and with nonlabeled cells. Cell suspensions were diluted in 2-(N-morpholino)ethanesulfonic acid (MES) buffers that had been filtered using a 0.2-mm-pore-size filter. Cells that had been incubated in control buffer were diluted in buffer containing 100 mM MES and 50 mM KCl at pH 6.5 (MES control buffer). Cells that had been incubated in stabilizing buffer were diluted in buffer containing 100 mM MES, 450 mM KCl, 20 mM $MgCl_2$, and 5% BSA at pH 6.5 (MES stabilizing buffer). Yellow-green fluorescent polystyrene microspheres with a diameter of 0.7 mm (Polysciences Europe GmbH; Eppelheim, Germany) were used to enable enumerations of cells in the FCM samples. FCM samples were prepared by mixing 2 μl of cell suspension, 100 ml of fluorescent bead suspension (1.335×10^7 beads per ml), and either MES control buffer or MES stabilizing buffer to a total volume of 1,000 μl. Thus, the concentration of fluorescent beads in the FCM sample was exactly 1.335×10^6 beads per ml and the concentration of cells was between 10^6 and10^7 cells per ml, depending on the cell incubation conditions.

Flow-cytometric analyses were performed with a FACSCalibur flow cytometer and data analysis software as described previously (5). A side scatter (SSC) threshold level was used to reduce background noise. The cell samples were delivered at the low flow rate, which gave 300 to 600 events per s. We used 2 min of data acquisition, which permitted measurement of on average 40,000 cells. For each cell, forward scatter (FSC), SSC, green fluorescence (515 to 545 nm), yellow-orange fluorescence (564 to 606 nm), and red fluorescence (>670 nm) were recorded. The data were analyzed using dot plots, i.e., bivariate displays in which each dot represents one measured event.

In the dot plot of FSC and SSC the cells and the beads gave distinct, nonoverlapping populations, and a cell region and a bead region were created for gating. The subpopulations of SYTO 9-labeled cells and PI-labeled cells were distinguished best in the red fluorescence histogram gated on cells. The beads gave a series of subpopulations with decreasing number at increasing FSC, SSC, and fluorescence signals, corresponding to single beads, double beads, etc. This was confirmed by fluorescence microscopic examination, which showed that the bead suspension contained mainly single beads but also some chains of two beads, three beads, and even four beads. The total number of beads was calculated by taking all bead subpopulations that gave distinct peaks in the green fluorescence histogram into account, usually up to four peaks. The concentrations of SYTO 9-labeled cells and PI-labeled cells were calculated from the ratios of cells to beads and the known concentration of beads.

The accuracy of counts is indicated by the coefficient of variation (CV). In a counting of n items, the associated standard deviation is $n^{1/2}$. The CV is the standard deviation over the mean. The CV is a common measure of precision (24).

Enzyme assays. L-Lactate dehydrogenase (LDH) activity was assayed by measurement of the decrease of A_{340} resulting from the pyruvate-dependent oxida- tion of NADH as described previously (31). For measurement of the total LDH activity cell extract was used. This was prepared by disruption of the cell sus-pension with zirconium beads by bead beating twice for 30 s each using a FastPrep FP120 (Bio 101, Savant Instruments, Holbrook, N.Y.). The disrupted cell suspension was centrifuged at 14,000 rpm for 5 min at 4°C, and the super- natant was carefully pipetted into another tube for use. Released LDH activity was determined by using supernatant of the centrifuged cell suspension.

Aminopeptidase N (PepN) activity was assayed as described previously (14). L-lysyl-*p*-nitroanilidedihydrobromide was used as the substrate because it is not permeant and therefore not hydrolyzed in a suspension of intact living cells at pH 6.5 (14). By incubating a whole-cell suspension with the substrate the accessible PepN activity was measured. Furthermore, the released activity was measured using supernatant of the centrifuged cell suspension.

CSLM of labeled cheese. Slices of 2-week-old Gouda cheese (5 by 5 by 2 mm) were cut with a razor blade and placed on a microscope slide. A staining mixture with 20 μM SYTO and 60 μM PI (25ml) was spread out on the freshly cut cheese surface, and a coverslip was placed on top. The cheese slice was thus incubated with the stains for 30 to 60 min in the dark at room temperature. The CSLM work was carried out using a Leica TCS SP confocal scanning laser microscope (Leica) with an argon-krypton laser (488- and 568-nm excitation) and a ×63 objective with a numerical aperture of 1.2. Maximum emission intensities were at 520 to 530 nm for SYTO 9 and 645 to 655 nm for PI. CSLM images were obtained 10 μm below the level of the coverslip.

RESULTS

Induction of lysis and stabilization of permeabilized cells. Model strain *L. lactis* subsp. *lactis* MG1363 was used as the test organism. Cells harvested in mid-exponential phase were resuspended in buffer containing 50 mM NaPi, pH 6.5 (control buffer), and mutanolysin was added to induce lysis to a final concentration of 10 or 100 U/ml or cells were incubated without mutanolysin. Also, cells were resuspended in a buffer with high protein and high salt concentrations (50 mM NaPi, 400 mM KCl, 20 mM MgCl2 , and 5% BSA, pH 6.5), referred to as stabilizing buffer, and incubated without mutanolysin or with 10 or 100 U of mutanolysin/ml. Cell suspensions were incubated for 22 h at 30°C.

A typical example of the effect of mutanolysin treatment on the turbidity of the cell suspension in control buffer is shown in Fig. 1A. Without mutanolysin there was only a slight decrease in the turbidity, caused by spontaneous lysis. With 10 U of

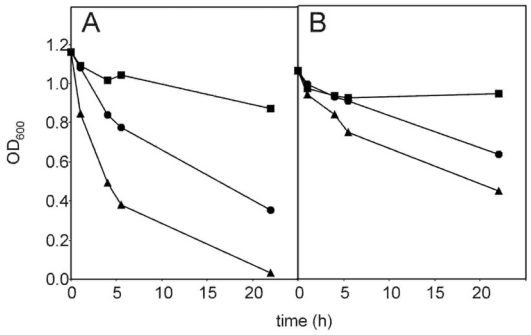

FIG. 1. Effect of mutanolysin treatment on the turbidity of the *L. lactis* subsp. *lactis* MG1363 cell suspension. Cells were incubated in control buffer (A) and in stabilizing buffer (B) at 30°C with 0 (squares), 10 (circles), or 100 (triangles) U of mutanolysin/ml. OD_{600}, optical density at 600 nm.

mutanolysin/ml the turbidity decreased considerably. With 100 U of mutanolysin/ml the turbidity decreased fast and the suspension was almost cleared after 22 h. In stabilizing buffer the turbidity decreased at a lower rate (Fig. 1B). In the example shown it decreased in 22 h only one-half as much as in control buffer. Both the mutanolysin-induced lysis and the spontaneous lysis were slower in stabilizing buffer.

The survival of the cells under the various incubation conditions was tested by plate counting (Table 1). The number of CFU decreased much more than the turbidity. Without mutanolysin the CFU decreased by 1/2 log unit. Mutanolysin caused a further decrease of CFU, down to a survival of less than 1%. There was no obvious difference between survival in control buffer and survival in stabilizing buffer.

Release of LDH. LDH is a cytoplasmic protein, and in most lactic acid bacteria it is a key enzyme in metabolism. It is commonly used as a marker for lysis. The LDH activities of supernatant and of cell extract were measured, and the ratio was taken as the fraction of LDH released. The LDH releases in stabilizing buffer were much lower than those in control buffer. In the example shown 21% of the LDH was released after 22 h of incubation in control buffer without mutanolysin as a result of spontaneous lysis (Fig. 2A). When cells were treated with 100 U of mutanolysin/ml, almost all LDH was released. In stabilizing buffer spontaneous lysis resulted in release of only 6% of total LDH after 22 h, and treatment with 100 U of mutanolysin/ml resulted in release of 29% of total LDH (Fig. 2B). For a control, the effect of buffer composition on the activity of LDH was measured. The presence of BSA, potassium, and magnesium had no influence on the activity level of LDH (data not shown).

TABLE 1. Effect of 22 h of mutanolysin treatment on *L. lactis* subsp. *lactis* MG1363 viability

Buffer[a]	Treatment	LG CFU/ml in expt: 1	2
Control	No incubation	9.3	9.2
	Incubation without mutanolysin	8.5	8.6
	Incubation with 10 U of mutanolysin/ml	8.1	6.9
	Incubation with 100 U of mutanolysin/ml	6.7	8.5
Stabilizing	No incubation	9.2	9.2
	Incubation without mutanolysin	8.7	8.8
	Incubation with 10 U of mutanolysin/ml	7.6	8.5
	Incubation with 100 U of mutanolysin/ml	7.8	8.0

[a] Control buffer: 50 mM NaPi, pH 6.5; stabilizing buffer: 50 mM NaPi, 400 mM KCl, 20 mM MgCl2 , 5% BSA, pH 6.5.

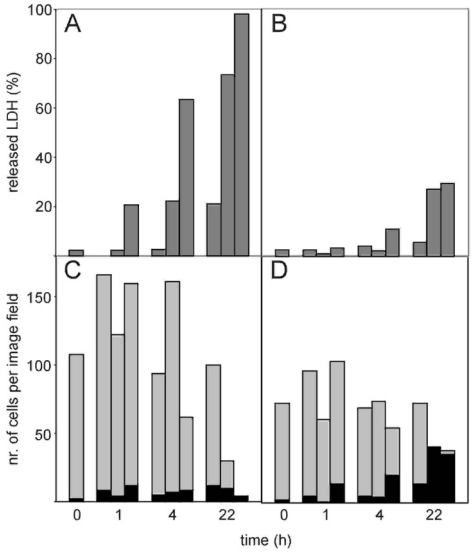

FIG. 2. Effect of mutanolysin treatment on LDH release and on total and permeable cell numbers of *L. lactis* subsp. *lactis* MG1363. Cells were incubated in control buffer (A and C) and stabilizing buffer (B and D) at 30°C with 0 (left bar), 10 (middle bar), or 100 (right bar) U of mutanolysin/ml. The released LDH percentage is the ratio of supernatant (released) and cell extract (total) activity (A and B). The microscopic counts of total cells (total height of the bars) and permeable cells (black part) were done by fluorescence microscopy image analysis using PI labeling. Average numbers of three image fields were calculated (C and D).

Fluorescence microscopy. Fluorescence labeling and microscopy allowed direct observations of the individual cells in the suspension. Samples were labeled with SYTO 9 and PI and analyzed with epifluorescence microscopy. Before incubation the concentration of cells was high and almost all cells were intact. After 20 h of incubation with 100 U of mutanolysin/ml in control buffer the number of cells was much lower. In contrast, in stabilizing buffer, the concentration of cells remained high but many of the cells were permeabilized as indicated by PI labeling.

Figure 2C and D show the results of a counting experiment using image analysis. In control buffer the total number of cells decreased as a function of time and mutanolysin concentration. The remaining number of cells after 22 h of incubation with 100 U of mutanolysin/ml was less than 5% of the number of cells before incubation. The numbers of permeable cells under these conditions were low. Under stabilizing conditions, on the other hand, the decrease of cell numbers was much smaller, with the lowest recorded values still remaining above 50%. However, the fraction of permeable cells increased with time and mutanolysin concentration. After 22 h of incubation with 10 or 100 U of mutanolysin/ml nearly all remaining cells

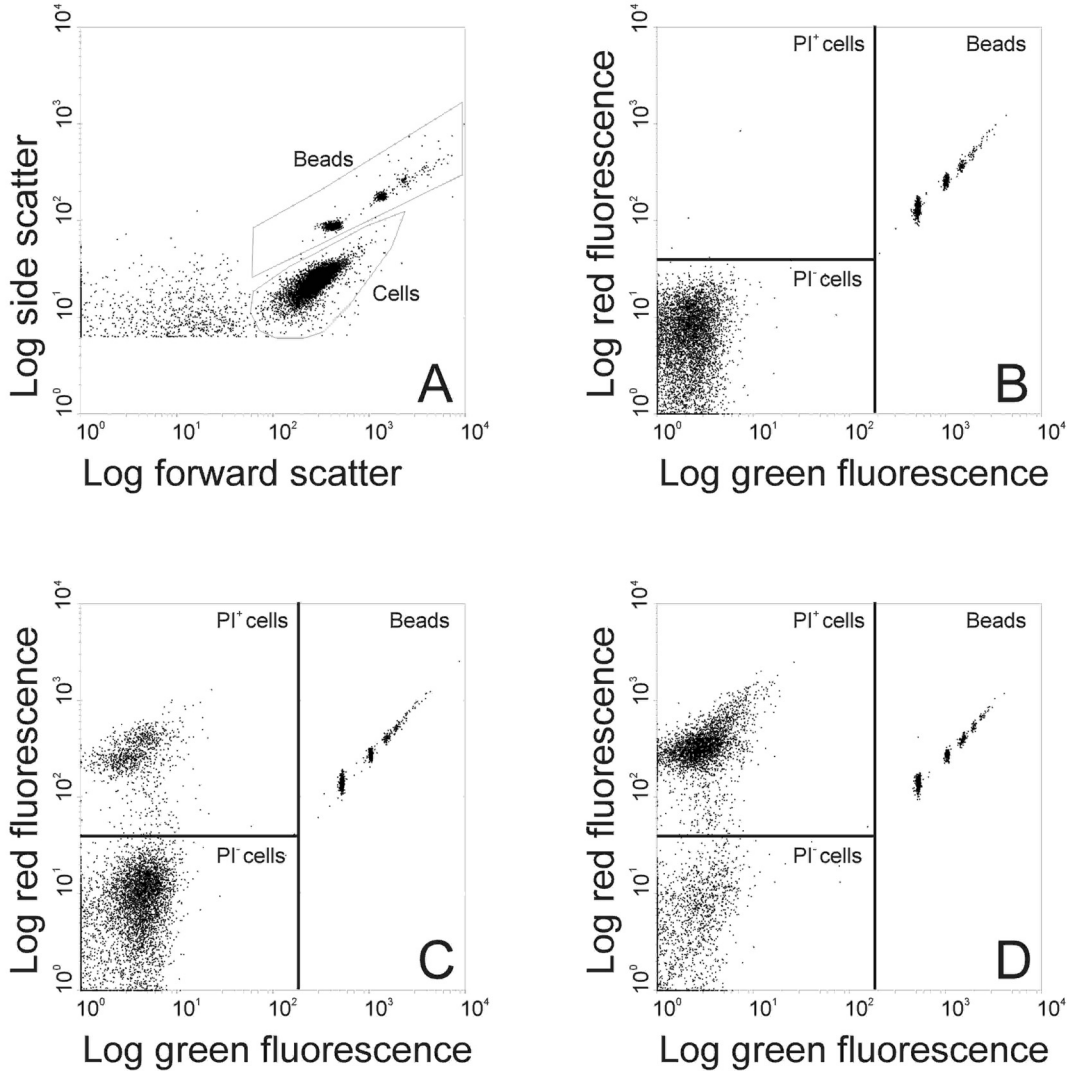

FIG. 3. Effect of mutanolysin treatment on cell number and membrane permeability of *L. lactis* subsp. *lactis* MG1363 shown by SYTO 9 and PI labeling and FCM. Shown are a dot plot of FSC and SSC (A) and dot plots of green and red fluorescence gated for cells and beads of an untreated cell suspension (B), a cell suspension incubated for 21 h in stabilizing buffer (C), and a cell suspension incubated for 21 h in stabilizing buffer with 100 U of mutanolysin/ml (D).

were labeled by PI. The difference between the total number of cells and the number of permeable cells represents the number of intact cells. In stabilizing buffer the number of intact cells was similar to the number in control buffer.

Comparisons of LDH release and microscopic countings show that the LDH release coincided with the decrease in total numbers of cells. The results indicate that mutanolysin treatment caused cell damage, rendering the cells permeable for PI but not for LDH. In control buffer, cell damage rapidly causes complete cell disruption, resulting in complete LDH release. Under stabilizing conditions, cell disruption is a much slower process, and therefore LDH appears much more gradually in the external medium.

FCM. Cell suspensions labeled with SYTO 9 and PI were diluted in MES-based buffers to 10^6 to 10^7 cells per ml, and yellow-green fluorescent polystyrene beads were added to enable calculations of cell numbers. The permeable cells were distinguished by PI labeling. Figure 3 shows typical FCM results. In the FSC-SSC plot (Fig. 3A) a region of beads and a region of cells were identified. Within the bead region subpopulations of single beads, double beads, etc., were observed. Figure 3B to D show dot plots of green fluorescence and red fluorescence gated for cells and beads of untreated cells (B), cells incubated in stabilizing buffer for 21 h without mutanolysin (C), and cells incubated for 21 h with 100 U of mutanolysin/ml (D). The beads gave distinct subpopulations in the upper left quadrant. The PI-stained cells gave a population with high red fluorescence and low green fluorescence (PI^+). The cells that were not stained by PI but by SYTO 9 gave a population with low fluorescence signals (PI^-). In untreated cell suspensions very few cells were stained by PI (Fig. 3B). The total number of cells did not change in stabilizing buffer, but the fraction of permeable cells increased with time and mutanolysin concentration. After 21 h of incubation without mu-

tanolysin 17% of cells were permeable as indicated by PI^+ staining (Fig. 3C), whereas the sample with 100 U of mutanolysin/ml contained 70% permeable cells (Fig. 3D). In control buffer (not shown) the total number of cells decreased as a function of time and mutanolysin concentration and the number of permeable cells remained low. After 21 h with 0, 10, and 100 U of mutanolysin/ml the total number of cells was decreased to 75, 68, and 37%, respectively. Of the remaining cells less than 10% were permeable.

As expected, FCM counting results corresponded with microscopic counting results, but the advantage of the FCM countings is that they give the actual number of cells per milliliter.

Furthermore, the FCM results are more accurate since more cells are counted. The average total cell count by FCM was 40,000. A count of 40,000 has a CV of 0.5%. When the PI-labeled subpopulation was as small as 5%, the CV of this subpopulation (2,000 events) was still only 2.2%. With fluorescence microscopy, on average 250 cells were counted as the total of three image fields. The CV of a total count of 250 is 6%, and the CV of a subpopulation that comprises 5% of the cells is 28%. The imprecision of the plate counts is similar to that of the microscopic counts, since the counted numbers were on the same order of magnitude.

The results show that fluorescence staining with SYTO 9 and PI and analysis with FCM constitute a highly accurate and straightforward method for assessment of total, intact, and permeable cell numbers.

Release and accessibility of PepN. Peptidases are an important group of enzymes in the generation of flavor components in cheese (18). Aminopeptidase N is an intracellular enzyme capable of catalyzing the hydrolysis of a wide range of substrates, and it has a strong debittering effect in cheese ripening. Cells have to be disrupted for release of the enzyme. To assay PepN activity, we used the artificial substrate L-lysyl-*p*-nitroanilidedihydrobromide, which releases the chromophoric (yellow) p-nitroaniline upon cleavage. Intact cells cannot catalyze this reaction since the substrate cannot permeate through the intact cell envelope. In a cell suspension, cleavage of the substrate can be a result of PepN released into the external medium or of intracellular PepN that has become accessible for the substrate because of cell permeabilization. We observed high cell-associated PepN activity specifically under conditions that resulted in permeabilization of the cells. Table 2 shows the results of an experiment using 21-h incubations with 10 U of mutanolysin/ml. In stabilizing buffer, 75% of the accessible enzyme activity was localized in the cell and only 25% was localized outside. In control buffer, 20% of the accessible enzyme activity was intracellular and 80% was released. The total measured PepN activity in stabilizing buffer was approximately the same as in control buffer, but the amount of activity released was much lower. The results show that intracellular enzymes become accessible for peptidase substrates when cells become permeable. This is specifically relevant for conditions found in cheese, where cell lysis is slow and many cells are present for prolonged periods of time in an intermediate, and permeable, stage of cell disruption.

Lysis in cheese. Thin slices of young, 2-week-old Gouda cheese were incubated with SYTO 9 and PI and were analyzed by CSLM. CSLM allowed clear observations of stained cells within the cheese matrix (Fig. 4). A majority of the starter cells were intact as shown by their green fluorescence. However, a substantial number of the cells were permeable as indicated by PI labeling. These results show that the cheese matrix supports permeable cells. As in stabilizing buffer, the lytic processes cause permeabilization but complete disruption is postponed by the stabilizing conditions. So the presence of permeable cells is an important feature during cheese ripening.

TABLE 2. Effect of mutanolysin treatment on the PepN activity of *L. lactis* subsp. *lactis MG1363*[d]

Buffer	Time (h)	PepN activity (ΔA_{450} /min/ml)					%PI labeled
		Total accessible[a]	Released[b]	(%)	Intracellular[c]	(%)	
Control	3	0.031	0.004		0.027		2
	21	0.184	0.147	(80)	0.037	(20)	26
Stabilizing	3	0.043	0.023		0.020		6
	21	0.197	0.049	(25)	0.148	(75)	84

[a]Total accessible activity was assayed by measuring activity of a whole-cell suspension.

[b]Released activity was assayed by measuring the activity of supernatant.

[c]Intracellular fraction of accessible activity was calculated by subtracting the released activity from the total accessible activity.

[d]Cells were incubated in control buffer and stabilizing buffer with 10 U of mutanolysin/ml.

DISCUSSION

In this paper we describe an effective method for measuring cell permeabilization in cheese. The measurement of lysis in cheese has always been a major problem (7, 9). Because the decrease of cell turbidity cannot be measured in cheese, other markers for lysis have been used such as decrease of viable counts, release of DNA, and release of intracellular enzymes, such as phospho-β-galactosidase, LDH, and different peptidases (2, 19, 20, 30). For all these methods, extraction procedures are required; these will, unquestionably, induce more lysis, thus causing overestimation of this process. The direct labeling with fluorescent dyes, as described in this paper, has great advantages since extraction procedures are not required. Furthermore, it can measure other cell characteristics in addition to the markers commonly used for lysis.

A number of fluorescence techniques for evaluating the physiological conditions of bacteria have been introduced over the last decade (for reviews see references 4, 10, and 16). Among the wide range of applications there are fluorescent dyes for measuring enzyme activities, membrane potential, redox potential, respiration activity, intracellular pH, membrane integrity, and viability of cells. Fluorescence microscopy enables direct visual analysis of labeled cell suspensions. However, FCM is often the method of choice for quantitative analysis. FCM measurements are made very rapidly on a large number of individual cells and give objective and accurate results (5, 11, 21, 24).

In the work described here, we used the fluorescent dyes of the LIVE/DEAD *Bac*Light bacterial viability kit of Molecular Probes. The kit contains two fluorescent nucleic acid stains: the permeant SYTO 9 (green) and the nonpermeant PI (red) (P. Haugland, Handbook of fluorescent probes and research chemicals, 7th ed., Molecular Probes). wen used in combi-

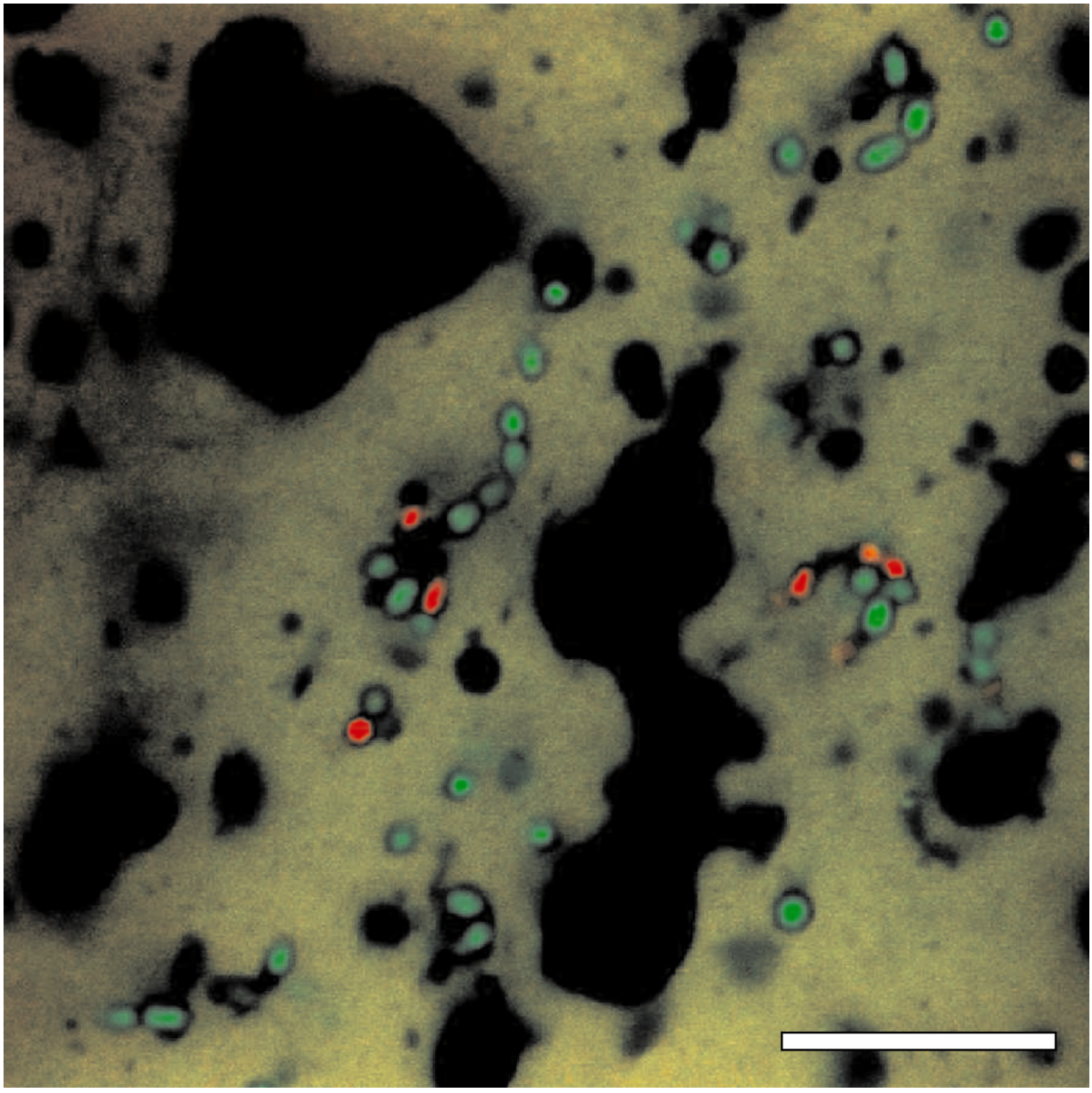

FIG. 4. CSLM image of 2-week-old Gouda cheese stained with SYTO 9 and PI. Bar, 10 μm.

nation intact cells are labeled green and cells with damaged membranes are labeled red. The *Bac*Light bacterial viability kit has been used for various bacterial species in pure culture, such as *Escherichia coli*, *Salmonella* spp., and *Listeria* spp. (11, 25, 26). Also, it has been used for bacteria in various environments, such as seawater, drinking water, biofilms, and also different food products (3, 13, 17). Recently, labeling of dairy products, including cheese curd, with the *Bac*Light kit has been used to investigate viability of probiotic bacteria (1). Also, CSLM of *Bac*Light-labeled cheese curd was applied to show the difference in viability between lytic and nonlytic *L. lactis* strains (23).

To study lytic processes, an aqueous system is preferred to facilitate extensive experimental analyses. However, lysis in a standard buffer may not represent lysis in cheese very well. In ripening cheese high concentrations of milk protein, fat globules, and salt are present, and the matrix becomes increasingly solid in time. The high osmolarity may well be an important factor in starter lysis during cheese ripening. Electron microscopy indicated this (7, 27, 29). Cells with various extents of wall lysis were observed, such as cells with protoplast membranes, which were maintained presumably by the osmotic stability in cheese, exploded protoplasts from which parts of the cell wall were released, and ghost cells whose walls were deformed. After complete lysis residual cell material remained detectable in the cheese for some time. The liberation of cytoplasmic material, including all intracellular enzymes, into cheese is commonly considered an essential step in protein degradation during production of fermented dairy products (20, 29, 30). However, Chapot-Chartier and coworkers measured a signifi-

cant amount of peptide hydrolysis without detecting lysis or enzyme release (7). It was proposed that cells become permeable to enzyme substrates at the beginning of ripening and that free amino acids are released from bacterial cells. However, electron microscopy cannot visualize this initial process of cell wall and membrane permeabilization. Nevertheless, bacterial cell disruption was still considered important for facilitating access of peptide substrates and acceleration of ripening (7).

Discrimination between intact and permeable cells by fluorescent stains has been used in many studies on bacteria, including a few recent applications on lactic acid bacteria. Injury of *Lactobacillus plantarum* by nisin treatment was observed with PI and carboxyfluorescein succinimidylester using FCM (28). Furthermore, the membrane permeability of *L. lactis* was measured with PI and carboxyfluorescein diacetate (cFDA) using spectrofluorimetry and fluorescence microscopy in a study of viability after various stress treatments (6). Also, permeable fractions in post-logarithmic-phase cultures of *L. lactis* were measured with PI using spectrofluorimetry (22). Finally, permeabilization by bile salts and acid with concomitant cell death of various lactic acid bacteria was measured with PI, TOTO-1, and cFDA (5). The fluorescence detection of permeable cells makes it reasonable to suggest that unlysed cells with permeabilized membranes may be significant in cheese ripening. Peptides from the cheese matrix may freely diffuse inside the permeable cells and be hydrolyzed by intracellular enzymes.

The present study demonstrated the presence of permeable cells and their relevance in peptidolytic activity under cheese conditions. To mimic the stabilizing conditions that occur in cheese, we used a buffer with high protein and salt concentrations. Lysis was induced by cell wall-digesting enzyme mutanolysin. Under stabilizing conditions mutanolysin addition resulted in smaller decreases of turbidity and total number of cells than were found with control buffer. The results of the fluorescence staining suggest that the process of cell disintegration is much slower under stabilizing conditions, resulting in the presence of permeable cells for long periods of time. The release of the marker enzyme LDH was much less in the stabilizing buffer than in control buffer. This LDH release did not correspond with the permeabilization but with the decrease in total cell number, indicating that LDH is too big to leak out of these permeable cells. However, permeabilization of the cells did make the intracellular peptidase PepN accessible for the extracellular, and impermeant, peptide substrates. Apparently, the cell damage caused by mutanolysin treatment is sufficient to make cells permeable for enzyme substrates, as well as for PI, but not severe enough for leakage of enzymes.

Our results clearly show the added value of using fluorescent stains for assessment of lysis, including CSLM analysis of cheese. The fluorescence method of cell counting using SYTO 9 and PI not only monitors the complete disintegration of cells, as is the case with traditional methods of measuring lysis, but also visualizes the permeabilization of the cell envelope upon cell wall digestion by lytic enzymes. Upon permeabilization intracellular enzymes are able to contribute to the process of protein degradation and flavor formation, as is shown for PepN activity. This clearly suggests that many cells in cheese could be present in permeabilized state. Traditional lysis techniques based on measurement of released intracellular enzymes into the cheese matrix overlook the contribution to the total peptidase activity by all the enzymes present in permeabilized cells.

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Christine Bunthof and Saskia van Schalkwijk contributed equally to this work.

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Flow Cytometric Assessment of Viability of Lactic Acid Bacteria

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Flow Cytometric Assessment of Viability of Lactic Acid Bacteria

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The viability of lactic acid bacteria is crucial for their applications as dairy starters and as probiotics. We investigated the usefulness of flow cytometry (FCM) for viability assessment of lactic acid bacteria. The esterase substrate carboxyfluorescein diacetate (cFDA) and the dye exclusion DNA binding probes propidium iodide (PI) and TOTO-1 were tested for live/dead discrimination using a *Lactococcus*, a *Streptococcus*, three *Lactobacillus*, two *Leuconostoc*, an *Enterococcus*, and a *Pediococcus* species. Plate count experiments were performed to validate the results of the FCM assays. The results showed that cFDA was an accurate stain for live cells; in exponential-phase cultures almost all cells were labeled, while 70°C heat-killed cultures were left unstained. PI did not give clear live/dead discrimination for some of the species. TOTO-1, on the other hand, gave clear discrimination between live and dead cells. The combination of cFDA and TOTO-1 gave the best results. Well-separated subpopulations of live and dead cells could be detected with FCM. Cell sorting of the subpopulations and subsequent plating on agar medium provided direct evidence that cFDA labels the culturable subpopulation and that TOTO-1 labels the nonculturable subpopulation. Applied to cultures exposed to deconjugated bile salts or to acid, cFDA and TOTO-1 proved to be accurate indicators of culturability. Our experiments with lactic acid bacteria demonstrated that the combination of cFDA and TOTO-1 makes an excellent live/dead assay with versatile applications.

Lactic acid bacteria (LAB) are applied in food production for their useful metabolic properties. They are used as starters and as probiotics. However, these applications imply that the LAB are exposed to various stress conditions that may affect the physiological status of the microbes. LAB are employed as starter cultures in the production of fermented foods, such as cheese, yogurts, wines, and fermented meats. The starter cultures are often stored in freeze-dried form, which decreases the number of CFU significantly (6). Cell proliferation and metabolic activity are crucial for success of fermentation processes, such as in cheese production. The starter bacteria multiply after being added to the curd, convert lactose to lactic acid, and degrade casein to peptides and amino acids. These are essential functions for the development of texture and flavor (9). At the same time the conditions of the fermentation process, in particular the decline of the pH, the temperature, and the high salt concentration, affect the physiological status of the bacteria.

Besides being used in dairy fermentations, several LAB species are employed as probiotics. Probiotics are living microorganisms which upon ingestion in certain numbers should exert health effects beyond inherent basic nutrition (17). The species are selected mainly on the basis of their potential health-associated properties, but it is well recognized that further criteria should also be fulfilled (14, 17, 20, 39). One of the requirements is resistance to technological processes, such as survival in fermented milk to provide a suitable shelf life period for the product. Another requirement is resistance to gastric acid and bile. This is necessary for persistence in the gastrointestinal tract to perform health-promoting actions (7, 13, 14). In studies on survival and stress response, the quantitative assessment of viability is important.

In concept, bacterial viability is the reproductive capacity, and survival is the maintenance of the viability (1). In operation, viability has to be demonstrated by replication in a validated laboratory system (1, 22). The conventional method for quantitative survival studies is the plate count technique, in which replication on an appropriate agar medium is tested. Although this is the only direct proof of culturability (1, 22), the plate count method has major drawbacks (3). For many species there is not (yet) a good growth medium. Furthermore, the plate count technique requires long incubation times (2 days to a few weeks). Alternative techniques for viability assessment are desired for fundamental as well as routine microbiology research, although they have to be rapid and reliable. Flow cytometry (FCM) is an appealing technique for fast viability assessment.

FCM is a rapid technique for cell-by-cell multiparameter analysis that is often used in combination with fluorescent labeling (37). Cells are analyzed at rates of 100 to 1,000 per s as they are carried within a fast-flowing fluid stream that passes a focused light beam. The forward-angle light scatter (FSC), the side-angle light scatter (SSC), and the fluorescence at selected wavelengths are measured. The analyses are done on large populations of cells, typically 5,000 to 10,000. Subpopulations can be identified and distinguished when they differ in light scatter or fluorescence characteristics. Also, subpopulations can be physically selected (sorted) for further study. FCM in combination with fluorescent labeling is increasingly applied in microbiology. It is used in counting the total number of bacteria and in detecting specific strains by 16S rRNA sequence or by antigen expression. It is also used for characterizing and quantifying cellular physiological

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parameters such as DNA content, enzyme activity, respiration, membrane potential, intracellular pH, and membrane integrity (11, 16, 27, 34, 35).

Various fluorescent probes are used for viability assessment (3, 18, 30, 35). Redox probes are used, such as tetrazolium salts that are reduced by the electron transfer chain. Also, mem-brane potential probes are used, such as anionic oxonol dyes and the cationic dye rhodamine 123. Furthermore, esterase substrates are used, such as fluorescein diacetate and calcein AM. These are nonfluorescent precursors that are taken up by the cell. The fluorescent products are positively charged, so they are retained in the cell provided that the membrane is intact. Thus, labeling indicates enzymatic activity and membrane integrity (4, 12). Finally, dye exclusion probes are used extensively, especially DNA binding compounds. The exclusion of such impermeant probes by cells with intact membranes is taken as an indicator of viability.

Two groups of dye exclusion probes are of importance: phenanthridium nucleic acid dyes and cyanine nucleic acid dyes. The phenanthridium nucleic acid dyes include ethidium bromide, propidium iodide (PI), and ethidium homodimer-1. These probes have been used almost exclusively to evaluate cell membrane integrity of bacterial as well as eucaryotic cells (5, 18, 28). Cyanine nucleic acid dyes are compounds that also have the chemical characteristics necessary for a viability assay based on dye exclusion. The group comprises the compounds of the monomeric TO-PRO series, the dimeric TOTO series, and the SYTOX series (Molecular Probes Inc., Eugene, Oreg.). These probes bind to DNA with little specificity, have very high fluorescence enhancement factors, and have a range of spectra covering the entire visible spectrum (18, 19). SYTOX-Green was described as a probe for bacterial viability and antibiotic susceptibility testing and has been applied and further investigated in several studies (25, 32, 36). In contrast, the TO-PRO and TOTO series compounds have been described mainly as probes for DNA gel electrophoresis and DNA analysis by FCM and laser confocal microscopy (18), whereas reports on their use as viability indicators are scarce. YOYO-1 and YOYO-3 have been applied for eucaryotic cells (2, 23). TO-PRO- 3 was used for investigating starving and resuscitating cultures of *Micrococcus luteus* (40). TO-PRO-1 was compared to PI and SYTOX-Green to study injured *Escherichia coli* (32).

The subject of this study was the rapid FCM analysis of LAB, in particular, the assessment of survival when LAB are exposed to bile salts or to acid. We aimed for an FCM assay that accurately indicates culturability, with proven validity for the given stress conditions. Plate counts were performed to ensure that the populations indicated as live by the FCM via-bility assay were indeed culturable while populations indicated as dead were not culturable. A selection of nine LAB species was tested, including species from the different genera of dairy LAB as well as species used in various dairy products and probiotics (8, 26, 33). We evaluated carboxyfluorescein diacetate (cFDA) as a live stain using the labeling protocol for *Lactococcus lactis* analysis by fluorescence microscopy devel-oped in an earlier study (5). Furthermore, the impermeant nucleic acid stains PI and TOTO-1 were evaluated for their capacity to stain dead LAB cells using FCM. PI was included because it is the probe most used for detection of dead cells and its spectroscopic properties make it suitable for FCM (18). TOTO-1 was chosen because the excitation and emission spectra are suitable for FCM, it has a high fluorescence enhancement, and its molecular mass is approximately twice as high as that of PI (18, 19). Possible applications of the developed FCM live/dead assay are discussed.

TABLE 1. Evaluation of FCM in combination with cFDA, PI, and TOTO-1 for identifying live and dead cells[a]

Organism	Result[b] with probe:		
	cFDA	PI	TOTO-1
Lactococcus lactis NCDO 712	+	-	+
Streptococcus salivarius subsp. *thermophilus*	+	+	+/-
Lactobacillus delbrueckii subsp. *bulgaricus*	+	+	+
Lactobacillus casei R	+	+	+
Lactobacillus helveticus T97	+	-	+
Leuconostoc mesenteroides DSM 20343	+	+	+
Leuconostoc lactis L60	+	-	+/-
Enterococcus faecium 93-828-3	+	-	+
Pediococcus acidilacti	+	+/-	+

[a]Nine LAB species were tested using exponential-phase cells that were not treated and cells that were heat killed at 70°C.

[b]The live and dead populations were well separated (+), overlapped each other somewhat (+/-), or overlapped each other considerably (-).

MATERIALS AND METHODS

Bacterial strains and culture conditions. The species used are listed in Table 1. *Leuconostoc lactis* L60, *Lactobacillus helveticus* T97, *Lactobacillus casei* R, and *Lactobacillus delbrueckii* subsp. *bulgaricus* 2 were supplied by NIZO Food Research, Ede, The Netherlands. The other strains are from the strain collection of our laboratory. The strains were maintained as freezer stocks at 280°C in 40% glycerol. After inoculation from the freezer stocks, the cultures were grown to stationary phase (16 to 30 h). *Lactococcus lactis, Enterococcus faeciu*m, and *Pediococcus acidilacti* were grown at 30°C in M17 broth (Unipath Oxoid, Basingstoke, United Kingdom) supplemented with 0.5% (wt/vol) lactose (LM17). *Streptococcus salivarius* subsp. *thermophilus* was grown at 42°C in LM17. *Leuconostoc mesenteroides* and *Leuconostoc lactis* were grown at 30°C in MRS broth (Merck, Darmstadt, Germany). *Lactobacillus casei* was grown at 37°C in MRS broth. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus helveticus* were grown at 42°C in MRS broth. The cultures were then diluted (1:9) in fresh medium and grown to mid-exponential phase. The cultures were harvested at an optical density at 620 nm (OD_{620}) of approximately 0.7 by centrifugation at 4,000 × *g* for 10 min at 10°C. Unless mentioned otherwise, 50 mM potassium phosphate (KPi) buffer adjusted to pH 7.0 was used for suspending, washing, and incubating cells. Harvested cells were washed twice, concentrated to an OD_{620} of 20, and kept on ice until use.

Treatments. Portions of 200 μl of concentrated cell suspension (OD620 of 20) were exposed to heat, acid, or bile salts. Cells were heat killed by exposure to 70°C for 10 min. Treatments with acid were done by incubating cells at 30°C for 60 min in 10 mM KPi adjusted with hydrochloric acid to pH 2.0, 3.0, or 4.0, or in 50 mM KPi adjusted to pH 5.0 or 6.0. Treatments with bile salts were done by incubation of cells at 30°C for 60 min with a final concentration of 0.05, 0.10, 0.25, 0.50, or 1.00% (wt/wt) deconjugated bile salts (50% sodium cholate, 50% sodium deoxycholate [Sigma-Aldrich, Steinheim, Germany]). As a control, cells were incubated at pH 7.0 and 30°C for 60 min. After the incubations, the cells were spun down, resuspended in buffer, and put on ice until use.

Measurement of culturability. Tenfold serial dilutions of control and treated samples were made in buffer, and triplicate aliquots of 100 μl of the appropriate dilution were spread out on agar plates. *Lactococcus lactis, E. faeciu*m, and *P. acidilacti* were plated on LM17 medium. The other species were plated on MRS medium. After 3 days of aerobic incubation at 30°C, the colonies were counted.

Fluorescence labeling. cFDA, PI, and TOTO-1 were purchased from Molec-ular Probes, Inc. TOTO-1 is 1,19(4,4,7,7-tetramethyl-4,7-diazaundecamethyl-ene)- bis-4-[3-methyl-2,3dihydro(benzo-1,3-oxazole)-2-methylidene]-1-(39-tri-methylammoniumpropyl)- pyridinium tetraiodide. cFDA is an esterase substrate yielding the fluorescent carboxyfluorescein (cF) upon hydrolysis. cF has an ex-citation maximum (λ_{ex}) of 492 nm and an emission maximum (λ_{em}) of 517 nm. PI and TOTO-1 bind to DNA. PI has a molecular mass of 668 g per mol and a fluorescence enhancement of 20- to 30-fold upon binding. The PI-DNA complex has a λ_{ex} of 535 nm and a λ_{em} of 617 nm. TOTO-1 has a molecular

mass of 1,303 g per mol and a very high fluorescence enhancement of 1,400-fold. The TOTO-1– DNA complex has a λ_{ex} of 514 nm and a λ_{em} of 533 nm. Stock solutions of 100 μM cFDA in 50 mM KPi buffer (pH 7.0), 1.5 mM PI in distilled water, and 100 μM TOTO-1 in dimethyl sulfoxide were prepared. For single-probe labeling, concentrated cell suspensions (OD_{620} of 10) were incubated with 50 μM cFDA, 30 μMPI, or 1 μM TOTO-1 at 30°C for 10 min. After incubation with cFDA, the cells were washed once. For double labeling, concentrated cell suspensions (OD620 of 10) were incubated with 50 μM cFDA and 1 μM TOTO-1 simulta-neously at 30°C for 10 min, after which the cells were washed once. All labeled cell suspensions were kept on ice until use.

Fluorescence microscopy. Labeled cell suspensions were diluted to approximately 10^9 cells per ml and microscopically analyzed with an Axioskop epifluorescence microscope equipped with a 12-V, 50-W halogen lamp for transmitted-light illumination, a 50-W mercury arc lamp for epifluorescence illumination, a fluorescein isothiocyanate filter set (excitation wavelength, 450 to 490 nm; emis-sion wavelength, >520 nm), a 100× 1.3-numerical-aperture Plan-Neofluar ob-jective lens, and an MC80 camera (Carl Zeiss, Oberkochen, Germany). Photomicrographs were made with simultaneous light and epifluorescence microscopy, a low transmitted-light intensity, and an exposure time of 15 s on Kodak 400 ASA color films. In these photomicrographs both the labeled cells and the nonlabeled cells were visible.

Spectrofluorimetry. To measure the cF labeling capacity, i.e., the amount of cF in the cells per milligram of protein, labeled cells were lysed by incubation at 70°C for 15 min and the debris was removed by centrifugation. The fluorescence of the supernatant was measured fluorimetrically (excitation at 490 ± 5 nm and emission at 515 ± 5 nm) with a Perkin-Elmer LS 50B luminescence spectrometer equipped with a plate reader by using computer-controlled data acquisition. The cF concentration was calculated from a calibration curve with a cF concentration range from 0 to 1.5 μM in 50 mM KPi buffer (pH 7.0). The cell protein concentrations were analyzed by the Lowry method.

FCM. FCM analyses were performed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.) equipped with a 15-mW, 488-nm, air-cooled argon ion laser and a cell-sorting catcher tube. Cell samples were diluted to approximately 10^6 cells per ml and delivered at the low flow rate, corresponding to 150 to 500 cells per s. FSC, SSC, and three fluorescence signals were measured. A band pass filter of 530 nm (515 to 545 nm) was used to collect the green fluorescence (FL1), a band pass filter of 585 nm (564 to 606 nm) was used to collect the yellow-orange fluorescence (FL2), and a long-pass filter of 670 nm was used to collect the red fluorescence (FL3). FSC was collected with a diode detector. SSC and the three fluorescence signals were collected with photomultiplier tubes. All signals were collected by using logarithmic amplifications. A combination of FSC and SSC was used to discriminate bacteria from background. Data were analyzed with the CELLQuest program (version 3.1f; Becton Dickinson) and the WinMDI program (version 2.8; Joseph Trotter, John Curtin School of Medical Research, Canberra, Australia [http:// jcsmr.anu.edu.au]).

Sorting. Sorting experiments were performed with *Lactococcus lactis*. Exponential- phase cell suspensions that were left unstained or incubated with cFDA, as well as 1:1 mixtures of exponential-phase cells and 70°C heat-killed cells labeled with cFDA or TOTO-1, were used. Furthermore, cell suspensions exposed to 0.10% bile salts labeled with cFDA or TOTO-1 were used. All sample handling was done aseptically. In the dot plot of FL1 and FL2, regions of nonlabeled, cF-labeled, and TOTO-1-labeled cells were defined to use as sort gates. One region was sorted at a time. Culturability was tested by plating 100-μl samples of the sorted subpopulations directly out of the sort collection tubes.

RESULTS

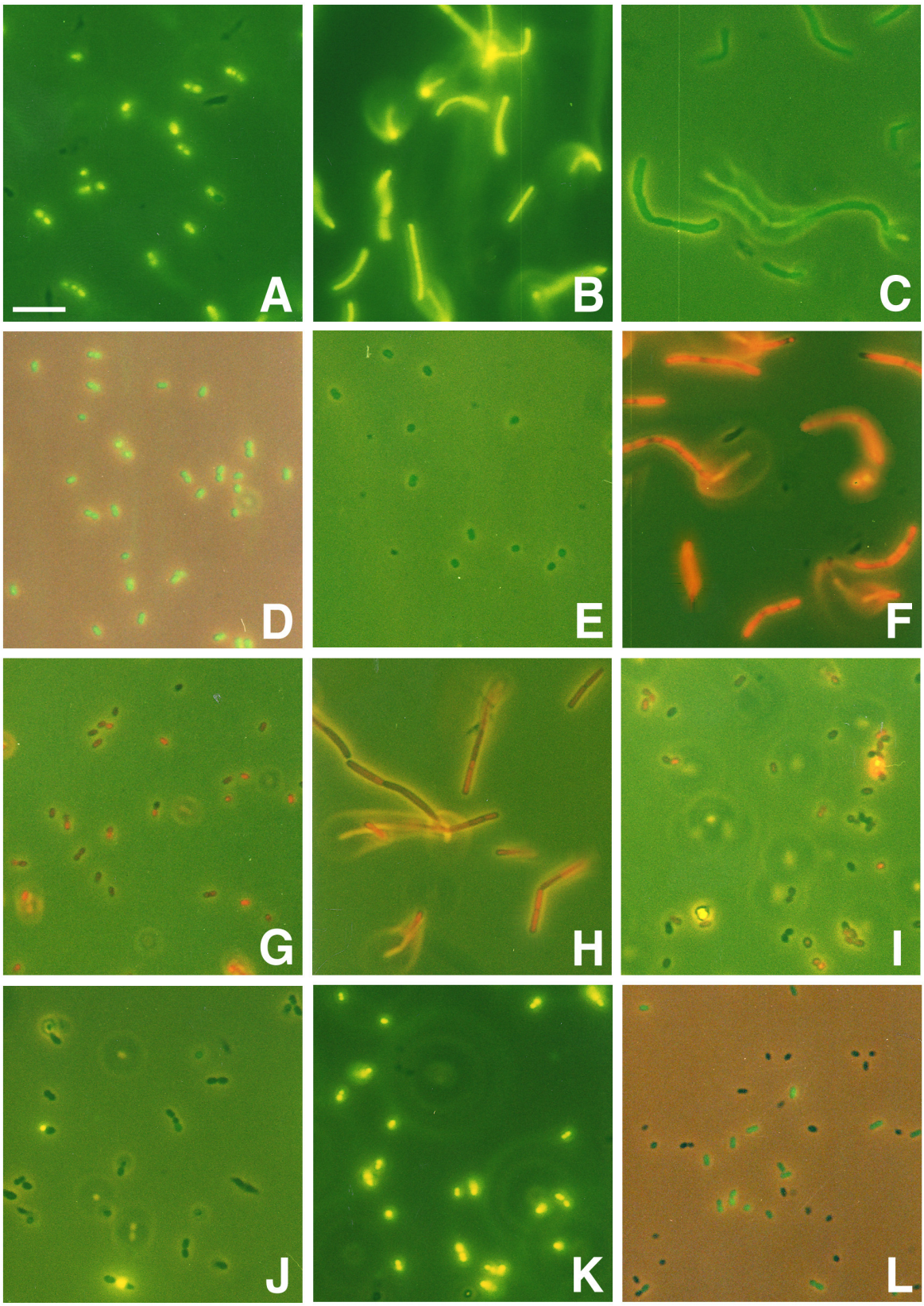
Staining with cFDA, PI, or TOTO-1. Differential staining of live and dead cells by cFDA, PI, and TOTO-1 was investigated by testing the probes on exponential-phase cells that were not treated and cells that were heated at 70°C for 10 min. The 70°C treatment killed all cells, as was confirmed by plating 100 μl of cell suspensions that contained 10^{10} CFU per ml before heat treatment. Samples were analyzed by FCM. For visual refer-ence, fluorescence microscopic photographs were made (Fig. 1A to K).

cFDA gave good results (Table 1). Incubation of exponential- phase cells of the nine selected LAB species with cFDA resulted in a high fraction of cF-labeled cells, in most cases above 90% (Fig. 1A to E), whereas no cells of 70°C heat-killed suspensions were labeled. In the FCM analysis of all species the labeled population gave a peak in the green fluorescence histogram, which was resolved from the signal of nonlabeled cells.

Notably, the intensity of cF fluorescence differed among the LAB species. The FCM histograms of cF-labeled control samples of exponential-phase cells (Fig. 2), as well as the photographs (Fig. 1A to E), show the differences in cF labeling intensity when the same protocol is applied for all species. This was confirmed by spectrofluorimetry, which revealed a greater-than-10-fold difference between *Lactobacillus delbrueckii* subsp. *bulgaricus* and *P. acidilacti*, the species with the highest and the lowest cF labeling capacities, respectively (data not shown). However, the results showed that the same standard protocol for labeling enables discrimination of live and dead cells of all tested species by FCM analysis

PI gave satisfactory results for only some of the tested species (Table 1). Good results were obtained for the species *S. salivarius* subsp. *thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus* (Fig. 1F), *Lactobacillus case*i, and *Lactobacillus mes-enteroide*s. All cells of the 70°C heat-treated suspension were brightly labeled as observed with microscopy, and the labeled population gave a peak in the FCM histogram distinct from that of nonlabeled cells. Of the exponential-phase cells, only a small fraction was labeled. The FCM results agreed with microscopic observations. However, for the other species PI labeling did not give clear FCM results. In addition, the estimates of the living and dead fractions with microscopy did not agree with FCM results. For *Lactococcus lactis* and *E. faecium* (Fig. 1G) the labeling intensity of the heat-killed cells was too low, which caused overlap of the peak of labeled cells with the peak of nonlabeled cells in the FCM fluorescence histogram. On the other hand, untreated *Lactobacillus helveticus* (Fig. 1H),

FIG. 1. Labeling of LAB with fluorescent probes. (A to E) Evaluation of cFDA as a stain for live cells. *S. salivarius* subsp. *thermophilus* (A), *Lactobacillus delbrueckii* subsp. *bulgaricus* (B), *Lactobacillus casei* (C), *Leuconostoc mesenteroides* (D), and *P. acidilacti* (E) exponential-phase cell suspensions were incubated with 50 mM cFDA and washed once. (F to I) Evaluation of PI as a stain for dead cells. *Lactobacillus delbrueckii* (F) and *E. faecium* (G) 70°C heat-killed cell suspensions and *Lactobacillus helveticus* (H) and *Leuconostoc lactis* (I) exponential-phase cell suspensions were incubated with 30 mM PI. (J and K) Evaluation of TOTO-1 as a stain for dead cells. Exponential-phase cells (J) and 70°C heat-killed cells (K) of *S. salivarius* subsp. *thermophilus* were incubated with 1 mM TOTO-1. (L) Application of labeling for viability assessment after bile stress. *Lactococcus lactis* was exposed to 0.10% deconjugated bile salts at 30°C for 60 min. After being washed, the cell suspension was incubated with 50 μM cFDA. All labeling incubations were at 30°C for 10 min. All photographs were made with simultaneous phase-contrast illumination and epifluorescence excitation and an exposure time of 15 s to visualize both labeled and nonlabeled cells. The composite was made with Adobe Photoshop 5.5. Bar, 10 μm.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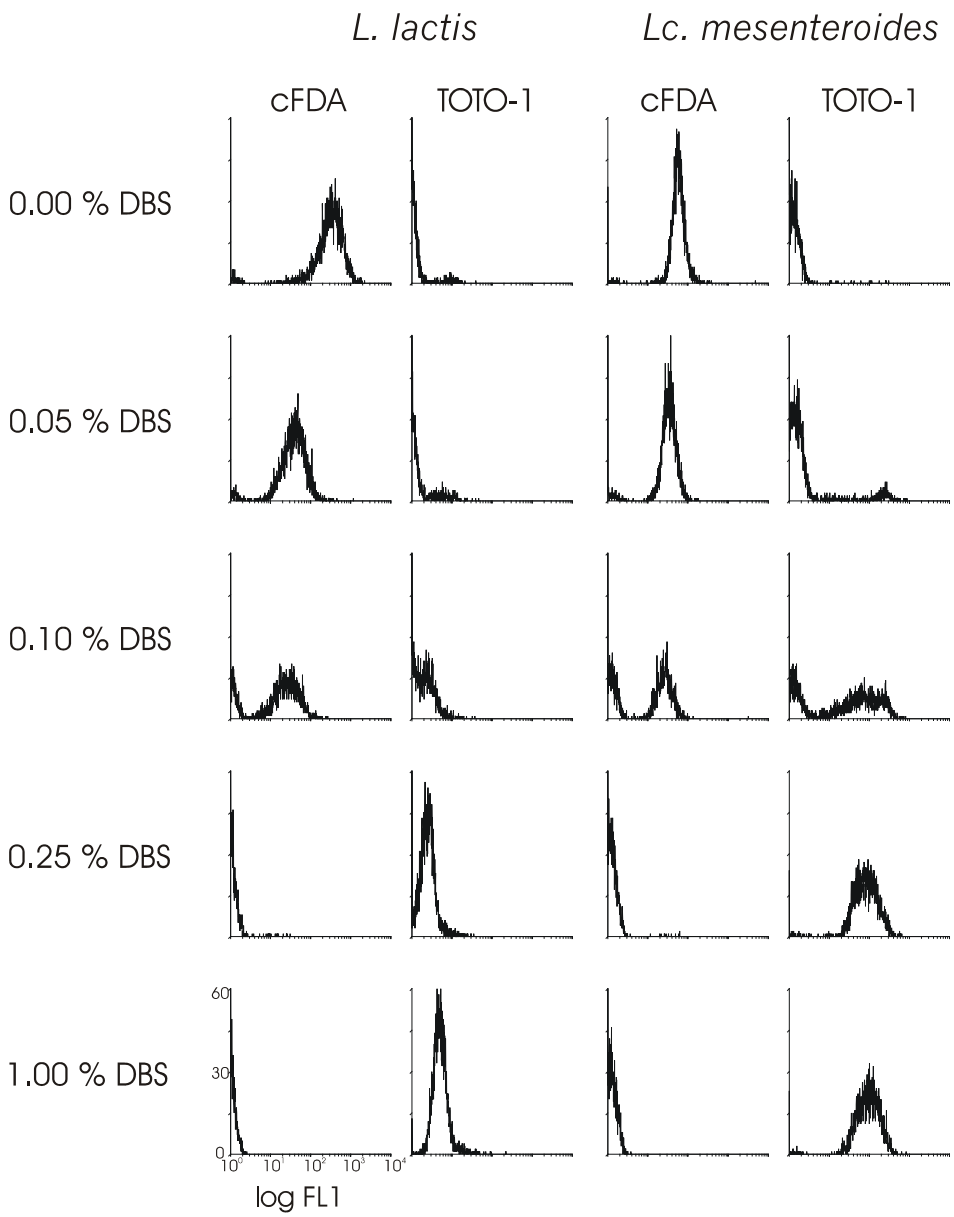

FIG. 2. Flow cytometry histograms of FL1 of *Lactococcus lactis* and *Leuconostoc mesenteroides* cell suspensions stained with cFDA or with TOTO-1 after exposure to deconjugated bile salts (DBS).

Leuconostoc lactis (Fig. 1I), and *P. acidilacti* cell suspensions showed PI labeling, although with low intensity. This low-intensity labeling resulted in peaks in the FCM fluorescence histogram distinct from that of nonlabeled cells and partly overlapping with the peak of 70°C heat-treated labeled cell suspensions.

TOTO-1 labeling enabled easy identification of the live and dead cells by FCM for most of the LAB species (Table 1). Dead cells were labeled with high-fluorescence intensity, while live cells were left unstained (Figs. 1J and K). The high fluorescence intensity of the dead cells resulted in peaks in the fluorescence histogram that were clearly resolved from the signal of nonlabeled cells. For *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Leuconostoc lactis* there was some overlap between the FCM fluorescence histogram peaks of labeled and nonlabeled populations. In general, the TOTO-1 labeling gave clearer results than the PI labeling, and peaks of live and dead cells in the FCM fluorescence histograms were better resolved. Because cFDA and TOTO-1 gave the best results and appeared to be useful for the different LAB species, these probes were chosen for further testing as stains for live and dead cells. The probes were tested separately and in combination using stressed cultures.

Viability assessment after treatment with bile salts or acid. Three LAB species, *Lactococcus lactis, Lactobacillus helveticu*s, and *Leuconostoc mesenteroide*s, were selected for

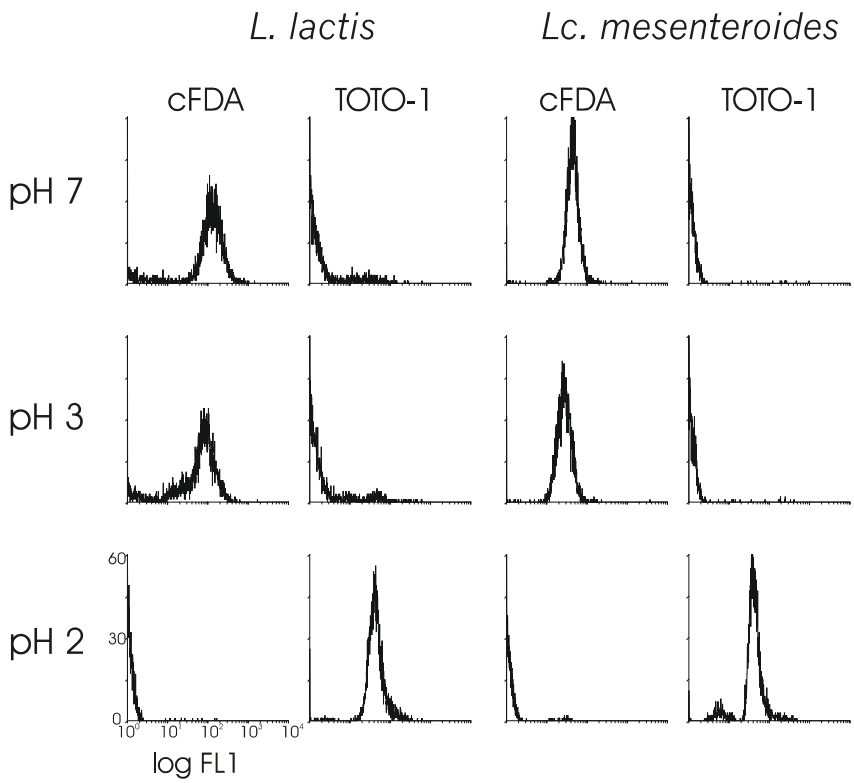

FIG. 3. Flow cytometry histograms of FL1 of *Lactococcus lactis* and *Leuconostoc mesenteroides* cell suspensions stained with cFDA or with TOTO-1 after exposure to acid.

more detailed analysis. FCM was applied for viability assessment using cFDA and TOTO-1 after exposure to deconjugated bile salts or acid. The results of the FCM were compared with the survival tested by plate counts. For visual impressions of labeling after stress, the cell suspensions were also analyzed by fluorescence microscopy (Fig. 1L). The microscopic observations agreed with the FCM results.

In the FCM analyses the bacteria were identified by their light scatter. In the dot plot of the FSC and the SSC, a region that comprised the cell population was created. Interfering particles that also had an SSC above the threshold value but were not in the created region were thus disregarded. Both cF (λ_{em}, 517 nm) and TOTO-1 (λ_{ex}, 533 nm) were best detected by the FL1 detector. Figures 2 and 3 display diagrams of the distribution of the cell population among 1,024 channels of fluorescence on a logarithmic scale. The height indicates the number of cells in a particular fluorescence channel.

In cell suspensions incubated without bile salts, between 90 and 98% of the cells were labeled by cFDA. This varied between the experiments and between the species. The results of TOTO-1 labeling were complementary to the results of cF labeling: 2 to 10% of the cells were labeled. Exposure to 0.05% bile salts hardly changed the number of labeled cells. Also, the number of CFU was similar to that of nontreated cell suspensions. However, the average fluorescence of the cF-labeled population, especially that of *Lactococcus lacti*s, was lower (Fig. 2). This suggests lower accumulation of cF caused by minor damage to the membrane. Exposure to higher bile salt concentrations resulted in lower fractions of cF-labeled cells and higher fractions of TOTO-1-labeled cells. Exposure to 0.10% bile salts resulted in heterogeneity in the populations. In *Lactococcus lactis* cell suspensions approximately 50% were labeled by cF. TOTO-1 staining resulted in overlapping peaks of labeled and nonlabeled cells (Fig. 2). The plate counts were at 42% compared to cell suspensions incubated without bile salts. In *Leuconostoc mesenteroides* cell suspensions both cFDA and TOTO-1 divided the population equally into labeled and nonlabeled subpopulations (Fig. 2). The plate counts were at 36%. In *Lactobacillus helveticus* cell suspensions cFDA labeled approximately 90% of the cells and TOTO-1 labeled 10% of the cells (not shown). The plate counts were at 89%. After exposure to 0.25% bile salts or more, almost no cells were labeled by cFDA. In agreement with this, almost all cells were labeled by TOTO-1. Correspondingly, the survival was low, 0.1% at maximum.

For cell suspensions exposed to acid the results of cF labeling and TOTO-1 labeling agreed with plate counts. The results for *Lactococcus lactis, Lactobacillus helveticu*s, and *Leuconostoc mesenteroides* were similar. Labeling with cF or TOTO-1 after exposure to pH 6.0, 5.0, 4.0, or 3.0 did not result in a change of the distribution of the subpopulations compared to that at pH 7.0 (Fig. 3). However, after exposure to pH 2.0, almost no cells were labeled with cF whereas almost all cells were stained with TOTO-1 (Fig. 3). Accordingly, the culturability was not affected until pH 3.0, but exposure to pH 2.0 resulted in at least a 3-log-unit reduction of the plate counts.

Fluorescent labeling in combination with FCM revealed stress-induced heterogeneity in the cultures. Most importantly, live and dead subpopulations could be distinguished.

Double staining with cFDA and TOTO-1. Live/dead assays with two differentially staining probes are attractive because detection is easier when all cells are labeled. Therefore, we tried double staining with cFDA and TOTO-1. Using FCM, the cF- and the TOTO-1-labeled populations could be spatially resolved in dot plots of FL1 and FL2, as illustrated by double-stained cultures that were stressed by exposure to 0.10% bile salts (Fig. 4). In the histograms of FL1 and FL2, there is considerable overlap between the peaks of cF-labeled and TOTO-1-labeled cells (Fig. 4). However, since the emission spectra of cF and TOTO-1 are different, the FL1/FL2 ratios are different. Therefore, the subpopulations are resolved in the dot plot of FL1 and FL2. For *Lactococcus lactis* exposed to 0.10% bile salts, the double labeling gave clear separation into two subpopulations (Fig. 4A), while single labeling with TOTO-1 did not give such clear results (Fig. 2). This illustrates the advantage of double staining. The cF-labeled cells and the TOTO-1-labeled cells were counted after double labeling by performing region analysis on FL1-FL2 dot plots. Figure 5 shows the results of these fluorescence counts in comparison with plate counts for cultures of *Lactococcus lactis, Lactobacillus helveticu*s, and *Leuconostoc mesenteroides* that were exposed to bile salts. The results of the labeling are in agreement with the plate counts. For cultures exposed to low pH, the FCM counts also agreed with plate counts (data not shown).

Cell sorting. To establish a direct relationship between labeling and culturability, the labeled and nonlabeled populations were sorted and plated. *Lactococcus lactis* exponentialphase nonlabeled cell suspensions gave a number of colonies corresponding to approximately 80% of the number of cells actually sorted. Similarly, cFDA-stained nontreated cell suspensions sorted on cF labeling gave a number of colonies corresponding to approximately 80% of the number of cells recovered in the sorting tube. The somewhat lower plate counts may have been caused by stress imposed during cell sorting. Standard regions for fluorescence-based sorting were then defined using mixtures of exponential-phase cells and 70°C heat-killed cells that were incubated either with cFDA or with TOTO-1 (Fig. 6). The labeled and nonlabeled subpopulations of the mixtures and of cell suspensions treated with 0.10% bile salts were then sorted and plated. When incubated with cFDA, the sorted cF-labeled subpopulation had high fractions of culturability, similar to that mentioned above. Accordingly, the nonlabeled subpopulation gave no colonies (Fig. 6). When incubated with TOTO-1, the labeled subpopulation was not culturable, whereas the nonlabeled subpopulation had a high culturability. The sorting experiments provided direct evidence that the FCM viability assay with cF and TOTO-1 indicates live and dead, i.e., culturable and nonculturable, subpopulations in stressed cultures.

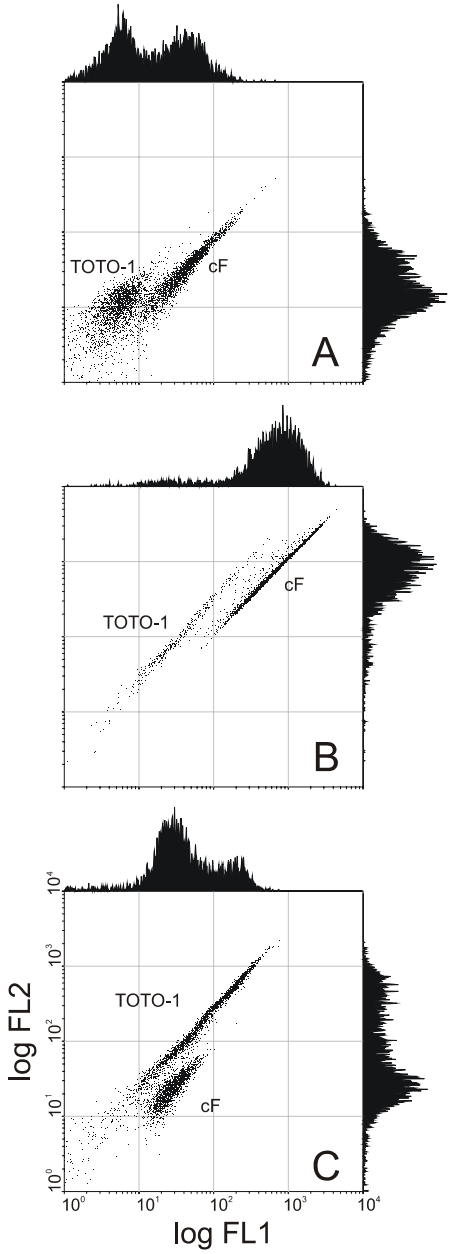

FIG. 4. Flow cytometry dot plots of FL1 and FL2 of *Lactococcus lactis* (A), *Lactobacillus helveticus* (B), and *Leuconostoc mesenteroides* (C) that were exposed to bile salts and stained with cFDA and TOTO-1. The cell suspensions were exposed to 0.10% deconjugated bile salts at 30°C for 60 min. After being washed, the cell suspensions were incubated with 50 μM cFDA and 1 μM TOTO-1. The cF-labeled and TOTO-1-labeled subpopulations are spatially resolved in the dot plots.

DISCUSSION

We examined the usefulness of FCM for viability assessment of LAB. To be useful, the method has to be reliable and rapid. Furthermore, it has to be useful for LAB of different genera and food applications. This was taken into account in selecting species for this study (8, 26, 33). Three fluorescent probes were tested for their usefulness for live/dead discrimination: cFDA as a stain for live cells and PI and TOTO-1 as stains for dead cells. The probes were tested using exponential-phase cells as a positive control and 70°C heat-killed cells as a negative control. Flow cytometry results were compared with plate counts. Labeled cell suspensions were also visually inspected by fluorescence microscopy. The

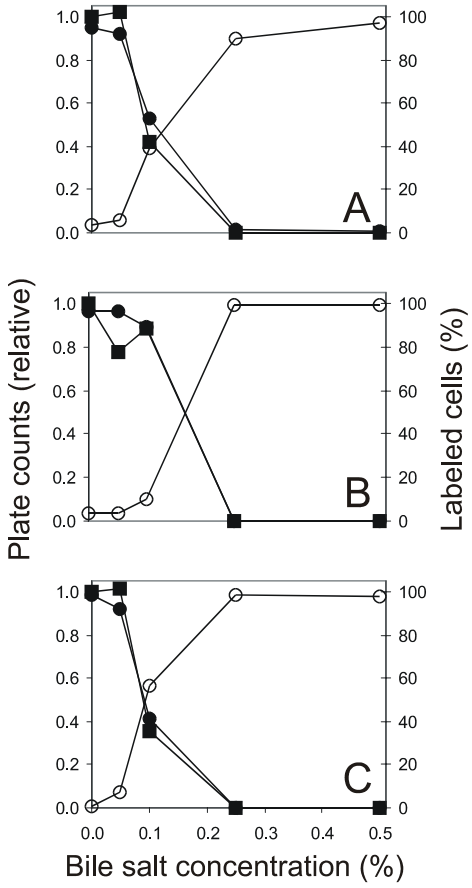

FIG. 5. Comparison of cFDA and TOTO-1 labeling with plate counts for bile-stressed LAB. *Lactococcus lactis* (A), *Lactobacillus helveticus* (B), and *Leuconostoc mesenteroides* (C) were exposed to 0.05, 0.10, 0.25, or 0.50% bile salts at 30°C for 60 min or incubated without bile salts as a control. After washing, the culturability was tested by plate counts (■) and by FCM using cFDA staining (●) and TOTO-1 staining (○).

results showed that cFDA was successful in labeling the live cells and leaving the dead cells unstained. TOTO-1 appeared to be better than PI as a stain for dead cells. For double staining, cF and TOTO-1 were shown to be an excellent combination for a flow cytometric live/dead assay, as was supported by sorting experiments with *Lactococcus lactis*. In further experiments the assay was successfully applied to deconjugated bile salt-stressed cultures and to acid-stressed cultures of *Lactococcus lactis, Lactobacillus helveticus*, and *Leuconostoc mesenteroides*.

Exposure to hydrochloric acid is often used as an in vitro condition to investigate the resistance of bacteria to a passage through the stomach (10). Generally, the viability is not affected when LAB are incubated with hydrochloric acid at pH 3.5 or higher, while at lower pH the survival decreases to less than 1%, at pH values that are dependent on species and strain (7, 24, 29). We found no decrease of culturability when cells were exposed for 60 min at 30°C to hydrochloric acid solutions with pHs as low as 3.0. At pH 2.0 there were hardly any surviving cells. The results of the labeling indicate that at pH 3.0 the membrane stays intact, while after exposure to pH 2.0 the membrane is damaged. Further studies using acid solutions with pHs between 3.0 and 2.0 could elucidate at what concentration of acid the membrane becomes compromised and how that relates to culturability.

In addition to resistance to acid, resistance to bile is recognized as an important feature for LAB used as probiotics (14, 17, 33). In the human intestinal tract the concentration is variable, with a maximum of 2% (10). The conjugated bile salts that are excreted are deconjugated by intestinal microorganisms, which makes them less effective as a detergent, but the deconjugated bile salts do kill bacteria at concentrations of below 0.5% (10, 15). The results of our experiments on survival in buffer show that the concentration of 0.10% deconjugated bile salts falls within the critical range, but different survival fractions were found for the three species. At 0.25% almost no surviving cells were detected. Different strains of one species can also have different levels of tolerance to bile, as reported in a study of six *Lactobacillus acidophilus* strains (24). By selective bile pressure, variants of *Lactobacillus acidophilus* that have a higher resistance to bile salts and that may be considered candidates for probiotic strains could be obtained (7, 38). Our labeling experiments indicated that membrane integrity is crucial for bile resistance. The detection of damage to membranes is indicative of the culturability, as was shown by the agreement between labeling results and plate counts.

cFDA was tested as a live-cell stain for LAB. cFDA is an esterase substrate that needs enzyme activity to yield the fluorescent compound and membrane integrity to keep the compound in the cell. Labeled and nonlabeled cells were distinguished successfully by FCM. One standard protocol was used for all species, which resulted in different fluorescence intensities of the different species. This diversity in labeling capacity might be explained by differences in permeability affecting the diffusion of cFDA, differences in esterase activity, or differences in esterase specificity. Adjusting the protocol can in principle optimize cF labeling intensities. *P. acidilacti* had the lowest labeling intensity, which made examination by microscopy difficult with our standard labeling protocol. However, even this relatively low labeling intensity was sufficient for accurate FCM analysis. Besides differences in labeling intensity, the possibility of cF efflux is a point to keep in mind (5, 31). To prevent cF efflux, the experiments were performed with washed cells and without fermentable sugars in the buffer. Under these conditions, no significant loss of cF from the cells occurred, as was checked by spectrofluorimetry. The retention of cF under nonenergizing conditions enables accurate FCM assays for all species. The labeling with cF gave clear discrimination between live and dead cells, as was confirmed by the sorting experiments.

PI and TOTO-1 were tested as counterstains for cFDA in the FCM viability assay. PI is a red fluorescent phenanthridinium intercalating dye used extensively for detecting dead cells (5, 18, 28, 29, 32). TOTO-1 is a yellow fluorescent dimeric cyanine dye. These dyes have the necessary properties for a dye exclusion probe but are hardly used as such (2, 18, 32, 40). In our experiments TOTO-1 proved to be superior to PI in discriminating intact and damaged cells. This may be because TOTO-1 is larger than PI; the molecular masses are 1,303 and 668 g per mol, respectively. Also, a lower partitioning into the

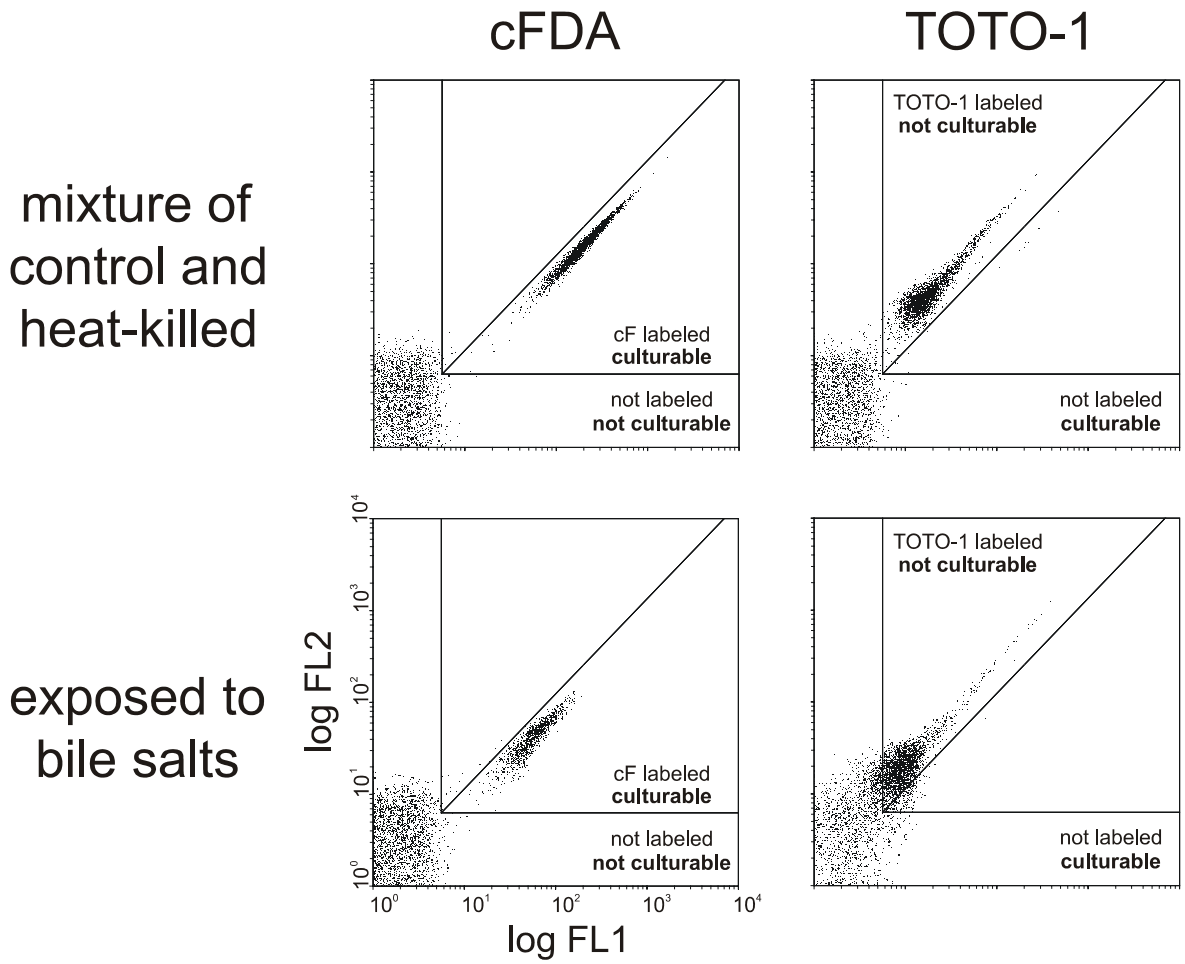

FIG. 6. Combined sorting and plating experiments with cFDA- and TOTO-1-stained *Lactococcus lacti*s. Regions of nonlabeled cells, cF-labeled cells, and TOTO-1-labeled cells were defined using 1:1 mixtures of exponential-phase cells and 70°C heat-killed cells that were incubated with cFDA or TOTO-1. These mixtures and cell populations stressed with 0.10% bile salts were sorted using the defined regions as sort gates. The culturability of the sorted subpopulations was tested by plate counting.

membrane could be a factor in favor of TOTO-1. Furthermore, the very high fluorescence enhancement of TOTO-1 enables good distinction of nonlabeled and labeled cells in the FCM. The labeling with TOTO-1 gave clear discrimination between live and dead cells, as was confirmed by sorting experiments.

Live/dead assays based on two probes have advantages over assays with one probe that labels either live or dead cells. In FCM, the identification of cells is facilitated when all cells are fluorescently labeled, especially when the background is high. In addition, total enumeration can be done together with viability assessment in the same assay when all of the cells are detectable. There are several probe kits from Molecular Probes developed for such assays (18). The live/dead viability/cytotoxicity kit for animal cells is based on two probes discriminating between live and dead cells: calcein AM and ethidium homodimer-1. Unfortunately, this probe combination appeared not to be generally suitable for use with bacterial cells (18, 21). The live/dead *Bac*Light viability kit for bacteria is also based on a combination of two probes, but only one of the probes, PI, discriminates between intact and damaged cells. SYTO 9 is a green fluorescent permeant nucleic acid stain that is included in the assay to have all of the cells labeled. PI is supposed to enter cells with compromised membranes only. PI displaces SYTO from the DNA because PI has a higher affinity for DNA. Both probes are excited by the blue laser used in many flow cytometers. The ViaGram Red$^+$ bacterial Gram stain and viability kit combines Gram staining using Texas Red-X wheat germ agglutinin with a viability assay using the permeant DNA stain DAPI (4',6'-diamidino-2-phenyl-indole) and the dead-cell stain SYTOX-Green. The combination of DAPI and SYTOX-Green acts on the same principles as the *Bac*Light probe combination. UV light is needed for excitation, which makes it unsuitable for a flow cytometer equipped with only a blue laser. The assay developed in this study combines two probes that individually discriminate between live and dead cells. cFDA acts as a stain for live cells because it needs hydrolysis by intracellular esterases and retention by an intact membrane. TOTO-1 acts as stain for dead cells because it is a nucleic acid binding probe excluded by cells with intact membranes. Thus, this assay acts on the same principle as the successful live/dead viability kit for animal cells. Instead of using a counterstain

only to have all of the cells labeled, both probes provide information on the cell status. Furthermore, this assay employs TOTO-1, which proved to be better than PI in our experiments. The green fluorescent cF-labeled cells and the yellow fluorescent TOTO-1-labeled cells are difficult to distinguish with microscopy; however, singly labeled cell samples can be used when a visual impression is required. In conclusion, the combination of cFDA and TOTO-1 makes a reliable live/dead assay for FCM assessment of bacterial viability.

This live/dead assay has many possible applications. In this study the application of viability assessment after exposure to bile or acid was validated. Likewise, the assay can be used for screening LAB that are possibly probiotic for tolerance against bile and acid under various conditions. FCM analyses are fast, and one standard labeling protocol appeared to be workable for all species in our selection, which makes the assay attractive for such studies. Furthermore, the viability of starters can be examined. Survival of starters after freeze-drying and after storage is of interest in dairy production (6). For the evaluation of different conditions FCM can be of use. The developed live/dead assay can also be applied as a fast screening method for assessment of susceptibility of bacteria to a wide range of antimicrobial compounds, including antibiotics. In summary, the probes cFDA and TOTO-1 make an excellent combination for bacterial live/dead assays by FCM with versatile applications.

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Development of a Flow Cytometric Method to Analyze Subpopulations of Bacteria in Probiotic Products and Dairy Starters

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Development of a Flow Cytometric Method to Analyze Subpopulations of Bacteria in Probiotic Products and Dairy Starters

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Flow cytometry (FCM) is a rapid and sensitive technique that can determine cell numbers and measure various physiological characteristics of each individual cell using appropriate fluorescent probes. Previously, we developed an FCM assay with the viability probes carboxyfluorescein diacetate (cFDA) and TOTO-1 for (stressed) lactic acid bacteria (C. J. Bunthof, K. Bloemen, P. Breeuwer, F. M. Rombouts, and T. Abee, Appl. Environ. Microbiol. 67: 2326-2335). cFDA stains intact cells with enzymatic activity and TOTO-1 stains membrane-permeabilized cells. Now we applied that assay to study viability of bacterial suspensions in milk, dairy fermentation starters, and probiotic products. To facilitate FCM analysis of bacteria in milk a commercially available milk clearing solution was used. The procedure was optimized to increase the signal-to-noise ratio. FCM enumerations were accurate down to 10^5 cells ml^{-1}. Retrieval of *Lactobacillus plantarum* WCFS 1 suspended in milk was high and viability was not affected by the procedure. The plate counts of cleared samples of untreated cell suspensions were nearly as high as total FCM counts and the correlation was strong (r>0.99). In dairy fermentation starters and in probiotic products FCM total cell counts were substantially higher than the number of CFU. Three functional populations could be distinguished: culturable cells, cells that are intact and metabolically active but not culturable, and permeabilized cells. The proportions of the populations differed between the tested products. This FCM method provides tools to assess the functionality of diffferent populations in fermentation starters and probiotic products.

In food industry lactic acid bacteria (LAB) are used for the production of fermented foods such as cheese, yogurt, wine and fermented meat products. Starter culture strains are selected for their fermentation capacity and their flavor formation characteristics. Other important criteria are the robustness of the cells during processing and the maintenance of the different strains in a mixed starter (5). LAB are also important as probiotics. Probiotics are live microbial food ingredients that are beneficial to health. Important issues in research and development of probiotics are growth and survival during production, shelf life, resistance to bile salts and acids, and adherence and survival in the gut (11, 14, 27). Obviously, enumeration and viability assessment are crucial in the research and selection of fermentation starter bacteria and probiotics.

The method most used for viability assessment of microorganisms is the plate count technique. Plate counting requires an incubation time of one or more days before the result can be scored, which is a disadvantage. Also, bacteria may occur in chains and clumps, resulting in underestimation of bacterial numbers. In addition, cell injury and dormancy may result in low viable counts (2, 18). Most importantly, the plate count method only displays the bacteria able to form colonies under the given circumstances on the medium that is used, but does not give a clue about the presence of bacteria that do not form colonies but are nevertheless metabolically active. Active but nonculturable starter cells might well contribute to fermentation. It has been established that when starter cultures are subjected to various forms of sublethal stress, some bacteria may be injured and may fail to grow on a medium adequate for the growth of unstressed cells, but are capable of growth when given a suitable environment (21, 33). Furthermore, it has been shown that permeabilization may lead to a higher acid production rate (21). In addition, many possible probiotic effects of bacteria depend on activity rather than culturability, and even dead cells can have some probiotic effect such as immunomodulation (22, 25). Therefore, gaining information about all individual bacteria and their physiological status is relevant. The aim of this work was to study bacteria in milk suspensions, fermentation starters, and probiotic products by flow cytometry (FCM).

FCM is a rapid and sensitive technique that measures each cell individually. Fluorescent stains are used with FCM to detect cells and to analyze population heterogeneity (31). For total cell enumeration permeant DNA stains are often applied. Specific bacterial species may be detected with antibodies or rRNA probes labeled with a fluorescent dye. A number of viability and metabolic activity probes are available to analyze physiological characteristics, such as membrane integrity, enzyme activities, membrane potential, intracellular pH, respiration, and antibiotic susceptibility (1, 4, 8, 16).

FCM has been used widely as a tool to investigate bacteria in laboratory cultures. Also, FCM has been applied for analysis of bacteria in environmental samples such as soil, air

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and especially water (31, 32, 37), and in clinical samples such as blood, urine, and feces (1, 31, 36). The potential of FCM for food industry has been recognized as well (19, 32, 35). Some applications were developed for FCM analyses of food products such as meat, fruit juices, eggs and milk. Considering milk, most attention was paid to the detection of specific pathogens and to bacterial quality control. Detection of the pathogen *Listeria monocytogenes* in raw milk was achieved using enrichment in broth and immunofluorescence staining (10). A recent approach for specific detection is the use of immunomagnetic separation and FCM, which was applied for detection of *E. coli* O157:H7 (29). For direct enumeration in milk staining with ethidium bromide and mithramycin was tried, however sensitivity was too low to detect 6×10^7 CFU added *E. coli* per ml because of high background signals (24). The sensitivity was increased by a sample clearing procedure using Promega's milk clearing reagents, which was applied for immunofluorescence detection of *Salmonella typhimurium* (20). Alternatively, enzymatic clearing with proteinase and savinase (for UHT milk) or savinase and Triton X-100 (for raw milk) was developed (13). This was used to enumerate total bacteria in milk from a dairy plant with the fluorescent probe SYTO BC (Molecular Probes). For routine analyses of milk quality an automated FCM instrument was developed: the Bactoscan-FC of Foss Electric (Hillerød, Denmark) (3, 34). This automated instrument uses ethidium bromide for staining and the disturbing milk components are reduced and dispersed by treatment with detergent and enzyme at 50°C (34).

FCM can also be applied for viability and metabolic activity analyses in food. This is particularly relevant for fermentation starter bacteria and probiotics. Previously, we used carboxyfluorescein diacetate (cFDA) and TOTO-1 for FCM viability assessment of various LAB species (6). cFDA is a nonfluorescent esterase substrate that enters the cell by passive diffusion. Once inside, the acetate groups are split off by enzymatic hydrolysis releasing the green-fluorescent carboxyfluorescein (cF). cFDA is commonly applied as live stain (4, 8, 31). TOTO-1 is a nucleic acid dye that is excluded by intact cells and thus only stains the cell when the membrane is damaged. In another study we applied the permeant and impermeant DNA stains SYTO 9 and propidium iodide (PI) of the *Bac*Light LIVE/DEAD Kit (Molecular probes) for analysis of permeabilization and lysis of *Lactococcus lactis* under simulated cheese ripening conditions using FCM (7).

In this study we optimized a milk clearing procedure for FCM analysis of bacteria in milk and investigated viability of bacteria in dairy starters and probiotic products. Staining with cFDA and TOTO-1 revealed three populations: culturable cells, cells that are intact and metabolically active but not culturable, and permeabilized cells. The proportions of the populations differed between the tested products. This FCM method allows for determination of the functionality of probiotic subpopulations and possible applications are discussed.

MATERIALS AND METHODS

Bacterial strain and sample preparation. The test strain in this study was *Lactobacillus plantarum* WCFS 1, which was obtained from the Wageningen Centre for Food Sciences, Wageningen, The Netherlands. Cells were cultured in MRS broth (Merck, Darmstadt, Germany) at 37°C and harvested in mid-exponential growth phase at an optical density at 600 nm (OD_{600}) of 1.0 by centrifugation at 13,000 rpm for 2 min. at 20°C (Biofuge Fresco Eppendorf centrifuge, Heraeus Instruments, Ostrode, Germany). This culture contained approximately 5×10^8 CFU/ml. The buffer that was used throughout the experiments was 100 mM 2-(N-morpholino) ethanesulfonic acid (MES) with 50 mM KCl adjusted to pH 6.5 and filtered using a 0.2-µm-pore-size filter. Cells were washed twice with buffer and resuspended to an OD_{600} of 1.0. To prepare cell suspension in milk the cells from a buffer suspension were spun down and resuspended and diluted in semi-skimmed pasteurized milk. To permeabilize cell membranes and cause cell death suspensions were heated at 70°C for 10 min.

Extraction procedure. A milk clearing solution (Promega; Leiden, The Netherlands) was used for extraction of bacteria and reduction of milk particles . This solution is commercially available as Reagents A of the Enliten milk assay kit for total viable organisms by ATP measurement. The clearing solution contains a nonionic detergent, a chelating agent and a microparticulate carrier (polystyrene beads with a diameter between 0.5 µm and 1.5 µm) (23). McClelland and Pinder (20) have shown that the clearing solution causes flocculation and coalescence of the micelles in milk. This increases the micelle size and therefore the fat moves readily to the surface of the milk during centrifugation. The clearing solution also lyses the somatic cells present in milk (23). That are mainly mammalian cells which come from the cow's udder into the milk during milking. Somatic cells were indeed observed in milk by fluorescence microscopy before clearing, but were lost by the clearing. The polystyrene beads sediment slightly slower than microbial cells during centrifugation and serve as visual indicator facilitating the removal of supernatant. FCM indicated that the polystyrene beads are uniform in size and that the concentration in clearing solution is approximately 10^9 ml^{-1}.

At first the procedure of extraction provided by the manufacturer was tried. In this procedure a sample of 1 ml bacterial suspension in milk was transferred to a 1.5 ml Eppendorf tube and 0.5 ml clearing solution was added. The tube was inverted 10 times for mixing, immediately followed by centrifugation at 13,000 rpm at 20°C for 5 min. The result was a cell pellet covered with polystyrene beads at the bottom of the tube, a clear supernatant, and a cream pad on top. Cream and supernatant were removed with an aspirator and the pellet was resuspended in MES buffer. This extraction procedure was optimized by two steps. Firstly, since the polystyrene beads interfered with the flow cytometry analyses, the beads were removed from the clearing solution before use by filtration over a 0.2-µm-pore-size filter. Secondly, since with the standard procedure some cream remained at the wall of the tube after the aspiration, which resulted in higher background counts, an extra procedure step was included. After aspiration of the supernatant and most of the cream the remaining cream was was removed with a cotton-tipped stick.

Fluorescent staining. Fluorescent stains used were cFDA, TOTO-1, and SYTO 9 (Molecular Probes Inc., Eugene, Oreg.). TOTO-1 is 1'(4,4,7,7-tetramethyl-4,7-diazaundecamethylene)-bis-4-[3-methyl-2,3dihydro(benzo-1,3-oxazole)-2-methylidene]-1-(3'-trimethylammoniumpropyl)-pyridinium tetraiodide. cFDA is an esterase substrate that yields the fluorescent carboxyfluorescein (cF) upon hydrolysis by cellular esterases. cF is retained in cells with an intact membrane and stains the cells green-fluorescent. cFDA staining was done by incubation with 50 µM cFDA at 30°C for 10 min. TOTO-1 is a membrane-impermeant nucleic acid stain with a high fluorescence enhancement that stains membrane-compromised cells yellow-green. Double staining with cFDA and TOTO-1 was done by incubation with 50 µM cFDA and 1 µM TOTO-1 at 30°C for 10 min. SYTO 9 is a membrane permeant nucleic acid stain, which was used for total cell enumeration of *Lb. plantarum* samples. This probe stains cells green-fluorescent and is part of the LIVE/DEAD *Bac*Light kit. SYTO 9 staining was done by incubation of samples with 6.5 µM SYTO 9 at room temperature for 10 min.

Fluorescence microscopy. Microscopic slides were analyzed using an Axioskop epifluorescence microscope equipped with a 12 V, 50 W halogen lamp for transmitted-light illumination, a 50 W mercury arc lamp for epifluorescence illumination, a fluorescein isothiocyanate filter set (excitation wavelength, 450 to 490 nm; emission wavelength, > 520 nm) and a × 100 1.3-numerical-aperture Plan-Neofluar objective lens. Fluorescence microscopy allowed direct observation of morphology of cells, presence of chains and clumps, labeling of cells, and presence and labeling of background particles. The effect of the extraction procedure on the cells was evaluated by comparison of labeled samples before and after extraction.

Flow cytometry. FCM analyses were performed with a FACSCalibur flow cytometer and data analysis software as described previously (6). Forward scatter (FSC), side scatter (SSC), and three fluorescence signals were measured. A band pass filter of 530 nm (515 to 545) was used to collect the green fluorescence (FL1), a band pass filter of 585 nm (564 to 606) to collect the yellow-orange fluorescence (FL2), and a long pass filter of 670 nm to collect the

red fluorescence (FL3). Fluoresbrite PC Red polystyrene latex microspheres (PC Red beads) with a diameter of 1.75 µm (Polyscience Europe GmbH; Eppelheim, Germany) were included in the FCM samples for enumeration of cells. The stock suspension (8.547×10^9 beads per ml) was sonicated and vortexed prior to use. In the evaluation of the extraction procedure and in the experiment with mixtures of nontreated and heat-treated milk with *Lb. plantarum* the FCM analyses were performed with detector settings FSC: E01; SSC: 400; FL1: 450; FL2: 450; and FL3: 600 using logarithmic gains. The threshold was set at a FSC signal of 250. In the experiments with *Lb. plantarum* in buffer and in milk at concentrations of 10^5-10^9 the FL1 and FL2 detector settings were adjusted to between 400 and 600, depending on the labeling of the sample. FCM samples were adjusted to an event rate of 200 to 700 per second at the low flow rate (approximately 10 µl min^{-1}). When possible, FCM samples that contained approximately 10^6 cells per ml were prepared , and 10,000 to 20,000 cells were measured during 2 min of data aquisition. For FCM samples prepared from milk with less than 10^7 cells per ml, longer data acquisition times were used (up to 10 min.), and lower numbers of cells were measured, but always at least 3,000.

Data analysis. The bead and cell populations were identified using dot plots, i.e. bivariate displays in which each dot represents one measured event. The bead population was easily identified both by scatter and by fluorescence signals. Windows were defined in the dot plot of FSC and SSC and in the dot plot of green fluorescence and red fluorescence. For calculating the number of beads the red fluorescence histogram of the bead population was used. On average 10 % of the beads was present in the form of double beads which gave rise to a small but distinct peak right of the main peak in the red fluorescence histogram.

In buffer samples the cells could easily be identified and windows were defined in the dot plot of FSC and SSC and in the dot plot of green fluorescence and red fluorescence. These windows were also used for milk samples. The fluorescence windows were adjusted when the fluorescence signals of the cells from the milk samples were lower or higher than in the buffer control sample. Cell countings were done by enumerating the events that were included both in the scatter window and in the appropriate labeled cell region. Cell concentrations were calculated using the count of the PC Red beads in the FCM sample.

The background count was defined as the FCM count of a labeled sample without added bacteria or beads. This count includes all events with a forward scatter above the threshold level, indicating the number of particles of similar size as cells or bigger.

The accuracy of counts is indicated by the coefficient of variation (CV). In a counting of n items, the associated standard deviation is $n^{½}$. The CV is the standard deviation over the mean. The CV is a common measure of precision (31)

Dairy Starters. The FCM assay was applied on three dairy starters: the cheese starter BOS, and two yogurt starters, RR and ISt (CSK Food Enrichment, Leeuwarden, The Netherlands). BOS starter is used for the production of Gouda cheese. It is a mixed culture containing mainly lactococci and some leuconostocs. After production BOS starter is concentrated 40 times, packed in plastic beakers and stored at –40°C. This mother starter can be added directly to milk for cheese production. However, in cheese production on industrial scale, low-fat milk is inoculated with mother starter for the production of bulk starter. After incubation this bulk culture is used for cheese fermentation. Therefore, we analyzed BOS starter directly (BOS mother starter) and after incubation (BOS-bulk starter). Both yogurt starters, RR and ISt, are used commonly in The Netherlands. Both contain *Lb. delbruecki* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* but RR contains other strains than ISt. After production these yogurt starters are concentrated 20 times, packed in plastic beakers and stored below –40°C. In industrial yogurt production the starter is added directly to milk. Therefore we analyzed RR and ISt starter directly.

The starters were thawn in a 20°C waterbath for one hour. BOS bulk starter was produced by inoculation of low-fat milk with 0.0125 % concentrated BOS mother starter and incubation at 20°C for 18 h. The concentrated starters were diluted to their original concentration in semi-skimmed milk. Samples were extracted with the optimized extraction procedure. Samples were diluted 10 times because of the high number of cells. Then, samples were incubated with 500 µM cFDA and 1 µM TOTO-1 at 30°C for 10 min. For easy total cell enumeration TOTO-1 staining of 70°C heat-treated samples was applied. The labeling was evaluated by fluorescence microscopy. FCM analyses were performed with detector settings FSC: E01; SSC: 400; FL1: 450; FL2: 450; and FL3: 600 using logarithmic gains. The threshold was set at a SSC signal of 200. Before and after extraction samples were plated on TSA agar.

Probiotic products. The FCM method was applied to three probiotic products: Orthiflorplus (Orthica B.V., Weesp, The Netherlands), Yakult (Yakult Europe B.V., Almere, The Netherlands), and Mona Vifit yogurt drink (Campina Melkunie, Woerden, The Netherlands). Yakult and Mona Vifit were purchased form a local supermarket, Orthiflorplus from a local reform shop.

Orthiflorplus is a probiotic food supplement sold in sachets of 3 g of powder that has to be suspended in water. According to the information leaflet one sachet contains in total at least 3×10^9 'germs' in approximately equal amounts of *Lb. acidophilus*, *Lb. casei*, *L. lactis*, *Enterococcus faecium* and *Bifidobacterium bifidum*. Furthermore, it contains oligofructose and fibers. One sachet was suspended in 100 ml of tap water. After 10 min waiting to allow the fibers to sink to the bottom, samples were taken from the almost clear liquid in the upper part of the glass. In this time span bacteria do not sink to the bottom as was confirmed by plate counts: CFU were as high as from samples taken directly after stirring. FCM was performed directly and after extraction of bacteria.

Yakult is a food supplement containing the probiotic *Lb. casei* Shirota, according to the supplier 6.5×10^9 per bottle of 65 ml. It is produced by fermentation of sterilized milk (made out of low-fat milk powder) with dextrose. After fermentation the drink is homogenized and water and glucose syrup are added. Experiments were done on the day of purchase, three weeks before the best before date. FCM was performed directly and after extraction of bacteria.

Mona Vifit is a yogurt (drink) that contains three probiotic bacteria: *Lb. acidophilus*, *B. bifidum*, and *Lb. rhamnosus* Goldin and Gorbach (LGG). The supplier recommends to drink at least a portion of 200 ml every other day. This should contain approximately 4×10^9 LGG bacteria. The amount of the other bacteria is not mentioned on the package. Experiments were done on the day of purchase, 8 days before the best before date. Samples were cleared prior to FCM analyses.

Fluorescent labeling, microscopy, and FCM analyses of the three probiotic products were done as described for dairy starters. Before and after extraction samples were plated on TSA agar.

Plate counts. Data obtained by flow cytometry were compared with plate counts. Samples were serially diluted in peptone physiological salt solution, and 50-µl portions of the appropriate dilution (resulting in 50 - 300 colonies) were spiroplated using an EddyJet (IUL Instruments; Barcelona, Spain). Samples of *Lb. plantarum* WCFS 1 were plated on MRS agar (Merck; Darmstadt, Germany). MRS is a medium for lactobacilli (9, 15). The plates were incubated anaerobic at 37°C for 48 h. Samples of dairy starters and probiotic products were plated on Tryptone Soya Agar (TSA), a general nonspecific laboratory medium (Oxoid Ltd; Basingstoke, UK). The plates were incubated anaerobic at 30°C for 72 h. for total anaerobic mesophilic colony counts.

RESULTS

Extraction of bacteria from milk. *Lb. plantarum* WCFS 1 cells were harvested in mid-exponential growth phase and suspended in semi-skimmed pasteurized milk and in buffer (control) at a concentration of 5×10^6 cells ml^{-1}. Fig. 1 shows dot plots obtained with SYTO 9 staining. A threshold on the FSC signal was used to include all particles of similar size or bigger than bacterial cells, and exclude smaller particles. The cell suspension in buffer was diluted 5 times, in order to give an appropriate event rate and the cell population was easily observed (Fig 1A and B). However, the cell suspension in milk had to be diluted 10,000 times in order to give an appropriate event rate, and thus the number of cells became too low to be detected. Milk particles gave a band in the dot plot of FSC and SSC that overlapped with the location of the signal of bacterial cells (Fig. 1C). However milk particles did not give much fluorescent signal (Fig. 1D). Milk contained more than 10^{10} particles ml^{-1} with a FSC signal above threshold level (Table 1). Such a high particle density affects the detection system because there is a high chance of scattering and absorption of excitation and emission light by the particles. Furthermore, milk caused problems with the flow system. To facilitate counting of bacteria in milk, a procedure was needed that reduced the signal from milk itself.

Milk clearing solution used according to the protocol of the supplier reduced the number of counted events approximately 100 times, to 2.3×10^8 events ml^{-1} (Table 1). Part of these events were remaining milk particles, the majority were

microcarrier polystyrene beads from the clearing solution. To decrease the number of particles detected by FCM the protocol was adjusted. Clearing solution was filtered to remove the polystyrene beads before adding it to the milk. Plate counts indicated that the yield of the extraction did not change when clearing solution without microcarrier beads was used (data not shown). Furthermore, the number of background counts from milk itself was reduced further by removal of cream that remained sticking to the wall of the eppendorf tube using a cotton-tipped stick. With this additional step the background counts were approximately 50 times lower than without removal of remaining cream (Table 1). In total, the optimized extraction procedure reduced the background from milk with a factor of approximately 10,000.

Figures 1E and F show dot plots obtained with a sample of *Lb. plantarum* in milk extracted with the optimized procedure. This sample needed to be diluted only 10 times in order to give an appropriate event rate. The remaining particles from milk still overlapped with the region of cells in the dot plot of FSC and SSC (Fig. 1E). Part of the milk particles had very low fluorescence signal while the other part was stained (Fig. 1F). Compared to the fluorescence dot plot of the suspension in buffer, the fluorescence of the cells extracted from milk is somewhat shifted towards a higher signal. It is possible that the extraction procedure has resulted in easier access for SYTO 9 by allowing faster diffusion accross the membrane.

TABLE 1. FCM of milk samples and optimization of extraction.

Sample	Background counts/ml[a]
Buffer	1.0×10^4
Milk	1.8×10^{10}
milk - standard extraction with milk clearing solution	2.3×10^8
milk - extraction with filtered milk clearing solution	9.4×10^7
milk - optimized extraction procedure[b]	2.1×10^6

[a]The detector settings were FSC: E01; SSC: 400; FL1: 450; FL2: 450; FL3: 600; all with logarithmic amplification. The threshold was set on FSC: 250.

[b]The optimized extraction procedure uses milk clearing solution free of polystyrene beads and includes removal of the rest of the cream pad with a cotton-tipped stick.

However, most importantly, the reduction of the number of milk particles was sufficient and the bacterial cells formed a distinguishable population in the fluorescence dot plot and could be enumerated.

Counting of bacteria in milk. Firstly, the bacterial background in milk was determined. The milk that was used was semi-skimmed pasteurized day-fresh milk, purchased on the day of the experiment, so low viable counts were to be expected. The milk was cleared with the optimized extraction procedure. Samples were stained with SYTO 9 for total counts. Also, samples were stained with cFDA and

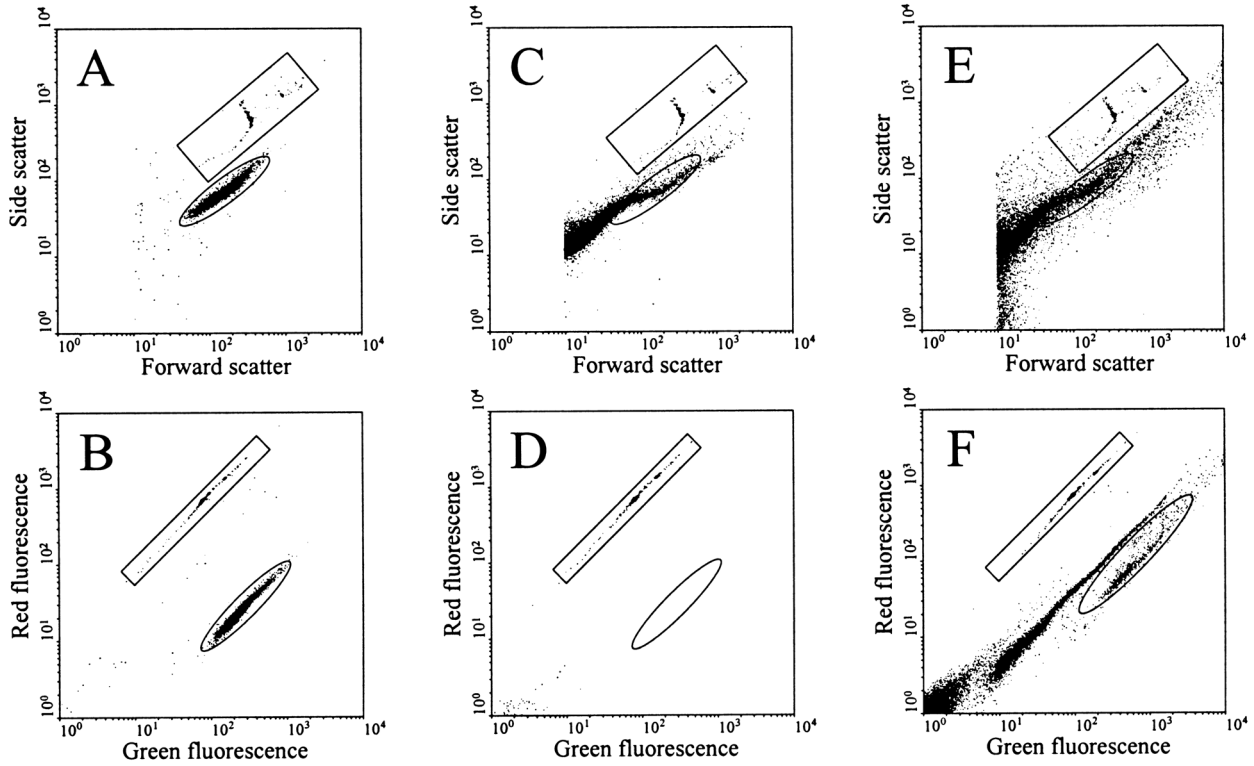

FIG. 1. Detection of bacteria in milk by FCM using SYTO 9 staining. *Lb. plantarum* was suspended at 5×10^6 cells ml^{-1} in either buffer or milk. Red-fluorescent 1.75 µm polystyrene beads of a known concentration were included in the FCM samples to enable counting. Dot plots show the results obtained from 5 µl appropriately diluted sample, the upper dot plots show FSC and SSC, the lower dot plots green fluorescence and red fluorescence. Rectangles indicate the location of the bead population and ellipses the location of the cell population. (A, B) Cell suspension in buffer; dilution 5 times. (C, D) Cell suspension in milk; dilution 10,000 times. (E, F) Cell suspension in milk extracted using the optimized procedure; dilution 10 times.

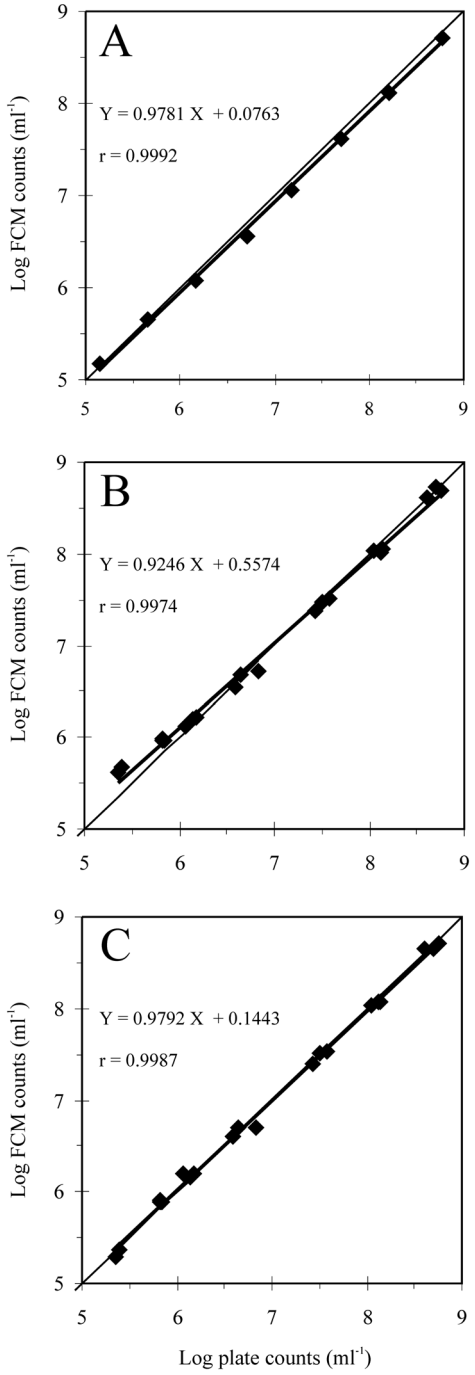

FIG. 2. (A) Correlation between plate counts and FCM counts using SYTO 9 staining of *Lb. plantarum* in buffer. (B) Correlation between plate counts and FCM counts using SYTO 9 staining of *Lb. plantarum* in milk extracted by the optimized procedure. (C) Correlation between plate counts and FCM counts using double staining with cFDA and TOTO-1 of *Lb. plantarum* in milk extracted by the optimized procedure. The thin lines represent 1:1 relationship. The bold lines show linear regression.

TOTO-1 to distinghuish intact cells and membrane-damaged cells. The sum of cF-stained and TOTO-1-stained cells also gives the total number of cells. Samples were plated on MRS agar before and after the extraction. The number of CFU before extraction was approximately 10^2 ml^{-1} (5-10 colonies on plates), after extraction it was below detection. In samples of milk stained with cFDA and TOTO-1 no cells could be detected with FCM because there were no events in the region of cF stained cells, and in the region of TOTO-1 stained cells there was too much background to count cells at concentrations below 10^6 ml^{-1}. With SYTO 9 staining the cell count was approximately 1×10^5 ml^{-1}. This was the number of events in a region in the dot plot of green fluorescence and red fluorescence, which was defined using milk with added *Lb. plantarum*. However, it should be realized that part of the 10^5 events from milk in this region might be particles other than bacteria. So, the bacterial background in milk is, at highest, 1×10^5 nonviable cells ml^{-1}.

Next, FCM counts and plate counts were performed of samples of *Lb. plantarum* suspended in milk at concentrations of 10^5-10^9, and, for comparison of samples of *Lb. plantarum* in buffer. The variability between replications of FCM counting was considerably lower than between multiple plate counts. With plating between 50 and 300 colonies were counted, so the CV was approximately 10%. With FCM at least 3,000 cells and at maximum 20,000 were counted, so the CV was between 0.7 and 1.8%. Thus, FCM counts are more accurate than plate counts. FCM counts of the cell suspensions in buffer with SYTO 9 staining gave a very strong correlation with plate counts and a regression line with nearly a 1:1 ratio (Fig. 2A). The suspensions of *Lb. plantarum* in milk were first cleared and then stained and analysed by FCM. FCM counts obtained with SYTO 9 staining (Fig. 2B) as well as the sum of cFDA and TOTO-1 staining (Fig. 2C) strongly correlated ($r > 0.99$) with plate counts of extracted samples. The regression lines approximate the 1:1 ratio closely, indicating that nearly all cells counted by FCM formed colonies on plate. The number of permeabilized cells in these samples was less then 2% as indicated by TOTO-1 staining.

Furthermore, viability staining of bacteria in milk was tested using heat treatment. Milk with 10^8 *Lb. plantarum* cells ml^{-1} was divided into two portions. One was not treated, the other was incubated at 70°C for 10 min. The heat treatment permeates the cells and kills them. Mixtures of nontreated and heat-treated milk were prepared. These milk samples were cleared, stained with cFDA and TOTO-1, and analyzed by FCM. Fig. 3 shows the mixture of 50% nontreated suspension and 50% heat-treated suspension. Nearly all cells in the nontreated suspension are intact and are thus stained by cFDA, while all cells of the heattreated suspension are permeabilized and thus stained by TOTO-1. FCM counts showed that extraction was as efficient for heat-killed cells as it was for non-treated cells and the correlation between FCM counts and the fractions of nontreated and heat-treated milk in the mixtures was very strong ($r > 0.99$).

FCM of dairy starters. The optimized extraction procedure and staining with cFDA and TOTO-1 were applied for FCM analyses of dairy starters (Table 2). The extraction procedure cleared the background very well. cFDA and TOTO-1 gave well-stained cell populations (high fluorescence intensities). Total cell counts were determined in 70°C heat-treated samples. After heat-treatment the cells have permeabilized membranes and are uniformly and highly stained by TOTO-1. The total number of detected cells in untreated samples was the same as in heat-treated samples, indicating that all cells

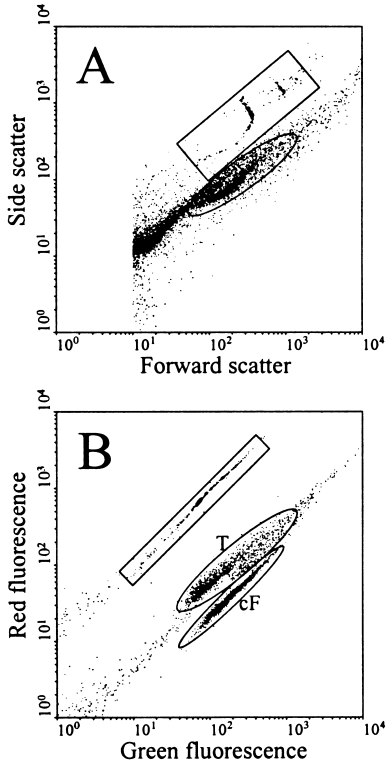

FIG. 3. Viability staining in milk by cFDA and TOTO-1 of a 1:1 mixture of nontreated and 70°C heat-treated milk containing 10^8 *Lb. plantarum* per ml. (A) FCM dot plot of FSC and SSC. (B) FCM dot plot of green fluorescence and red fluorescence. The rectangles indicate the location of the bead population and ellipses the location of the cell populations. The cF-stained population (cF) and the TOTO-1-stained population (T) are distinguished from each other by their location in the dot plot of green fluorescence and red fluorescence.

were also detected in untreated sample. Note that the counts for BOS, RR and ISt are the numbers present in the concentrated mother starters, BOS has been concentrated 40 times and RR and ISt 20 times. The total FCM counts of BOS, BOS-bulk and RR were approximately three times as high as the respective plate counts (Table 2). Total FCM counts of ISt were approximately five times as high as plate counts. A considerable fraction of the cells lacked membrane integrity as was indicated by 30 to 60% TOTO-1-stained cells. Still, the number of intact and enzyme active cells, as indicated by cF staining, was substantially higher than the number of CFU.

FCM of probiotic products. Three probiotic products were selected for this study: a powder (Orthiflorplus), a drink (Yakult), and a yogurt (Mona Vifit yogurt drink). Some details about these products are described in Materials and Methods. The effects of the clearing procedure on the product matrix and on the bacteria were tested first by FCM and fluorescence microscopy. This showed that Orthiflorplus suspension did not contain many particles other than bacteria, so clearing was not needed. Moreover, the procedure increased the number of membrane-damaged cells in Orthiflorplus substantially. In Yakult there were many particles besides bacteria, mostly particles somewhat smaller than the bacteria. Clearing did decrease the number of these particles, but it also damaged the Yakult bacteria. However, FCM analyses could be done without clearing the samples. In Mona Vifit yogurt drink there was very much background. The clearing decreased that, but not as well as it did for milk. In this product the procedure hardly increased the number of membrane-damaged bacteria. It was decided to do FCM analyses of Orthiflorplus and Yakult with untreated samples, while Mona Vifit samples were cleared before use.

In each of the probiotic products the bacteria were heterogeneous with respect to membrane integrity. FCM with cFDA and TOTO-1 quantified the intact and membrane-damaged cells. Plate counts quantified the culturable cells, which is a subpopulation of the intact cells. Results are shown in Table 3. When results of FCM and plate counts are combined three populations are revealed. The first is the culturable population. The second is the population of cells that are intact and metabolically active but not culturable (FCM count with cFDA minus plate count). The third is the permeabilized (dead) population. The proportions of these populations differed among the tested products.

(i) Orthiflorplus. According to the supplier Orthiflorplus powder contains at least 3×10^9 'germs' per sachet, i.e. 3×10^7 per ml of suspension. The measured number of CFU was somewhat lower, 9.15×10^6. This might be due to sticking of cells, or to loss of viability during production and storage. The total cell number was 4.29×10^7 ml^{-1}, of which approximately 21% was culturable, 27% was intact and active but not culturable, and 52% was permeabilized.

(ii) Yakult. According to the supplier Yakult drink contains 6.5×10^9 *Lb. casei* Shirota bacteria per bottle of 65 ml, i.e. 1×10^8 per ml. The measured number of CFU was somewhat higher, 4.76×10^8 per ml. The total cell number was 1.83×10^9. Most cells were stained by cF and only a small population

TABLE 2. Bacterial numbers determined by plating and FCM method in dairy starters.

Dairy starter[a]	Plate count/ml	FCM count/ml		
		cFDA	TOTO-1	total[b]
BOS	1.43×10^{10}	2.84×10^{10}	1.29×10^{10}	4.13×10^{10}
BOS-bulk[c]	2.35×10^8	3.67×10^8	4.02×10^8	7.70×10^8
Ist	3.49×10^9	8.49×10^9	8.35×10^9	1.68×10^{10}
RR	5.69×10^9	7.55×10^9	1.10×10^{10}	1.85×10^{10}

[a]The starter cultures were cleared before analyses, extraction yield was on average 0.78.
[b]Total counts were obtained from heat-treated samples using TOTO-1 staining.
[c]BOS-bulk was produced by inoculation of low-fat milk with 0.0125 % concentrated BOS mother starter and incubation of 18 hr at 20°C

TABLE 3. Bacterial numbers determined by plating and FCM method in probiotic products.

Probiotic product	Plate count/ml	FCM count/ml		
		cFDA	TOTO-1	total[a]
Orthiflorplus [b,c]	9.15×10^6	2.04×10^7	2.25×10^7	4.29×10^7
Yakult[b]	4.76×10^8	1.78×10^9	5.50×10^7	1.83×10^9
Mona Vifit[d]	1.93×10^7	9.85×10^8	1.43×10^8	1.13×10^9

[a]Total counts were obtained from heat-treated samples using TOTO-1 staining.
[b]The products Orthiflorplus and Yakult were used directly (without clearing).
[c]One sachet of Orthiflorplus (3 g) was suspended in 100 ml tap water.
[d]Mona Vifit was cleared, the extraction yield was 0.82.

was stained by TOTO-1. The average fluorescence signal of the cF-stained cells was however quite low and the cF population was not separated from the background. Thus, intact cells could not be enumerated directly by cF-staining. The difference between the total number of cells determined from a heat-treated sample, and the number of TOTO-1 stained cells in untreated sample was used to calculate the number of intact cells. This was done for all samples of probiotics and starters, but only for Yakult the number of intact cells calculated by this method was substantially higher than the count of the cF-stained region for untreated sample. The proportions of culturable, intact and active but not culturable, and permeabilized cells were 21%, 76% and 3%, respectively.

(iii) Mona Vifit. According to the supplier Mona Vifit yogurt drink contains three probiotic bacteria: *Lb. acidophilus*, *B. bifidum*, and LGG. Since it is a yogurt it should contain also at least 10^7 yogurt bacteria per ml (*Lb. delbruecki* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*).

Only for the probiotic LGG an amount is mentioned on the package; there should be approximately 2×10^7 per ml. Our assays do not distinguish between species, so we do not know in what proportion the two yogurt species and the three probiotic species occur in Mona Vifit. The number of CFU of extracted samples was 1.93×10^7 per ml. FCM counted in total 1.13×10^9 cells per ml. The number of background events was high, but the populations of cF-stained and the TOTO-1-stained cells were well detectable. Fluorescence intensities of the cF-stained cells were homogeneous and high. Approximately 2% of the cells was culturable, 85% was intact and active but not culturable, and 10% was permeabilized.

DISCUSSION

In a previous study we developed an FCM viability assay with the fluorescent probes cFDA and TOTO-1 (6). The assay was used on various LAB species, which were harvested in exponential growth phase and suspended in buffer. Plate counts were in agreement with relative FCM counts, and the assay was validated for cells stressed by exposure to acid or bile. FCM viability assessment has many possible applications. In the present study we show the usefulness for bacterial suspensions in milk, fermentation starters, and probiotic products.

Bacteria are small compared to mammalian cells and in FCM analysis the signal-to-noise ratio is of greater concern for bacterial cell samples than for mammalian cell samples, especially in a medium such as milk. Milk contains a very high concentration of colloidal and other particles that are in the size range of bacteria. Furthermore, milk might cause clogging of the flow system and turbulence of the flow. The analysis of low concentrations of bacteria in milk is not possible because a high dilution factor is needed for direct FCM of milk samples. A procedure is needed that reduces the milk background, but does not affect the bacteria. To do this we choose a method based on a commercially available clearing solution, which was used before by McClelland and Pinder for immunofluorescent detection of *Salmonella* in milk (20). The clearing solution contains a nonionic detergent, a chelating agent and polystyrene beads (23). We optimized the method by using clearing solution free from polystyrene beads and by removing cream that remained at the wall of the tube with a cotton-tipped stick. The optimized method cleared milk very well. The counts of particles of similar or bigger size than bacteria were decreased enormously. The extraction yield of bacteria was high and the culturability and membrane integrity were not affected by the clearing step.

FCM indicated that the bacterial background in the pasteurized milk was, at highest, 10^5 bacteria per ml, and that these are all dead. This is a rather normal result for milk, also measured by plate counts, total FCM counts, and direct epifluorescence filter technique viability counts just before pasteurization (13, 26). FCM counts of *Lb. plantarum* added to milk at a concentration of 5×10^5 per ml or higher were highly accurate, especially with cFDA and TOTO-1 double staining. FCM counts of permeable cells were also accurate, since these cells were extracted by the clearing procedure with the same yield as intact cells.

This work is a step in the development of FCM methods for rapid monitoring of fermentation processes and other applications of measurement of bacteria in milk suspensions. A complete FCM assay can be done within an hour including extraction and labeling, because no long incubation times are required. This makes FCM favorable to plate counts, which needs incubation of two days or more before any result can be obtained. Furthermore, FCM can be used for total cell counts, for specific detection of strains, and for measurement of various aspects of cell viability on all individual cells.

FCM has been used before for bacteria in milk, but mostly for pathogens and for bacteriological quality control (10, 13, 20, 24, 29, 34). FCM can also be used to measure the fermentation starter bacteria. Here we applied FCM with cFDA and TOTO-1 to investigate viability of dairy fermentation starters. All samples were cleared, though this was not strictly necessary for the concentrated mother starters since they contained very high cell numbers ($>10^{10}$). The results showed that approximately 50% of the cells in the mother starters were permeabilized. This must have occurred mainly during fermentation and (almost) not during freezing, since the freshly produced BOS-bulk starter contains approximately the same proportion of permeable cells. The other 50% of the cells were intact and active. The number of intact cells in the starters was higher than the number of colonies on plates. Between 40 and 65% of the intact cells was culturable. Cells, which are not culturable, might well contribute to fermentation processes (21, 33). Enzymes present may still be active and cells may even still have the capacity to synthetize enzymes. Hence, metabolic routes may still be functional and these cells may be involved in the fermentation.

We also applied cFDA and TOTO-1 to investigate probiotic products with FCM. Three different types of probiotic products were selected: a powder (Orthiflorplus), a drink (Yakult), and a yogurt (Mona Vifit yogurt drink). Probiotics have been defined as living microorganisms, which upon ingestion in certain numbers, exert health affects beyond inherent basic nutrition (12). A number of criteria have been stated for probiotic microorganisms, including human origin, resistance to technological processes, resistance to gastric acid and bile, modulation of immune response, influence on metabolic activities, and certain persistence in the gastrointestinal tract (11, 27). *In vitro* and *in vivo* studies are important to elucidate effects of stress factors on the performance of probiotics and mechanisms of probiotics on

human health. It is difficult to say in what numbers probiotic microorganisms should be ingested, because the knowledge about survival, activity, and dose-response is limited. The minimum therapeutic dose per day is suggested to be 10^8 to 10^9 living microorganisms and a criterion has been suggested of a minimum of 10^6 CFU ml^{-1} probiotic bacteria at the expiry date (17, 30). However, 10^6 CFU ml^{-1} might not be enough, considering that only part of the bacteria reaches the intestines alive. Based on results of *in vitro* survival studies, a daily portion of 10^9 to 10^{10} CFU is assumed to be needed for probiotic effects (28). Our plate counts indicated that the numbers of CFU per recommended portion were approximately $\times 10^9$ for Orthiflorplus (per sachet), 3×10^{10} for Yakult (per bottle of 65 ml), and 5×10^9 for Mona Vifit (per portion of 200 ml). These numbers are high enough to be an effective probiotic according to the criterion of 10^9 - 10^{10} CFU per daily portion.

Most studies on probiotics only use plate counts to assess the viability of the microorganisms. However, cell viability is more complex than whether cells are culturable or not. Only for prolonged persistence dividing cells would be a prerequisite. Cells that are active but not culturable might contribute to many of the proposed health effects. For actions such as lactose conversion, production of antibacterial compounds, assimilation of cholesterol, and antioxidant effects, the microorganisms need to be active but not necessarily culturable. For some probiotic effects the microorganisms even don't have to perform metabolic activities. It has been reported that nonviable forms of probiotic bacteria can adhere to intestinal mucus, and have immunomodulatory effects (22, 25). Because of the complexity in the possible contributions of different cell populations in proposed probiotic effects, FCM is a very appropriate tool to study probiotics. FCM can detect all individual cells and can distinguish between various populations.

The FCM viability assay with cFDA and TOTO-1 gave the total cell count and distinguished permeable cells from intact cells. In combination with plate counts three cell populations were revealed: culturable cells, cells that are intact and metabolically active but not culturable, and permeabilized cells. The proportions of the populations differed between the tested products.

The FCM viability assay could be used for various further purposes. For example to study the effect of prolonged storage on probiotic products. It has been shown that the decrease in CFU depends on factors such as pH, temperature, the level of inoculum, and type of product (17, 28, 30). It would be interesting to measure also the effects on total number of cells and the number of active cells after prolonged storage. Furthermore, FCM could be used in investigation of the effect of gastrointestinal tract passage on probiotic microorganisms, and in strain selection. A combination of viability probes and probes for specific detection of a species would be needed to study one probiotic strain in a mixed culture, such as in Orthiflorplus and Mona Vifit yogurt. This may also be done by FCM.

In conclusion, this study demonstrates the usefulness of FCM for viability analysis of bacterial suspensions in milk, dairy fermentation starters, and various types of probiotic products. The FCM assay with cFDA and TOTO-1 is very accurate and highly sensitive and provides tools to assess the functionality of different populations in fermentation starters and probiotic products.

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7

Summary and Concluding Remarks

Aim

In food industry there is a perceived need for rapid methods for detection and viability assessment of microbes. Fluorescent staining and flow cytometry provide excellent tools for microbial analysis. The aim of the PhD-research described in this thesis was to develop fluorescent techniques for the viability assessment of lactic acid bacteria. This chapter summarizes the experimental research and discusses the potential of the developed methods for application in food industry

Background

Fluorescent probes can be used for counting the total number of bacteria in a suspension using a dye that stains all cells, for example a permeant nucleic acid dye. Furthermore, specific species or phylogenetic groups can be detected using antibodies or oligonucleotide probes tagged with a fluorescent dye. Also, various fluorescent dyes are available that can characterize and quantify cellular physiological parameters such as membrane integrity, enzyme activity, DNA content, protein content, respiration, intracellular pH and membrane potential. Such assays provide information on the physiological state of bacteria. However, it should be noted that you need to validate whether such a 'viability probe' may be taken as an indicator of culturability. Fluorescently stained bacterial samples may be analyzed with fluorescence microscopy, which allows direct observation of the labeling, or with flow cytometry.

With flow cytometry multiple parameters of thousands of individual cells in a suspension are measured within a few minutes. The cells are carried by a liquid stream at high rate along a measuring point where the cells are illuminated by a focussed light beam and where light scatter and fluorescence are detected. A large population of individual cells is measured which allows analysis of the population heterogeneity. Furthermore, selected populations can be physically sorted for further analysis.

A novel two-step assay combining staining with cFDA and active cF-extrusion

The first study (Chapter 3 of this thesis) concerned viability assessment of *Lactococcus lactis* using carboxyfluorescein diacetate and propidium iodide. *Lactococcus lactis* is an important organism in dairy fermentations and a model organism for genetic and physiological studies. Carboxyfluorescein diacetate and propidium iodide are commonly used to assess bacterial viability. Propidium iodide is a nucleic acid dye that is used to determine membrane integrity by dye exclusion. When the membrane is compromised, propidium iodide can enter the cell and bind to nucleic acids forming a red-fluorescent complex. Carboxyfluorescein diacetate is a nonfluorescent esterase substrate. It enters the cell by passive diffusion. Once inside, the acetate groups are split off by enzymatic hydrolysis releasing the green-fluorescent carboxyfluorescein. Carboxyfluorescein is one of the probes for which active extrusion had been reported.

Lactococcus lactis subsp. *lactis* ML3 could easily be labeled with carboxyfluorescein and retained the probe well when not energized. But upon addition of lactose the cell immediately and rapidly extrudes cF. Dissipation of the proton motive force did not inhibit this, which indicates that the

cF-efflux pump is an ATP-driven system. Therefore, we hypothesized that this probe efflux could be used in viability assessment.

We aimed to develop fluorescent viability assessment applicable under a wide range of conditions. Therefore, *Lactococcus lactis* was exposed to various stresses, including heating, freezing, low pH, and bile salts. A number of traditional and fluorescent viability assays were performed testing the stressed cell suspensions. First of all culturability was tested using standard plate counting. Furthermore, the acidification capacity was tested by a fermentation starter acidification assay. In this test skimmed milk medium is inoculated with the lactic acid starter and after an incubation of 6 hours the amount of acid formed is determined by titration to pH 7.0 with NaOH. During the incubation time the cells may grow and divide. This test is commonly applied in dairy industry in The Netherlands to control the quality of starter cultures. The acidification capacity corresponded well with the culturability. This indicates that the 6-hour-acidification depends on the number of cells that survived the stress condition and suggests that injured but culturable cells recover fast and contribute to the acidification. Also, the initial rate of acidification upon addition of lactose in a suspension with low buffering capacity was determined. This initial rate was taken as a measure for glycolytic activity and reflects the vitality of the cell suspension. The stress treatments had more impact on the glycolytic activity than on the acidification capacity. This was rationalized by the fact that the glycolytic activity was tested immediately after the stress treatment, before injured cells had recovered and improved their physiological condition.

The fractions of cells stained by carboxyfluorescein or by propidium iodide were determined by microscopic counting, because we aimed for a method that would not necessarily need expensive equipment. Furthermore, a number of fluorescent parameters that assess the culture as a whole were determined. The first was the internal cF concentration averaged over the total cellular mass, which was measured by spectrofluorimetry. The second was the esterase activity, determined by incubation of cell extract with cFDA and spectrophotometric monitoring of the increase of cF concentration. The third and last was the cF-efflux, monitored by measuring the increase of cF in the supernatant and therefrom calculating the decrease in intracellular concentration.

Treatment at 70°C killed the cells as was indicated by loss of culturability and activity. Accordingly, the fluorescent staining showed that the membrane was permeabilized by this treatment. However, after treatment up to 60°C the fraction of cells stained by cF remained high, whereas the fraction of culturable cells decreased above 50°C and survival was completely lost for those cell suspensions treated at 60°C. Furthermore, cF staining deviated from culturability when cells had been exposed to conjugated bile salts.

Surprisingly, none of the treatments completely abolished the esterase activity. Even after treatment at 70°C there was some remaining activity in the cells (15% compared to nontreated cells). Thus, whether a cell was labeled or not by cF depended on membrane integrity in these experiments. This was confirmed by correspondence of cF labeling with PI exclusion. The concentration of cF in the labeled, thus intact, cells was however considerable lower after stress treatments. After labeling with cF the cells were energized by adding lactose and the subsequent efflux of cF was monitored. Strikingly, the 60°C treatment, which killed the cells (no colonies), but did not permeabilize the

membrane (still cF labeling), had resulted in a nearly complete loss of the efflux capacity. For some stress conditions, notably the freezing and exposure to 1% conjugated bile salts, efflux results indicated that part of the intact subpopulation also had the capacity to extrude cF while the rest was more severely injured and had lost the efflux capacity. The results indicated that the fraction of cells capable of labeling with cF and of active extrusion upon energizing is the best measure to assess the physiological condition of the culture since this combined information agreed best with culturability.

To summarize, our results showed that the fraction of cF labeled cells was not a general indicator of culturability and acidification, nor were PI-exclusion, the average intracellular cF concentration or the esterase activity. For decisive viability assessment a two-step assay was needed. In the first step cells are incubated with cFDA to determine the fraction of intact cells. In the second step the lactose-energized efflux is determined. This combined assay proved to be a good and rapid indicator for culturability and acidification capacity.

The novel assay combining the measurement of the fraction of cF-labeled cells and the subsequent measurement of the fraction of cF efflux has potential for physiological research of bacteria and for applications in dairy industry.

Staining with cF is generally taken as prove of membrane integrity and of enzyme activity. However, it should be noted that esterase enzyme activity is energy-independent and it is quite persistent. Cells still show esterase activity after severe stress treatments and the activity persists for some time in cells that have lost other activities and even membrane integrity. Thus, the detection of esterase activity really only indicates that the cell was active not too long ago. Measuring the esterase activity quantitatively may provide information on the vitality of the cells, but such an assay needs to be validated before conclusions can be drawn. Therefore, cF labeling is in the first place a tool to assess membrane integrity. It does give some clue about the physiological status but does not prove much. Though an intact membrane may be regarded as the first prerequisite for cell viability, it does not guarantee that the cell is indeed metabolically active, let alone capable of cell division.

The additional value of the subsequent efflux assay is that cells that are capable of performing glycolytic activity and getting energized upon sugar addition are distinguished from the cells that are not. Obviously, this additional test only has use for species that do posses cF efflux capacity. We tested a number of lactic acid bacteria and all were found to extrude cF when energized (unpublished results), suggesting that this property is common. Still, efflux capacity of a specific strain or mixture should be tested prior to application of the combined assay.

The assay could be used in selection of strains to test their physiological response under various conditions, for example storage by freezing or exposure to acid or salt. The assay may also be used as an indicator for acidification capacity of cheese starters, provided that the whole starter mixture is capable of efflux. Combining the cF labeling and efflux assay with flow cytometry would enable fast and accurate measurements.

Fluorescent monitoring of lysis

The study described in Chapter 4 was committed to investigate the lysis process of cheese starter bacteria on the single-cell level. Starter bacteria initiate the fermentation by acidification of the milk through conversion of the milk sugar lactose into lactic acid. In later stages, during cheese ripening, the proteolytic, peptidolytic, and amino acid-converting enzymes of the starter bacteria are responsible for the generation of flavor components. It is generally considered that lysis results in leakage of intracellular enzymes in the cheese curd and, thus, plays an important role in ripening and flavor formation.

The measurement of lysis in cheese has always been a major problem. Because decrease of cell turbidity can not be measured in cheese, other markers have been used such as decrease in viable counts and release of intracellular enzymes such as lactate dehydrogenase (LDH) and different peptidases. However, these tests require extraction procedures, which, unquestionable, induce more lysis, thus causing overestimation of this process. The direct labeling with fluorescent dyes has great advantages since extraction procedures are not required and a number of fluorescent probes are available for measuring various aspects of cell physiology.

To monitor the permeabilization and lysis the LIVE/DEAD *Bac*Light kit of Molecular Probes was applied. This kit combines propidium iodide, which was also used in the first study, and the membrane-permeant green fluorescent nucleic acid dye SYTO 9. This combination stains cells with compromised membranes fluorescent red and intact cells fluorescent green. Thus, all cells are stained and can easily be detected and counted, and the intact and permeable cells can be distinguished. Cells of *L. lactis* MG1363 were incubated under different conditions and subsequently labeled with *Bac*Light and analyzed by flow cytometry as well as by epifluorescent microscopy. Lysis was induced by treatment with the cell wall-hydrolyzing enzyme mutanolysin. Cheese conditions were mimicked by incubating cells in a buffer with a high concentration of protein, potassium and magnesium, which stabilizes the cells.

As expected, flow cytometry results corresponded with fluorescence microscopy results. Microscopy allowed direct observation of the individual cells in the suspension. However, the advantage of flow cytometry is that the number of cells (total, intact and permeable) could be calculated per ml of sample and that the counts were highly accurate since approximately 40,000 cells were counted in a flow cytometry experiment while at maximum 500 cells were counted using microscopy.

Under nonstabilizing conditions a high concentration of mutanolysin caused complete disruption of the cells. This resulted in a decrease of the total number of cells and in release of LDH. In stabilizing buffer mutanolysin caused membrane damage as well, but the cells disintegrated at a much lower rate. The damaged cells were supported by the stabilizing buffer, as indicated by a high number of cells stained by propidium iodide. In addition, permeable cells did not release the intracellular aminopeptidase N, but increased enzyme activity was observed with the externally added, nonpermeant peptide substrate lysyl-*p*-nitroanilide. Apparently, the cell damage caused by mutanolysin treatment was sufficient to make the cells permeable for enzyme substrates, as well as for access of propidium iodide, but not severe enough for leakage of enzymes.

Also, the *Bac*Light kit was applied to assess permeabilization and lysis of starter bacteria in a Gouda cheese that had been ripening for two weeks. Thin slices of cheese were incubated with SYTO 9 and PI and analyzed with confocal scanning laser microscopy (CSLM). CSLM allowed clear observation of stained cells within the cheese matrix. The majority of the observed starter cells were intact, but a substantial number of cells were permeable as indicated by PI labeling. These results show that the cheese matrix supports permeable cells. As in stabilizing buffer, the lytic processes cause permeabilization but complete disruption is postponed by the stabilizing conditions.

In conclusion, this study demonstrated the presence of permeable cells under cheese conditions and showed that intracellular enzymes will be able to contribute in the process of protein degradation and flavor formation upon permeabilization, as was demonstrated for aminopeptidase N activity. Also, the potential of the fluorescent staining for direct observation of intact and permeable bacteria in the cheese matrix was shown.

This study clearly showed the added value of using fluorescent stains for assessment of lysis. The fluorescent method of cell counting using SYTO 9 and PI does not only monitor the complete disintegration of cells, as is the case with traditional methods of measuring lysis, but it also visualizes the permeabilization of cells. Traditional lysis techniques based on measurement of released intracellular enzymes into the cheese matrix overlook the contribution to the total peptidase activity by permeable cells. As demonstrated fluorescent staining and CSLM allow for direct assessment of lysis in cheese. In dairy research, the *Bac*Light kit may be applied with flow cytometry as a routine method to test starter strains and mixed starter cultures for their lytic capacity, and to test the effect of changes in the fermentation conditions on the performance of the starters. Furthermore, these fluorescent probes can be used for monitoring cheese ripening.

Viability assessment by flow cytometry and staining with cFDA and TOTO-1

The next study (Chapter 5) concerned fluorescent viability assessment of lactic acid bacteria using flow cytometry. In particular, survival upon exposure to bile salts or to acid was measured by plate counts and compared with the indication of viability provided by fluorescent staining. The aim was to provide a method for rapid and reliable viability assessment of lactic acid bacteria from different genera, including species used in various dairy fermentations and probiotics. Therefore, a broad selection of species was used including a *Lactococcus*, a *Streptococcu*s, three *Lactobacillus*, two *Leuconostoc*, an *Enterococcus*, and a *Pediococcus* species.

Carboxyfluorescein diacetate was evaluated for staining live cells and the impermeant nucleic acid stains PI and TOTO-1 were evaluated for their capacity to stain dead cells. PI was included in this study because it is the probe most used for detection of dead cells and its spectroscopic properties make it suitable for FCM. TOTO-1 was chosen because the excitation and emission spectra are suitable for FCM and it has a high fluorescence enhancement. Plate counts were performed to test whether the population indicated as live by the FCM viability assay were indeed culturable and the population indicated as dead was not.

First, the capacity of the probes for live/dead discrimination was evaluated using exponential phase cells as the positive control and 70°C heat-killed cells as the negative control. The results showed that cFDA was an accurate live stain; in exponential phase cultures almost all cells were labeled, while 70°C heat-killed cultures were left unstained. PI did not give clear live/dead discrimination for some of the species. TOTO-1 on the other hand gave clear discrimination between live and dead cells. The combination of cFDA and TOTO-1 gave the best results. Well-separated subpopulations of cF stained cells and TOTO-1 stained cells could be detected with FCM.

Further experiments using cultures of *L. lactis*, *Lb. helveticus*, and *Lc. mesenteroides* showed that the cF/TOTO viability assay could be applied successfully to acid stressed cultures and deconjugated bile salt stressed cultures. Exposure to hydrochloric acid was chosen because this is often taken as an *in vitro* condition to investigate the resistance of bacteria to a passage through the stomach. Furthermore, in dairy fermentations the starter cultures acidify their environment and thus also impose an acid stress on themselves. In the experiments cells that were exposed for 1 hour at 30°C to hydrochloric acid at pH values down to 3.0 remained culturable, but after exposure to pH 2.0 there were hardly any surviving cells. The results of the cF/TOTO staining indicated that at pH 3.0 the membrane stays intact, while by exposure to pH 2.0 the membrane is damaged. Thus, labeling results were in agreement with plate count results.

Besides the resistance to acid, the resistance to bile is recognized as an important feature for LAB used as probiotics. Our results show that 0.10% deconjugated bile salts falls within the critical range for survival, but different survival fractions were found for the three species. At 0.25% almost no surviving cells were detected. The labeling experiments indicated that the membrane integrity is crucial for bile resistance. The detection of damage to membranes was indicative for the culturability as was shown by the agreement between labeling results and plate counts.

Plating of sorted subpopulations provided direct evidence that the FCM viability assay with cF and TOTO-1 indicates live and dead, i.e. culturable and nonculturable subpopulations in stressed cultures.

The assay developed in this study combining cF and TOTO-1 may be compared with the *Bac*Light bacterial viability kit (SYTO 9 and PI, see previous part). The novel cF/TOTO-1 assay combines two probes that individually discriminate between intact and membrane compromised cells. Under the conditions tested, cF acts as a stain for live cells because it needs hydrolysis by intracellular esterases and retention by an intact membrane. TOTO-1 acts as a stain for dead cells because it is a nucleic acid binding probe excluded by cells with intact membranes. Instead of using a counterstain only to have all the cells labeled, both probes provide information on the cell status. Furthermore, this assay employs TOTO-1, which proved to be better than PI in our experiments. It should be noted that the green-fluorescent cF labeled cells and the yellow fluorescent TOTO-1 labeled cells may be difficult to distinguish with microscopy. On the other hand, single labeled cell samples can be used when a visual impression is required.

In the final study, described in Chapter 6, the novel cF/TOTO assay for physiological assessment of bacteria was applied to suspensions of bacteria in milk, and to dairy starter cultures and probiotic products.

Milk contains a very high concentration of colloidal and other particles that are in the size range of bacteria. Furthermore, milk causes clogging of the flow system and turbulence of the flow. The direct analysis of low concentrations of bacteria in milk is not possible because a high dilution factor is needed for direct flow cytometry of milk samples. A treatment is needed that reduces the milk background, but does not affect the bacteria. To facilitate FCM analysis of bacterial suspensions in milk a commercially available milk clearing solution was used. The active components of this solution are a nonionic detergent and a chelating agent. The standard procedure was optimized to increase the signal-to-noise ratio. The optimized method cleared milk very well. The counts of particles of similar or bigger size than bacteria were decreased enormously. FCM enumerations were accurate down no 10^5 cells/ml. Retrieval of *Lactobacillus plantarum* WCFS 1 suspended in milk was high and viability was not affected by the procedure. The plate counts of cleared samples of untreated cell suspensions were nearly as high as total FCM counts and the correlation was strong ($r>0.99$).

The FCM assay was applied on three dairy starters: the cheese starter BOS, and two yogurt starters RR and ISt (CSK Food Enrichment, Leeuwarden, The Netherlands). The BOS starter is a mixed culture containing mainly Lactococci and Leuconostocs and it is used for the production of Gouda cheese. Both yogurt starters contain *Lactobacillus delbruecki* subps. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus* but RR contains other strains than ISt. After production the starters are concentrated and stored below -40°C. The concentration of cells in these concentrated starters is very high, approximately 10^{10} per ml. Therefore, extraction is not strictly necessary to enable flow cytometry. However, the optimized extraction procedure cleared the background of the starter cultures very well, and it did not affect the bacterial staining. Thus, clearing facilitated easy analysis of the samples. cFDA and TOTO-1 gave well-stained cell populations (high fluorescence intensities). The total number of cells in the BOS and RR starters were approximately three times as high as the respective plate counts. Total counts of ISt were approximately five times as high as plate counts. A considerable fraction of the cells lacked membrane integrity as was indicated by 30 to 60% TOTO-1 stained cells. Still, the number of intact and esterase active cells, as indicated by cF staining, was substantially higher than the number of CFU.

Furthermore, the assay was applied to probiotic products. The selected products were Yakult (Yakult Europe BV), Mona Vifit yogurt drink (Campina Melkunie) and Orthiflorplus (Orthica BV) Yakult is a liquid food supplement containing the probiotic *Lactobacillus casei* Shirota. Mona Vifit is a yogurt drink that contains in addition to the normal yogurt bacteria three probiotic species: *Lactobacillus acidophilus*, *Bifidobacterium bifidum* and *Lactobacillus rhamnosus* Goldin and Gorbach (LGG). Orthiflorplus is a powder formula that has to be suspended in water. It contains five probiotic species. Yakult and Orthiflorplus were analyzed without pretreatment because microscopic analysis showed that the clearing procedure increased the number of membrane-damaged cells and because the background was sufficiently low to allow counting of the bacteria. The background signal of Mona Vifit yogurt drink however was high. Clearing decreased the background, but not as well as it did for

milk, and clearing did hardly increase the number of membrane-damaged cells. When results of FCM and plate counts were combined three populations were revealed, like for the dairy starters. The first is the culturable population, the second is the population of cells that are intact and have enzyme activity but are not culturable, and the third is the permeabilized (dead) population. The proportions of the populations differed between the three tested probiotic products.

In conclusion, this study demonstrated the usefulness of cFDA/TOTO-1-staining and flow cytometry for viability analysis of bacterial suspensions in milk, dairy fermentation starters, and various types of probiotic products. The assays are very accurate and highly sensitive and provide tools to assess the functionality of different populations in fermentation starters and probiotic products.

The novel live/dead assay using the combination cFDA and TOTO-1 may have many applications. In Chapter 5 the application for viability assessment after exposure to bile or to acid was validated by comparison of labeling results with plate count results. Thereby the assay has proven its use for screening bacteria that may be used as probiotic for tolerance against bile and acid under various conditions. The standard labeling protocol that was used was suitable for all tested species, which makes the assay attractive for routine studies. The assay may be applied to examine the viability of single species and mixed lactic acid starter cultures. In addition, it can be applied as a fast screening method of susceptibility of bacteria to a wide range of antimicrobial compounds, including antibiotics.

It should be kept in mind that the assay is mainly based on membrane integrity. The assay needs to be validated for its reliability as indicator of culturability for each condition imposing a possibly lethal stress on the cells. Under conditions that injure the membrane leading to cell death, the viability, as indicated by this assay, will correlate with plate counts. However, under conditions that abolish the capacity of cell division by other mechanisms but leave the membrane intact, survival assessment can not be done with cF/TOTO staining. Examples of such conditions are UV radiation leading to lethal mutations and exposure to a certain range of heat stress (50-60°C for *Lactococcus lactis*, as was shown in the first study).

In Chapter 6 the usefulness is demonstrated of the novel fluorescent assay for physiological assessment of dairy starters and probiotic products. The added value of the fluorescent probes was clearly demonstrated. Three populations were revealed by combining fluorescent count and plate count results as explained above. The assay could be applied to various further purposes. For example to study the effect of prolonged storage on probiotic products.

Use of cF/TOTO as viability assay is by no means restricted to lactic acid bacteria. It may be applied to other species as well. The novel cF/TOTO combination may be considered as an alternative of the *Bac*Light bacterial viability kit for flow cytometry analysis.

General concluding remarks

The fluorescent probes used in this study mainly assessed membrane integrity. Other physiological parameters, such as membrane potential, intracellular pH and gene expression can also be measured by fluorescent probes. Thus, various cellular functions can be studied by fluorescent techniques. The power of flow cytometry is that it performs single-cell multi-parametric analysis, that it is rapid, and

sensitive. Flow cytometry enables the study of microbes as a heterogeneous population of individual cells. Flow cytometry has found application in all fields of microbiology and a continuing rise of its use is anticipated.

In the line of research described in this thesis various potential applications may be recognized. Fluorescent viability assessment can be used for analysis of probiotic bacteria as well as fermentation starter bacteria. For example for the screening of probiotic strains for acid and bile tolerance, development of new products and improvement of the production processes. Furthermore, viability assessment may be combined with specific detection using FISH techniques. This would allow for study of population dynamics in starter cultures and for the effect of stress conditions on the various species in a mixed starter culture. The combination of species specific detection and viability assessment also has great potential for the study of food pathogens. Finally, viability assessment may be used in online monitoring of a fermentation process by flow cytometry. This may be done with an integrated and automated system. Online monitoring by a flow cytometry system will be a valuable to provide detailed knowledge about fermentation processes and it is a sophisticated tool that allows direct regulation of process conditions in order to optimize the production.

8

Samenvatting:
Het wel en wee van de bacteriën in de zuivelindustrie en hoe je dat meet

door Wieger Wamelink en Christine Bunthof

Introductie

Eén van de belangrijkste dingen die een mens doet, elke dag weer, is het eten en drinken van voedsel. Zonder drinken houdt een mens het een paar dagen uit, zonder eten iets meer dan een maand. Terecht behoort voedsel dan ook tot de eerste levensbehoeften; eigenlijk is het de belangrijkste levensbehoefte. Een groot deel van het leven van de meeste mensen staat in het teken van het verkrijgen van voedsel. Direct door de productie en verwerking ervan, of indirect door het verkrijgen van geld om het te kunnen kopen. Het is daarom logisch dat er veel geld wordt gestoken in onderzoek naar de productie, verbetering en veiligheid van voedsel.

Inhoud van het proefschrift

Het proefschrift begint met een algemene introductie over bacteriën, fluorescentie en flow cytometrie. Het eerste hoofdstuk beschrijft ook de geschiedenis van de microscopie en flow cytometrie in de microbiologie. Hoofdstuk 2 geeft een overzicht hoe je met fluorescente kleurstoffen bacteriën kunt onderzoeken en hoe je dat kunt toepassen in de levensmiddelenmicrobiologie. Dit hoofdstuk bevat een samenvatting van de literatuur. De hoofdstukken 3 tot en met 6 beschrijven de experimenten die in het kader van dit onderzoek zijn uitgevoerd. In Hoofdstuk 3 staat beschreven hoe de bacteriesoort *Lactococcus lactis* reageert op stress en hoe je dat op een snelle manier kunt meten met behulp van fluorescente technieken. Hoe je kunt meten wat er met de bacteriën tijdens het maken van kaas gebeurt staat beschreven in Hoofdstuk 4. Het thema van Hoofdstuk 5 is hoe je met behulp van fluorescente kleurstoffen kunt bepalen of een bacterie dood of levend is. In Hoofdstuk 6 is met behulp van de flow cytometer geteld hoeveel bacteriën de probiotische producten Yakult, Mona Vifit en Orthiflorplus bevatten en bepaald of de bacteriën intact of beschadigd zijn. Ditzelfde is bepaald van bacteriemengsels gebruikt bij de productie van Goudse kaas en yoghurt.

Al deze hoofdstukken hebben gemeen dat ze gaan over het onderzoeken van bacteriën, waarbij gebruik wordt gemaakt van fluorescente kleurstoffen en een elektronisch apparaat: de flow cytometer. In de onderstaande paragrafen wordt daarom eerst ingegaan op wat bacteriën zijn en wat ze doen, hoe in dit onderzoek met fluorescente kleurstoffen is gewerkt en wat een flow cytometer is. Daarna wordt van elk van de vier onderzoeken een samenvatting gegeven.

Wat zijn bacteriën?

Een bacterie is een levend organisme dat met het blote oog niet kan worden waargenomen. De grootte varieert van 0,5 tot 5 µm (1 µm is éénduizendste millimeter). Afzonderlijke bacteriën zijn alleen met een microscoop waarneembaar. Daarom werden bacteriën pas ontdekt na de uitvinding van de microscoop en voor het eerst beschreven door Antonie van Leeuwenhoek (1632-1723), die het overigens over *animalculi* had. Hij beschreef niet alleen wat wij later bacteriën zouden noemen, maar de groep van organismen waar de bacteriën toe behoren; de micro-organismen. Hieronder vallen ook de gisten, schimmels, algen en protozoa (pantoffeldiertjes). Al gauw werd duidelijk dat er ontelbaar veel bacteriën zijn, dat er veel verschillende soorten zijn en dat ze overal voorkomen. Een theelepel zand bevat al gauw miljarden bacteriën. Bacteriën komen voor in en op het menselijk lichaam (zo'n 100 biljoen). Maar ze komen ook voor in extreme milieus waar geen ander leven mogelijk is, zoals bij

vulkanen bij zeer hoge temperaturen en diep in oceanen onder enorme druk. En bacteriën zijn nog ergens anders kampioen in: zij waren de eerste levende organismen op aarde.

Bacteriën zijn eencellige organismen. Ze hebben een celwand, die de binnenkant (het cytoplasma) beschermt en de vorm van de bacterie stabiel houdt. Tussen het cytoplasma en de celwand zit het celmembraan. Ook deze biedt bescherming tegen de buitenwereld en wordt gebruikt als aanhechtingsplaats voor allerlei eiwitten. Het celmembraan 'regelt' welke stoffen in en uit de bacterie kunnen. Zo kunnen voedingsstoffen het celmembraan passeren en gaat afval naar buiten, maar worden gifstoffen buitengesloten. Als het membraan wordt beschadigd en lek raakt verliest de bacterie snel allerlei stoffen en de energievoorraad raakt dan snel uitgeput, waardoor de bacterie dood gaat. Het handhaven van een intact membraan is dus erg belangrijk voor het kunnen functioneren van de bacterie. In het cytoplasma worden voedingsstoffen omgezet in energie en bouwstenen door eiwitten die als kleine fabriekjes voor het functioneren van de bacterie zorgen (enzymen). Het genoom (drager van de erfelijke eigenschappen; het bouwplan van de cel) van de bacterie bevindt zich in het cytoplasma als een cirkelvormige DNA keten. Daarnaast kunnen bacteriën ook nog DNA in plasmiden hebben. Dit zijn kleine ronde stukjes DNA die uitgewisseld kunnen worden met andere bacteriën. Plasmiden kunnen in veelvoud aanwezig zijn in een bacterie. Een schematische voorstelling van een bacterie wordt gegeven in figuur 1.

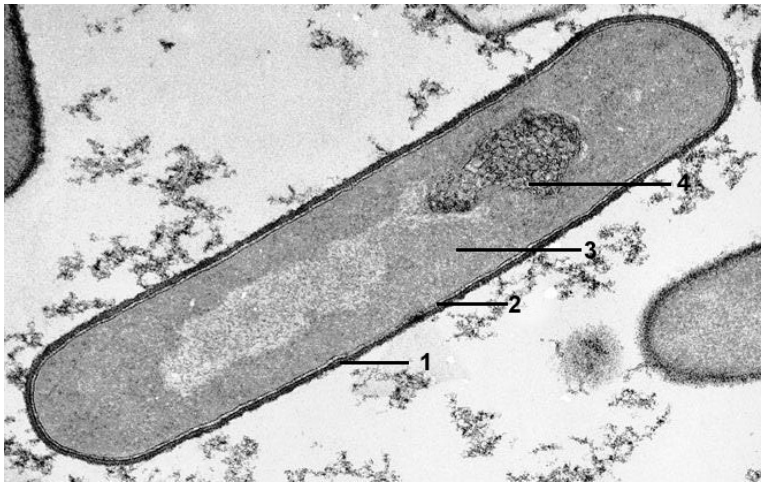

Fig. 1. Microscopische vergroting van een *E coli* bacterie. 1 = celwand, 2 = celmembraan, 3 = cytoplasma en 4 = DNA.

Zoals gezegd, individuele bacteriën zijn onzichtbaar voor het blote oog, maar toch kun je bacteriën zichtbaar maken en tellen zonder dat daar een microscoop voor nodig is. Dat heeft te maken met de manier waarop bacteriën zich vermeerderen. Anders dan bij de meeste planten en dieren planten bacteriën zich niet voort via seks, maar via deling. Daarbij splitst een bacterie zich daadwerkelijk in tweeën en ontstaan er twee nieuwe bacteriën uit één oude. De oude bacterie gaat dus niet dood, maar gaat over in twee nieuwe organismen. Als er voldoende voedingstoffen zijn dan zullen er vele delingen volgen. Op een vaste voedingsbodem zal er uit één bacterie een hele kolonie ontstaan en na verloop van tijd is de kolonie zo groot dat ze met het blote oog zichtbaar is. Dit kan bijvoorbeeld gebeuren als eten te lang buiten de koelkast wordt bewaard. Na verloop van tijd worden er micro-

organismen zichtbaar, een overduidelijk signaal dat het voedsel beter niet meer kan worden gegeten (zie fig. 2).

Bij celdeling wordt er geen genetisch materiaal (DNA) uitgewisseld met andere bacteriën, iets dat bijna alle planten en alle dieren wel doen. Kunnen bacteriën dan geen genetisch materiaal uitwisselen? Ja, dat kunnen ze wel, namelijk door middel van de plasmiden. De genen op de plasmiden kunnen bijvoorbeeld informatie bevatten over hoe een bacterie een antibioticum zoals penicilline kan weerstaan. Wanneer zulke plasmiden worden uitgewisseld met andere bacteriën worden die ook resistent tegen het antibioticum. Daarnaast kan het DNA van een bacterie veranderen door een mutatie. Vaak is zo'n mutatie ongunstig en gaat de bacterie dood. Soms is zo'n mutatie gunstig en heeft de bacterie er voordeel van. De bacterie zal het dan beter doen en zich sneller vermeerderen dan andere bacteriën en die langzamerhand weg concurreren (natuurlijke selectie).

Fig. 2. Tortellini bedorven door micro-organismen.

Wat doen bacteriën?

Net als bij alle andere organismen is alle activiteit van bacteriën gericht op het voortbestaan van de soort. De bacterie neemt voedingsstoffen op en scheidt afvalstoffen uit. Beschadigingen aan de bacterie worden gerepareerd, nieuwe onderdelen worden gemaakt, van één bacterie worden er twee gemaakt, enzovoort. Daar waar bacterie en mens met elkaar in aanraking komen hebben bacteriën zowel positieve als negatieve effecten.

Negatief voor ons is het als bacteriën van ons voedsel eten en het daardoor bederven. Wanneer dit bederf veroorzaakt wordt door ziekteverwekkende bacteriën kan dat in sommige gevallen zelfs levensgevaarlijk zijn. Denk bijvoorbeeld aan voedselinfectie veroorzaakt door het eten van met *Salmonella* besmette bavarois, wat de dood tot gevolg kan hebben. Dysenterie wordt veroorzaakt door voedselvergiftiging met de bacteriesoort *Shigella*. Een ander voorbeeld is *E. coli* O157:H7 die mensen binnen kunnen krijgen als vlees (bijvoorbeeld hamburgers) te rauw gegeten wordt, bijvoorbeeld met

barbecuen. Minder ernstige effecten als misselijkheid en diarree zijn ook vaak terug te voeren op het eten van bedorven voedsel. Ziekteverwekkende bacteriën kunnen dus via bedorven voedsel ons lichaam binnen komen en zich daar gaan vermeerderen. Ziekteverwekkende bacteriën kunnen ons ook op andere manieren infecteren zoals via drinkwater of via de lucht. Uiteraard is zo'n infectie geen gezonde situatie en ons lichaam zal reageren door het immuunsysteem te activeren en een tegenaanval lanceren. Deze toestand, die als een staat van oorlog in ons lichaam kan worden omschreven, ervaren wij als ziek zijn. Het is ook mogelijk dat we ziek worden door stoffen die de bacterie produceert en die schadelijk zijn voor ons lichaam (toxines). De bacterie of zijn afval vergiftigt als het ware ons lichaam.

Van het grote aantal bacteriesoorten zijn er eigenlijk maar relatief weinig die ziektes verwekken. Er zijn ook heel veel positieve effecten van bacteriën. Sterker nog, zonder bacteriën zouden wij niet

Fig. 3. Voedsel dat met behulp van micro-organismen is geproduceerd. Het voedsel wordt daardoor langer houdbaar, makkelijker verteerbaar en wellicht lekkerder.

kunnen leven. Bacteriën spelen een belangrijke rol bij de afbraak van dode planten en dieren. Zij zorgen ervoor dat voedingstoffen weer beschikbaar komen voor bijvoorbeeld planten. Zonder dit proces zou al het dode materiaal niet verteren, maar zich ophopen en er zou uiteindelijk geen voedsel meer beschikbaar zijn voor planten. Hierdoor zouden dieren niets meer te eten hebben en uiteindelijk ook de bacteriën zelf niets meer te eten hebben en als laatste uitsterven. Wat er over zou blijven is de aarde met een gigantische berg 'afval'. Kortom, bacteriën zijn een essentieel onderdeel van de kringloop op aarde.

De mens heeft ook direct op een positieve manier met bacteriën te maken. Verschillende soorten bacteriën worden door de mens gebruikt voor de productie van voedsel. De productie van voedsel valt grofweg in twee groepen uiteen: het telen van planten voor directe consumptie (primaire productie) en het fokken van dieren voor de productie van vlees (secundaire productie). Voor de productie van vlees wordt gebruik gemaakt van de eetbare delen van planten als voedsel voor de dieren. In de productie van heel wat voedselproducten wordt gebruik gemaakt van micro-organismen (fig. 3). Zij kunnen

zowel plantaardige als dierlijke producten omzetten in producten die voor ons beter verteerbaar en langer houdbaar zijn. Bekende voorbeelden hiervan zijn de productie van bier uit granen door gist, de productie van wijn uit druiven door gist en de productie van yoghurt en kaas uit melk door melkzuurbacteriën. In dit proefschrift is veel onderzoek gedaan met en naar de melkzuurbacterie *Lactococcus lactis*. Deze bacteriesoort is een belangrijke component in starterculturen die worden gebruikt voor de productie van kaas (fig. 4).

Een ander positief effect is dat bacteriën in ons lichaam helpen bij de vertering van voedsel in de darm. In de darm komen zeer veel bacteriesoorten voor, zowel 'goede' als 'slechte'. Bij een goede balans voorkomen de goede bacteriën dat de slechte bacteriën veel kans krijgen en bijvoorbeeld diarree veroorzaken. Bacteriën kunnen ook andere gunstige eigenschappen hebben, zoals het stimuleren van de immuunrespons en betrokken zijn bij de preventie van darmkanker. Bacteriën met dergelijke gunstige eigenschappen worden probiotische bacteriën genoemd. De laatste tijd is er een trend producten met probiotische bacteriën toe te voegen aan onze voeding, wat tot een betere gezondheid zou leiden. Voorbeelden hiervan zijn Yakult en Mona Vifit (fig. 4).

Fig. 4. Een kaasstarter en twee probiotische producten.

Dead or alive? Hoe meet je dat bij een bacterie?

Het vaststellen van het dood of levend zijn, is voor dieren niet zo moeilijk, voor planten kan dat al wat lastiger zijn, maar voor bacteriën is dat vrij lastig. Dit komt deels omdat bacteriën zo klein zijn. Ze kunnen wel onder de microscoop worden bekeken, maar dan zie je nog niet direct of ze dood of levend zijn. Bacteriën die delen en kolonies vormen zijn duidelijk levend. Soms kunnen bacteriën niet meer delen, maar nog wel actief zijn en bijvoorbeeld fermentatie processen uitvoeren of gifstoffen produceren. Ook is het mogelijk dat bacteriën tijdelijk op non-actief gaan, om bijvoorbeeld ongunstige omstandigheden te kunnen overleven (een soort winterslaap). Wanneer de omstandigheden verbeteren, kunnen zulke bacteriën weer actief worden en gaan delen. Het onderscheid tussen levende en dode bacteriën is dus een complexe zaak. In veel toepassingen is vooral de activiteit van bacteriën van belang en gaat het erom of ze goed functioneren bijvoorbeeld bij de fermentatie van voedsel of bij gebruik als probioticum.

Een belangrijk deel van onze voedingsindustrie is afhankelijk van de goede werking van bacteriën. Het is daarbij belangrijk om van tevoren en tijdens het productieproces te meten hoe goed de bacteriën het doen. Ook bij het toepassen van bacteriën als probioticum willen we graag weten of ze wel leven en of ze doen wat er van ze verwacht wordt. Al meer dan een eeuw is hiervoor een methode beschikbaar. De te onderzoeken bacteriecultuur wordt op een voedingsbodem gebracht.

Bacteriën die delen vormen na verloop van tijd kolonies die met het blote oog zichtbaar zijn en dus kunnen worden geteld (zie fig. 5). De bacteriecultuur is hiervoor zover verdund dat elke kolonie uit slechts één enkele bacterie is ontstaan. Het aantal delende bacteriën in de onderzochte bacteriecultuur kan dan worden berekend. Deze methode is goed beschreven, makkelijk en wordt al heel lang gebruikt. Er kleven echter ook een aantal belangrijke nadelen aan plaattellingen. Het duurt lang voordat de uitslag van de test bekend is (minimaal een dag) en het is bewerkelijk. Misschien wel het belangrijkste nadeel is dat je met plaattellingen alleen delende bacteriën kunt aantonen en niet alle actieve bacteriën. Hierboven is al beschreven hoe belangrijk het verschil kan zijn tussen delende en niet delende maar wel actieve bacteriën. Onderzoek met behulp van fluorescente kleurstoffen en flow cytometrie biedt een aantrekkelijk alternatief. Dat is snel en nauwkeurig, individuele cellen kunnen worden bekeken en verschillende eigenschappen van bacteriën kunnen worden gemeten.

Fig. 5. Voedingsbodem van agar met voedingstoffen voor de kweek van bacteriën. De 'puntjes' zijn de bacteriekolonies.

Hoe werkt fluorescentie en wat kun je er mee?

Fluorescentie is kortweg het oplichten van een stof in een andere kleur dan het licht dat er op schijnt. Dit oplichten van de stof gebeurt met een zeer kleine vertraging. Fluorescentie lijkt heel veel op fosforescentie waarbij ook licht wordt uitgestraald, maar met meer vertraging. Bij fluorescentie en fosforescentie wordt de energie die het licht bevat als het ware opgenomen door de stof en later weer afgegeven, ook weer in de vorm van licht. Omdat hierbij energie verloren gaat heeft het uitgezonden licht een andere kleur dan het licht waarmee beschenen wordt. Als voorbeeld voor fosforescentie kun je denken aan figuurtjes die in kinderkamers aan de muur worden geplakt en in het donker nog een tijdje licht blijven geven. In de mergelgrotten in Zuid-Limburg kan op sommige plekken hetzelfde worden gezien.

Er zijn veel fluorescente stoffen bekend met elk zijn karakteristieke eigenschappen, zoals de kleur licht die de stof absorbeert en de kleur licht die het uitzendt. Er zijn verschillende fluorescente stoffen die biologisch materiaal kleuren, bijvoorbeeld stoffen die aan DNA of eiwitten binden en het

daardoor fluorescent maken. Ook zijn er stoffen die eerst door enzymen moeten worden omgezet om fluorescent te worden. Bacteriën kunnen worden gekleurd met verschillende fluorescente stoffen waardoor verschillende eigenschappen van de bacteriën onderzocht kunnen worden. Fluorescerende bacteriën kunnen met een fluorescentiemicroscoop worden bekeken (zie fig. 1 in Hoofdstuk 5).

Bepaalde fluorescente stoffen kunnen door bacteriën afhankelijk van hun conditie opgenomen of buiten gehouden worden of in de bacteriën al dan niet worden omgezet. Bacteriën die een fluorescente kleurstof hebben opgenomen fluoresceren als de geschikte kleur licht er op wordt geschenen. Bacteriën die geen kleurstof hebben opgenomen fluoresceren dus niet. Zo valt er onderscheid te maken tussen bacteriën en kan er ook iets worden gezegd over hun toestand. Door een geschikte combinatie van fluorescente stoffen te gebruiken kunnen bijvoorbeeld zowel de actieve als de niet-actieve bacteriën gekleurd worden, waarbij de actieve in een andere kleur oplichten dan de niet-actieve bacteriën. Met een fluorescentiemicroscoop kan dit dan worden bekeken en gefotografeerd. Met een flow cytometer kan heel snel de fluorescentie van veel afzonderlijke bacteriën gemeten worden en kunnen de actieve en de niet-actieve bacteriën worden onderscheiden en geteld.

Wat is een flow cytometer en hoe werkt het?

In figuur 6 is een flow cytometer afgebeeld. Om duidelijk te maken hoe een flow cytometer werkt wordt hieronder beschreven welke weg een bacterie aflegt door de flow cytometer en hoe de meting ervan in zijn werk gaat. Een vloeistof met bacteriën behandeld met een fluorescente kleurstof wordt aangeboden aan het apparaat. Laten we aannemen dat een deel van de bacteriën de fluorescente stof heeft opgenomen en dat de bacterie die we volgen er zo één is. Via een dunne naald wordt continu vloeistof opgezogen. De vloeistof bevat zeer veel bacteriën en de flow cytometer meet elke bacterie afzonderlijk. Als onze bacterie is opgezogen gaat hij een weg afleggen door het apparaat. De vloeistof met bacteriën wordt meegenomen door een ‘loopvloeistof’ én daardoor in een heel smal en heel snel stromend straaltje gedwongen. Daarom is onze bacterie meestal niet omgeven door andere bacteriën. Die zitten een stukje voor of achter hem, maar niet vlakbij of naast hem. De vloeistofstroom komt langs een laser die een bepaalde kleur licht geeft. Onze bacterie, die de kleurstof heeft opgenomen fluoresceert dan. De bacteriën die de kleurstof niet hebben opgenomen fluoresceren niet wanneer ze de laser passeren.

De flow cytometer heeft detectoren die lichtbreking meten en daardoor alle bacteriën die de laser passeren meten en andere detectoren die specifiek de fluorescentie meten. De lichtbreking en de fluorescentie van onze bacterie wordt opgevangen door de detectoren en gemeten, waarna er signalen worden afgegeven aan de computer. De computer houdt bij hoeveel bacteriën er voorbij komen en wat de fluorescentie van elke bacterie was. Als er nu twee fluorescente bacteriën tegelijk langs de laserstraal zouden komen dan zou dit slechts als één bacterie worden gemeten door de detectoren, omdat deze geen onderscheid kunnen maken tussen een of meerdere bacteriën die tegelijkertijd een lichtsignaal geven. Na de detectie kan de flow cytometer fluorescerende bacteriën zoals de onze, nog scheiden van de niet-fluorescerende bacteriën (sorteren). Na de plek waar de bacteriën de laser passeren wordt dan op het juiste moment heel even een opvangbuisje in de stroom geplaatst. Zo kan onze fluorescente bacterie in een potje voor fluorescerende bacteriën opgevangen worden.

Fig. 6. De flow cytometer, 'FACSCalibur' die gebruikt is in dit onderzoek. 1 = monsters, 2 = naald voor de monsteropname, 3 = lade een containers voor loopvloeistof en afvalvloeistof, 4 = laser, 5 = meetcel, 6 = sorteerbuisjes, 7 = controlepaneel, 8 = elektronica, 9 = kap (normaal dicht) en 10 = computer voor de aansturing van de flow cytometer en de data verwerking.

Is het effect van stress op de bacterie *Lactococcus lactis* te meten met fluorescente technieken?

Lactococcus lactis is een melkzuurbacterie die gebruikt wordt in de zuivelindustrie. Deze bacterie wordt ook veel gebruikt in microbiologisch onderzoek. Zuivelbacteriën krijgen vaak te maken met ongunstige, stressvolle omstandigheden, die zij moeten zien te overleven om hun werking te kunnen blijven houden. Voor opslag worden bacteriën vaak gedroogd of bevroren, tijdens de fermentatie staan bacteriën bloot aan veel zuur (lage pH), bij het rijpen van kaas is de temperatuur laag en is de zoutconcentratie hoog. Voor probiotische bacteriën geldt dat ze eerst het zuur in onze maag moeten overleven en daarna de hoge concentratie gal in de dunne darm om de dikke darm te bereiken en hun werk te kunnen doen.

In dit onderzoek is bekeken of je met fluorescente technieken kunt bepalen hoeveel bacteriën overleven bij verschillende soorten stress. In dit onderzoek is gebruik gemaakt van de fluorescente stoffen carboxyfluoresceine diacetaat (cFDA) en propidiumjodide (PI). cFDA zelf is niet fluorescent, maar in de bacterie wordt het door enzymen die esterverbindingen splitsen (esterases) omgezet in de groen fluorescerende stof carboxyfluoresceine (cF). Bacteriën met enzymactiviteit en een intact membraan worden fluorescent bij het gebruik van cFDA. Als het membraan beschadigd is lekt het door enzymen geproduceerde cF naar buiten en wordt de bacterie niet fluorescent. PI kan in principe alleen een bacterie binnen komen als het membraan niet meer intact is. Het bindt zich aan DNA en de bacterie fluoresceert dan rood. cFDA en PI kleuren dus bacteriën met respectievelijk intacte en lek

geraakte membranen. Vaak wordt een intact membraan gezien als een indicatie dat de bacterie levend is. Wanneer cFDA en PI worden toegevoegd dan zouden alle bacteriën fluorescent moeten worden, de levende door cF (intact membraan), de dode door PI (lek membraan).

In dit onderzoek bleek echter dat deze manier van bepalen of bacteriën levend zijn niet onder alle condities overeenkomt met de groei van kolonies op een voedingsbodem. Een intact membraan, aangetoond door kleuring van de bacterie met cF, bleek niet zo'n algemene indicator voor het kunnen groeien en delen te zijn als men gebruikelijk aanneemt. Bacteriën gedood bij 70^0C gaven wel het verwachte resultaat, ze werden niet gekleurd door cF, maar wel gekleurd door PI. Maar wanneer bacteriën waren gedood bij 50 à 60^0C bleek hun membraan nog steeds intact te zijn en konden de levende bacteriën niet door cF of PI worden onderscheiden van de dode.

Om de bruikbaarheid van cF en PI verder te testen werden de bacteriën ook blootgesteld aan verhitting, bevriezing, zuur of galzouten. Het effect van deze stresscondities op de bacteriën is onderzocht door het uitplaten op voedingsbodem (bepaling van de delingscapaciteit), de meting van verzuring van melkmedium na 6 uur bij 30^0C (verzuringcapaciteit van de bacteriën), meting van de beginsnelheid van verzuring (maat voor de glycolitische activiteit), fluorescente kleuring door cF (intact membraan) en fluorescente kleuring door PI (lek membraan) en tenslotte de bepaling van het naar buiten kunnen pompen van cF door de bacteriën (cF efflux). Voor het naar buiten pompen van cF heeft de bacterie energie nodig. Daarom veronderstellen we dat cF efflux gebruikt kan worden om te testen of de bacterie energie kan produceren als indicatie voor het actief zijn.

De verzuringcapaciteit kwam goed overeen met de plaattellingen. De glycolitische activiteit was gevoeliger voor stress dan de delings- en verzuringcapaciteit. Onder alle condities, zelfs na doding bij 70^0C bleek er voldoende esterase activiteit te zijn voor omzetting van cFDA in cF. De kleuring door cF bleek eigenlijk alleen maar afhankelijk van het intact zijn van het bacteriemembraan. Kleuring met cF, het buiten houden van PI en de interne cF, na toedienen van cFDA, concentratie bleken geen algemeen bruikbare indicatoren te zijn voor de delingscapaciteit of de verzuringcapaciteit van de bacteriën. Hiervoor bleek een methode met twee stappen nodig te zijn. In deze nieuwe methode wordt eerst bepaald wat het percentage intacte bacteriën is door middel van cF kleuring. Daarna wordt bepaald hoeveel van deze gekleurde bacteriën het cF naar buiten kunnen pompen. Daarvoor wordt aan de bacteriën een suiker gegeven zodat ze energie kunnen produceren en wordt gemeten hoeveel van het cF uit de bacteriën wordt gepompt. Deze nieuwe gecombineerde methode bleek een goede en snelle indicator te zijn voor het bepalen van de delings- en verzuringcapaciteit van melkzuurbacteriën.

Hoe meet je lysis van zuivel starter culturen met fluorescentie?

Net als in het hierboven beschreven onderzoek is gebruik gemaakt van de melkzuurbacterie *L. lactis* als modelbacterie voor melkzuurstarters. Melkzuurbacteriën worden bij de productie van kaas gebruikt, ze brengen als het ware het eerste proces van het kaasmaken op gang (vandaar de naam starter). De melkzuurbacteriën zetten het in de melk aanwezige melksuiker (lactose) om in melkzuur. Later, tijdens het rijpen van de kaas, zorgen de starterbacteriën voor de productie van smaakstoffen. Tijdens deze essentiële stap in de kaasproductie lyseren de bacteriën. Hierbij gaan de membranen en celwanden van de bacteriën kapot en er wordt verondersteld dat daardoor de enzymen naar buiten

lekken en daar, dus buiten de bacterie, hun werk kunnen doen. De mate en snelheid van lysis en de smaak van de kaas is afhankelijk van welke melkzuurbacteriën er worden gebruikt. De lysis wordt veroorzaakt door de processen gedurende het maken van kaas en het rijpingsproces.

In dit onderzoek is de lysis van melkzuurbacteriën onderzocht met fluorescente stoffen. Hierbij is gebruik gemaakt van flow cytometrie en fluorescentie microscopie. De meeste metingen zijn gedaan aan bacteriën in een oplossing die de condities in kaas zo goed mogelijk nabootsten. Door te werken met een vloeibaar model systeem kon flow cytometrie gebruikt worden en konden gemakkelijk bacteriën worden uitgeplaat en enzymactiviteiten worden bepaald. Met de fluorescente stoffen PI en SYTO 9 is het lek raken en het uiteenvallen van de bacteriën gemeten. PI werd ook in het vorige onderzoek gebruikt en zoals in de vorige paragraaf uitgelegd, kleurt het beschadigde bacteriën rood. Wanneer samen met PI SYTO 9 wordt gebruikt kleurt dat de overige bacteriën groen. Dit maakt het tellen van het totale aantal bacteriën en het onderscheid tussen intacte en beschadigde bacteriën gemakkelijk.

Om lysis op gang te brengen is het enzym mutanolysine toegevoegd, dat de celwand van de bacterie afbreekt. Hierdoor nam in een gewone buffer het aantal bacteriën snel af en kwamen enzymen vrij. Wanneer echter de kaasomstandigheden werden benaderd door een hoge concentratie eiwitten, magnesium en kalium (stabiliserende buffer) nam het aantal bacteriën veel langzamer af. Deze buffer stabiliseerde vooral de bacteriën met een beschadigd membraan en voorkwam daarmee dat de door mutanolysine beschadigde bacteriën direct verder lyseerden en verdwenen. De beschadigde bacteriën konden zich hierdoor geruime tijd handhaven. Kleuring van een groot aantal van de aanwezige bacteriën met PI liet dit zien. Met flow cytometrie kan gemakkelijk het aantal intacte en gepermeabiliseerde (lekke) melkzuurbacteriën in oplossing worden bepaald. Kleuring met PI en SYTO 9 bleek ook geschikt te zijn om de permeabilisatie van bacteriën in kaas zelf te analyseren (zie fig. 4 in Hoofdstuk 4). Hierbij werd een plakje kaas die nog maar twee weken had gerijpt gekleurd met de fluorescente stoffen en bekeken met confocale laser scanning microscopie.

Dit onderzoek heeft laten zien dat het met fluorescente technieken mogelijk is om het rijpen van kaas beter te volgen en te onderzoeken.

Is flow cytometrie geschikt om te bepalen of melkzuurbacteriën levend of dood zijn?

In de paragraaf over de flow cytometer is de noodzaak voor een alternatieve methode voor het uitplaten van bacteriën al genoemd. Samenvattend is het bezwaar tegen de uitplaatmethode dat het lang duurt voordat er een resultaat bekend is en dat fysiologisch actieve, maar niet delende bacteriën niet kunnen worden gedetecteerd. Flow cytometrie geeft in principe de mogelijkheid voor een snellere en nauwkeurigere detectie; het kan meer informatie geven over de bacteriën. In dit onderzoek is dat getest aan de hand van negen verschillende melkzuurbacteriesoorten. De fluorescente stoffen die getest zijn, zijn cFDA dat intacte bacteriën groen kleurt, PI dat permeabele bacteriën rood kleurt en TOTO-1 (TOTO-1 jodide) dat in principe werkt zoals PI maar geel fluoresceert. Flow cytometrie tellingen zijn vergeleken met plaattellingen.

Delende bacteriën en bij 70^0C gedode bacteriën zijn gebruikt om de geschiktheid van flow cytometrie te testen. De verwachting was dat dode cellen door PI en TOTO-1 worden gekleurd en dat

de flow cytometer deze kan onderscheiden van de levende bacteriën die door cFDA worden gekleurd. cFDA bleek inderdaad zeer geschikt om onderscheid te maken tussen dode en levende cellen; dode bacteriën werden niet gekleurd, levende bacteriën wel. Ook TOTO-1 gaf zeer goede resultaten, dode cellen werden gekleurd en levende cellen niet. PI daarentegen was niet bij alle melkzuurbacteriën goed in staat om onderscheid te maken tussen dode en levende cellen. Het beste resultaat gaf een combinatie van cFDA en TOTO-1. De flow cytometer had geen enkele moeite om de dode en levende bacteriën met deze combinatie te onderscheiden. De populaties met cFDA en TOTO-1 gekleurde bacteriën zijn ook met de flow cytometer gesorteerd om ze daarna uit te platen. Uiteraard was de verwachting dat de met cFDA gekleurde populatie wel deelt en kolonies vormt en de populatie die door TOTO-1 gekleurd wordt niet deelt en dus geen kolonies vormt. De plaattellingen gaven duidelijk aan dat dit het geval was.

De kleuring met cFDA en TOTO-1 is daarna toegepast op bacteriën die waren blootgesteld aan verschillende concentraties zoutzuur (pH 6, 5, 4, 3 en 2) of aan gal (gedeconjugeerde galzouten in een concentratie van 0,05, 0,10, 0,25, 0,50 en 1,00 %). De flow cytometrie resultaten zijn vergeleken met plaattellingen. Bacteriën gingen niet dood bij pH 3 of hoger, maar bij pH 2 wel. Na blootstelling aan deze hoge concentratie zuur konden ze niet meer delen en kolonies vormen. In overeenstemming hiermee liet de fluorescente kleuring zien dat alleen bij pH 2 het membraan lek was geraakt. Bij een oplopend galzoutconcentratie gaan er steeds meer bacteriën dood en ook dit kwam goed overeen met de flow cytometrie resultaten. De conclusies van dit onderzoek waren dat (1) cFDA en TOTO-1 te gebruiken zijn als indicator voor dode en levende bacteriën, ook na blootstelling van bacteriën aan zoutzuur of aan gedeconjugeerde galzouten, (2) de flow cytometer zeer geschikt is om onderscheid te maken tussen cFDA en TOTO-1 gekleurde bacteriën en (3) flow cytometrie het voordeel heeft veel sneller te zijn dan plaattellingen: de benodigde tijd voor een flow cytometrie experiment is slechts ongeveer 1 uur, terwijl bij uitplaten twee à drie dagen incubatietijd nodig is.

Kunnen bacteriën in zuivelstarter culturen en probiotische producten met flow cytometrie onderzocht worden?

In het laatste onderzoek is de kleuringsmethode die beschreven is in de vorige paragraaf gebruikt om bacteriën te detecteren in melk, probiotische producten en starterculturen. In melk zitten onder andere minuscule vetbolletjes en cellen afkomstig van de uier van de koe. Zulke componenten storen de detectie van bacteriën door de flow cytometer. Met behulp van een detergens (‘zeep’) kunnen zulke componenten worden verwijderd. In dit onderzoek is gebruik gemaakt van een commercieel verkrijgbare ‘milk clearing solution’ die onder andere een zeep bevat. Bij het gebruik volgens het meegeleverde standaard voorschrift bleek er echter toch nog veel achtergrondsignaal (storende ruis) van melkcomponenten te zijn. Daarom is eerst de zuiveringsmethode verbeterd zodat het achtergrondsignaal werd teruggebracht tot een aanvaardbaar niveau. Hierbij is natuurlijk belangrijk dat deze zuiveringstap niet ten koste gaat van de activiteiten van de bacteriën, maar dat bleek niet het geval.

Een interessant resultaat voor zowel de probiotische producten als de zuivelstarters was dat het aantal door de flow cytometer getelde ‘levende’ bacteriën behoorlijk hoger was dan het aantal delende

Tabel 1. aantal bacteriën (in miljoenen per ml) in probiotische producten geteld door middel van uitplaten op een voedingsbodem en door middel van flow cytometrie.

Product	plaattellingen	telling door de flow cytometer		
		cFDA	TOTO-1	totaal
Orthiflorplus[a]	9	20	23	43
Yakult[b]	476	1780	55	1835
Mona Vifit[c]	19	985	143	1128

[a]Orthiflorplus is een product met vijf probiotische bacteriesoorten in poedervorm. Een zakje poeder (3 gram) is in 100 ml water gedaan en na goed roeren gebruikt in de experimenten.
[b]Yakult bevat de probiotische bacterie *Lactobacillus casei* Shirota.
[c]Mona Vifit yoghurt drink bevat yoghurt starter cultuur bacteriën en drie probiotische bacteriesoorten, waaronder *Lactobacillus rhamnosus* Goldin and Gorbach (LGG).

bacteriën bepaald met plaattellingen. Dit duidt erop dat een deel van de bacteriën activiteit vertonen, maar niet meer kunnen delen. Door het gebruik van plaattellingen en flow cytometrie tellingen met cFDA en TOTO-1 kleuring konden drie functionele groepen van bacteriën worden onderscheiden:

1. Bacteriën die kunnen delen en dus kolonies vormen.
2. Bacteriën die nog wel intact zijn en activiteit vertonen maar niet meer kunnen delen en dus geen kolonies meer vormen.
3. Bacteriën met kapotte celmembranen die dus dood zijn en geen activiteit meer vertonen.

Het is aannemelijk dat de tweede groep nog wel een bijdrage levert aan de fermentatie (i.g.v. zuivelstarters) of aan de gezondheidsbevorderende effecten (i.g.v. probiotische producten). Dit laat nog maar weer eens zien dat flow cytometrie iets extra's te bieden heeft ten opzichte van plaattelingen. Met plaattelingen wordt de tweede groep bacteriën niet opgemerkt, terwijl die nog wel veel invloed kan hebben en een belangrijk deel vormt van de bacteriën die aanwezig zijn in de onderzochte producten.

Tabel 1 laat de resultaten zien van de probiotische producten. Het aandeel van de verschillende functionele groepen verschilt tussen de producten. De plaattellingen laten zien dat alle producten op zich voldoende levende bacteriën bevatten om een werkzaam probioticum te kunnen zijn. Van de bacteriën in Orthiflorplus kleurt ongeveer de helft met cFDA en de andere helft met TOTO-1, wat dus betekent dat ongeveer de helft van de bacteriën intact is. Het aantal intacte bacteriën is ongeveer tweemaal zo groot als het aantal delende bacteriën. Voor Yakult geldt dat de meeste bacteriën kleuren met cFDA en dus voor het overgrote deel intacte bacteriën bevat. Het aantal intacte bacteriën is ongeveer viermaal zo groot als het aantal delende bacteriën. Ten slotte bevat Mona Vifit yoghurt drink ongeveer 90% intacte bacteriën. Het aantal intacte bacteriën was maar liefst vijftig maal zo hoog als het aantal delende bacteriën.

Yakult bevat per ml veel meer delende bacteriën dan Mona Vifit en Orthiflorplus zoals de plaattellingen laten zien. De flow cytometrie tellingen geven echter een ander beeld. Het aantal intacte bacteriën per ml is in Yakult slechts ongeveer tweemaal zo groot als in Mona Vifit, terwijl het aantal intacte bacteriën in Orthiflorplus behoorlijk veel lager is. Dit verschil zit vooral in het aandeel bacteriën dat wel kleurt met cFDA maar niet deelt (de tweede functionele groep), dat voor Yakult en Mona Vifit fors groter is dan voor Orthiflorplus. Wie Yakult drinkt krijgt dus per milliliter tweemaal

zoveel intacte bacteriën binnen als iemand die eenzelfde hoeveelheid Mona Vifit drinkt en veel meer dan iemand die eenzelfde hoeveelheid Orthifloplus drinkt. Vergelijking tussen deze producten zegt nog niet direct iets over hoe goed deze als probioticum zijn. Orthiflorplus, Yakult en Mona Vifit bevatten verschillende bacteriesoorten en zijn verschillende typen van producten. Mona Vifit is een yoghurt drink en bevat dus gewone yoghurt bacteriën en daarnaast extra probiotische bacteriën, terwijl Yakult en Orthiflorplus alleen maar probiotische bacteriën bevatten. Ook is het belangrijk dat de waarden hier zijn gegeven per milliliter en het aantal bacteriën dat iemand binnenkrijgt afhangt van de hoeveelheid die gedronken wordt. Verder kunnen er verschuivingen optreden tussen de verschillende functionele groepen wanneer de producten langer bewaard worden. In dit onderzoek zijn de producten meteen na aankoop onderzocht.

Wat dit onderzoek laat zien, is dat met flow cytometrie verschillende functionele groepen van bacteriën onderscheiden kunnen worden en dat het kan worden toegepast op zowel starters gebruikt in de zuivelindustrie als op zuivelproducten en probiotica verkrijgbaar in de winkel. Het zou interessant kunnen zijn om in toekomstig onderzoek in producten met meerdere bacteriesoorten de soorten te onderscheiden en per soort te onderzoeken wat het aandeel intacte en delende bacteriën is. Ook dit is mogelijk met fluorescente kleurstoffen en flow cytometrie.

Nawoord

Zo, het proefschrift is bijna af, het laatste wat rest is het schrijven van dit nawoord.

Na mijn afstuderen als moleculair wetenschapper zes jaar geleden wilde ik graag verder met onderzoek en academische ontwikkeling. Een promotieonderzoek dus. Door een vacature in De Volkskrant is dat een OIO-onderzoek bij levensmiddelenmicrobiologie geworden. Een drukke periode van niet alleen labwerk maar ook veel 'bureauwerk' ligt nu achter me en is gedeeltelijk weergegeven in dit proefschrift.

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Wageningen, 14 maart 2002

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Curriculum vitae

Christine Johanna Bunthof werd geboren op 9 juli 1971 te Gerwen (gemeente Nuenen, Gerwen en Nederwetten). Op haar zesde ging ze naar de plaatselijke lagere school. Op haar twaalfde begon ze de middelbare school opleiding aan het Eckartcollege te Eindhoven waar ze in juni 1989 het VWO diploma behaalde. In september dat jaar, op haar achttiende, startte ze met de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit Wageningen. In september 1995 is ze cum laude afgestudeerd met specialisatie in biochemie, moleculaire biologie en onderwijskunde. Afstudeeronderzoeken heeft ze verricht bij de vakgroep Biochemie in Wageningen en bij de vakgroep Biochemistry and Biophysics van de University of Göteborg. Tijdens de studie heeft ze practicum 'Algemene en Fysische Chemie' gegeven en werkgroepen 'Oriëntatie Duurzaamheid' begeleid voor eerstejaars studenten van diverse studierichtingen. Op haar vierentwintigste, in mei 1996, startte ze als onderzoeker in opleiding aan de Wageningen Universiteit in samenwerking met NIZO food research. De resultaten van het onderzoek zijn beschreven in dit proefschrift. Dit jaar, op haar dertigste, is het promotieonderzoek afgerond en sinds 1 april 2002 is ze werkzaam als postdoc in het NWO-Biomoleculaire Informatica onderzoeksprogramma Phytoinformatics bij de leerstoelgroep Moleculaire Biologie van Wageningen Universiteit in combinatie met de business unit Genomics van Plant Research International.

Addendum

This work was carried at out Wageningen University, Department of Agrotechnology and Food Sciences, Laboratory of Food Microbiology, P.O. Box 8129, 6700 EV Wageningen, The Netherlands, and at NIZO food research, Microbial Ingredients Section, P.O. Box 20, 6710 BA Ede, The Netherlands.

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