Lactobacillus plantarum 44A as a live feed supplement for freshwater fish

Adolfo Bucio Galindo

Promotoren
Prof. dr. ir. F.M. Rombouts
Hoogleraar in de levensmiddelenhygiëne en –microbiologie
Wageningen Universiteit

Prof. dr. J.A.J.Verreth
Hoogleraar in de Visteelt en Visserij
Wageningen Universiteit

Co-promotor
Dr. ir. Ralf Hartemink
Universitair docent, leerstoelgroep levensmiddelenmicrobiologie

Promotiecommissie
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Prof. dr. ir. M.H. Zwietering, Wageningen Universiteit

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Contents

1. General Introduction

1.1. Intestinal bacteria in fish

In the last years, aquaculture has become a very fast growing sector in animal production. However, the number of outbreaks of bacterial diseases in cultured fish has also increased. Microbiological research has been generally focused to detect the harmful intestinal bacteria, and the methods to control them. Very little research has been done to detect the beneficial bacteria, especially the lactic acid bacteria group and its applications. Beneficial bacteria, in the best cases, could be used to substitute the use of antibiotics, as preventive agents of disease (Nikoskelainen et al., 2001) and as growth promoters (Byun et al., 1997).

1.2. *Lactobacillus*

Lactobacillus is a genus of Gram-positive, non-sporulating and catalase negative rods that ferment various carbohydrates mainly to lactate and acetate. Various amino acids, vitamins and minerals are essential for their growth (Kandler and Weiss, 1986). They are commonly associated with nutritious environments like foods, decaying material and the mucosal surfaces of the gastrointestinal and urogenital tract (Kandler and Weiss, 1986; Havenaar et al., 1992a). The ecological role played by *Lactobacillus* in the gastrointestinal tract among others include: production of antimicrobial substances; lowering of O/R potential; enhancement of the immune response, increase of availability of nutrients and use of some non-digestible carbohydrates (Fuller, 1989; Havenaar et al., 1992a).

Lactobacillus is present in the gastrointestinal tract of various vertebrates, including freshwater fish. In fish, most of the studies of *Lactobacillus* have been done for cold water fish and marine fish like *Salmonidae* (Ringø, 1995; Ringø and Gatesoupe, 1998; Gonzalez et al., 2000). Little information is available on the presence of *Lactobacillus* in other fish (Kvasnikov et al., 1977) and it is not clear

whether lactic acid bacteria are present in other fish taxa. There is a lack of information on the effect of modern conditions of animal husbandry (for example using high standards of water quality, and balanced diets) on the presence of *Lactobacillus* and host performance.

1.3. Probiotics

Probiotics are described as preparations of living microbial cells that, when ingested in high enough concentration, beneficially affect the host's health by improving the intestinal microbial balance (Fuller, 1989). Selection of probiotic strains is achieved by screening procedures for several characteristics *in vitro*, such as inhibitory activities against several pathogens, resistance to gastric secretions, bile tolerance and growth in faecal material. They should resist processing and storage conditions and be alive and active even after gastrointestinal passage. They should be safe and impart benefits to the host ((Fuller, 1989; Havenaar et al., 1992a).

1.4. Outline of this thesis

The aim of this thesis was to perform quantitative studies on the presence of intestinal lactobacilli in different fish hosts; to study biochemical and inhibitory properties of the isolated lactobacilli strains *in vitro*, to assess the survival rate of a selected *Lactobacillus* after exposure to low water content feed and gastrointestinal fluids; and to determine the microbiological impact after using the selected strain as a live supplement for farmed freshwater fish. The content of each chapter is as follows: **Chapter 2**, is a review on the bacteria of fish of different ages and environments; **Chapter 3**, is a comparative study on *Lactobacillus* populations in fish from natural and a farm environment; **Chapter 4**, is a study on the screening and selection of various *Lactobacillus* strains as candidates to be used as probiotics; **Chapter 5**, is a study on the survival rates of *Lactobacillus plantarum* 44a during dehydration in feed, and after storage and exposure to gastrointestinal fluids *in vitro*. **Chapter 6**, is a study on the *L. plantarum* 44a supplementation in tilapia and the kinetics of its excretion in faeces and a mathematical model; **Chapter 7**, is a study on multiple administration of *L. plantarum* 44a to tilapia and its impact on the *Lactobacillus* population of fish and its mathematical modelling; **Chapter 8**, is a concluding section.

2. Bacteria of freshwater fish and its environment: A review

2.1. General bacteria

The bacteria of fish eggs

Eggs from healthy fish are almost sterile before spawning (Hansen and Olafsen, 1999), thereafter, the eggshell is normally colonised by bacterial genera present in the water (Hansen and Olafsen, 1999; Ringø and Birkbeck, 1999). The glycoproteinaceous nature of the eggshell is well suited for adhesion and colonisation by bacteria (Hansen and Olafsen, 1999). Since the oxygen concentration is critical for successful hatching, bacterial overgrowth on the egg surface may also affect hatching (Hansen and Olafsen, 1999). Other damages on the eggshell affecting the developing embryo may be due to the presence of extracellular proteolytic enzymes, exotoxins or toxic metabolites released by the epiflora. The eggs of Atlantic cod are normally colonised by *Pseudomonas, Alteromonas, Aeromonas* and *Flavobacterium* (Hansen and Olafsen, 1999). Pathogenic *Vibrionaceae* may hinder mass production in the fish hatchery (Gatesoupe,1994). Studies on bacteria colonising eggs have been done only in coldwater fish species.

The microflora of fish fry and larvae

At the time of hatching, most aquatic species contain few, if any, bacteria in the gastrointestinal tract. Bacteria which first colonize the gastrointestinal tract of fish larvae originate from the resident epiflora of the eggs at the time of hatching, and the bacteria present in the water (Hansen and Olafsen, 1999). Under normal conditions most fish larvae at the onset of exogenous feeding have a developing stomach in which alkaline pH prevails (Verreth et al., 1992). Feeding implies a free access for practical all microorganism present in the feed and water into the larval

gut. Once feeding begins, the intestinal flora is derived from the ingestion of feed/prays ingested rather than the bacteria present in water (Ringø and Birkbeck, 1999). Because of the immature nature of the immune system in fish larvae, they have to rely on non-specific defence mechanisms, of which a protective intestinal microflora intimately associated with the gut mucosa will constitute a primary barrier. It is accepted that a primary microflora will become established at the larval stage and develop into a persistent flora at the juvenile stage or after metamorphosis.

As fish grows, the microflora must adapt to varying conditions of nutrient composition, pH, anaerobiosis, concentration of bile salts and digestive enzymes, the host's immune system, and the mutual influences of other members of the intestinal bacterial community (Hansen and Olafsen, 1999). In general, *Aeromonas* spp., *Pseudomonas* spp. and bacteria of the *Flavobacterium/Cytophaga* group are the most common bacteria in the intestines of freshwater fish (Ringø and Birkbeck, 1999). There are some reports indicating the presence of lactic acid bacteria in larvae and fry intestines (Kvasnikov et al., 1977; Ringø and Birkbeck, 1999).

The intestinal microflora of young and adult fish

The intestinal microflora of fish reflects the bacterial content of ingested food and of the environment (Hansen et al., 1992, Gatesoupe, 1999). The microflora varies with salinity, use of antibiotics, diet and dietary components (Ringø et al.,1995). According to the review of Ringø et al. (1995) the predominant bacterial genera/species isolated from most fish guts are aerobic or total anaerobic microorganisms.

In general, the bacterial species isolated from the fish intestines are also found in the water samples of the environment (Hansen et al., 1992, Gatesoupe, 1999; Okpokwasili and Alapiki, 1990; Apun et al., 1999; Spangaard et al., 2000). The dominant bacteria in trout were *Citrobacter*, *Aeromona* and *Carnobacterium* and other *Proteobacteria*. Species which are regarded as autochtonous in salmonids are *Acinetobacter* spp., *Enterobacter* spp., *Pseudomonas* spp., *Aeromonas* spp., *Flavobacterium* spp. and *Lactobacillus* spp. In average 50% of the trout microflora could be cultured in plates (Spanggaard et al., 2000) and bacteria belonging to the gamma subclass of Proteobacteria (of the genera *Citrobacter*, *Aeromonas* and *Pseudomonas*), and the Gram-positive bacteria with low GC content (such as

Carnobacterium). In another study *Carnobacterium* was found as a psychrotroph in wild trout gills (44.4%), gut (37.5%), and skin (22.2%) while *Enterobacteriaceae* were absent (Gonzalez et al., 1999). The common intestinal bacteria in carp are *Aeromonas hydrophila*, *Citrobacter freundii*, *Escherichia coli, Enterobacter aerogenes*, *Klebsiella pneuomoniae*, *Pseudomonas* spp., *Bacillus* spp., and *Staphylococcus* spp.(Apun et al., 1999).

2.2. Bacterial pathogens of fish

Importance

One of the major problems in aquaculture is disease (Austin and Allen-Austin, 1985). Some authors consider that bacterial diseases are a major cause of mortalities in hatcheries (Gomez-Gil et al., 2000). It is particularly prevalent in intensive aquaculture where fish are stocked at high intensities, and subjected to stress.

Basic hygiene, which is necessary for maintaining good water quality, is often overlooked and may lead to the start of a disease (Austin and Allen-Austin, 1985). Bacterial diseases are mainly caused by opportunistic (facultative) bacterial pathogens, which can reside in the environment or on/in apparently normal fish (latent carriers) (Wedemeyer, 1996). Interactions between fish and microorganisms that are present in the aquatic environment are a potentially serious source of mortality but under standard conditions of hygiene are often harmless. Infections are often precipitated by some stress (e.g. overcrowding, low dissolved oxygen, high ammonia, high temperature, or various pollutants) that upsets the natural defenses (Noga, 1996) and allow bacteria to colonize, penetrate, and invade host tissues (Hansen and Olafsen, 1999).

Of all the known bacteria, species from fewer than 25 genera are responsible for disease problems in fish cultured in either marine or freshwater systems (Austin and Allen-Austin, 1985), however there are some emerging pathogens (Austin and Gibb, 1993). More than 100 bacterial pathogens of fish and shellfish have been reported more recently (Alderman and Hastings, 1998).

Fish pathogens in the environment

Opportunistic facultative pathogens are normally ubiquitous in natural waters, thriving on suspended organic matter. Some opportunistic pathogens are

Aeromonas hydrophila (motile aeromonas septicaemia), *Flexibacter columnaris* (columnaris disease), *Edwardsiella ictaluri* (enteric septicaemia of catfish), many external protozoan parasites (e.g. Trichodina spp.), and fungi such as *Saprolegnia* spp.

Obligate pathogens require a living host in order to grow and reproduce. They remain infectious suspended in the water column or attached to the sediment particles. When shed into the water by an infected host, however, they usually do remain viable long enough to be transmitted horizontally (fish to fish). Few bacteria such as *Aeromonas salmonicida* (furunculosis), *Yersinia ruckeri* and *Renibacterium salmoninarum* (bacterial kidney disease) are obligate pathogens.

2.3. Methods to control fish pathogens in farmed fish

Disinfection.

Different methods are available to reduce the populations of fish pathogens in aquaculture, including chemical disinfection of eggs, equipment (nets, boots, etc), earth ponds and basins. In hatcheries, water is treated employing UV, ozone or the combination of both; chlorine, or other disinfectants to reduce obligate or facultative fish pathogens. However, in pond culture, and in most large hatcheries operated under flow-through conditions, water is not treated. Bacterial disease problems can be minimized by managing rearing conditions to meet the physiological needs of the fish and provide a low-stress environment (Wedemeyer, 1996).

Control of fish pathogens

During outbreaks of disease, populations are treated by exclusion of infected or dead fish, chemotherapy, use of antibiotics, or vaccination. Antibiotics can be used to eliminate bacteria before or during a disease outbreak. Sometimes these are added in little quantities to the feeds in a prophylactic way and as growth promoters. However, this practice has several negative aspects, such as antibiotic residues in the tissues and the generation of antibiotic resistant bacteria (Hansen et al., 1992; Gildberg et al., 1997; Byun et al., 1997; Ringø and Birkbeck, 1999). The latter may develop into an ecological threat to fish populations sustainability (Gildberg et al., 1997).

Although some diseases are prevented by vaccination, it is unlikely that vaccines will be developed to protect fish against facultative pathogens like *Aeromonas hydrophila* because of its ubiquitous presence and because of its numerous serotypes. Furthermore, larvae and early fry are too small to be vaccinated (Gildberg and Mikkelsen, 1997), and are very susceptible to dying due to proliferation of pathogens (Gildberg and Mikkelsen, 1997). Moreover, vaccines are not available for all the farmed fish species, including most tropical species.

Effective vaccines have been developed to prevent diseases caused by *Aeromonas salmonicida, Vibrio anguilarum, Vibrio salmonicid* and *Yersinia ruckeri*. Until now, antibacterial drugs still remain necessary to treat occasional outbreaks of bacterial diseases.

The restrictive use of antibiotics as growth stimulants for farm animals and the concern about the side effects of their use as therapeutic agents, has produced a climate in which both consumer and manufacturer are looking for alternatives.

2.4. Probiotics

A growing concern about the high consumption of antibiotics in aquaculture has initiated a search for alternative methods of disease control (Gildberg et al., 1997) and growth promotion (Byun et al., 1997). Improved resistance against infectious diseases can be achieved by the use of probiotics (Gildberg et al., 1997). Probiotic are living preparations of microbial cells that, when ingested in high enough concentration, beneficially affect the host's health and growth by improving the intestinal microbial balance (Fuller, 1989; Havenaar et al., 1992ab). Selection of probiotic strains is achieved by screening procedures for several characteristics *in vitro*, such as inhibitory activities against several fish pathogens and gastric and intestinal secretions (Byun et al., 1997; Jöborn et al.,1997). Once the strains have been selected, a way to supply them to the host via feeds or water needs to be developed. This is a very important aspect as great losses in viability during processing and storage are generally reported (Havenaar et al., 1992b)

Some experiments *in vivo* have shown that the inoculation of some probiotic strains, mainly lactic acid bacteria, increase fish survival after being challenged with fish pathogens while lactic acid bacteria populations in the gut increase, and in one study lactic acid bacteria inoculation was related with an increase of fish growth rate (Cai et al., 1998).

Probiotic strains

A more detailed description of the most extensively studied strains is described below.

Carnobacterium divergens

One of the most extensively studied strains is *Carnobacterium divergens* isolated from Atlantic salmon by Strøm, initially identified as *Lactobacillus plantarum* (Ringø and Gatesoupe, 1998). *In vitro* tests showed that *C. divergens* inhibited *Vibrio anguillarum* and *Vibrio salmonicida*, as well as *Proteus vulgaris* (Strøm, 1988, cit. by Ringø and Gatesoupe, 1998).

In vivo tests showed that *C. divergens* given to 5 days old larvae, was present as 70% of the total flora in the experimental larvae while the bacterial flora of unsupplemented larvae was dominated by opportunistic groups of *Cytophaga/Flexibacter* (45%) and *Pseudomonas* sp. (45%) (Strøm and Ringø, 1993).

In another experiment, the same strain of *C. divergens* was also able to attach and colonise the gut of turbot larvae at the time of hatching, showing the highest percentage of survival after 15 days (20% versus 13.5% in larvae not exposed to exogenous bacteria); 7.5% in larvae exposed to *Vibrio pelagius* + *Carnobacterium divergens*; and 5% in larvae exposed to *Vibrio pelagius* (Ringø, 1999).

Carnobacterium sp. K1

Another strain is *Carnobacterium* sp. K1 isolated from the gastrointestinal tract of juvenile salmon by Jöborn et al (1997). It showed in vitro antagonistic activity against *Aeromonas salmonicida*, *Vibrio anguillarum* (Jöborn et al., 1997), *Aeromonas hydrophila*, *A. salmonicida*, *Flavobacterium psychrophilum*, *Photobacterium damselae*, *Streptococcus milleri*, *Vibrio anguillarum* and *V. rodalii* (Robertson et al., 2000).

Carnobacterium sp K1 was able to adhere in vitro to intestinal mucus of Atlantic salmon, and was metabolically active in faeces (Jöborn et al., 1997) inhibiting *Vibrio anguillarum* and *Aeromonas salmonicida*. *In vivo* tests showed that *Carnobacterium* sp K1 orally administrated to fingerling rainbow trout survived and

persisted for several days (>10^6 CFU/g faeces) in comparison with the control which had no lactic acid bacteria (Jöborn et al., 1997).

Salmonids (weighing ≈15 g) fed daily on diets containing *Carnobacterium* sp. K1 (≈5 x 10^{10} cells/ml) contained ≈10^5 CFU *Carnobacterium* sp. K1 /g gut in the lower intestine after 7 feeding days and ≈ 10^6 CFU/g gut after 28 feeding days. After interruption of *Carnobacterium* sp. K1 administration, its presence rapidly declined to zero within six days.

In another experiment, rainbow trout previously fed either with or without the probiotic, were transferred to the same water tank to be challenged with infected fish. An improvement in survival was consistently recorded in the fish previously fed on the probiotic. After infecting with *A. salmonicida*, the survival in the probiotic treatment was 84% vs 32% in the control. After fish were challenged with *Yersinia ruckerii*, those fish fed previously on probiotic survived for 100% vs 36% in the control.

Lactobacillus rhamnosus (ATCC 53103)

Another strain with probiotic effects is *Lactobacillus rhamnosus* (ATCC 53103) originally isolated from a human source (Nikoskelainen et al., 2001). The bacterium was administered at two different doses (10^9 and 10^{12} CFU/g feed) to rainbow trout (average weight = 32.2g) for 51 days. Sixteen days after the start of the *Lactobacillus* feeding, the fish were challenged with *Aeromonas salmonicida* ssp. *salmonicida*, which causes furunculosis. During the challenge trial the mortality was monitored. *L. rhamnosus* reduced the fish mortality significantly, from 52.6% in the control to 18.9% and 46.3% in the 10^9 CFU/g feed and the 10^{12} CFU/g feed groups, respectively. From this, it was concluded that the tested strain might be a promising probiotic for fish without subsequent risk for human consumption.

Lactobacillus sp. DS-12 later classified as Weissella hellenica

Another interesting strain is *Lactobacillus* sp. DS-12 (Byun et al., 1997) later classified as *Weissella hellenica* (Cai et al., 1998) isolated from flounder intestines. The strain had antagonistic activity *in vitro* against *Edwardsiella tarda*, *Pasteurella piscida*, *Aeromonas hydrophila*, and *Vibrio anguillarum* and showed bile resistance (10%) and acid resistance (pH 3; 90 min). It was assumed that the antagonistic activity against fish pathogenic bacteria was due to acid production. When the strain was given to juvenile fish, its presence in intestinal faecal samples was high

(≈log 7 CFU/g intestinal sample) while none were detected in control fish. The oral administration of *W. hellenica*. DS-12 to the fish for 30 days was related to an increase of the fish mean weight gain of 64.2 g against 35.7g in the control group.

Conclusions

Fish in all the life-stages have interactions with bacteria from the environment. Some relations are detrimental and others are beneficial. Current methods of control of pathogens in the fish farms should be improved by studying the beneficial bacteria.

3. Presence of *Lactobacillus* spp. in the intestinal content of freshwater fish from a river and from a farm with a recirculation system

Adolfo Bucio, Ralf Hartemink, Johan W. Schrama and Frank M. Rombouts

Abstract

The presence of *Lactobacillus* in the gastrointestinal tract is considered beneficial in a wide range of hosts. In fish, the presence of *Lactobacillus* has been reported mainly in salmonids and marine fish. Few studies have described lactic acid bacteria in other freshwater fish. In particular, there are no reports on the presence of *Lactobacillus* in the intestines of young and adult freshwater fish inhabiting river environments and from fish reared in aquaculture units with water recirculation systems. In this study we show that various species of *Lactobacillus* are present in relatively high number in the intestines of fresh water fish from a river, especially in the warm season but in low numbers in the intestines of farmed fish reared in a recirculation system in warm water. *Lactobacillus* are reported for the first time from the intestines of *Anguilla anguila*, *Perca fluviatilis*, *Scardinius erithrophthalmus*, *Gymnocephalus cernuus*, *Alburnus alburnus*, *Blicca bjoerkna*, *Leuciscus cephalus*, *Silurus glanis* and *Clarias gariepinus*. The two first species, commonly named eel and perch and the last one are highly valuable species for aquaculture. Additionally, improved methods for storage and bacteriological analysis of fish intestinal content are described.

3.1. INTRODUCTION

Lactic acid bacteria are Gram-positive, non-sporulating and catalase negative rods or cocci that ferment various carbohydrates mainly to lactate and acetate. Various amino acids, vitamins and minerals are essential for their growth (Kandler and Weiss, 1986). Accordingly, they are commonly associated with nutritious environments like foods, decaying material and the mucosal surfaces of the

gastrointestinal and urogenital tract (Kandler and Weiss, 1986; Walstra et al., 1999; Havenaar et al., 1992a), where they enhance the host protection against pathogens (Havenaar et al., 1992a). Various authors have shown that lactic acid bacteria are also part of the normal intestinal flora of fish (Ringø and Gatesoupe, 1998) Most of the evidence comes from salmonid species like Arctic charr (*Salvelinus alpinus*), Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhyncus mykiss*) (Ringø and Gatesoupe, 1998; Ringø et al., 1995; Ringø and Olsen, 1999; Spanggaard et al., 2000 and Gonzalez et al., 2000). Few studies have described lactic acid bacteria in other freshwater fish. (Kvasnikov et al, 1977; Cai et al., 1999). Kvasnikov et al., (1977) described the presence of lactic acid bacteria, including *Lactobacillus* in the intestines of various fish species at larval, fry and fingerling stages inhabiting ponds in Ukraine, giving information on the changes in their composition as a function of the season of the year and life-stage of the fish. However, it was discussed that some human activities like artificial feeding in ponds would have had an effect on the bacterial composition and load in some fish, like carp (*Cyprinus carpio*) which showed the highest content of lactic acid bacteria in the intestines.

In another study, the lactic acid bacteria from *Cyprinus carpio* collected from the Thajin river in Thailand, were described, reporting the presence of *Enterococcus* spp. and the dominance of *Lactococcus garviae*, an emerging zoonotic pathogen that has been isolated from cattle, fish, and humans (Cai et al., 1999).

In particular, there are no reports on the presence of *Lactobacillus* in the intestines of freshwater fish inhabiting river environments and from fish reared in aquaculture units with water recirculation systems, whereas other groups of bacteria have been better studied (Cahill et al., 1990). Knowledge on the presence of *Lactobacillus* as a natural flora in fish may lead to further applications to improve fish health.

The aim of this study was to make a survey of the presence of *Lactobacillus* in the intestinal content of various freshwater fish from a river and from a farm, basically to make a bank collection of strains for further screening research. To fullfil our aim, we improved the methods to store samples and to quantify lactic acid bacteria in fish. Some factors which affect the presence of lactic acid bacteria and *Lactobacillus* in the sampled fish are discussed.

3.2. MATERIAL AND METHODS

Fish

102 fish in young and adult stage and from various species (*Perca fluviatalis*, *Abramis brama*, *Scardinius erithrophthalmus*, *Gymnocephalus cernuus*, *Anguilla anguilla*, *Alburnus alburnus*, *Blicca bjoerkna*, *Leuciscus cephalus*, *Stizostedium lucioperca*, *Leuciscus idus* and *Silurus glanis*) were collected by a fisherman from the Maas river, and in the lateral canal near Venlo, Limburg province, The Netherlands in summer, autumn and winter. Identification of the fish species was done by the fisherman and checked up in the laboratory by examining morphological characteristics like shape and coloration of the body and fins of the specimens according to a field guide (Nijssen et al., 1998). In general all fish studied were randomly picked. Fish were weighed and measured in length before dissection. Thirty other fish (*Tilapia nilotica* and *Clarias gariepinus*) from tropical origin, but cultured for several generations in aquaria, were obtained from a hatchery with a recirculation system.

Dissection

Fish were dissected under aseptic conditions in a room at 4^{o}C. The gall bladder, liver and fat deposits surrounding the gut were removed. The intestinal content was squeezed out using sterile forceps. The intestinal content was collected into previously weighed flasks containing storage medium (see section below)

Storage Medium

The storage medium consisted of 50 ml of modified Buffered peptone water (Buffered peptone water (BPW, Oxoid CM 509, Hampshire, England) 20g/l; cysteine·HCl (C-7880, Sigma) 0.5g/l; resarzurin 1 tablet/l BDH, England; tween 80 1 ml/l)(Hartemink et al., 1997). Intestinal content in storage medium was homogenized using a Vortex mixer. 1 ml was transferred to reduced neutralized bacterial peptone (NBP, Oxoid L34) 0.5g/l, NaCl 8g/l, Cysteine.HCl 0.5g/l, pH adjusted to 6.7 (Hartemink and Rombouts, 1999). Afterwards serial dilutions were spread on plates of selective media.

To investigate whether freezing is a suitable technique to preserve intestinal samples, some samples were examined both fresh and after being frozen. Frozen samples were prepared as follows: 1 ml of modified Buffer peptone water containing intestinal content was transferred to sterile vials containing 300 µl

glycerol, which were frozen at -20°C for 24 h. After thawing for one hour at room temperature, the vial content was transferred to 8.7 ml of reduced bacterial peptone to complete the second decimal dilution. Thereafter, successive dilutions were made and bacteria were enumerated by the plate counting method on the media described below. Fresh and freeze-thawed samples were compared quantitatively by differences in bacterial content. Comparison of means of fresh and frozen thawed intestinal samples were analyzed by Student's t-test as independent samples with two tailed distribution. Moreover, for every sample, the logarithm of the concentration of cells from the thawed vials (Y) was plotted against the logarithm of the concentration of the cells from the fresh samples (X) A linear regression analysis was performed using the program SPSS 11.0 (SPSS Inc., Chicago, USA).

Culture medium for bacteria

Columbia Blood Agar (CBA; Oxoid CM 331 with 50 ml/l of sheep blood added) incubated anaerobically at 30°C for 48h was used to study total anaerobic bacteria[1], MRS (MRS, Merck, Darmstadt, Germany) with 1.5% agar and pH lowered to 4.2 (MRS 4.2) and incubated anaerobically at 30°C for 96 h was used as selective medium for lactic acid bacteria but not for *Carnobacterium*; as this genus does not grow in MRS.

LAMVAB (*Lactobacillus* spp. Anaerobic MRS with Vancomycin and Bromocresol green) incubated at 30°C for 96 h was used as as elective and selective medium for *Lactobacillus* spp. (Hartemink et al, 1997).

Anaerobic incubation of the three media was made in anaerobic jars with a mixture of 80% N_2, 10% H_2 and 10% CO_2 using the Anoxomat system (Mart, Lichtenvoorde, The Netherlands).

LAMVAB broth incubated for 48 hours at 30^0C was used to detect *Lactobacillus* spp. following the Most Probable Number techniques for fish samples in which *Lactobacillus* spp. were not found using the plate counting method (i.e. *Clarias*

[1] Although the term of total anaerobic bacteria is used in the whole thesis, it should be noted that certain oxygen sensitive obligate anaerobes could be killed by the handling procedures.

gariepinus). Samples studied using "Most Probable Number" techniques were the intestinal content of 13 young catfish (*Clarias gariepinus*).

Table 3.1. Comparison of means of fresh and frozen thawed intestinal samples by paired t-student test

	Mean of intestinal samples Log CFU/g		No. of samples	Independent Student's t-test* P
Medium	Fresh	Frozen-thawed		
MRS 4.2	4.72 ± 1.40	4.90 ± 1.22	17	0.59
LAMVAB	5.01 ± 1.40	5.25 ± 1.30	15	0.65
Columbia Blood Agar	8.09 ± 0.43	7.75 ± 0.53	13	0.21

*Differences were not significantly different because p values were > 0.05. Fish intestinal samples used were from perch (*Perca fluviatalis*) and carp (*Abramis brama*); a carnivorous and a herbivorous freshwater species, respectively.

Tryptone Soy Agar (Oxoid CM13, Hampshire, England) was also used primarily to find out whether any other lactic acid bacteria could be recovered from intestinal samples of catfish as Gonzales et al (2000) found in brown trout. As lactic acid bacteria was not found in 30 isolates, no further analysis was considered necessary.

Seasonal variations in bacterial counts

Means (Analysis of variance) of total anaerobic bacteria, lactic acid bacteria and *Lactobacillus* from intestinal samples from fish in different seasons was carried out on data gathered from all the fish species collected for each season. Therefore, the effect of fish species was not considered, basically because of the low number of samples.

Isolation of lactic acid bacteria

For some fish, 10 different typical yellow or green colonies from LAMVAB agar (Hartemink et al., 1997) or yellow-green broth with white color precipitate were taken from the second lowest countable dilution and purified on the same medium.

Purity was examined by microscopic observations. All the isolates were checked for morphology, Gram reaction, oxidase and catalase tests, and CO_2 production in MRS-Durham tubes and growth in acetate agar pH 5. Isolates were stored in vials with 30 % glycerol (v/v) at -80^{o}C.

Table 3.2. Average (Log CFU/g intestinal content) of some wild freshwater fish.

Fish species, Scientific name and common name	Season	No.	CBA (CFU/g)	LAMVAB (CFU/g)	MRS 4.2 (CFU/g)
Carnivorous					
Perca fluviatalis (Perch)	Summer	8	9.02	7.60	6.64
"	Autumn	20	8.51	4.84	4.43
"	Winter	4	7.03	3.56	-
Anguilla anguilla (Eel)	Summer	2	7.84	5.10	4.75
Gymnocephalus cernuus(Ruffe)	Summer	1	8,89	7.85	7.40
Silurus glanis (Somnul)	Winter	1	6.45	2.20	1.13
Herbivorous					
Abramis brama (Carp)	Summer	1	8.21	7.31	5.63
"	Autumn	9	7.56	4.97	4.67
"	Winter	13	6.23	1.67	0.97
Omnivorous					
Scardinius erithrophthalmus (Rudd)	Summer	1	8.67	7.31	6.84
"	Autumn	16	7.85	3.89	3.29
"	Winter	7	6.47	1.63	1.29
Alburnus alburnus (Bleak)	Autumn	1	9.16	5.22	5.70
Blicca bjoerkna (Silver bream)	Autumn	1	7.72	3.49	3.63
Leuciscus cephalus (Chub)	Autumn	3	7.07	2.71	3.17
Stizostedion lucioperca (Pike perch)	Autumn	1	7.44	3.14	3.62
Leuciscus idus(Orfe)	Autum	8	7.53	4.46	3.76

CBA = Columbia Blood Agar; LAMVAB = *Lactobacillus spp.* Anaerobic MRS with Vancomycin and Bromocresol green; MRS 4.2. Nd, not determined.

Identification procedures for lactic acid bacteria

72 strains were randomly selected for identification procedures based on phenotypical characteristics. Fermentation of carbohydrates was determined using API 50 CHL strips according to the manufacturer's instructions (Biomerieux, Marcy l' Etoile, France). Ammonium production as hydrolysis of arginine was tested in modified MRS broth without meat extract that contained 0.3% (w/v) arginine, 0.2% (w/v) sodium citrate and glucose 0.05% and after 5 days of aerobic incubation at 30^{o}C, a few drops of Nessler's reagent were added to detect ammonium presence. Isolates were also tested by growth at 15 and 45^{o}C. The configuration of the isomers of lactic acid produced from glucose were determined enzymatically in the cell-free supernatant from 24 hours cultures in MRS broth (Boehringer Mannheim, Darmstadt, Germany). Some of the isolates were analysed by presence of meso diaminopimelic acid in the cell walls. Identification was done according to Kandler and Weiss (1986); Kuiper (1999); and Hammes et al. (1995).

Characterization of other genus

29 strains from farmed fish were randomly isolated from Tryptone Soy Agar and characterized until level genus by morphology, motility, use of glucose (oxidative/fermentative), cytochrome c oxidase test and catalase according to Spanggaard et al.(2000).

3.3. RESULTS

Storage procedure

Counts on CBA, MRS 4.2 and LAMVAB from frozen samples were not significantly different ($p > 0.05$) from the values obtained from fresh samples (Table 3.1). Data from fresh and frozen samples were highly correlated with regression lines with slopes significantly close to one ($p \leq 0.05$) and intercepts significantly close to 0 ($p \geq 0.05$) (Fig.3.1a, 3.1b and 3.1c). The results shown above indicate that freezing is a suitable method to store samples for further bacteriological analysis.

Table 3.3 Biochemical characteristics of *Lactobacillus* species isolated from the intestines of several fish species with various feeding habits.

Presumptive *Lactobacillus* species	*L. alimentarius*	*L. coryneformis subs coryneformis*	*L. coryneformis subs torquens*	*L. casei subs. paracasei*	*L. sakei*	*L. pentosus*	*L. plantarum*	*L.brevis*	*L. oris*
No. isolates	**12**	**6**	**2**	**1**	**30**	**2**	**1**	**5**	**15**
Diaminopimelic acid	nd	nd	nd	nd	nd	+	+	nd	nd
CO_2 from glucose	-	-	-	-	-	-	-	+	+
NH_3 from arginine	-	-	-	-	-	-	-	+	+
15ºC	+	+	+	+	+	+	+	+	+
45ºC	2	-	-	-	-	-	-	-	-
Glycerol	1	-	-	+	-	+		-	-
L-Arabinose	2	-	+	-	+	+	+	2	+
Ribose	+	-	+	+	+	+	+	+	+
D-Xylose	-	-	-	-	26	-	-	-	+
Galactose	-	-	-	+	29	-	-	-	-
Rhamnose	-	+	-	+	-	+	-	2	-
Inositol	-	-	-	+	-	-	-	-	
Mannitol	-	5	-	+	-	+	+	+	+
Sorbitol	-	-	-	+	-	1	+	-	-
1-Methyl-D-mannoside	-	-	-	-	-	+	+	-	-
1-Methyl-D-glucoside	7	-	-	-	-	-	+	+	+
N Acetyl glucosamine	+	+	+	+	28	+	+	+	+
Amygdaline	+	-	-	+	10	+	+	-	+
Arbutine	+	-	-	+	1	+	+	-	+
Esculine	+	+	+	+	+	+	+	1	+
Salicin	+	-	-	+	+	+	+	-	+
Cellobiose	+	-	-	+	27	+	+	-	+
Maltose	+	-	-	+	19	+	+	+	+
Lactose	+	-	-	+	26	+	+	-	+
Melibiose	2	+	+	-	+	+	+	+	+
Sucrose	8	+	+	+	+	+	+	+	+
Trehalose	+	-	1	+	+	+	+	-	+
Melezitose	-	-	-	+	-	+	+	+	-
D-Raffinose	2	-	+	-	29	+	+	+	+
Starch	-	-	-	+	-	+	-	-	-
Xylitol	-	3	-	-	-	-	-	-	-
2-Gentiobiose	+	-	+	+	+	+	+	-	+
D-Turanose	-	-	-	-	-	+	+	+	+
D-Tagatose	+	-	-	+	1	-	-	-	-
D-Arabitol	-	5	-	-	-	+	-	-	-
Gluconate	+	-	+	+	+	+	+	+	+
2-keto-gluconate	2	1	1	-	-	+	-	-	+
5-keto-gluconate	1	-	-	+	-	+	-	-	+
Lactic acid configuration	DL	DL	D	DL	DL	DL	DL	DL	D

+, Positive reaction of all the isolates. Numbers are the positive isolates. All isolates fermented D-Glucose, D-Fructose, D-Mannose. No fermented erythrol, D-Arabinose, L-Xylose, Adonitol, 2-Methyl-xyloside, L-Sorbose, Dulcitol, Inulin, Glycogen, D-Fucose, L-Fucose, L-Arabitol. nd, Not data.

Wild freshwater fish

Culture media

LAMVAB

LAMVAB was highly selective to quantify lactobacilli as 99% of 143 randomly picked colonies and purified isolates were confirmed as *Lactobacillus* spp. according to (Kandler and Weiss, 1986). Counts of intestinal lactobacilli ranged from approximately 10^2-$10^{7.5}$ CFU/g of intestinal content.

Lactobacillus spp. was found in fish with several feeding habits, like carnivorous (*Perca fluviatalis*, *Anguilla anguilla*, *Gymnocephalus cernuus*, *Stizostedium lucioperca* and *Silurus glanis*) or herbivorous (*Abramis brama*), and omnivorous species (*Scardinius erythrophthalmus*, *Leuciscus idus*, *Leuciscus cephalus*, *Blicca bjoerkna* and *Alburnus alburnus*) (Table 3.2). The physiological and biochemical characterization of *Lactobacillus* isolates and the presumptive identification of the species found for various fish species are shown in Tables 3.3. From 72 isolates, 2 metabolic groups of *Lactobacillus* were recovered: facultative heterofermentatives and obligate heterofermentatives. *L. sakei* and *L. alimentarius* were the most often found isolates. *L. sakei* was found in both carnivorous and herbivorous fish. *L. alimentarius* was found only in *A. anguilla* (Table 3.4).

MRS 4.2

MRS 4.2 was suitable to quantify *Lactobacillus* as 30 randomly picked colonies on the highest dilution were identified as *Lactobacillus*; coccoid forms were not found. Means of counts of 73 samples were not statistically different to LAMVAB counts in the Student's t-test ($p < 0.29$) and were correlated with LAMVAB counts ($r^2 = 0.85$, $p < 0.001$). The correlation of counts on MRS 4.2 with those on LAMVAB and the absence of coccoids suggest that *Lactobacillus* were the most important acidophilic lactic acid bacteria in the samples analyzed.

Columbia Blood Agar

In this medium, total anaerobic flora grew as the highest counts in the samples. Most of the colonies were round shaped and white.

Seasonal variations.

Counts of intestinal bacteria isolated from all the fish species on media Columbia Blood Agar, MRS 4.2 and LAMVAB were different among seasons, being the highest counts in summer and the lowest in winter (Table 3.5).

Farmed freshwater fish

Media for bacterial collection

LAMVAB

Lactobacillus were not recovered from LAMVAB plates neither from tilapia (*Tilapia nilotica*) nor from catfish (*Clarias gariepinus*) reared in a recirculation system. In some individuals of catfish, low numbers of *Lactobacillus* (< 2.01 Log CFU/g) were detected using Most Probable Number methods (Table 3.3).

MRS 4.2

Lactic acid bacteria were not recovered from MRS 4.2 plates from fish reared in a recirculation system.

Columbia Blood Agar

Total anaerobic counts from farmed fish were in average (Log 7.11 CFU/g ± 0.79) Most of the colonies were accompanied by a green discoloration likely caused by the reduction of hemoglobin in the red blood cells to metmyoglobin.

TSA

Lactic acid bacteria were not recovered from the isolated colonies from TSA plates from catfish or tilapia reared in the recirculation system. TSA was primarily used to find out whether any lactic acid bacteria could be recovered in a similar way as described by Gonzales et al. (2000) However, we were not able to detect any lactic acid bacteria.

Some isolates from TSA plates from catfish (*Clarias garipinus*) belonged to the family *Vibrionaceae* (71%). However, it was not studied whether they were pathogens.

Lactic acid bacteria were neither detected in the different parts of the recirculation system nor in the fish feed (6 determinations).

Table 3.4.*Lactobacillus* species isolated from the intestines of several fish species with various feeding habits.

Presumptive *Lactobacillus* species		*L. alimentarius*	*L.coryneformisk subs coryneformis*	*L. coryneformis Subs. torquens*	*L. casei subs. paracasei*	*L. sakei*	*L. pentosus*	*L. plantarum*	*L.brevis*	*L. oris*
	Carnivorous									
	S.erithrophthalmus	X					X			
	A. anguilla	X	X			X		X		
Fish	*P. fluviatalis*			X		X				X
	Herbivorous									
	A. brama		X			X				
	Omnivorous									
	C. gariepinus				X				X	
Season										
Spring					X				X	
Summer		X				X		X		
Winter			X	X			X			X

X = Presumptive species present.

Table 3.5. Comparison of Means (Analysis of variance) of bacteria counted in Columbia Blood Agar, MRS 4.2 and LAMVAB (Total anaerobic bacteria, lactic acid bacteria and *Lactobacillus* respectively) from intestinal samples from fish in different seasons of the year.

Media	Summer	Autumn	Winter	Probability
CBA	$8.74^{a} \pm 0{,}20$	$7.95^{b} \pm 0{,}60$	$6.46^{c} \pm 0{,}47$	< 0.00
LAMVAB	$7.19^{a} \pm 1.01$	$4.39^{b} \pm 1.66$	$2.38^{c} \pm 1.15$	< 0.00
MRS 4.2	$6.34^{a} \pm 1.2$	$4.03^{b} \pm 1.46$	$1.96^{c} \pm 1.43$	< 0.00*

* Different letters in rows means significant difference between season means ($p < 0.05$). Variances of the dependent variable following the Levene's test were assumed equal ($p > 0.01$) across seasons: CBA, $p = 0.483$; LAMVAB, $p = 0.015$; and MRS 4.2, $p = 0.196$).

3.4. DISCUSSION

Conventional methods to count, isolate and identify *Lactobacillus* from fish intestinal samples are usually laborious, limiting the number of samples that can be handled (Ringø and Gatesoupe, 1998).

This report provides some suitable techniques to study *Lactobacillus* from fish intestines. It was possible to freeze a high number of samples, and thaw them to be plated out on LAMVAB, which is a highly selective and reliable medium to count and isolate *Lactobacillus* from fish with various feeding habits, gastrointestinal morphologies and digestive physiology. Homofermentative and heterofermentative *Lactobacillus* species could be identified. None of the *Lactobacillus* species recovered have been reported as pathogens for fish.

Lactobacillus are reported for the first time from the intestines of *Anguilla anguila, Perca fluviatilis, Scardinius erithrophthalmus, Gymnocephalus cernuus, Alburnus alburnus, Blicca bjoerkna, Leuciscus cephalus, Silurus glanis* and *Clarias gariepinus*. The two first species, commonly named eel and perch are highly valuable species for aquaculture (Juell et al., 2001; Madsen et al., 2000) and fishing in Europe (Nijssen et al., 1998). The last species is also important for aquaculture in Africa. In this study we confirm the presence of *Lactobacillus* in

Abramis brama, *Leuciscus idus* and *Stizostedion lucioperca* previously described (Kvasnikov et al., 1977).

Lactobacillus species found in this study were entirely different to the species described in (Kvasnikov et al., 1977), even though some host species were common in both studies. These authors found as most frequently occurring isolates: *L. plantarum*, *L. casei, L. leichmannii, L. acidophilus, L. fermenti, L. cellobiosus and L. buchneri*. In this study, the only species matched with Kvasnikov et al (1977), was *L. plantarum*. On the contrary, the most common species in this study were *L. alimentarius, L. sakei*, and *L. oris* which were not found by Kvasnikov et al (1977). This observation may be the result of different hosts, different habitats or totally different identification procedures.

The biochemical characteristics used for identification of *Lactobacillus* may suggest some ideas in relation to the occurrence of the strains in nature.

Most of *Lactobacillus* examined in this study (80%) had the capacity to ferment lactose, and galactose. Generally, most lactobacilli are able to ferment lactose, by uptake of this disaccharide by a specific permease and splitting it by ß-galactosidase for further phosphorylation of galactose and glucose (Kandler, 1983). Because, lactose is only present in milk and milk derivates, it is possible that these strains have evolved from environments related with mammals, as was suggested for other lactose positive *Lactobacillus* (Garvie, 1984). Lactose may be present or was present in the environment as a waste; resulting from livestock production, and disposal effluents from dairy factories.

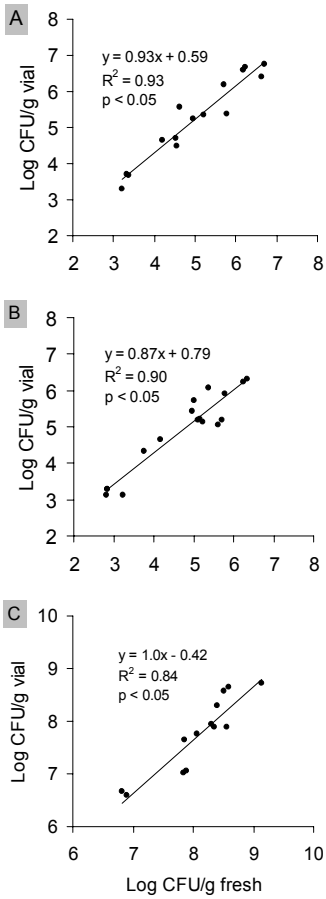

Fig. 3.1. Counts (Log CFU/g) of intestinal content of several fish species in (a) LAMVAB, (b) MRS 4.2 and (c) Columbia Blood Agar from paired samples plated both fresh (X axis) and after thawed from the vials with glycerol (Y axis) The ability of the mathematical model to predict the actual values was tested by a linear regression analysis. The coefficients of the straight line are shown in the graphs. It was tested with a Student's t-test if the slope ≈ 1 and the intercept ≈ 0 as a final measurement of the precision and accuracy. In all the cases, the null hypothesis was accepted with p≥ 0.05 for both the slope and the intercept, meaning that fitted curve does not significantly differ from the actual values, thus y = x. (a), LAMVAB, Model parameter ***b***, (intercept) = 0.587, SE ± 0.378, p = 0.144 and ***m*** (slope) = 0.929, SE ± 0.073, p > 0.05. (b), MRS 4.2. Model parameter ***b***, (intercept) = 0.788, SE ± 0.364, p = 0.047 ≈ 0.05 and ***m*** (slope) = 0.871, SE ± 0.075, p > 0.05. (c), CBA. Model parameter ***b***, (intercept) = -0.420, SE ± 1.093, p 0.708 and ***m*** (slope) = 1, SE ± 0.135, p > 0.05.

Another component, often fermented by the strains was the amino-sugar N-acetyl-glucosamine, a compound present in peptidoglycans; in blood, chitin and as one of the main constituents of mucus in the gastrointestinal tract (Hicks et al., 2000). The carbohydrate portion constitutes above 40% of the weight of the mucus (Stephen, 1985) or higher values (Hicks et al., 2000). The ability of many strains to ferment N-acetyl-glucosamine may partly explain the presence of *Lactobacillus* in some carnivorous fish, like eel and perch whose diet is low in carbohydrates.

The seasonal changes of lactic acid bacteria in the intestines of freshwater fish in this study - highest counts in summer and the almost absence counts in winter- was similar to what was found in fingerlings of pond fish by Kvasnikov et al (1977). However, the fish species analyzed in that study were *Ciprinus carpio*, *Hypophthalmichtus molitrix* and *Aristichthus nobilis*, different to the 12 species analyzed in this study (Table 3.2 and 3.4) which were collected from a river environment.

An interesting point was that lactic acid bacteria were found in extremely low numbers or absent in catfish and tilapia respectively reared in the recirculation system. Similarly, lactic acid bacteria were absent in the water and the sediments of the different parts of the recirculation system and in the feed. The composition of the diet which beside being essentially sterile, as mentioned above, is different from the diets found in nature, and do not contain glucose or other readily fermentable carbohydrates for lactobacilli. The most abundant carbohydrates in fish feed is starch, which can be better used by other groups of bacteria which produce amylases, like many members of the *Vibrionaceae* family, like *Aeromonas hydrophila*.

An explanation of the absence of lactic acid bacteria in the rearing water may be that water supplied in the inlet of the aquariums is exposed to some treatment of sterilization (ultraviolet or ozonization) before entering the tanks. Furthermore it contains very little suspended organic matter and oxygen to saturation. Because lactic acid bacteria require a very nutritious environment to grow (Kandler and Weiss, 1986), it is feasible that they can hardly thrive in the aquaria which limits their possibility to reach the gastrointestinal tract of those farmed fish. In other studies, low numbers of lactic acid bacteria (10^0 to 10^3 CFU/g) have been found in fish reared in hatcheries (Spanggaard et., 2000; Ringø et al., 2000). In this study, the main bacteria found in fish in the recirculation system were Gram negative

bacteria, like some members of the *Vibrionaceae* family, which have been reported to be able to survive suspended as apparently non-culturable but viable bacteria for long periods of time (Nyunt et al., 1996). Moreover, many species have been reported resistant to UV radiation in the hatcheries environment (Litved and Landfold, 1996). These abilities may explain the presence of this group as one of the most ubiquitous bacteria in water and consequently their likelihood to reach the fish intestines is very high. They have been reported as important inhabitants of the fish intestines (Austin and Austin, 1985; Sugita et al., 1996; Spanggaard et al., 2000). In conclusion, improved methods for storage and bacteriological analysis of fish intestinal content are described, showing among others, that various species of *Lactobacillus* are present in relatively high number of wild fresh water fish, but in low numbers in farmed fish.

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4. Screening of *lactobacilli* from fish intestines to select a probiotic for warm freshwater fish[2]

Adolfo Bucio, Ralf Hartemink, Johan W. Schrama and Frank M. Rombouts

Abstract:

The aim of this study was to select a *Lactobacillus* with probiotic abilities suitable for *in vivo* studies in farmed freshwater fish. 55 *Lactobacillus* isolated from the intestines of freshwater fish were screened for inhibition of fish and human pathogenic bacteria *in vitro*; and some selected strains by absence of production of biogenic amines and resistance to gastric and intestinal fluids (GIF) in a simulation model. A strain was studied in co-cultures with a pathogen in fish feed extract. Selected strains were tentatively identified as *Lactobacillus plantarum* 44a, whose mechanism of inhibition was based on acid production, and *L. brevis* 18f which was detected as a high H_2O_2 producer, because its supernatant adjusted to pH 6 strongly inhibited *Aeromonas hydrophila*. This activity was not observed when supernatant was treated with catalase. In the exposure of cells to GIF, *L. plantarum* 44a survived better than the other strains to pH 2, 2,5 and 3 and pepsin. *L. brevis* 18f had a very low survival in GIF. *L. plantarum* 44a co-cultured with *A. hydrophila* in fish feed extract with an initial ratio $\approx 10^3$:10^7 and 10^7: 10^3 respectively, started killing the pathogen when pH was around 5.5. *L. plantarum* 44a has potential applications as probiotic in freshwater fish. *L. brevis* 18f as a H_2O_2 producer may have application as a possible fish pathogen antagonist in the upper gastrointestinal tract, the skin, the gills and eggs where oxygen is available.

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4.1. INTRODUCTION

In the last years there has been an increasing interest in intestinal lactic acid bacteria (LAB) as beneficial flora that can improve resistance of fish against bacterial pathogens (Byun et al., 1997; Gatesoupe; 1999, Gildberg et al., 1997; Jöborn et al., 1997; Nikoskelainen et al., 2001; Ringø and Gatesoupe, 1998). Some strains, like *Carnobacterium divergens* K1 isolated from the gastrointestinal tract of Atlantic salmon (Jöborn et al., 1997), *Lactobacillus rhamnosus* from human origin (Nikoskelainen et al., 2001), and *Lactobacillus* DS-12, later classified as *Weisella hellenica* DS-12 isolated from the flounder intestines, a marine fish (Byun et al., 1997) have been selected as a result of a screening procedure for several characteristics studied in vitro, such as inhibitory activities against several fish pathogens (Byun et al., 1997; Jöborn et al., 1997; Nikoskelainen et al., 2001); acid tolerance (Byun et al., 1997), bile tolerance (Byun et al., 1997, Jöborn et al., 1997, Nikoskelainen et al., 2001) and growth in fecal material (Jöborn et al., 1997). *In vivo* trials with these strains have shown a positive role of LAB in the protection of fish when challenged with bacterial pathogens (Nikoskelainen et al., 2001; Robertson et al., 2000) or in the reduction of some undesirable bacteria already present in fish (Byun et al., 1997). Reports about screening lactic acid bacteria for warm freshwater fish species are almost absent in the literature. In this study we are reporting the screening and selection of a probiotic *Lactobacillus* for warm water fish based on inhibitory activities of a wide range of bacterial pathogens, using several substrates present in commercial fish feed, including low amounts of monosaccharides and presence of polysaccharides; survival during a simulation of a fish gastrointestinal tract passage and absence of biogenic amine production under limitation of glucose. Fish is very sensitive to some of these compounds (Fairgrieve et al., 1994) that under certain circumstances may be produced by some lactic acid bacteria (Cid and Holzapfel, 1999; Joosten et al., 1989).

4.2. MATERIAL AND METHODS

Lactobacillus

A set of 55 strains from a strain collection of 156 strains isolated from fish intestines was used for the screening experiments (Table 4.1). The strains were stored at –80^{o}C before use. Prior to use, they were thawed at room temperature, and inoculated in MRS (Merck, Darmstadt, Germany).

Pathogens

To detect antagonistic activities of *Lactobacillus*, the following bacteria were used as indicators: *Aeromonas hydrophila* (Dept. Food Microbiology, WUR), *Edwardsiella tarda* 524362 and *Yersinia ruckerii* were isolated from diseased fish (ID-Lelystad /Institue for Animal diseases) and *Staphylococcus aureus* 196E (human isolate, enterotoxin A producer) obtained from the National Institute of Public Health and the Environment, The Netherlands). These strains belong to species that have been reported as being often harbored in fish intestines (Austin and Austin, 1985; Austin and Gibb, 1993; Sugita et al., 1996).

Antimicrobial activity assay

For our initial screening approaches, we used the agar spot assay, with previous growth of the *Lactobacillus* strains before pathogens are seeded (Schillinger and Lücke, 1989). Modified MRS agar with varying glucose concentration (2.0; 0.2 and 0% w/v) was used as bottom medium.

The spots were made by pouring 10 μl of a well grown overnight culture of the strains in the center of the agar plates. The plates were incubated overnight at 30^{o}C and the growth of the strains was checked next day.

After the spots were developed, the plates were poured with soft agar (≈50^{o}C) composed of Tryptone Soya Broth (TSB, Oxoid CM129, Hampshire, England) + 0.7% agar bacteriological (Oxoid L11, Hampshire, England) containing 5% of an overnight culture of a pathogen in TSB. Inhibition was recorded by measuring absence of growth of the pathogens around the spots. Selected strains were spotted onto the surface of Nutrient Agar (NA, Oxoid CM3, Hampshire, England) plates with 1% either of glucose, maltose, dextrin or starch and 0.01% of bromocresol purple as pH indicator. They were incubated aerobically and anaerobically for up to 72 h. After growth was detected on the spots, the plates

were poured with soft agar (≈50°C) composed of NA + 0.7% agar bacteriological containing 5% of an overnight culture of *A. hydrophila* previously grown in TSB. All tests were performed in duplicate.

Table 4.1. Source of the intestinal *Lactobacillus* screened to select a probiotic for warm water fish: fish hostess, habitat and season of collection of fish; log CFU of intestinal *Lactobacillus* in the intestinal content and number of isolates screened.

Common name of fish	No.of fish analyzed	Habitat of fish[1]	Season of collection	Log CFU/g intestinal content[3]	No. of isolates
African catfish	2	Farm [2]	spring	< 2.00	6
European eel	3	River Maas	summer	5.10	19
Bream	2	River Maas	summer	7.30	7
Perch	3	River Maas	summer	7.30	18
Rudd	2	River Maas	summer	7.60	5

Scientific names of the fish and codes of the bacterial isolates used in the screening experiments: African catfish (*Clarias gariepinus*), 7 (a,j) and 18 (b,c,f,j); European eel (*Anguilla anguilla*), 43 (c,d,e,f,h,j), 44 (a,b,c,d,e, h, j) and 45(a,b,d,f,g,h); Bream *(Abramis brama*), 38(a,b), 46 (d,e,i) and 47 (c, h,i), Perch (*Perca fluviatalis*), 48 (a, c,d,e,f,h,i) and 49 (a,b,d,f,g,h,i,j); Rudd (*Scardinius erypthothalmus*), 50(a,f,i) and 51 (a,b,d,j). [1]All the sites were localized in the Netherlands. [2]Farm with a recirculation system of water. 3Approximated values described in chapter 3.

The well diffusion agar test was used to study the effect of the cell-free supernatant from the previously selected *Lactobacillus* cultures on growth of pathogens. *Lactobacillus* strains were cultured as overnight cultures in MRS broth containing 0.2, 0.4, 0.8, 1.6 and 2% glucose. The supernatant free of cells was obtained after centrifuging and filtering through 0.2μm membranes (Schleicher and Schuell, Dassel, Germany). The supernatants were assayed in plates seeded with pathogens, into which 9 mm-holes had been punched and filled with the sample to be tested. Pathogen plates were prepared as follows: an overnight culture of pathogens ($OD_{600} \approx 1$) in TSB was inoculated at 3% v/v in NA and poured in plates.

After solidification; 4 wells were made in each plate with a sterile 9 mm stainless steel tube. 200 µl of the supernatant of *Lactobacillus* cultures was used to fill the wells. Plates were incubated at 30°C. Inhibition was recorded after overnight incubation by measuring the diameter of absence of growth of the pathogens around the wells. All tests were performed in duplicate.

Mechanisms of inhibition

The supernatants of the six most promising strains were adjusted to pH 6.0 with 2M NaOH to determine whether the inhibitory activity on the pathogens remained. In case of remaining inhibition at pH 6, catalase (Sigma C-30, St Louis MO, USA) was added (1000 U/ml, incubation 37°C for 3 h) to test presence of H_2O_2. This test was also carried out on supernatants obtained from presumptive H_2O_2 producer strains grown in agitation (150 rpm for 48 h) at 30°C. Agitation was used to promote H_2O_2 production. In case that inhibition was only observed at low pH, and presumptively due to lactic acid in the case of facultative heterofermentative strains, a control assay was performed using pure lactic acid at the same concentration and pH of acid produced by *L. plantarum* 44a (196 mM), which was the highest concentration of acid produced by the selected strains. For the reference solution, sodium acetate and ammonium citrate were added in the same concentration as present in MRS and presumptively in the culture medium. pH values of supernatants and chemical solution were adjusted to 3.7; 4.5, 5 and 6 using 2 M HCl.

Amine production

Most inhibitory strains were screened according to Joosten and Northold (1989) and Cid and Holzapfel (1999) to detect decarboxylation of arginine, lysine, histidine and tyrosine. All of the plates were incubated either aerobically or anaerobically at 30°C for up to 7 days.

Table 4.2. Inhibition of Gram-negative and Gram-positive bacteria in the agar spot test by *Lactobacillus* isolates. Bottom layer in the test was MRS modified with varying glucose concentration (0, 0.2 and 2%).

	Pathogens											
	Gram-positive						Gram-negative					
	Staphylococcus aureus			*Aeromonas hydrophila*			*Edwardsiella tarda*			*Yersinia ruckeri*		
	Glucose (%)			Glucose (%)			Glucose (%)			Glucose (%)		
MRS	0	0.2	2	0	0.2	2	0	0.2	2	0	0.2	2
Strains												
L. brevis 7j	+	+	+++	—	+	+++	—	—	++	—	—	+++
L. brevis 18b	+	++	+++	—	+	+++	—	+	++	—	—	++
L. brevis 18f	++	+	+++	—	—	+++	—	+	+++	—	—	+++
L .plantarum 44a	—	+	+++	—	+	+++	—	+	+++	—	—	+++
L. sakei 49b	—	—	++	—	++	++	—	+	+++	—	—	+++
L. sakei 38a	—	+	+++	—	—	++	—	—	+++	—	—	++
Other 46 strains	—	—	+++	—	—	+++	—	—	+++	—	—	+++

Symbols denote the zone of inhibition as follows: (-), no inhibition; (+),1-5 mm; (++),6-20 mm; (+++), 21-60 mm.

Identification procedures

The most promising *Lactobacillus* were examined by morphology, growth in acetate agar, presence of meso-diaminopimelic acid in the cell walls (Hammes and Vogel, 1995), CO_2 production from glucose in MRS broth without ammonium citrate, ammonium production as hydrolysis of arginine, in modified MRS broth (without meat extract and with added arginine 0.3% w/v, sodium citrate 0.2% w/v and glucose 0.05%) and adding a few drops of Nessler's reagent to detect the presence of ammonia after 5 days of aerobic incubation at 30^{o}C (Sánchez et al., 2000). Growth was tested at 15 and 45^{o}C. Fermentation of carbohydrates was determined three times using API 50 CHL strips (Biomerieux, Marcy l' Etoile,

France). The configuration of the isomers of lactic acid produced from glucose were determined enzymatically in the cell-free supernatant from 24 hour cultures in MRS broth (Boehringer Mannheim, Germany). Identification of major characteristics of the isolates was done according to manuals of Hammes and Vogel (1995), and Kandler and Weiss (1986) and the report of Takahashi et al. (1999).

Gastrointestinal tract simulation

Four selected *Lactobacillus* strains were cultured overnight in 25 ml MRS broth incubated at 30ºC. The grown cultures were washed three times in an isotonic solution, 0.1 M PBS, pH 6 (Charteris et al., 1998) centrifuging at 2765 x *g* for 15 min at 4°C. The cell suspension contained between 8-9 log CFU/ml depending on the strains.

Simulation of stomach passage. 5 ml of the washed cell suspension was poured in flasks containing 20 ml 0.1 M PBS, pH6 (Charteris et al., 1998). The flasks were kept in a water bath with thermostat adjusted to 30^{o}C. The content was kept spinning gently with a magnetic stirrer. After mixing the suspension, 13 mg of pepsin (≈ 50,000 U) (Sigma P6887, St Louis MO, USA) previously dissolved in 1 ml of 0.5% NaCl was added to the flasks. The pH was adjusted to pH 2.0 (treatment a), 2.5 (treatment b) and 3.0 (treatment c) adding approximately 1.62 mmol, 0.85 mmol and 0.5 mmol HCl respectively. Little differences in these amounts were used to obtain accurate pH values for the different strains. pH values were checked every 30 minutes and adjusted when necessary. Exposure time for simulation gastric juice was 2 hours.

Simulation of intestine passage.

After 2 h of stomach simulation time, the pH was raised to pH 6 by adding 2.5, 1, and 0.1 ml of 0.6 M NaOH (pH 6.7) to the respective treatments and approximately 10 ml of 0.2 M PBS (pH 8). Thereafter 100 mg pancreatin (Sigma, P 1750) dissolved in 2 ml 0.5% NaCl solution and 0.3% bile extract (Sigma B-8631) (w/v) were added to the treatments according to the final volume. For the treatment c, previously exposed to pH 3, instead to use bile extract, 1% v/v of pasteurized fish bile was added. Fish bile was collected fresh from the gallbladders of healthy adults of African catfish cultured in a hatchery.

Although pepsin, pancreatin and bile extract used in the simulation of the intestinal fluids were from pig origin, it is worth to mention that fish enzymes and

bile have been reported to be analogous to those found in the gastrointestinal tract of some freshwater fish (Guillaume and Choubert, 1999; Yeh and Hwang, 2001).

Enumeration of *Lactobacillus* was performed on MRS agar. Serial dilutions of samples were prepared using Reduced Peptone Solution (Hartemink and Rombouts, 1999). Plates were incubated at 30°C in anaerobic jars with a mixture of 80% N_2, 10% H_2 and 10% CO_2 using the Anoxomat system (Mart, Lichtenvoorde, The Netherlands). Results were reported by multiplying the number of CFU by the dilution factor after exposure to the stomach simulation and multiplying by the accumulated dilution factor after exposure to the intestine simulation to make standard values in reference to the initial concentration of cells given.

Co-cultures.

L. plantarum 44a was cultured with *A. hydrophila* in flasks containing 100 ml fish feed extract adjusting the initial ratio *Lactobacillus*: *A. hydrophila* to about 10^3: 10^7 CFU/ml and 10^7: 10^3 CFU/ml respectively. The given CFU values were chosen basically to study the potential of *L. plantarum* 44a to inhibit the growth of *A. hydrophila* using the nutrients available in the feed extract.

They were prepared by diluting a previously grown culture 1:100 to achieve about 10^7 CFU/ml of fish feed extract and 1:1000,000 to achieve about 10^3 CFU/ml of fish feed extract. Inoculum of *Lactobacillus* was grown in MRS broth and *A. hydrophila* in TSB both as overnight cultures. Cells were washed 3 times in PBS pH 6 and the re-suspended pellets were used to make the dilutions and to inoculate the fish feed. Feed extract was made by grinding feed T-2 Trouvit (Trouw, Fontaines les vervins, France) in a hammer mill (Retsch, bv, Ochten, The Netherlands) using a grinding screen with openings of 0.1 mm diameter. The powder was suspended in water 20:80 w/v and centrifuging 2620 x *g* for 5 min at 25°C. The precipitate was discarded and the supernatant was sterilized at 120°C for 15 min. Enumeration of *Lactobacillus* was performed at time 0, 4, 8, 12, and 24h on MRS agar as described previously. Enumeration of *A. hydrophila* was performed on Starch Ampicillin Agar (Palumbo et al., 1985). Serial dilutions of samples were prepared using Reduced Peptone Solution. Plates were incubated at 30°C in aerobic conditions for 18-20h.

4.3. RESULTS

Screening of Lactobacillus for inhibition of pathogens by the agar spot test

For our initial screening approaches, we used the classical agar spot assay, with previous growth of the *Lactobacillus* strains before pathogens are seeded (Schillinger and Lücke, 1989). Fifty-five *Lactobacillus* strains were used. First analysis showed that 52 out of 55 of the *Lactobacillus* isolates cultured with 2% glucose were able to inhibit growth of either *A. hydrophila* or *S. aureus* and in most of the cases to both pathogens. Fifty-two *Lactobacillus* isolates were characterized as homofermenters of glucose and only 3 strains as heterofermenters of glucose; thus lactic acid, in the case of homofermenters and lactic acid, acetic acid, CO_2, and ethanol in the case of heterofermenters may have played an important role in the inhibition.

For the evaluation of the inhibitory potential of the *Lactobacillus* isolates under limitation of glucose, the 55 *Lactobacillus* strains were spotted on MRS 0.2% glucose. Six *Lactobacillus* strains inhibited one or more of the following pathogens *A. hydrophila*, *E. tarda*, and *S. aureus*. Three of the isolates were also able to inhibit *S. aureus* without glucose (Table 4.2).

Six of the isolates were phenotypically identified as presumptive *L. brevis* 7j; *L. brevis* 18b *L. brevis* 18f, *L. plantarum* 44a, *L. sakei* 49b, and *L. sakei* 38a. The three strains of *L. brevis* showed a carbohydrate fermentation pattern close to *L. brevis* JCM 1562 (Takahashi et al., 1999). Cells were rod shaped, characterized by heterofermentation of glucose, D- and L- lactic acid production; acid production from cellobiose, fructose, galactose, glucose, maltose, mannose, N-acetyl-glucosamine, ribose, sucrose, trehalose, xylose; acid was not produced from L-arabinose, lactose, melebiose, melezitose, raffinose, sorbose, sorbitol and starch. *L. brevis* 18f can be distinguished from *L. brevis* JCM 1562 and from other *L. brevis* described (Hammes and Vogel, 1995; Kandler and Weiss, 1986) in producing acid from rhamnose. *L. plantarum* 44a was characterized by the presence of meso-diaminopimelic acid in the cell wall, homofermentation of glucose producing D- and L- lactic acid. Acid was produced from amygdalin, arabinose, cellobiose, gluconate, mannitol, melezitose, melibiose, raffinose, ribose, sorbitol and sucrose. Acid was not produced from glycerol and xylose. Esculin was hydrolyzed. *L. sakei* 49b and *L. sakei* 38a were characterized by absence of meso-diaminopimelic acid in the cell wall, homofermentation of glucose in D- and L- lactic acid. Acid production from

amygdaline, L-arabinose, cellobiose, D-fructose, galactose, β-gentibiose, gluconate, D-glucose, D-mannose, melibiose, N-acetyl-glucosamine, ribose, saccharose, trehalose and D-xylose. The six *Lactobacillus* strains also inhibited *Aeromonas hydrophila* using maltose and dextrin (Table 4.3), but not starch.

Mechanisms of inhibition of cell free supernatants in the well diffusion assay.

Although some strains showed inhibitory activities against some of the pathogens at low glucose level in the agar spot test, the supernatants from these strains grown in MRS with 0.2% glucose were not inhibitory against the pathogens in the well diffusion assay. Only *L. plantarum* 44a was able to inhibit pathogens using ≥ 0.4 % glucose; the rest of the strains were only able to inhibit pathogens with 1.6% of glucose.

Inhibition of pathogens by *L. plantarum* 44a supernatant was compared with pure lactic acid adjusted to pH 3.7, 4.5, 5 and 6 as this strain is supposed to produce lactic acid as the main end product of the glucose fermentation. Pure lactic acid in the same concentration as it was titrated in *L. plantarum* 44a supernatant (196 mM) gave similar diameter halos of inhibition of *L. plantarum* 44a supernatant at pH 3.7, however; *L. plantarum* 44a supernatant was more inhibitory than pure acids at pH 4.5 (Table 4.4). Although some other metabolites may be acting in the supernatant pH ≥ 4.5, it may be considered that the larger inhibition of this strain is due to the acid presence at low pH. For *L. sakei* 49b and *L. sakei* 38a acid production was also likely the main mechanism of inhibition because supernatants were not inhibitory above pH 4.5 (data not shown).

Table 4.4. Inhibition of *Aeromonas hydrophila* in the well diffusion agar using lactic acid and *L. plantarum* 44a supernatants adjusted to several pH values. Diameter of inhibition (mm) measured in two independent tests and averaged.

	pH			
	3.7	4.5	5.0	6.0
Acids[1]	11.3 ± 0.7	5.7 ± 0.16	-	-
L .plantarum 44a Supernatant	12.4 ± 1.3	7.3 ± 0.90*	-	-

[1]Acids: Pure lactic acid (196 mM) + sodium acetate + ammonium citrate in the same concentration as in MRS. Values are means ± SD. Significantly different from control at the same pH value: *p < 0.05.

Inhibition by L. brevis 18f supernatant

L. brevis 18f was detected as a high H_2O_2 producer, since its supernatant adjusted to pH 6 inhibited *A. hydrophila*, *E. tarda*, and *S. aureus*, while this inhibitory activity was entirely lost after incubation with catalase (Table 4.5). Catalase mediates the dismutation of H_2O_2 to H_2O and O_2 (Voet et al., 1998). *L. brevis* 7j, and *L. brevis* 18b also produced some H_2O_2, however, diffusion zones were turbid and hardly measurable (data not shown).

Table 4.5. Inhibition of some pathogens in the well diffusion agar by *L. brevis* 18f supernatant at pH 6.0.

	Pathogens			
Strain	*A. hydrophila*	*E. tarda*	*S. aureus*	*Y.ruckerii*
L. brevis 18f	—	—	—	—
L. brevis 18f (a)	11.2 ± 1.16	2.0	8.9 ± 2.1	—
L. brevis 18f (a + cat)	—	—	—	—

(*a*), cultured under aereation; (*cat*), supernatant exposed to catalase. Zone of inhibition shown in mm, - no inhibtion detected.

Screening for biogenic amine production by Lactobacillus

None of these strains showed decarboxylation activity of arginine, histidine and lysine neither in aerobic nor in anaerobic conditions. However, tyrosine was decarboxylated by *L. brevis* 7j; *L. brevis* 18b, *L. brevis* 18f and *L. sakei* 38a under anaerobic conditions according to the procedure described by Joosten and Northold (1989), which led to the presence of tyramine, evidenced as the presence of a transparent halo surrounding the spots (Cid and Holzapfel, 1999). *L. plantarum* 44a and *L. sakei* 49b did not showed decarboxylation activity.

Effect of gastrointestinal simulation on the viability of Lactobacillus

L. plantarum 44a, *L.sakei* 49b, *L. brevis* 7j and *L. brevis* 18f were selected on basis of overall performance to be screened in a dynamic model of the gastrointestinal tract, first by acid resistance in presence of pepsin and after that by bile resistance in presence of pancreatin. As a result, a variable response of the different strains was found (Fig. 4.1A, 4.1B and 4.1C). Homofermenters of glucose (*L. plantarum* 44a and *L.sakei* 49b) were more resistant to the gastric fluids than heterofermenters of glucose (*L. brevis* 7j and *L. brevis* 18f). Among homofermenters, *L. plantarum* 44a showed a strikingly higher survival than other strains. *L. plantarum* 44a showed little or no decrease in viable cell numbers even after 4 h incubation at pH 3.0 (data not shown) and the lowest reduction at lower pH. This strain was not affected by the presence of 1% fish bile.

L. plantarum 44a was selected to make a kinetic study cultured with *A. hydrophila* because *L. plantarum* 44a displayed inhibitory activities at low and high glucose levels, it did not produce biogenic amines and was the most resistant strain in the passage through a simulated gastrointestinal tract. *L. plantarum* 44a and *A. hydrophila* were cultured in fish feed extract as single cultures and as mixed cultures, adjusting the initial ratio *Lactobacillus*: pathogen to about 10^3: 10^7 CFU/ml and 10^7: 10^3 CFU/ml. In single cultures, both strains *L. plantarum* 44a and *A. hydrophila* grew until reach about log 8.0 CFU/ml after 24 h when they had been inoculated in a low initial concentration (Fig. 4.2A) and reach the late stationary phase by the same time when they had been inoculated in a high initial concentration (Fig. 4.2B). In mix cultures, however; *L. plantarum* 44a strongly

inhibited *A. hydrophila*. When *L. plantarum* 44a was initially dominant, inhibition of *A. hydrophila* was evident from the 8th hour and following hours (Fig. 4.2C). When *L. plantarum* 44a was initially in low concentration, and *A. hydrophila* in higher concentration, inhibition of *A. hydrophila* took more time, being evident only at the end of the experiment (Fig. 2D). pH values in both mix cultures at the time when the inhibition had started were around 5.5.

4.4. DISCUSSION

The present study was carried out to select from a large number of strains isolated from various fish species those with the best functional characteristics in relation to pathogen inhibition and survival to low pH and bile to be studied as probiotic candidates for freshwater fish reared at temperatures around 30^{o}C.

Most of *Lactobacillus* isolates, both homofermenters and heterofermenters, were able to inhibit pathogens by acid production when using a high glucose concentration. A few strains also inhibited both Gram-positive and Gram-negative fish and human pathogens with low (0.2%) concentration of glucose in the medium. Microbial inhibition under limitation of glucose is a rather unique property displayed by some *Lactobacillus* strains. Inhibitory activities of these strains have been usually detected against related species (Schillinger and Lücke, 1989), related genera like *Clostridium* or other Gram-positive organisms like *Staphylococcus aureus*. But, only few *Lactobacillus* growing at low glucose concentration have been reported to inhibit a broader range of microorganisms, including Gram-negative foodborne and human pathogens. Some of these are currently used as probiotics (Jacobsen et al., 1999).

Selected pathogens inhibited at low glucose concentrations were *Aeromonas hydrophila*, *Edwardsiella tarda*, common pathogens in freshwater fish belonging to *Vibrionaceae* and *Enterobacteriaceae* families, respectively. *S. aureus*, and *A. hydrophila* are both pathogens in humans, and may be harbored in fish intestines (Austin and Austin, 1985; Austin and Gibb, 1993; Sugita et al., 1996). Inhibition of some of these pathogens by selected *Lactobacillus* spp. at high and low glucose concentrations is a desirable characteristic for probiotic candidates as it increases the chance of inhibition within the gastrointestinal tract where carbohydrate availability is fluctuating.

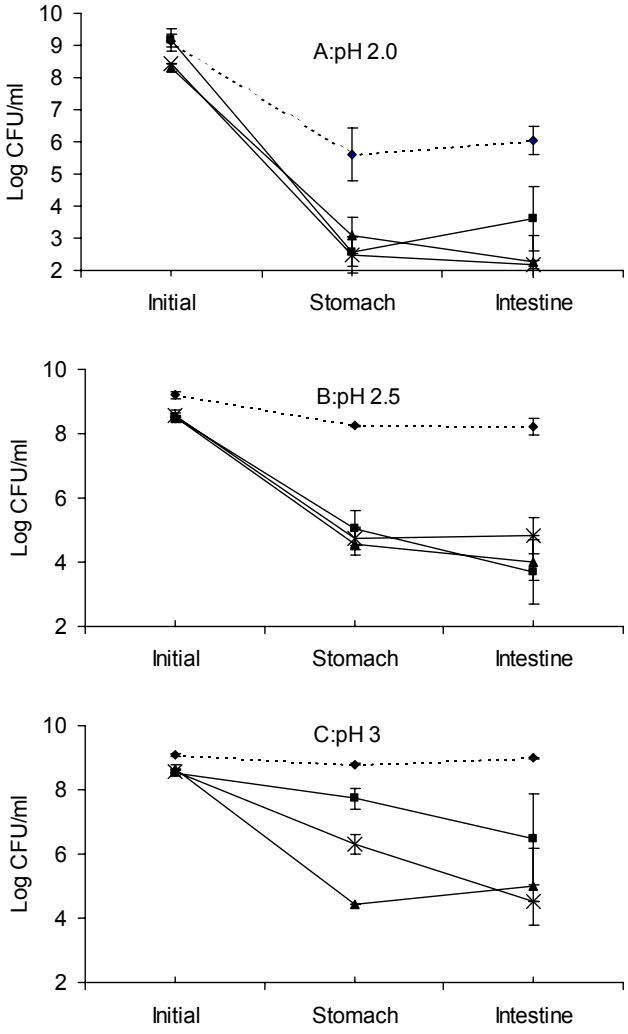

Fig. 4.1. Survival of various *Lactobacillus* after simulation of exposure to stomach fluids for two hours at A, pH 2; B, pH 2.5 and C, pH 3.0 and thereafter simulation of exposure to intestinal fluids for two hours. Strains: ▲, *L. brevis* 7j, ×, *L. brevis* 18f., ◆, *L. plantarum* 44a; ■, *L. sakei* 49b.

Although other groups of lactic acid bacteria like *Carnobacterium* have been reported to display inhibitory activities against Gram-positive and Gram-negative fish pathogens *in vitro* (Jöborn et al., 1997; Nikoskelainen et al., 2001; Ringø and Gatesouope, 1998; Robertson et al., 2000), they are psychrotrophic bacteria, usually present in cold water fish species and act against cold water pathogens at low temperature. Although *Lactobacillus rhamnosus* isolated from human origin was an effective probiotic for trout (Nikoskelainen et al., 2001); its effectiveness for tropical fish has not been reported.

Acid production was the main mechanism of inhibition by *L. plantarum* 44a, *L. sakei* 49b and *L. sakei* pH38a. Acid inhibition is largely contributed to the undissociated form of the acids which is predominant at low pH depending on the pK_a of the acid type present. Acid production was previously described as the main mechanisms of inhibition of pathogens by *Lactobacillus* sp. DS-12 isolated from flounder intestines, a marine fish (Byun et al., 1997); *Lactobacillus* sp. DS-12 was considered as a probiotic strain because, when orally supplemented to flounder, led to a decrease in the number of total aerobes and in a trial improved the body weight gain of fish (Byun et al., 1997)

Hydrogen peroxide was the main mechanism of inhibition detected in *L. brevis* 18f supernatant and its activity was pH independent. In the case of *L. brevis* 7j and 18b, the effect of H_2O_2 was only observed at pH $\leq$ 5.5 (data not shown). Although, it seems that this the first study where H_2O_2 production by intestinal fish *Lactobacillus* has been reported, H_2O_2 production by *Lactobacillus* spp. from other habitats has been reported previously (Lindgren and Dobrogosz, 1990). H_2O_2 is inhibitory to a wide spectrum of Gram-positive and Gram-negative bacteria. The antimicrobial effect of H_2O_2 has been attributed to its strong oxidizing effect on the bacterial cell (Lindgren and Dobrogosz, 1990), to the oxidation of sulfhydryl groups causing denaturing of a number of enzymes, and to the peroxidation of membrane lipids thus affecting membrane permeability (Kong and Davidson, 1980).

The production of H_2O_2 by *L. brevis* 18f may be explained by the availability of oxygen, and the lack of catalase activity (Lindgren and Dobrogosz, 1990). *Lactobacillus* spp. are aero-tolerant bacteria, and most of the species can survive and grow in presence of oxygen. Some species partially reduce O_2 to yield reactive oxygen species like the superoxide radical $O_2^{\cdot -}$ which can be reduced into relatively less toxic H_2O_2 with an NADH Oxidase, and the enzyme superoxide dismutase (Marty Teysset et al., 2000). It is possible that H_2O_2 was also the inhibitory compound produced by *L. brevis* 18b and *L. brevis* 18f when cultured in the

previously described agar spot test in MRS at low glucose level, and even without glucose. Jaroni and Brashears (2000) found that *Lactobacillus delbrueckii* subsp. *lactis* inoculated in phosphate buffer containing 0% glucose produced significantly more H_2O_2 than cultures containing 1% or 10% glucose at refrigeration temperatures. Although cells had been previously propagated in a medium that contained a carbohydrate source, H_2O_2 was produced under starvation conditions.

One disadvantage of *L. brevis* 18f to be used as a probiotic is a very low survival to low pH in the gastrointestinal simulation model. However, this strain may be tested to be used as protective agents for other sites of action with higher pH and where oxygen is available like the fish skin, gills, fish eggs and fish larvae. H_2O_2 has been successfully used for the prophylactic treatment of fish eggs, to control fungus and to improve hatching success (Rach et al., 1998), it has been recommended for the control of fungal infections in all fish life stages; and it has been approved by the U.S. Food and Drug Administration.

In the simulation of the gastrointestinal tract, *L. plantarum* 44a was the best survivor among the strains tested. In a study, *Lactobacillus* sp. DS-12 showed high bile and acid resistance (pH 3, 90 min) and had also high survival in flounder intestines (Byun et al., 1997). On the other hand, *L. plantarum* 44a had several characteristics that led us to select it as the best candidate for probiotic intestinal action, as it could inhibit pathogens at low and high glucose concentrations, had the highest survival in the simulation of gastrointestinal passage and did not decarboxylate amino acids. In mixed cultures in fish feed extract it starts to kill *A. hydrophila* at pH around 5.5 (Fig. 4.3).

As feed supplied to fish is the only exogenous source of nutrients available for bacterial growth in the gastrointestinal tract of farmed fish; we might expect an advantage of this *Lactobacillus* strain over other flora competing for nutrients. It has been stated that the ability to compete for limiting nutrients is one of the most important factors that determine the composition of the gut flora, in which the species that are unable to compete are eliminated (Fooks and Gibson, 2002). Despite the neutral pH conditions of the large intestine, which probably are not favorable for the antimicrobial action of lactic acid in general, it could be assumed that probiotic lactic acid bacterial strains might be beneficial in combating Gram-negative pathogens in the intestine. This could happen through local production of relevant concentrations of lactic acid in the microenvironments, with inhibition of

harmful Gram-negative strains by the combined action of lactic acid and bile salts (Alakomi et al., 2000).

Although, *L. plantarum* 44a and *L. brevis* 18f were part of the predominant intestinal lactic acid bacteria in the host from which they were collected (Table 4.1), they were not dominant in the total anaerobic population. However, according to the criteria employed to screen the strains in this research they were selected for further studies.

In conclusion we are proposing *L. plantarum* 44a and *L. brevis* 18f as candidates for further probiotic studies *in vivo*.

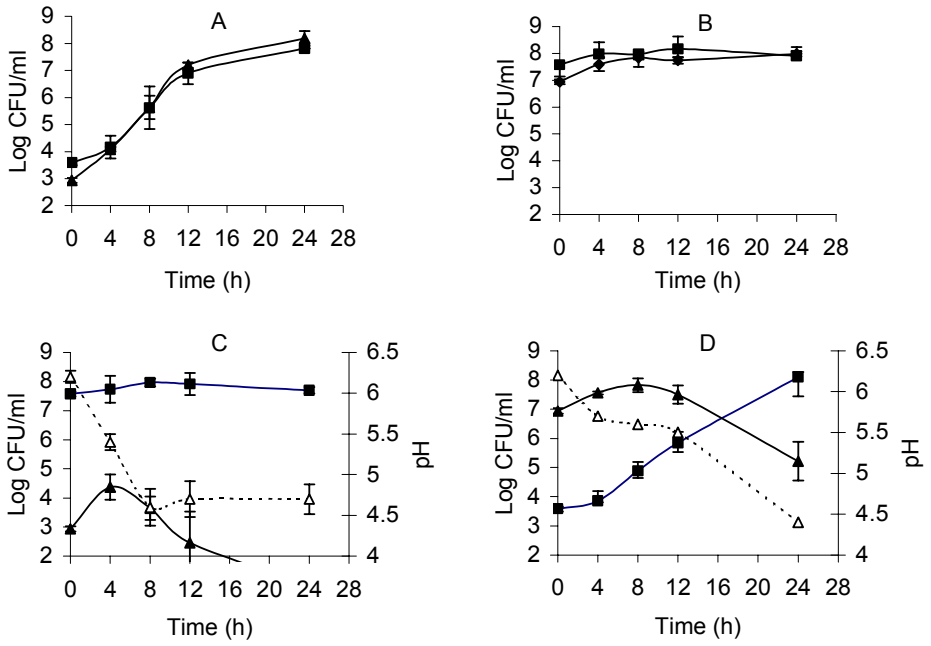

Fig. 4.2. Growth of *Lactobacillus plantarum* and *Aeromonas hydrophila* in fish feed extract incubated at 30°C in single cultures (A and B) and in a simulation of a disease-prevention situation C) and a simulation of a disease-recovery situation D). Bacterial strains: ■, *L. plantarum* 44a ▲, *Aeromonas hydrophila* --∆--, pH of mixed culture.

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5. Survival of *Lactobacillus plantarum* 44a after spraying and drying in feed and during exposure to gastrointestinal tract fluids *in vitro*

Adolfo Bucio, Ralf Hartemink, Johan W. Schrama, Johan Verreth and Frank M. Rombouts

Abstract:

A good probiotic strain should be able to survive the conditions of handling and storage to be delivered in high concentration to the host. That is especially important when stressful conditions are prevalent in the carrier, for instance in low water content foods like animal feed. The aim of this research was to study the survival of the probiotic candidate *Lactobacillus plantarum* 44a after spraying and drying in feed, and during storage and exposure to gastrointestinal tract fluids *in vitro*. In addition, the viability of the strain during exposure to distilled water and 2% NaCl was studied. Feed was sprayed with a suspension of ≈ 2 x 10^{10} CFU of *L. plantarum* 44a in 10, 15, 20, 25 and 30 % v/w of the feed and dried to constant weight (6% moisture) in a convective oven at 25°C. *L. plantarum* 44a survived 15, 36, 52, 79, and 100% respectively in relation to the original % v/w of the bacterial suspension applied to the feed. After three weeks of storage at 25°C, survival was similarly low in all the treatments. *L. plantarum* 44a stored in feed containing 13% of moisture, vacuum-packaged and stored in refrigeration, maintained high viability (≈100%) after 1 year of storage. Survival was not affected after feed-containing *L. plantarum* 44a was exposed to gastrointestinal fluids in a simulation model. Viability of *L. plantarum* 44a as a cell suspension in PBS added directly to distilled water or distilled water with 2% NaCl was maintained up to 48 h; after 72 h, viability started to decline. As conclusions, *L. plantarum* 44a maintained high viability after drying and storage in feed even after exposure to gastric and intestinal fluids *in vitro*.

5.1. INTRODUCTION

Probiotics are microorganisms that when ingested in high enough numbers beneficially affect the host's health. To reach these beneficial results, it is necessary to maintain good viability of the probiotic strains during processing, storage and consumption. Stress conditions that usually affect viability of probiotics during production are the following: large concentration of fermentation by-products (e.g., lactic acid in the culture medium), harvesting (centrifugation, ultrafiltration), freezing, and drying (during freeze-drying process). In addition, the strains encounter the following stress conditions in the gastrointestinal tract: re-hydration in an acidic environment, long exposure time to stomach acidity, sometimes in the presence of antimicrobial compounds (in certain foods) and thereafter to the exposure to bile acids (Siuta Cruce and Goulet, 2001).

Probiotics are usually added to animal feed as freeze-dried cultures which sometimes are mixed with lipids to be added as top dressings in the feed (Robertson et al., 2000, Nikoskelainen et al., 2001). Fatty acids may be also used to encapsulate freeze-dried probiotics to enhance their viability (Siuta Cruce and Goulet, 2001).

Convective drying has been suggested as another means to preserve lactic acid bacteria starters with less expensive equipment than freeze drying (Linders et al., 1996; Kets et al., 1996) and also seems to be an attractive possibility to dry and preserve lactic acid probiotics added to the feed. However, little information on this topic has been described.

In order to maintain viability, several parameters in relation to thermal and drying inactivation (fermentative capacity) as well as culture conditions of the strains have to be optimized, to allow cells to adapt physiologically to the de-hydration process (Linders et al, 1996; Kets et al., 1996; Wouters et al., 1998). For instance *L. plantarum* cultured at pH 5 had a higher resistance to osmotic pressure than cells cultured at pH 7 (Wouters et al., 1998). Survival of *L. paracasei* increased during spray drying as a result of preadaptation with heat or salt (NaCl) compared with non-treated controls (Desmond et al., 2001). Bacteria also should interact positively with the food or carrier matrix to use (which depends on feed composition, pH, a_w, etc.) and to the extrinsic factors like temperature, O_2, relative humidity, etc (Linders et al., 1997).

The study of survival rate of bacteria when exposed to various levels of stress have led to define a term called "the adaptative response", that is when cells are exposed to a moderate level of stress, they acquire increased resistance to a subsequent exposure to a more severe level of the same or other stress that otherwise would be lethal (Kim et al., 2001). Adaptative response has not been reported as a mechanism of survival of lactic acid bacteria when subjected to dryness at physiological temperature, particularly, when incorporated as probiotics in animal feed, like fish feed.

In the present investigation, we studied the survival rate of *L. plantarum* in function of re-hydration and dehydration in low moisture feed to try to define some optimal process parameters to maximize viability. The viability of cells in the best treatment was studied during storage and exposure to simulated gastrointestinal tract fluids. In addition, the viability of the strain during exposure to distilled water and 2% NaCl was studied as it is also possible to administer probiotic strains through (drinking) water.

We selected *Lactobacillus plantarum* 44a for our studies because it is a probiotic candidate for warm freshwater fish as it displays inhibitory activities against common fish pathogens, and it is tolerant to intestinal environment being acid tolerant and fish bile tolerant (Chapter 4)

5.2. MATERIAL AND METHODS

Strains

L. plantarum 44a was isolated from the intestinal content of European eel (*Anguilla anguilla*) (Chapter 3) and a strain of *Lactobacillus salivarius* (win) was supplied by Winclove Bioindustries b.v., Amsterdam, The Netherlands. *L. salivarius* (win) was used as a comparative strain for some experiments because that strain has a similar acidification capacity as *L. plantarum* 44a, but differs in its peptydoglycan type (Information from Winclove and Kandler and Weiss, 1986).

Feed

Fish feed was Trouvit-2 (Trouw, Fontaines, Les Vervins, France) manufactured by Nutreco, France - taken from the fish farm De Haar Vissen - belonging to Wageningen University. Feed was prepared from fish meal, soybean and other grain products. Composition of the feed in (%) is crude protein, 55; crude fat, 16;

ash, 12; Vit.A, 10,000 U; Vit. D3, 1,500 U; Vit E, 250 U and butylated hydroxytoluene (BHT) as antioxidant.

Optimization of volume of the cell suspension (inoculum) in relation to feed weight

L. plantarum 44a was grown as a 24 h culture at 30°C in MRS broth (Merck, 1.10661, Darmstadt, Germany), washed 3 times in 0.1 M potassium phosphate buffer PBS pH 6 and harvested by centrifugation (2760 x g for 15 min). The cells were re-suspended in the same buffer. The cell suspension was divided into 5 equal portions which were adjusted with (PBS) pH 6 to represent 10%, 15%, 20%, 25% and 30% v/w of the final feed (100g). Cells were sprayed on fish feed and dried at 25°C up to 96 hours to completely dry the feed. The relative humidity (RH) in the convective oven was about 40%. Samples were also analyzed after 3 weeks of maintenance in the same oven.

Samples of 1g of feed were collected during drying time or in the final product (dry feed). Enumeration of *Lactobacillus* was performed on MRS agar. Serial dilutions of samples were prepared using Reduced Peptone Solution (Hartemink and Rombouts, 1999). Plates were incubated at 30°C in anaerobic jars with a mixture of 80% N_2, 10% H_2 and 10% CO_2 using the Anoxomat system (Mart, Lichtenvoorde, The Netherlands). The percent survival at each of the treatments was calculated as follows: % survival = (A_0/A_1) x 100, where A_0 is the number of bacteria per gram of dry mass before drying and A_1 is the number of bacteria per gram of dry mass after drying.

The moisture content and dry mass of the feed before and after drying was determined by grinding an aliquot of about 1g in a mortar and drying in an oven at 103°C. The results were expressed in % of the moisture and dry mass content.

Feed with the highest viability of bacteria at 24 h was selected to study the kinetics of a_w during the first 24 h of drying time.

Water activity was measured using a Novasina a_w box (RS 232, Axair, Pfäffikon, Switzerland) up to 24 hours.

Comparative survival of L. plantarum stored at 25 and 4°C

Feed with the highest viability of *L. plantarum* 44a after 24 h of drying was selected to study storage life. Feed was placed in closed flasks or in vacuum packages and stored at 25 and 4°C. As a reference to survival in refrigeration, the *L. salivarius* (win) strain was also tested. *L. salivarius* (win) was cultured and harvested as described for *L. plantarum* 44a. *L. salivarius* (win) was also used in lyophilized form, which was prepared by suspending 0,1g in 30 ml of PBS pH 6. All the cell suspensions were sprayed at 30% of the feed weight, dried for 24h and stored as described previously.

Viability of Lactobacillus after gastrointestinal simulation

These experiments were carried out using feed inoculated either with *L. plantarum* 44a or *L. salivarius* (win) both in lyophilized form and as overnight cultures. The three kinds of inoculated feed had previously been stored in refrigeration for three weeks.

For the experiment, 0.5 g of of each feed was weighed and put in flasks containing 25 ml PBS pH 6 and a magnetic rod. After 2 hours of agitation in a water bath at 30°C, 0.11g pepsin (Sigma P6887) previously dissolved in 1ml 0.5% NaCl was added. Then, the pH was adjusted to 3.0, using 8 ml 0.1M HCl. After 15 minutes pH was adjusted again to this value adding 0.5 ml 0.1M HCl. The suspensions with the cells were kept spinning with magnetic rods for 2 hours. After this, 1 ml of the solution was taken for microbiological analysis.

For the intestinal simulation, 10 ml PBS pH 8 was added to the flasks and the pH was adjusted to 7 with 2.5 ml 0.6 M NaOH. Then 0.100g pancreatin (Sigma, P 1750) was dissolved in 1 ml 0.5% NaCl and added to the solution. The total volume of the solution was 45.5 ml, then 0.14 g of bile extract (Sigma B-8631) previously dissolved in 1 ml 0.5%NaCl was added to the suspension. The flasks were maintained in the water bath at 30°C for another 2 hours, while stirring with magnetic rods. Experiments were done in duplicate.

Viability of L. plantarum 44a in distilled water and distilled water with 2% NaCl

A cell suspension of *L. plantarum* 44a was prepared from a 24-h culture in MRS and washing 3 times in PBS as described previously. 1 ml of the cell suspension was added to 999 ml of distilled water or distilled water containing 2% NaCl. Flasks were maintained at 25°C and viable counts were estimated at 24 h intervals using

Reduced Peptone Solution to make dilutions and MRS to determine viability as described earlier.

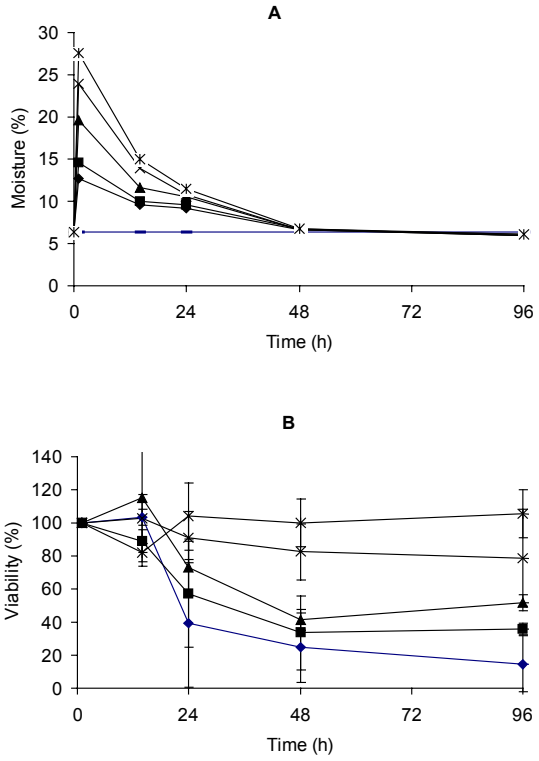

Figure 5.1. Variation of moisture (5.1A) and *Lactobacillus* viability (5.1B) (based on initial dry mass) during dehydration at 25oC. Feed was sprayed in all the treatments with a suspension of ≈ 2 x 1010 CFU of *L. plantarum* 44a using PBS in various amounts in relation to feed weight: ◆, 10 % PBS; ■, 15 % PBS; ▲, 20 % PBS; X, 25 % PBS; ∗, 30 % PBS. _, original moisture content of the feed. Each point represents the mean value of 3 measurements ± SD. Original moisture content of the feed.

5.3. RESULTS

Optimization of volume of the cell suspension (inoculum) in relation to feed weight

Fig. 5.1 shows the kinetics of the re-hydration and dehydration process of feed sprayed with *Lactobacillus* and the associated viability of the strain for the same intervals of time.

It can bee seen in Fig. 5.1A that a complete de-hydration of fluid was achieved after 48 hours. *Lactobacillus* viability appears to be relatively constant in two of the treatments (treatments with the highest initial moisture content) throughout the drying time (Fig. 5.1B). *Lactobacillus* viability in the other three treatments began to fall after 14 h of drying and appeared to be stabilized between 15 and 50% after 48 hours.

Standard deviations of viability were relatively high for all the treatments, suggesting that *Lactobacillus* were not homogeneously distributed in the feed samples. Despite that variability, the regression line generated with the data plotted in 5.2, shows that viability of *Lactobacillus* was directly related to the amount of fluid used to spray the feed and its associated moisture content. However, viability decreased to approximately the same levels for all the treatments after 3 weeks of storage of the dried feed at 25^{o}C and 40% RH (Fig. 5.2), which shows that the loss of viability was delayed in the treatment with the higher initial moisture content.

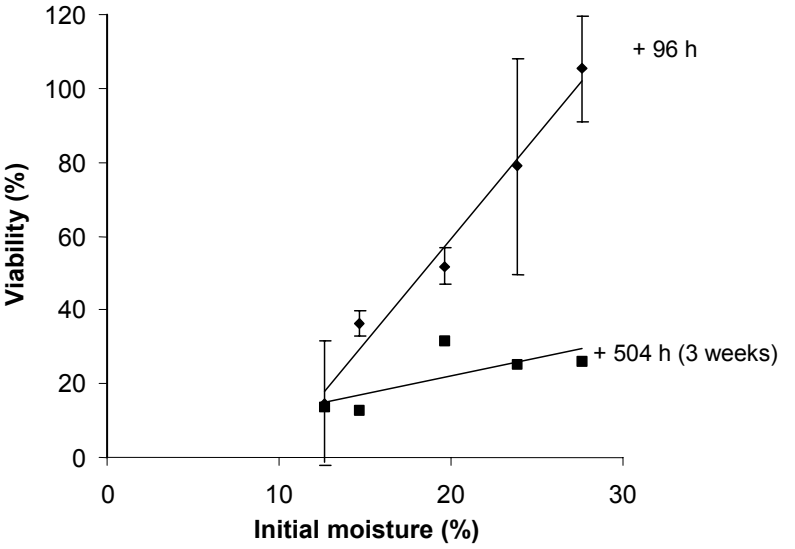

Fig. 5. 2. Regression line between initial moisture and viability of strain in feed dried to values of non inoculated feed. Vertical lines show standard deviation of each of the means for 96h.

Fig. 5.3 shows the kinetics of water activity of the inoculated feed (30% v/w) during drying at 25°C along with the survival of *L. plantarum* 44a. In an amount of 30% v/w of suspension as compared to the dry feed, the initial a_w of the feed of 0.5 increased to 0.9. After 24 h of drying at 25°C, a_w had dropped to just below 0.6, with a very high survival rate of the inoculum. Although, a_w was not measured in the other treatments it may be inferred that their initial value and final values were lower leading to more severe levels of osmotic stress to the strain.

Viability of L. plantarum 44a during feed storage at two temperatures

Viability of *L. plantarum* 44a in feed stored in refrigeration either in vacuum packages or in glass flasks was kept constant during 2 weeks, and after one year in

the case of vacuum packaged feed (Fig. 5.4). When feed was stored in glass flasks at higher temperature (25°C), viability started to decline by the second week (Fig. 5.4). Thus refrigeration temperature was used in further experiments.

Viability of L. salivarius (win) after drying and during feed storage in refrigeration

L. salivarius (win) added to feed either as freeze-dried culture or as cell suspension (% v/w) showed a considerable loss in viability after drying in the first 24 hours. That viability was maintained during the 15 days of storage in refrigeration, but was almost completely lost after 1-year (Fig. 5.4).

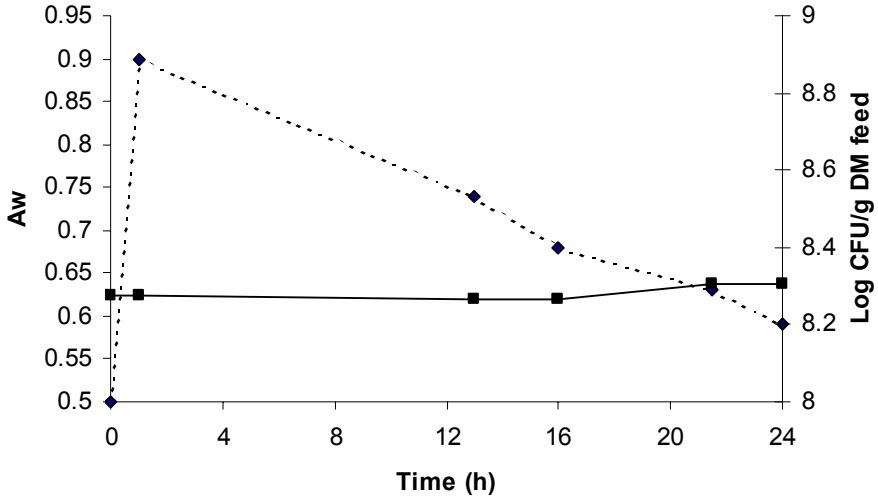

Figure 5.3. Water activity and counts of *L. plantarum* 44a during drying at 25°C in duplicates. Symbols:◆, water activity; ■, *L. plantarum* 44a counts.

Viability after gastrointestinal passage simulation

Survival of both strains after gastrointestinal passage simulation was very high (Fig. 5.5). Practically no reduction was detected. However, because the initial concentration of *L. plantarum* 44a in the feed was higher than that of *L. salivarius (win)*, the counts were higher after the gastrointestinal simulation. *L. salivarius (win)* in freeze-dried form survived less than freshly cultured forms.

Viability of cells inoculated in water

Fig. 5.6 shows CFU of *L. plantarum* 44a after exposure to distilled water or distilled water with 2% NaCl. There was no statistically significant difference in CFU between cells exposed to distilled water or distilled water with 2% NaCl. CFU remained without changes after 24 and 48 h exposure to the treatments. After 96 h of exposure time, a lower CFU of the cells was observed in both treatments.

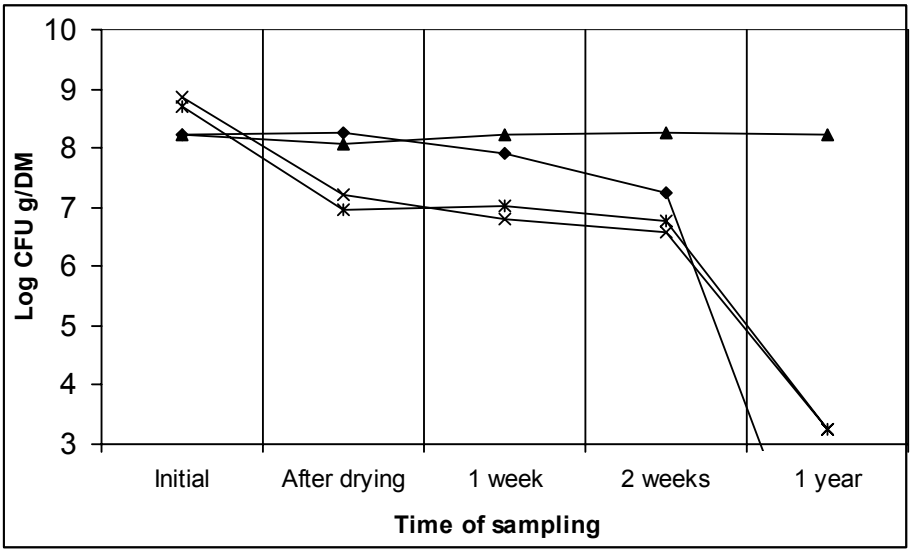

Figure 5.4. Survival of *L. plantarum* 44a and *L. salivarius* (win) during drying, and storage in feed. Symbols: ▲, *L. plantarum* 44a vacuum packaged and refrigerated; ◆, *L. plantarum* 44a stored at 25°C in closed flasks; X, *L. salivarius* (win) added as freshly harvested, vacuum-packaged and stored in refrigeration, ✳ *L. salivarius* (win), added as a freeze dried culture, vacuum-packaged and stored in refrigeration. Moisture content for all the samples after drying and during storage ≈ 13% (a_w 0.55).

5.4. DISCUSSION

Maintenance of viability of probiotic products before consumption is a very important aspect to realize the claimed benefits of probiotic supplementation (Havenaar et al., 1992b). Major stress factors for probiotic strains in feeds are the

high osmotic pressure generated by the low a_w in the feed; the re-hydration in an acidic environment like the stomach and afterwards the exposure to bile and pancreatic fluids.

Fish feed is usually manufactured with a low water activity to prevent microbial deterioration over several months and to confer floatability of the feed in the water. Therefore, it is a great challenge to find a suitable method to inoculate the strains without losses of viability, maintaining the physicochemical characteristics of the feed.

In this study, we can see the importance of temporarily increased initial moisture content of the feed to maintain the viability of the strain, likely due to both a reduction in the initial osmotic pressure in the feed, and probably to an adaptative response of the strain to the progressive reduction in water content. That practice allowed to achieve a high survival during the drying process, with a final moisture content close to the original values of the feed. Probiotic strain viability thus could be maintained in low water content feed during storage without experiencing considerable additional reduction of viability.

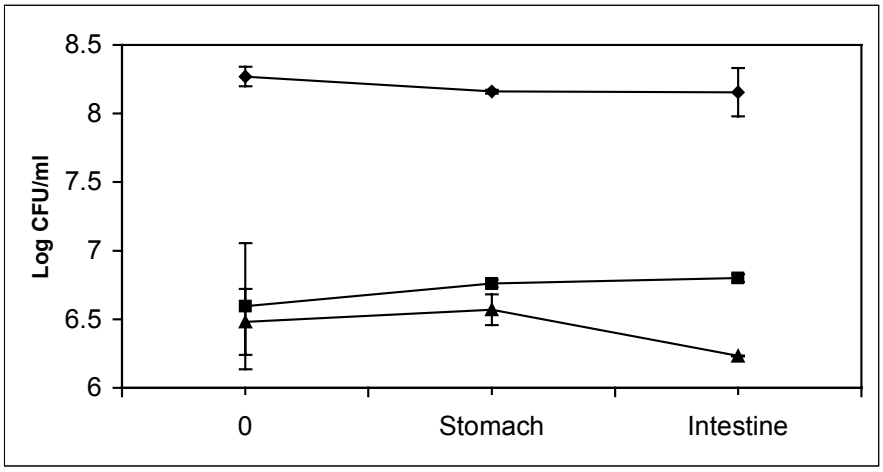

Figure 5.5. Counts of *L. plantarum* 44a and *L. salivarius (win)* inoculated in feed, and stored for 3 weeks and before and after 2 h of gastric and 2 h intestinal passage simulation. Symbols:◆, *L. plantarum* 44a, ▲, *L. salivarius (win)* inoculated as lyophilised culture or ■ freshly harvested and feed stored in refrigeration. Moisture content ≈ 12%.

Several adaptation mechanisms to withstand the osmotic stress have been described for *L. plantarum*. One of the most important is the flexibility of the cell wall to stretch as water flows into the cell and to shrink when water flows out of the cell (Poolman and Glaasker, 1998; Delcour et al.,1999). This elasticity and rigidity of the cell wall has been associated to the peptide cross-links between the glycan threads and between the concentric glycan layers (Delcour et al., 1999). Other adaptation mechanisms of *L. plantarum* to withstand the osmotic stress consist of the accumulation or release of compatible solutes to i) counteract the extracellular osmotic pressure; ii) to stabilize proteins (Glaasker et al., 1996; Kets et al., 1996; Glaasker et al., 1998) and iii) probably to maintain integrity of biological membranes (Kets and de Bont, 1997). *L. plantarum* has a limited capacity to synthesise compatible solutes (Glaasker et al., 1996; Kets and de Bont, 1997); however, it can accumulate them by specific transport systems activated by osmotic shock in response to osmotic sensing mechanisms (Konings, 2002). Some compatible solutes are potassium, trimethyl aminoacids like betaine and carnitine or some amino acids like proline, which are widely distributed in feedstuffs from animal and plant origin (Kets and de Bont, 1997) and probably were present in the feed used in the experiments which is composed mainly of fish meal, soy meal, and wheat meal.

Another adaptation of *L. plantarum* to resist the osmotic pressure is due to the phospholipids in the lipid bilayer of the cell membrane which maintains fluidity upon dehydration, in the presence of some carbohydrates like some maltodextrines present in the feed (Linders et al., 1997). Fluidity perhaps could also be maintained by the presence of some triacylglycerols present in the feed. Triacylglycerols can act as protectants of *Lactobacillus* against osmotic pressure (Siuta Cruce and Goulet, 2001).

The presence of the meso-diaminopimelic acid as the third residue of the oligopeptide cross-link of *L. plantarum* glycan might be related with its high resistance to the osmotic pressure. On the contrary, the presence of lysine as the third residue of the oligopeptide cross-link of *L. salivarius* (win) glycan might be related with its low resistance to the osmotic pressure. Lysine requires less energy to be hydrolyzed from the cell wall than the mesodiaminopimelic acid (Kandler and Weiss, 1986) and is likely more labile to osmotic pressure.

Viability of *L. plantarum* 44a was maintained in refrigeration for up to one year. Feed is generally stored in refrigeration several weeks before use. Other studies

have also shown higher survival rates at lower storage temperatures (Gardiner et al., 2000). At low temperature, such as in the refrigerator, bacterial metabolism decreases, and the accumulation of toxic wastes from the metabolism is likely minimized. However, feed may be exposed for some time to room temperature without compromising viability (Fig. 5.3).

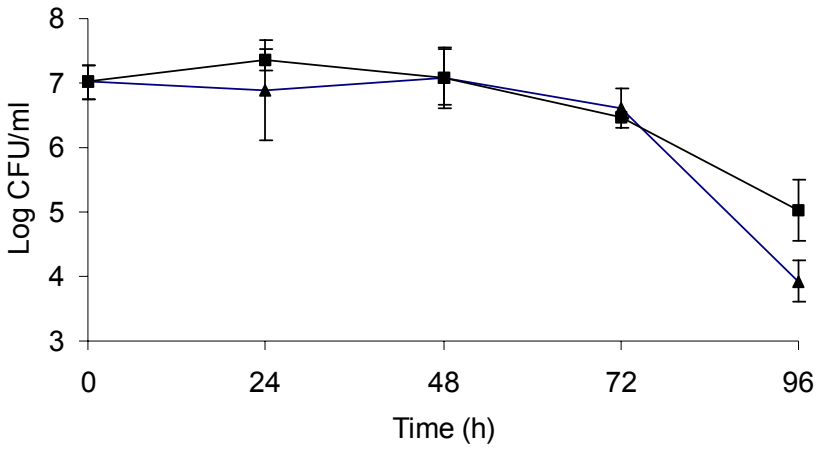

Figure 5.6. Counts of *L. plantarum* maintained in distilled water or distilled water with 2% NaCl for up to 96 hours. Symbols: ▲, distilled water; ■ distilled water + 2% NaCl. Differences between treatments are not significant according to Student's t-test (p = 0.15).

Another observation that makes *L. plantarum* 44a suitable as a probiotic is that after being stored in refrigeration, *L. plantarum* 44a can survive with little reduction the stresses found in the gastrointestinal tract, like low pH and pepsin, and subsequently to bile and pancreatin.

It is apparent from the results of this study that *L. plantarum* 44a and the method to inoculate and to store feed, makes this strain suitable to survive the technological barriers that have been reported to reduce viability in probiotic preparations. However, certain constraints exist for industrial adoption of a process of re-hydration and dehydration of *Lactobacillus* in feed, which need to be addressed in current and future research in the area. Adding large amounts of fluid is costly, however the probiotic may be added to the feed when the ingredients

have been pelleted and are being dried. However, temperatures for water removal should be reduced to ambient temperatures, to minimize losses of viability. Since osmotic dehydration is an inherently slow process, several improvements need to be done to increase the rates of osmotic mass transfer.

The results also showed that this strain had a high likelihood to survive stress as in the gastrointestinal tract like low pH and pepsin, and bile presence and pancreatin. Moreover, *L. plantarum* 44a can also survive to tap water or salty water, being suitable as a probiotic strain using water as a vehicle of inoculation.

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6. Kinetics of *Lactobacillus* in tilapia (*Oreochromis niloticus*) faeces after its intake in the feed

Adolfo Bucio, Ralf Hartemink, Johan W. Schrama, Marcel Zwietering and Johan Verreth

Abstract

The aim of this research was to investigate the viability of *L. plantarum* 44a as a feed supplement after drying and storage, and its kinetic of passage throughout the tilapia gastrointestinal tract. The growth parameters of tilapia were studied as potential health effect. The following single dosages: 10^{12} CFU; 10^{9} CFU; 10^{6} CFU; 0 CFU were given in duplicates to eight groups of fish, with 26 fish per group with an average body weight of 70g per fish. After ingestion of the inoculated feed, faeces were periodically collected in sedimentation tanks and analyzed for its *Lactobacillus* and total anaerobic flora content. *L. plantarum* 44a had a very high viability after drying and storage. After intake of inoculated feed by tilapia, *L. plantarum* 44a appeared in faeces as pulses excreted after a mean retention time, and height not significantly affected by the dose. Nevertheless, a comparison between the numerical data of the pulses demonstrated similarity between duplicates of each dose and differences in response to the doses. Subsequently, the recovery of *Lactobacillus* population in the faeces of all the treatments followed an exponential decay pattern with detectable levels of organisms after some days. The highest dose of *L. plantarum* 44a ingested, reached the same level as the total anaerobic flora. All randomly picked isolates from the highest dilution plates gave a fermentation pattern typical of *L. plantarum* 44a. *L. plantarum* 44a has a high potential to pass the gastrointestinal tract alive and to be present in faeces in very high numbers with potentially growth promotion effects in tilapia.

6.1. INTRODUCTION

Lactobacillus is a common inhabitant of the gastrointestinal tract of a wide range of animals. The ecological roles played by *Lactobacillus* in the gastrointestinal tract

include amongst others: production of antimicrobial substances; lowering of O/R potential; modulation of the immune system, fermentation of some non-digestible carbohydrates and increase of availability of nutrients (Fuller, 1989).

Various authors have reported the presence of *Lactobacillus* in various fish taxa such as *Salmonidae* (Ringø et al., 1995; Ringø and Gatesoupe, 1998; Gonzalez et al., 2000), *Cyprinidae* (Kvasnikov et al., 1977; Chapter 3), *Percidae* (Kvasnikov et al., 1977; Chapter 3) and *Anguillidae* (Chapter 3).

In our studies, lactic acid bacteria were absent in the intestinal content of tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*) reared in a recirculation system, suggesting the absence of important mechanisms in those fish hosts to maintain intestinal health by the presence of these bacteria.

Administration of exogenous *Lactobacillus* or other lactic acid bacteria has been suggested as a way to reduce the possibilities of pathogen infections in salmonid fish (Gildberg et al., 1997; Gildeberg and Mikkelsen, 1998; Jöborn et al., 1997; Ringø and Gatesoupe, 1998; Robertson et al., 2000; Nikoskelainen et al., 2001) and to improve the host's health in marine fish (Byun et al., 1997). Administration of *Lactobacillus* can be done by adding viable microorganisms to the feed (Robertson et al., 2000; Nikoskelainen et al., 2001). Recovery of the strains from intestinal content is the usual method to assess survival of the strains (Byun et al., 1997; Jöborn et al., 1997; Robertson et al., 2000).

Information on the application of lactic acid lactic bacteria as probiotic feed supplements in warm-water fish is scarce. In a previous paper (Chapter 4) we selected *Lactobacillus plantarum* 44a as a probiotic candidate for warm-water fish after *in vitro* screening of several strains for inhibitory activities against common pathogens like *Aeromonas hydrophila* and *Edwardsiella tarda*, and by its high survival in a gastrointestinal tract model, absence of amine production, and high viability during feed storage (Chapter 5).

The aim of this research was to study the viability of *Lactobacillus plantarum* 44a before consumption by tilapia and the kinetics of passage in faeces after intake in various single dosages ($\approx 10^{12}$ CFU; 10^9 CFU; 10^6 CFU; 0 CFU). The use of different dosages of *Lactobacillus* allowed us to study possible positive or negative implications for further dosage recommendations. Therefore, fish growth performance as function of *Lactobacillus* dosage was also studied. We selected

tilapia as fish host for our experiments as tilapia is one of the most cultured freshwater fish world-wide.

6.2. MATERIALS AND METHODS

Bacterial strain

L. plantarum 44a had been previously isolated from the intestinal content of European eel (*Anguilla anguilla*) (Chapter 3).

Preparation of* L. plantarum *44a for the feeding trail

L. plantarum 44a was grown in an overnight culture at 30°C in 4 L MRS broth (Merck, 1.10661, Darmstadt, Germany), washed 3 times in 0.1 M Potassium phosphate buffer (Kpi) pH 6 and harvested by centrifugation (2760g for 15 min). The cells were resuspended in 100 ml of the same buffer. Ninety-nine ml were sprayed directly onto 200 g of feed to have approximately 10^{10} CFU/g, and one ml was diluted 1:1000 to have 10^7, and also diluted 1:1,000,000 for 10^4 per gram of feed. Feed was sprayed with the cell suspension and dried overnight in a flow cabinet at room temperature.

Enumeration of *Lactobacillus* was performed on MRS agar (Merck, Darmstadt, Germany). Serial dilutions of samples were prepared using Reduced Peptone Solution (Hartemink and Rombouts, 1999). Plates were incubated at 30°C in anaerobic jars with a mixture of 80% N_2, 10% H_2 and 10% CO_2 using the Anoxomat system (Mart, Lichtenvoorde, The Netherlands).

The moisture content of the feed inoculated with *Lactobacillus* was determined by grinding the feed and drying it in an oven at 103°C until constant weight. The results were expressed in % of the dry mass content.

Accommodation of fish

Tilapia (*Tilapia oreochromis*) GIFT (Genetically improved female tilapia) with an average weight ≈ 70 g were randomly allocated in eight tanks of 70 L (26 fish/tank): Two tanks were used as control (duplicate). Six tanks were used to make 3 treatments in duplicate for testing the dose-response of *Lactobacillus*. Every tank was connected to a settling tank for collecting faeces without disturbing the fish.

Environmental parameters:

Fish were reared in a recirculation system with aeration in each aquarium, under a 12L :12D light regime. Water quality parameters were: Temperature, 28^{o}C; pH, between 7-7.5; conductivity, 1500µS; NH_4^+, <2 mg·l^{-1}; NO_2^- , <6 mg·l^{-1} NO_3^-, <500 mg·l^{-1}. $O_2 > 4$ mg·l^{-1} of water. Water flow rate through the aquarium was 5 l · min^{-1}.

Feed type

A commercial feed (T-3 Nutreco, France) was used to feed animals. Inoculation of feed with *L. plantarum* 44a was done at the Food Microbiology Department prior to the experiment

Feeding method and regime

This experiment was divided in two trials with four groups (tanks) of fish in each trial. The first trial lasted 21 days and the second trial lasted 30 days. The adaptation period for the first trial was 13 days and 22 days for the second trial. The flushing period was eight days for both trials. All experimental groups were fed restrictively according to their body weight at a level of 20 g /kg$^{0.8}$ /d. The metabolic weight (kg) was calculated as follows: (fish weight (g) /1000)$^{0.8}$ x N, where N is the number of fish and 0.8 is a constant related to the metabolic rate in relation to the body mass (Gillooly et al., 2001). Fish were fed twice a day by hand at 8:30 h and 16:30 h. The amount of feed was similar during the morning and the afternoon feeding. On the day of inoculation (day 0), six groups of fish were fed with standard diet during the morning feeding but inoculated with *L. plantarum* 44a. The total dose of *L. plantarum* 44a consumed differed between the experimental treatments: ≈10^{12} CFU; 10^9 CFU; 10^6 CFU; one group was fed without *Lactobacillus*. The evening feedings and all subsequent feedings were given in the usual way with a standard diet at a level of 20 g/kg$^{0.8}$d for eight days. The two control groups were fed in the usual way all the time.

Device for collection of faeces

In each aquarium, faeces were collected from a plastic sedimentation tank (AquaOptima, Trondheim, Norway) with a total volume of 17 liters, having the following dimensions: total height 45 cm; internal diameter, 24.5 cm; lower diameter 5 cm. Faeces were collected in flasks of 50 ml connected to the settling tank. At

sampling, the total volume of the flasks (faeces + water) were poured into a sterile plastic bag and thereafter homogenized in a stomacher. The homogenized samples were transferred into 100 ml sterilized flasks containing 15 ml glycerol and 15 ml Buffered peptone water (Buffered peptone water (BPW, Oxoid CM 509, Hampshire, England) 20g/l; Cysteine.HCl (C-7880, Sigma) 0.5g/l; resarzurin 1 tablet/l BDH, England; Tween 80 1 ml/l)(Hartemink et al., 1997). Samples were immediately frozen at -20 ^{o}C.

Accumulation of faeces

In all the samples, faeces were accumulated continuously in the sedimentation tanks for 4 hours (for instance from 8: 30 until 12:30). For the first sample, faeces were collected some minutes before the inoculation time. Thereafter, the collection of faeces was done in intervals of 4 hours until complete 72 hours, and every 24 hours until the 8th day. The sedimentation tanks were quickly cleaned after sampling.

Microbiological analysis of faeces

At the end of the second trial, the samples were thawed during 1 hour in a water bath at 30^{o}C. 1 ml was transferred to reduced neutralized bacterial peptone (NBP, Oxoid L34) 0.5g/l, NaCl 8g/l, Cysteine.HCl 0.5g/l, pH adjusted to 6.7 (Hartemink and Rombouts, 1999). Afterwards serial dilutions were spread on plates of selective media.

Columbia Blood Agar (Oxoid CM 331 with 50 ml/l of sheep blood added) was used to count total anaerobic bacteria. Enumeration of *Lactobacillus* was performed on LAMVAB (Hartemink et al. 1997). Plates were incubated under anaerobic conditions at 30^{o}C for 48 h and for 96 h respectively. Plates containing between 10-100 colonies were counted and expressed as colony forming units (CFU) per ml of sample.

Lactobacillus and total anaerobic counts were determined and expressed on fecal dry mass (DM) basis of the faeces according to the following formula:

$$C_d = C_V \cdot DM_{ml}^{-1}$$

where

C_d = CFU per dry mass (CFU/g).

C_v = CFU per volume (CFU/ml).

DM_{ml} = Dry mass (g/ml)

Dry mass analysis

About 50 ml of the faecal sample was centrifuged at 2660g/4°C for 15 min and the precipitated was weighed (wet precipitate). One gram of the wet precipitated was dried at 115°C for 24 hours. Dry mass per ml was calculated as follows:

$$DM_{ml} = \frac{W_p \times DM}{V_s}$$

where,

DM_{ml} = Dry mass per ml (g/ml).
W_p = Wet precipitated (g).
DM = Dry mass (g/g).
V_s = Volume of the sample (ml).

The dry mass per sample (x_i), was obtained as follows:

$$x_i(g) = DM_{ml} \times V_s$$

***Cumulative excretion of* Lactobacillus**

Cumulative excretion of *Lactobacillus* (*L*) in faeces was estimated according to the following formula,

$$L = \sum_{i=1}^{n} c_{d\,i}\, x_i \qquad (1)$$

where,

L = Cumulative excretion of *Lactobacillus* (CFU).

C_{di} = CFU of *Lactobacillus* per dry mass of faeces (CFU/g) at time *i.*

x_i = Dry mass (g) per sample of faeces at time *i*: 0,4,8…72h.

Recovery of* Lactobacillus *in relation to the dose

The recovery of *Lactobacillus* (*R*) in faeces in relation to the dose was estimated by using the formula:

$$R(\%) = (L/Dose) \times 100 \qquad (2)$$

where

R = recovery of *Lactobacillus* (CFU).

Dose = Dose of *Lactobacillus* given (CFU).

This recovery can be < 100 meaning inactivation or > 100, meaning growth in the intestines.

An estimation of recovery was also performed by using the model described in the next section.

Dynamic model of* Lactobacillus *excretion

The pattern of the *Lactobacillus* population in the faeces as a function of time was described using a model of two ideal mixers in series. The number of organisms was predicted for every hour during the experiment. This model can be mathematically described as follows:

$$Y = A_o \cdot t/\theta \cdot e^{-t/\theta} \qquad (3)$$

where

Y = number of *L. plantarum* 44a in faeces at time *t* (CFU/h).

θ = mean retention time of *Lactobacillus* in one of the mixers (h); θ is also the time value at which the pulse reaches its peak.

A_o = Cumulative excreted *Lactobacillus* (*L*) divided by θ, (CFU/θ).

t = Time (*h*).

The model (3) was tested by estimating the *Lactobacillus* content in the accumulation of the faeces during 4h. So the measured data were compared to the cumulative data of the model over four hours.

$$Y_{4h} = \sum_{i=1}^{4} (A_0 e^{-t_{i-4}/\theta} \cdot t_{i-4}/\theta) \quad (4)$$

where,

Y_{4h} = Predicted numbers of *L. plantarum* 44a in continuous periods of 4 hours (CFU/4h).

Although the cumulative excretion of *Lactobacillus* was calculated as described in (1), a value was calculated to verify the mathematical model.

Cumulative excretion of* Lactobacillus *according to the model

It was calculated by the integral of the model (4),

$$\int_0^{\infty} A_0 e^{-t/\theta} t/\theta \, dt = A_0 \cdot \theta \quad (5)$$

and checked by the predicted values of the model,

$$A_o \theta = \sum_{i=4}^{n} Y_{4h_i} \cdot = L_{Model} \quad (6)$$

where,

Y_{4h}= predicted numbers of *L. plantarum* 44a in continuous periods of 4 hours (CFU/4h) at time *i*.

L_{model}= cumulative excretion of *Lactobacillus* according to the predicted values (CFU).

Estimation of* Lactobacillus *recovery in relation to the dose according to the model

$$R_{model} = (L_{Model}/Dose) \cdot 100\% \quad (7)$$

where,

R_{model} = Estimated *Lactobacillus* recovery from faeces *(%).*

Dose= Total dose *of Lactobacillus* given to each treatment (CFU).

Statistics

The model was fitted to the data by determining a value for θ that minimized the residual sum of squares (SSR) between the observed data and the values of the model. θ was determined by using the solver algorithm in Microsoft® Excel 97 and checking it in TableCurve™ 2D. The graphs were plotted in logarithmic scale. The results were also reported in logarithmic scale. A linear regression of actual and estimated log CFU was done. It was tested with a Student's *t-test* if the slope = 1 and the intercept = 0 as a final measurement of the precision and accuracy. Differences between θ and A_o among treatments were done by independent Student's *t-test*. Differences were considered significant when P < 0.05. Additionally, differences between the data of the pulses in the inter and intra-treatments were examined by paired Student's t-tests. The statistical analyses were carried out using SPPS 11 (SPSS Inc, Chicago, Illinois, USA).

Growth parameters of fish

All fish per tank were weighed at the beginning and at the end of the experiment. Growth parameters were calculated according to the formulas:

$$SGR = (\ln BW_t - \ln BW_0) / t \times 100\%$$

where *SGR* is the Specific Growth Rate (*%d*$^{-1}$); BW_0 is the initial body weight (*g*); BW_t is the final body weight (*g*); t is the time (*d*).

No feed refusal occurred during the experiment, consequently the feed intake equalled the feed given. The Feed ration (*R*) was calculated by:

$$R = F / BW_g \times 100\%$$

where *R* is the feed ration (*%d*$^{-1}$); *F* is the feed intake (g ·d^{-1}), calculated by the cumulative amount of feed (g)/ no. of fish per tank/no days; BW_g is the mean geometric body weight = *exp* ((*ln* BW_t + *ln* BW_o)/2).

Feed conversion efficiency (%) was calculated as follows:

$$FCE(\%) = ((BW_t - BW_0)/F) \times 100\%$$

where *FCE* is the Feed Conversion Efficiency (%)

The effect of *Lactobacillus* doses in the parameters of growth and growth in relation to the feed was analyzed using ANOVA. A trial effect was considered as a possible source of variation. This statistical analysis was also carried out using SPPS 11 (Chicago, Illinois, USA).

6.3. RESULTS

Recovery of L. plantarum *44a in feed*

Viability of *L. plantarum* 44a in the feed remained as high as the inoculum in the dried feed and during storage (Fig. 6.1). Survival during drying and storage is the first prerequisite of effective probiotic cultures.

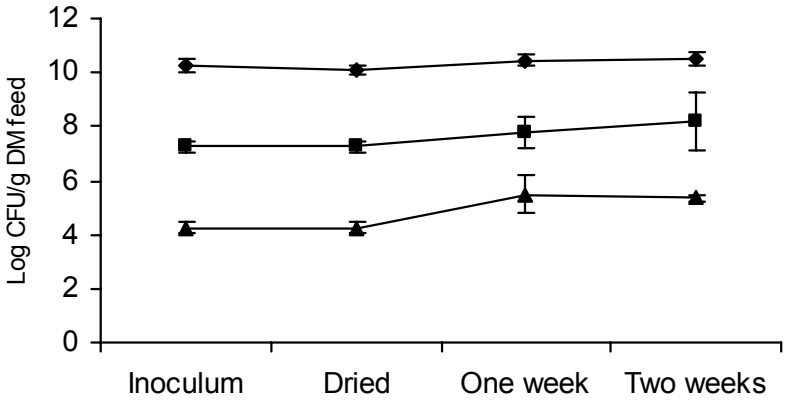

Fig. 6.1. Viability of *L. plantarum* in 3 initial concentrations after drying and during storage in feed. ◆, 10^{10}CFU/g DM; ■,10^{7}CFU/g DM; ▲, 10^{4}CFU/g DM.

Counts of Lactobacillus *in faeces*

In Fig. 6.2 the data of *Lactobacillus* excreted throughout the time of the three treatments are shown. For the treatments of the two highest dosage of *Lactobacillus*, a good description of the data was given by the two compartments

ideal mixers model (Fig. 6.2A,B,C and D). In this model, after *L. plantarum* 44a consumption, they appeared in faeces as pulses excreted after a mean retention time, which was not significantly affected by the dose (Table 6.1). Although the pulses had different heights, the heights were not statistically different in function of the dosage of *Lactobacillus* ingested (Table 6.1). However, differences between the data points of the pulses in function of the dosage of *Lactobacillus* ingested were detected using a paired t-student test (Fig. 6.2). After reaching the peaks, the *Lactobacillus* population was excreted following an exponential decay pattern with detectable levels of organisms retained after some days. For the treatments on the lowest dosage, no model fit was possible due to the small number of points above the detection level. To illustrate the shape of the curve in real scale of the highest dose, the microbial counts of *Lactobacillus* of one of the treatments throughout time are shown in arithmetic scale (Fig 6.3). It can be seen that the pulses occur in very high concentration in a very short time.

All 10 randomly picked isolates from the highest dilution plates gave a fermentation pattern typical of *L. plantarum* 44a. Because *Lactobacillus* were not present in the host nor in the water before the experiment, no further analysis of additional isolates was considered necessary.

Table 6.1. Kinetic parameters of *Lactobacilllus* excretion in the faeces[1,2,3]

Lactobacillus(log CFU) per ration	Parameters of the ideal mixer model of two compartments		
	Log (A_0)	Log (L)= Log $A_0\theta$	θ (h)
≈ 8.80	6.7 ± 0.60	7.42 ± 0.48	5.2 ± 1.0
≈11.65	8.4 ± 0.15	8.96 ± 0.11	3.6 ± 0.4
Independent Student's t-test (P)	0.38	0.124	0.243

[1] n = 2 experimental units per treatment. An experimental unit was an aquarium containing 26 fish.

[2] Results are shown in means ± standard deviation

[3] Differences were considered significant when P < 0.05.

A_0, cumulative excretion of *Lactobacillus*, CFU/h(L/θ); θ, Mean retention time (h).

Recovery of* Lactobacillus *in relation to the dose

The *Lactobacillus* recovery of the three treatments is shown in Table 6.2. Recovery of *Lactobacillus* was variable and lower than 5% (Table 6.2). The recovery value of the mathematical model agreed very well as expected.

Growth performance of tilapia

Feed intake, specific growth rate and feed conversion efficiency of tilapia was not significantly different among treatments at the level of 0.05 (Table 6.3). However, specific growth rates differed among the treatments at 0.06 level of significance, finding the highest specific growth rate in the treatment fed on the highest dose of *Lactobacillus*. No health problems were detected in any group of fish.

Table 6.2. Kinetic parameters of *Lactobacillus* excretion in the faeces[1,2]

Lactobacillus(log CFU) per ration	Recovery[4] (%)	Recovery $_{model}$ (%)[5]
≈ 5.79	3.83	-
≈ 8.80	4.45	4.45
≈11.65	0.23	0.23
SEM[3]	1.96	-
P dose	0.382	-

[1]n=2 experimental units per treatment. An experimental unit was an aquarium containing 26 fish.

[2] Differences were considered significant when $P < 0.05$.

[3]SEM, Standard Error of the mean.

[4]The recovery was calculated using the equation 2

[5]The recovery was calculated using the equation 7

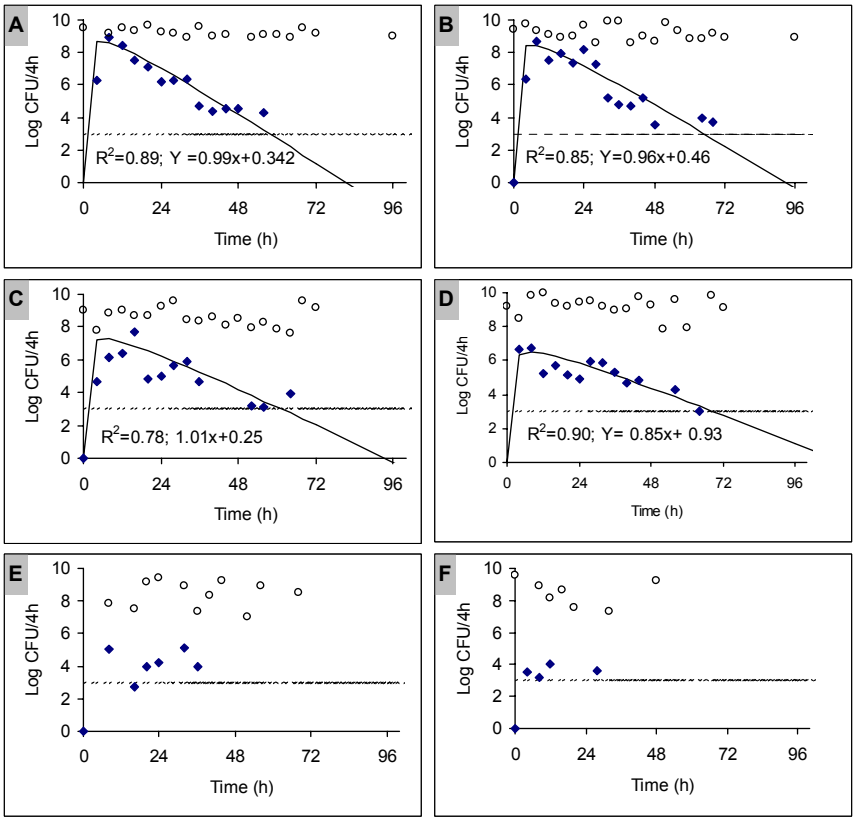

Fig. 6.2. Pattern of *Lactobacillus plantarum* 44a appearance in the faeces after feeding to tilapia. Pattern fitted to an ideal mixer model with two compartments in series. ◆,Observed *Lactobacillus* data; continuous line, the fitted model for *Lactobacillus* data; ○, total anaerobic flora. Dashed line is the detection level. Dose of *L. plantarum* 44a applied per tank: A and B, $\approx 10^{12}$ CFU; C and D, $\approx 10^{9}$ CFU; E and F, $\approx 10^{6}$ CFU. Starting values for time zero were plotted as zero as *Lactobacillus plantarum* 44a was not added yet to the fish at this time, and similarly they were never detected in the fish before feeding. R^2, are the coefficient of determination between the actual and the estimated values. The ability of the mathematical model to describe the actual values was tested by a linear regression analysis. The coefficients of the straight line are shown in the graphs. It was tested with a Student's t-test if the slope ≈ 1 and the intercept ≈ 0 as a final measurement of the precision and accuracy. In all the cases, the null hypothesis was accepted with $p \geq 0.05$ for both the slope and the intercept, meaning that fitted curve does not significantly differ from the actual values, thus y = x. According to the paired t-student test for the data of the pulses in the figure: (A=B)≠(C=D) with 95% of probability.

Level of contamination

Lactobacillus were detected in some samples of the control group in values around 10^3 CFU/g, very likely as a result of a contamination, as *Lactobacillus* was not detected in the samples before the experiment had started nor in the samples

that were collected in the last part of the experiment. The values of CFU of contaminant *Lactobacillus* in the control were subtracted from the values of *Lactobacillus* present in the faeces of fish supplied with *Lactobacillus* for each sampling time.

6.4. DISCUSSION

We verified that *Lactobacillus plantarum* 44a had a high viability after drying and storage as a pre-requisite in an effective probiotic culture. Thereafter, we studied the kinetics of *L. plantarum* 44a after intake by the fish by measuring its presence in the faeces. Faeces were collected in sedimentation tanks, stored by freezing, and analyzed for *Lactobacillus* content by using a selective medium. Data were analyzed by mathematical and statistical modeling. The results obtained provided information with means and variations at the population level studied (26 fish randomly picked).

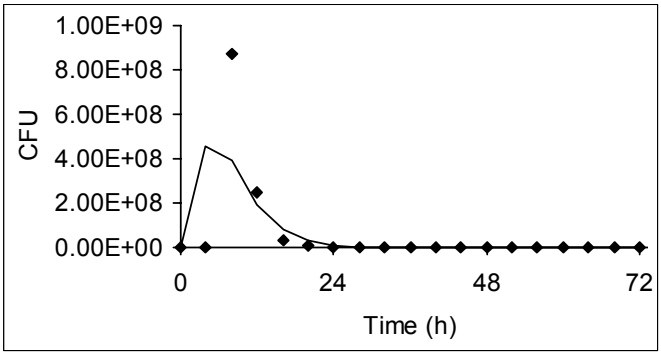

Fig. 6.3. Pattern of a *L. plantarum* pulse in the faeces: in arithmetic scale for a tank with fish fed the highest concentration of *Lactobacillus*. ◆, Observed data; Continuous line, model.

The kinetics of displacement of *Lactobacillus* from feed to faeces followed a sequential trajectory of a pulse-signal, with a zero *Lactobacillus* population at time zero, and after its ingestion a sudden appearance of *Lactobacillus* and thereafter an exponential disappearance of *Lactobacillus* from the faeces. The description of *Lactobacillus* kinetics by the ideal mixer model had high regression coefficients. This pulse-signal, indicated certain viability of the strain within the host, but also indicated low retention time within the host.

Viability and adherence are some of the most important criteria for probiotic selection (Fuller, 1989). Viability of bacteria depends on resistance of bacteria to gastric secretions, such as acid and enzymes which constitute the primary defense mechanism against ingested microorganisms; bile, especially bile salts are the second important defense mechanism but other digestive secretions have also influences. Gastrointestinal motility constitutes the third major defense mechanism of the gut by which bacteria are expelled along with non-digested materials (Marteau and Vesa, 1998). Gastrointestinal motility in fish depends on feed intake, feed composition, temperature, previous nutritional history, fish size and stress (Jobing, 1987; Storebakken et al., 1999).

In our study, the cultured fish of the eight tanks had a similar size and were reared under similar conditions (feed intake, feed composition, temperature, previous nutritional history, and stress). It is therefore likely that the retention time would be similar between treatments. This is suggested by observing that the estimated θ values in the two treatments (Table 6.1) were not affected by effect of the dose. Because feed intake by the fish of the eight tanks was near to satiation during the adaptation period and after *Lactobacillus* intake, it is possible that *Lactobacillus* were flushed out along other non-digested materials and non-adherent materials ingested at the same time. Stomach content in tilapia of similar size has been reported to be completely evacuated in about 9-13 h in fish naturally occurring in ponds at water temperatures of 29-33^{o}C (Yousuf-Haroon et al., 1998). However, no data are available on the mean time to evacuate the gastrointestinal tract.

Table 6.3. Growth parameters of tilapia administered with *Lactobacillus* in various single dosages[1,2]

Growth parameters		Dose (Log CFU *L. plantarum*)				
	Control	≈ 5.79	≈ 8.80	≈11.6	SEM[3]	P[4]
SGR (% · d^{-1})	3.24	3.15	3.34	3.37	0.035	0.058
Feed ration (% · d^{-1})	2.60	2.69	2.60	2.64	0.055	0.674
FCE (%)	127.7	119.55	131.42	131.22	3.40	0.218

[1] n = 2 experimental units per treatment. An experimental unit was an aquarium containing 26 fish.

[2] Means in a column without a common letter differ significantly, $p < 0.05$.

[3] Standard error of the mean

[4] Probability of difference between at least two treatments.

C, Control. (no *Lactobacillus* added)

SGR, Specific growth rate; FCE, Feed conversion efficiency

L. plantarum 44a was not retained for a long time within the host, and this seems to be the rule for most of the probiotics (Marteau and Vesa, 1998). For successful application as a microbial ingredient in fish, other characteristics are essential, such as: retention of high viability during processing, storage, and certain viability after gastrointestinal transit. We did not observe any negative side effects related with the ingestion of *Lactobacillus*, not even with the intake of *L. plantarum* 44a in the highest dose (10^{12} CFU/dose). At this highest dose, *Lactobacillus* became part of the dominant biota in faeces some hours after ingestion, and this dose was associated with some improvements in the specific growth rate, suggesting some health effects in these fish reared in absence of *Lactobacillus* for several generations. In spite of the high concentration of *Lactobacillus* ingested in some fish groups, it seems that the resident flora exerted a strong “colonization resistance” occupying the niche of the gastrointestinal tract while the exogenous probiotic was being flushed out. Resident flora is adapted to many environmental factors such as host secretions and sometimes is adapted to attach to the mucosa.

In relation to the survival of *L. plantarum* 44a after gastrointestinal passage, this was statistically similar for all the treatments (Table 6.1) which shows absence of a

dose-effect. Similar percentages, have been previously reported for *Lactococcus lactis* (Klijn et al., 1995). In a previous study, we found that the survival of *L. plantarum* 44a was more dependent on the exposure to gastric fluid than on exposure to intestinal fluids (Chapter 4). This study suggests that an advantage can be obtained by inoculating this strain in very high numbers to achieve a survival fraction that can equal the numbers of the resident flora (total anaerobic flora). A dose of *Lactobacillus* near to 4.46×10^{11} was sufficient to achieve the number of $\approx 10^{8.5}$ found in the resident total anaerobic flora. However, further studies are necessary to confirm these findings. Also, it would be interesting to use the model developed in this chapter in a study in which dosages of *Lactobacillus* are repetitively fed to fish and to evaluate the biological effects of such treatments.

6.5. CONCLUSIONS

Lactobacillus plantarum 44a had a high viability after drying and storage and pass through the gastrointestinal tract as a relatively short pulse depending on the dose and has the potential to appear in faeces in numbers as high as the total anaerobic flora. The effect on specific growth rate was near significant at the highest dose. This shows to be promising for trials with repetitive dosage.

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7. Kinetics of *Lactobacillus plantarum* 44a in the faeces of tilapia (*Oreochromis niloticus*) in response to the frequency of supplementation in the feed

Adolfo Bucio, Ralf Hartemink, Johan W. Schrama, Frank M. Rombouts, Marcel Zwietering and Johan Verreth

ABSTRACT

The aim of this study was to determine the effect of the frequency of *Lactobacillus plantarum* 44a on the kinetics of passage of *Lactobacillus* through the gastrointestinal tract of tilapia and its potential effect on the growth parameters of the fish. Feed containing 1.07 x 10^{11} *Lactobacillus* per ration were supplied as follows: once in 14 days; 5 times in 14 days and 14 times in 14 days. Faeces were periodically collected in sedimentation tanks and analyzed for *Lactobacillus* content. A suspension of 2.2 x 10^{8} spores of *Bacillus stearothermophilus* per feed ration was used as biomarker in the same feed inoculated with *Lactobacillus*. For the analysis of the kinetic data, a previously described mathematical model for single dosages was used. The kinetic parameters of *Lactobacillus* passage were not significantly affected by the frequency of *Lactobacillus* intake. The mathematical model for a single dose predicted well the data for multiple dosage. The estimated values were highly correlated with the data. The number of *Lactobacillus* in faeces during the period of supplementation did not differ significantly from the total anaerobic flora. After suspending the supplementation of *Lactobacillus*, its presence decreased, until complete disappearance. Growth parameters of the fish were not affected by the frequency of *Lactobacillus* supplementation.

7.1. INTRODUCTION

Probiotics are "live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance" (Fuller, 1989). Probiotic

supplementation has been proposed as a means to increase the numbers of beneficial bacteria and to reduce the numbers of harmful bacteria (Gildberg et al., 1997). Probiotics can influence physiology and health through direct or indirect effects in the gastrointestinal tract (Marteau and Vesa, 1998). For most of the applications, it is recommended to use probiotic strains able to survive the gastrointestinal passage, and preferentially with the capacity to adhere to the host intestinal tissues in order to prolong their health effects (Fuller, 1989; Havenaar et al., 1992b; Ouwehand et al., 1999). Most of the probiotics, however; are completely excreted in the following days after its ingestion (Marteau and Vesa, 1998; Robertson et al., 2000). If a probiotic does not colonize, they should be regularly administered to obtain its effects (Marteau and Vesa, 1998). Knowledge on their pharmokinetics is needed to answer the questions how much probiotic should be consumed, how often, for how long; and what concentrations of probiotics should be present in the commercial preparations (Marteau and Vesa, 1998); and also to correlate the effects with the concentration of the probiotic at the target site (Marteau and Vesa, 1998).

In a previous study, we supplied tilapia with *L. plantarum* 44a describing the kinetics of excretion by using an ideal mixer model with two compartments (Chapter 6). In that study, the kinetic parameters were not affected by the dosage of *Lactobacillus* ingested. In the present study the aims were to investigate how our previous model for single dosages predicted the data for multiple dose of *L. plantarum* 44a supplemented to tilapia. We used spores of *Bacillus stearothermophilus* as a biomarker. The spores of *B. stearothermophilus* have been reported to be stable during gastrointestinal passage of several hosts and do not germinate below a temperature of approximately 50^{o}C (Marteau and Vesa, 1998). Another aim in this research was to test the ability of *L. plantarum* 44a to be excreted in counts as high as the total anaerobic flora, an aspect rarely observed with a probiotic. Total anaerobic flora may contain some Gram negative and Gram positive pathogens. A previous study (Chapter 6) showed a potential effect of a single high dose on specific growth rate of tilapia. Therefore it was tested if such an effect could be reproduced with repetitive doses.

7.2. MATERIAL AND METHODS

Overview

Eight groups of fish, with 30 fish per group of an average weight of 57g per fish were randomly placed in 8 aquaria and fed in duplicate with feed inoculated with *L. plantarum* 44a and *B. stearothermophilus* as a biomarker in the same feed ration. Three treatments consisted of tilapia supplied with feed containing both *L. plantarum* 44a (≈1.07e + 11 CFU per daily ration) and *B. stearothermophilus* (≈2.2e +08 CFU per daily ration) in the following frequency: 1 dose/14 days (1/14), 5 doses/14 days (5/14), and 14 doses/14 days (14/14). 2 groups were added as control. During the other days of the experiment, the diet consisted of feed without *Lactobacillus*. The faeces were periodically collected in sedimentation tanks to analyze the *Lactobacillus* content. Fish was weighed before starting and after finishing the experiment. At the end of the experiment (day 28), 3 randomly taken fish from each aquarium were dissected to examine the *Lactobacillus* content in the intestinal fluids.

Bacterial strains

L. plantarum 44a had been isolated from the intestinal content of European eel (*Anguilla anguilla*) (Chapter 3) and selected from an array of fish-borne strains by its inhibitory activities against some common fish and human pathogen, and high survival in a gastrointestinal tract model, absence of amine production, and high viability during feed storage and viability within the gastrointestinal tract of tilapia. For the present experiment, *L. plantarum* 44a was grown as an overnight culture in 7 l of MRS (Merck, 1.10661, Darmstadt, Germany) at 30^{o}C, washed 3 times in 0.1 M Potassium phosphate buffer (PBS) pH 6 and harvested by centrifugation (2760g for 15 min). The cells were resuspended in 600 ml of the same buffer. A spore suspension (1.11 x 10^{10} spores) of *Bacillus stearothermophilus* (Merck, 1.11499, Darmstadt, Germany) was also added to the *Lactobacillus* suspension in PBS. The suspension was sprayed onto 2000g of feed and dried for 24 h and stored in refrigeration until use. Final concentration of *Bacillus stearothermophilus* was 5.5 x 10^{6} spores/gram of feed.

The dry mass content of the feed with the cell suspension was determined by finely grinding feed in a mortar and drying it in an oven at 103^{o}C until constant weight.

Culturing conditions of fish

Tilapia (*Tilapia oreochromis*) GIFT (Genetically improved female tilapia) (average weight ≈ 57 g) were randomly allocated in 8 tanks of 70 L (30 fish/tank). Two tanks were used as control in duplicate. Every tank was connected to a settling tank for collecting faecal material.

Environmental parameters:

Fish were reared in a recirculation system with aeration in each aquarium, under a 12L:12D light regime. Water quality parameters maintained were: Temperature, 28^{o}C; pH, between 7 and 7.5; Conductivity, < 1500µS; NH_4^+, <2 mg·l^{-1} NO_2^- , <6 mg·l^{-1} NO_3^-,<500 mg·l^{-1}. Flow rate was 5 liters min^{-1}. $O_2 > 4$ mg·l^{-1} of water.

Feed type and feeding method and regime

Feed used for all the treatments was troutvit ME-2 (Pellet size 2.4 mm) manufactured by Nutreco, France. The 8 groups of fish were fed manually twice a day. Because fish was in an adaptation period after being allocated to the experimental aquaria, the feed ration was 10g kg $^{0.8}$ d^{-1}, with increments of 5g each day until reach the level of 23 g/kg$^{0.8}$ d^{-1} by the 6th day. The feed amount by that day was 80g. From that day onwards, the diet was given on a fixed amount of 80g, because the tank culture of tilapia had reached the maximum carrying capacity to maintain oxygen within the safety level for the fish (>0.4mg/l). As fish wastes generate a high biochemical oxygen demand, the excretion was minimized by supplying only 80g of feed to the tilapia. Fish was supplied with feed supplemented with *L .plantarum* 44a in the following frequency: once in 14 days; every 3 days for 14 days; and 14 days in 14. Two groups were the control. During the other days of the experiment, the diet consisted of normal feed.

Collection of faeces devise

In each aquarium, faeces were collected and stored from a plastic sedimentation tank (AquaOptima, Trondheim, Norway) according to the methods described in chapter 6.

Accumulation of faeces

All the samples consisted of faeces accumulated continuously in the sedimentation tanks for 4 hours (for instance from 8: 30 until 12:30). For the first sample, faeces were collected some minutes before the inoculation time and thereafter eight hours after *Lactobacillus* was supplied for the first time to one of the fish treatments. After that time, the faeces were collected in periods of 24 hours during 14 days. At the end of that period, when *Lactobacillus* was supplemented for the last time for all the treatments, collection intervals were as follows: every 4 hours for 72 hours, and for another 14 days, every 24 hours. The sedimentation tanks were flushed and cleaned (4 h before sampling time).

The *Lactobacillus* content was determined, and expressed on dry fecal basis. At the end of the second experiment, 3 randomly picked fish (from of each tank) of both trials were anaesthetized by using a solution with Tricaine Methane Sulfonate (TMS, Crescent Research Chemicals, Phoenix, AZ, USA) 0.4g/l buffered with 0.8g $CaCO_3$. After 15 minutes they were dissected to extract their intestinal contents which were stored in sterile and weighed flasks containing 18 ml storage medium. Three fish was analyzed by bacteriological content as described in the following section.

Microbiological analysis

At the end of the trial, the fecal samples were thawed during 1 hour in a water bath at 30^{o}C. 1 ml was transferred to reduced Neutralized Bacterial Peptone (NBP, Oxoid L34) 0.5g/l, NaCl 8g/l, Cysteine.HCl 0.5g/l, pH adjusted to 6.7 (Hartemink and Rombouts, 1999). Afterwards serial dilutions were spread on plates of selective media.

Columbia Blood Agar (Oxoid CM 331 with 50 ml/l of sheep blood added) was used to count total anaerobic bacteria. Enumeration of *Lactobacillus* was performed on LAMVAB (Hartemink et al. 1997). Plates were incubated in anaerobic jars with a mixture of 80% N_2, 10% H_2 and 10% CO_2 using the Anoxomat system (Mart, Lichtenvoorde, The Netherlands at 30^{o}C for 48 h and for 96 h respectively.

Enumeration of *Bacillus stearothermophilus* was done using Nutrient agar (NA, Oxoid CM3, Hampshire, England) with 0.006% bromocresol purple incubated for 24 h at 65^{o}C (Pochart, et al., 1992). Plates containing between 10-100 colonies were counted and expressed as colony forming units (CFU) per ml of sample.

Lactobacillus and total anaerobic counts and *B. stearothermophilus* were determined and expressed on dry mass (DM) basis of the faeces according to the following formula:

$$C_d = C_V \cdot DM_{ml}^{-1}$$

where
C_d = CFU per dry mass (CFU/g).
C_v = CFU per volume (CFU/ml).
DM_{ml} = Dry mass (g/ml).

Dry mass analysis

Faeces were obtained by centrifugation of 2 ml of the sample content at 13,000 rpm for 5 minutes. The precipitate was weighed, and a measured aliquot dried at 103°C for 24 hours. Total dry mass per ml (DM_{ml}) was calculated as follows:

$$DM_{ml} = \frac{W_p \times DM}{V_s}$$

where,

DM_{ml} =	Dry mass per ml (g/ml).
W_p =	Wet precipitated (g).
DM =	Dry mass (g/g).
V_s =	Volume of the sample (ml).

The dry mass per sample (x_i), was obtained by the following formula:

$$x_i\,(g) = DM_{ml} \times V_s$$

***Cumulative excretion of* Lactobacillus**

The fraction of *L. plantarum* 44a collected in faeces for each aquarium was estimated by the data from the last 72 hours after the last supplementation according to the following formula,

$$L = \sum_{i=1}^{n} c_{d\,i}\, x_i \qquad (1)$$

where,

L = Cumulative excretion of *Lactobacillus* (CFU).

C_{di} = CFU of *Lactobacillus* per dry mass of faeces (CFU/g) at time i.

x_i = Dry mass (g) per sample of faeces at time i: 0,4,8…72h. The amount of faeces in all the samples were the resultant of a continuous accumulation of faeces starting 4 h before the collection was taken.

Recovery of* Lactobacillus *in relation to the dose

An estimation of recovery of *Lactobacillus* (*R*) in faeces in relation to the dose ingested was carried out by using the data generated following the last day of supplementation of feed containing *Lactobacillus*, and for this pulse the recovery was calculated,

$$R_{data} = (L_{last\ pulse} / Dose_{last\ pulse}) \cdot 100\% \qquad (2)$$

where

R_{data} = Recovery from the last pulse onwards.

$L_{last\ pulse}$ = Bacteria cumulatively excreted from the last pulse (CFU).

$Dose_{last\ pulse}$ = Dose given in the last pulse (CFU).

Dynamic model of* Lactobacillus *excretion

The output (excretion) of *Lactobacillus* population in faeces was a dynamic function depending on the input of *Lactobacillus* throughout the time. This output was described by a model of two ideal mixers in series, as previously was described for single dosages (Chapter 6). However, this time an "Y_o" component was included describing the cells already present at the moment of the new dosage. The equation can be mathematically described as follows:

$$Y = \sum_{i=1}^{4} (A_0 \cdot t_{i-4} / \theta + Y_0)\, e^{-t_{i-4}/\theta} \qquad (3)$$

where,

Y= Predicted output of bacteria at time t (CFU). Initially, the predicted values were calculated by hour, thereafter; the predicted values corresponding to t =1,2,3,4h were pooled to represent the accumulation of faeces after periods of 4 hours. Such estimation, allowed to compare the actual values with the predicted values for the same intervals of time.

A_o = For the treatment 1/14, A_o was the bacteria cumulatively excreted (L) divided by θ, (L/θ).
For the other two treatments 5/14 and 14/14, A_o was the average of bacteria recorded during 5 and 14 days respectively divided by θ.

θ = Predicted mean retention time of bacteria in faeces. It is the time when bacterial concentration reaches the highest value.

t = Time (h).

Y_o = Number of cells present in faeces at time zero for the new dosage.

Estimation of cumulatively excreted* Lactobacillus *using the model

For the last pulse of each aquarium, the cumulative excreted *Lactobacillus* were calculated by the integral of the model (3), In the case of treatments 5/14 and 14/14, the integral was calculated both, from the last pulse and from all the pulses.

$$\int_0^{\infty} A_0 e^{-t/\theta} t/\theta \, dt = A_0 \cdot \theta \qquad (4)$$

and checked by

$$A_o \theta = \sum_{i=1}^{n} L_{Model} \qquad (5)$$

where,

L_{model} = Cumulative output of *Lactobacillus* (CFU) using the mathematical model.

Lactobacillus recovery (%) in relation to the dose according to the model

The *Lactobacillus* recovery was calculated both for the last pulse of each aquarium. In the case of treatments 5/14 and 14/14, the recovery was calculated both, from the last pulse and from all the pulses.

$$R_{model} = (L_{Model}/Dose) \cdot 100\% \quad (6)$$

where,

R_{model} = *Lactobacillus* recovery (%).

L_{model} = Cumulative output of *Lactobacillus* (CFU) using the mathematical model. In the case of treatments 5/14 and 14/14, the integral was calculated both, from the last pulse and from all the pulses

Dose = The input of the dose of *Lactobacillus* (CFU). In the case of treatments 5/14 and 14/14, the dose was calculated both, from the last dose supplied and by using the cumulative dosage supplied.

Comparison between* Lactobacillus *and total anaerobic flora counts

Counts of the *Lactobacillus* and total anaerobic flora collected from samples from the fish supplied with *Lactobacillus* 14 times in 14 days were compared by Student's paired t test.

Statistics

Initially, the average of θ value from the previous experiment (Chapter 6) was used to evaluate the model's predictive power. Thereafter, the model was fitted to the data by determining the value for θ that minimized the residual sum of squares (SSR) between the observed data and values of the model. θ was determined by using the solver algorithm in Microsoft® Excel 97. For each experimental unit, A_o was calculated by using the average of CFU of the daily pulses recorded depending on the frequency of supplementation.

The graphs were plotted in logarithmic scale. The results were also reported in logarithmic scale. A linear regression of actual and estimated log CFU was done. It was tested with a Student's t-test if the slope = 1 and the intercept = 0 as a final measurement of the precision and accuracy. Differences between θ and A_o among treatments were detected by using ANOVA. Mean comparisons were made using least significant differences. Differences were considered significant when $P < 0.05$. The statistical analyses were carried out using SPPS 11 (SPSS Inc, Chicago, Illinois, USA).

Growth parameters of fish

All fish were weighed per tank at the beginning and at the end of the experiment. Growth parameters were calculated according to the formulas:

$$SGR = (\ln BW_t - \ln BW_0)/t \times 100\%$$

where *SGR* is the Specific Growth Rate ($\%\cdot d^{-1}$); BW_0 is the initial body weight (*g*); BW_t is the final body weight (*g*); t is the time (*d*).

No feed refusal occurred during the experiment, consequently the feed intake equalled the feed given. The Feed ration (*R*) was calculated by:

$$R = F/BW_g \times 100\%$$

where *R* is the feed ration ($\%\cdot d^{-1}$); *F* is the feed intake ($g \cdot d^{-1}$), calculated by the cumulative amount of feed (g)/ No. of fish per tank/No days; BW_g is the mean geometric body weight = $exp((\ln BW_t + \ln BW_o)/2)$.

Feed conversion efficiency (%) was calculated as follows:

$$FCE(\%) = ((BW_t - BW_0)/F) \times 100\%$$

where *FCE* is the Feed Conversion Efficiency (%)

Statistical analysis of growth parameters in relation to the frequency of *Lactobacillus* supplementation were done using an ANOVA using SPPS 11(Chicago, Illinois, USA).

7.3. RESULTS

Predicting the excretion of Lactobacillus using a previously calculated θ and calculating A_0as the result of the average of the peaks

As a first analysis, we explored the prediction power of the model of two ideal mixers to describe multiple dosages using as a constant θ = 4.39 (Chapter 6). A pulse from a single dosage (1/14) can be seen in Figs. 7.1A and B. The pulse appears with a sharp peak after the ingestion of *Lactobacillus* disappearing after three days of intake of *Lactobacillus*. For the other treatments (5/14 and 14/14), the number of pulses in the curves depends directly on the number of days that fish were supplemented with *Lactobacillus* (Fig. 7.1C,D,E, and F). The model predicted well the kinetic behavior using the earlier estimated model parameter θ, however, some deviations can be seen.

In the case of the single dosage of this study (Fig.7.1A), the predictive model was highly and positively correlated with the data. The slope was close to one and the intercept close to zero.

For the other two treatments (5/14 and 14/14) the prediction lines did not match with the data points exactly (Figure 7.1C,D,E and F). Those predictive lines were characterized by intercepts significantly different from zero giving less accuracy to the predictive model.

Dynamic model of excretion of Lactobacillus calculating θ and using the last pulse of the curve to estimate A_o as the result of the average of the peaks.

In an attempt to improve the accuracy of the model, and to have a better descriptive line matching to the data points in the last pulse, we calculated the peak of the curve A_0, from data of cumulative recovery of *Lactobacillus* only from the last pulse. This model was not accurate enough (Fig. 7.2C,D,E, and F), having deviations for the time before the last dose.

Dynamic model of excretion of Lactobacillus calculating θ and calculating A_o, the peak of the dose

In Fig. 7.3, it is shown the data fitted with a calculated θ, and A_o on basis of the average of all the peaks for each treatment. In this model, the slopes were close to one (with the exception of Fig. 7.3E and F where little deviation occurred) and five

out of six intercepts were close to zero. This model was used to describe the kinetic parameters of the three treatments in Table 7.1.

Parameters of the best descriptive model

The mean kinetic parameters A_0 and θ of *Lactobacillus* excretion in the faeces, from the three treatments were not affected by the frequency of *Lactobacillus* supplementation (Table 7.1). The recovery of *Lactobacillus* in relation to the dose (%) was also similar between the three groups, and the estimated values of the model did not differ from the real data (Table 7.2).

Table 7.1. Kinetic parameters of *Lactobacillus* excretion in the faeces[1,2]

Frequency of supplementation (days/days)	Daily dose (Log CFU)	Cumulative dose (Log CFU)	A_0 (log CFU)	θ (h)
1/14	11.03	11.03	8.31	4.28
5/14	11.03	11.72	8.70	3.60
14/14	11.03	12.17	8.40	4.02
SEM[3]			0.20	0.23
P dose[4]			0.47	0.26

A_0, is a scale parameter defining the height of the curve. (L/θ); θ, Mean retention time.

[1]n = 2 experimental units per treatment. An experimental unit was an aquaria containing 30 fish.

[2] Means in a column without a common letter differ, $p < 0.05$.

[3] Standard error of the mean

[4] Probability of difference between at least two treatments

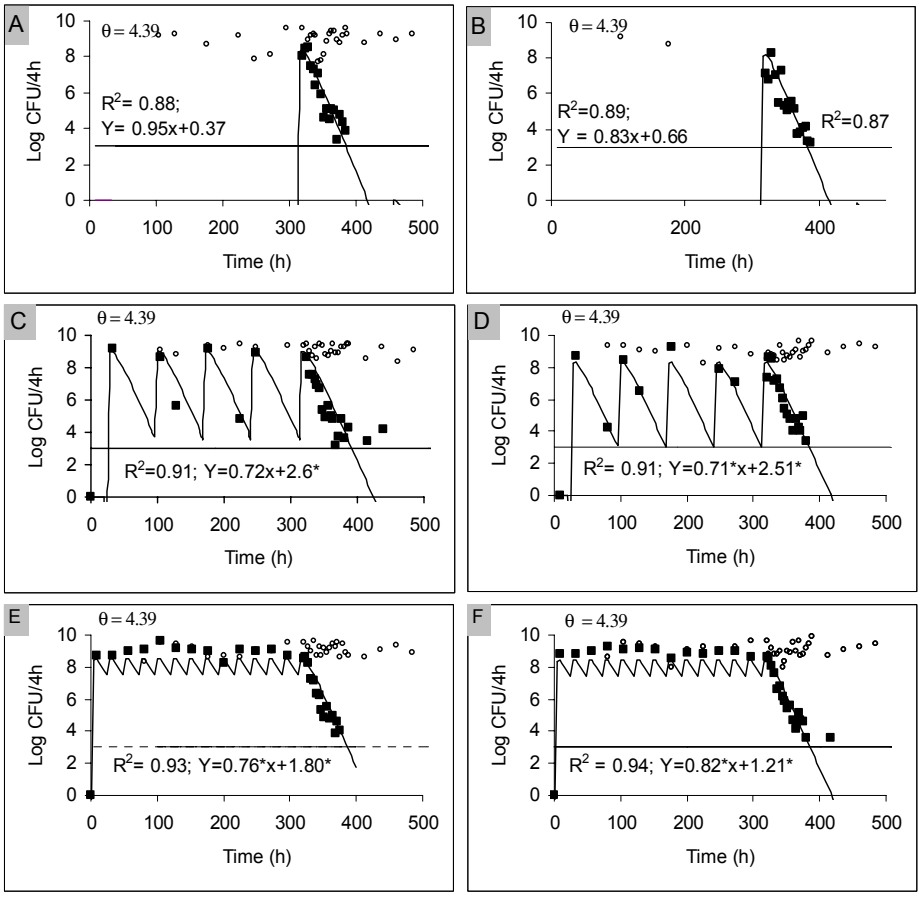

Fig. 7.1. Pattern of *Lactobacillus plantarum* 44a appearance in the faeces of tilapia during the experiment, predicted by the ideal mixer model with two compartments in series. For the prediction line, the ideal mixer model of two compartments was used. The value of the parameter $\theta \approx 4.39$ from previous experiments (Chapter 6) was used as a constant. A_0 was calculated as it is described in the equation 3. ■,Observed *Lactobacillus* data; continuous line, the prediction of the model for *Lactobacillus*. Open small circles, the counts of total anaerobic flora. Detection level = 3, also illustrated by the horizontal line. Dose of *L. plantarum* 44a applied per tank: A and B, single dose of ≈1.07e+11 CFU of *Lactobacillus*; C and D, five times a dose of ≈1.07e+11 CFU of *Lactobacillus*; E and F, ≈1.07e+11 CFU of *Lactobacillus* daily, for 14 days. The ability of the mathematical model to predict the actual values was tested by a linear regression analysis. The coefficients of the straight line are shown in the graphs. It was tested with a Student's t-test if the slope ≈ 1 and the intercept ≈ 0. The null hypothesis was accepted with p≥ 0.05 for both the slope and the intercept, meaning that fitted curve does not significantly differ from the actual values, thus y = x. *, means rejection of null hypothesis either for the slope or for the intercept.

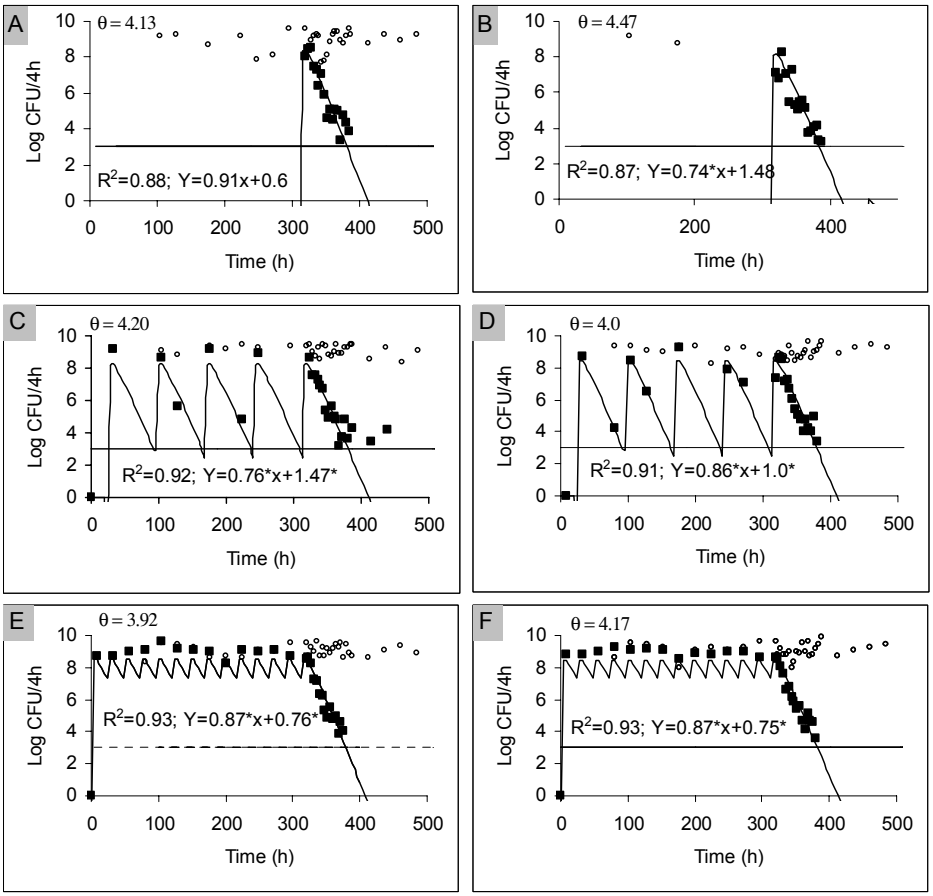

Fig. 7.2. Pattern of *Lactobacillus plantarum* 44a appearance in the faeces of tilapia fitted to an ideal mixer model with two compartments in series. The retention time (θ) was calculated for every treatment. In addition, the parameter A_0, which defines the height of the curve was calculated by using the data of continuous cumulative recovery from the last pulse; ■,Observed *L. plantarum* 44a data; continuous line, model. Dose of *L. plantarum* 44a applied per tank: A and B, single dose of ≈1.07e+11 CFU of *L. plantarum* 44a; C and D, five times a dose of ≈1.07e+11 CFU of *L. plantarum* 44a; E and F, ≈1.07e+11 CFU of *L. plantarum* 44a daily, for 14 days. Open small circles, the counts of total anaerobic flora. Detection level = 3, also illustrated by the horizontal line. The ability of the mathematical model to predict the actual values was tested by a linear regression analysis. The coefficients of the straight line are shown in the graphs. It was tested with a Student's t-test if the slope ≈ 1 and the intercept ≈ 0. The null hypothesis was accepted with p≥ 0.05 for both the slope and the intercept, meaning that fitted curve does not significantly differ from the actual values, thus y = x. *, means rejection of null hypothesis either for the slope or for the intercept.

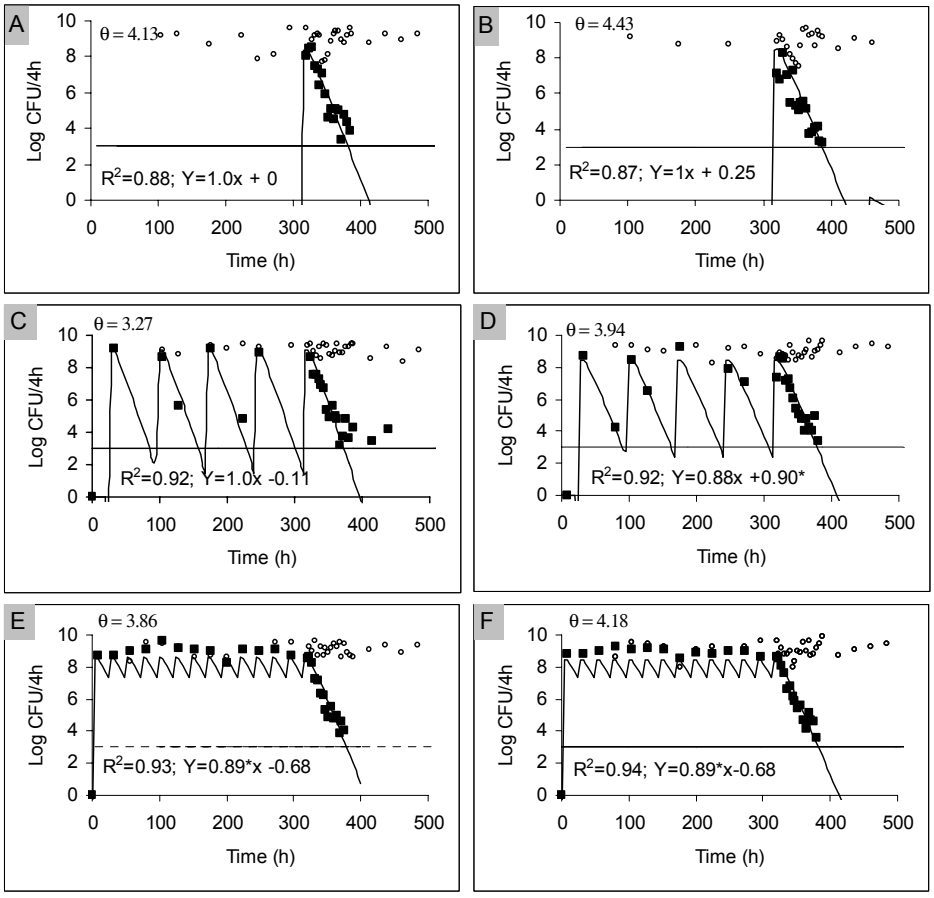

Fig. 7.3. Pattern of *Lactobacillus plantarum* 44a appearance in the faeces of tilapia during the experiment, fitted to an ideal mixer model with two compartments in series. For this figure all the parameters were estimated: A_0 and retention time (θ).A_0 was calculated using the average of the height of all the peaks for each treatment. ■,Observed *Lactobacillus* data; continuous line, model. Dose of *L. plantarum* 44a applied per tank: A and B, single dose of ≈1.07e+11 CFU of *Lactobacillus*; C and D, five times a dose of ≈1.07e+11 CFU of *Lactobacillus*; E and F, ≈1.07e+11 CFU of *Lactobacillus* daily, for 14 days. Open small circles, the counts of total anaerobic flora. Detection level = 3, also illustrated by the horizontal line. The ability of the mathematical model to predict the actual values was tested by a linear regression analysis. The coefficients of the straight line are shown in the graphs. It was tested with a Student's t-test if the slope ≈ 1 and the intercept ≈ 0. The null hypothesis was accepted with p≥ 0.05 for both the slope and the intercept, meaning that fitted curve does not significantly differ from the actual values, thus y = x. *, means rejection of null hypothesis either for the slope or for the intercept.

Table 7.2. Recovery of *Lactobacillus* excretion in the faeces (%)

Frequency of supplementation (days/days)	R_{data} [1] (%)	R_{model} [2] one pulse	R_{model} [2] all pulses
1/14	0.58	0.58	0.58
5/14	0.62	0.91	0.57
14/14	0.89	0.66	0.85
SEM[2]	0.14		
P dose[3]	0.27		

[1] Recovery calculated according to the equation 2.

[2] Recovery calculated according to the equation 6

Dynamic model of excretion of Bacillus stearothermophilus calculating θ and using all the data available to estimate A_o, the peak of the dose.

The counts of germinated spores of *B. stearothermophilus* fitted relatively well to the ideal mixer model of two compartments as is shown in Fig. 7.4. The number of pulses depends on the frequency of *Bacillus* supplementation. Retention time (θ) of *B. stearothermophilus* spores was longer than the retention time of *Lactobacillus*. Retention time was not significantly affected by the frequency of supplementation (Table 7.3). Survival was variable (Table 7.4).

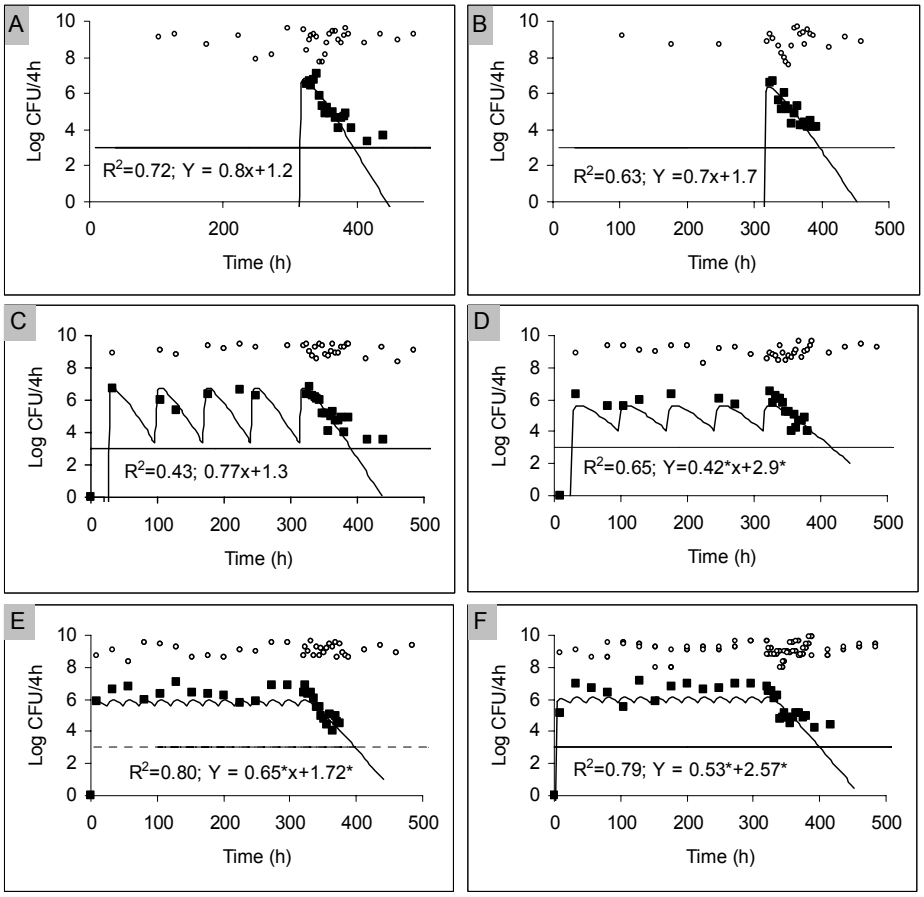

Fig. 7.4. Pattern of spores of *Bacillus stearothermophilus* in the faeces of tilapia, fitted to an ideal mixer model with two compartments in series. ■,Observed *Bacillus* data; continuous line, the fitted model for *Bacillus* data. Dose of *B. stearothermophilus* applied per tank: A and B, single dose of ≈2.2e+08 CFU of *B. stearothermophilus*; C and D, five times a dose of ≈2.2e+08 CFU of *B .stearothermophilus*; E and F, ≈2.2e+08 CFU of *B.stearothermophilus* daily for 14 days. R^2, the linear regression values between the actual and the estimated values. Open small circles, the counts of total anaerobic flora. Detection level = 3, also illustrated by the horizontal line. The ability of the mathematical model to describe the actual values was tested by a linear regression analysis. The coefficients of the straight line are shown in the graphs. It was tested with a Student's t-test if the slope ≈ 1 and the intercept ≈ 0. The null hypothesis was accepted with p≥ 0.05 for both the slope and the intercept, meaning that fitted curve does not significantly differ from the actual values, thus y = x. *, means rejection of null hypothesis either for the slope or for the intercept.

Lactobacillus and total anaerobic flora

An important information from Fig. 7.1-7.3 is that the *Lactobacillus* counts reached the numerical levels of the total anaerobic flora. In fact, the mean number of *Lactobacillus* recovered from the treatment of fish supplied with *Lactobacillus* 14 times in 14 days (from the first 14 daily samples) did not differ from the numbers of total anaerobic flora (log 8.98 vs log 9.01; P = 0.80 and 8.94 vs 9.10; P=0.24; respectively).

After suspending the supplementation of *Lactobacillus*, the *Lactobacillus* content decreased until complete disappearance. This fact suggests that *L. plantarum* 44a disappears from the faeces as predicted by the ideal mixer model of two compartments. On the other hand, the lack of establishment of *Lactobacillus* within the host can also be related to a possible lack of nutrients or growing factors necessary for retention within the gastrointestinal tract. Clearly, more detailed studies are necessary to describe which factors are relevant to extend retention time.

Table 7.3. Kinetic parameters of *Bacillus stearothermophilus* excretion in the faeces[1,2]

Frequency of supplementation (days/days)	Log *Dose* (Log CFU)	Log A_0 (Log CFU)	θ (h)
1/14	8.34	6.4	7.1
5/14	9.04	6.0	8.7
14/14	9.48	5.8	8.3
SEM[3]	0.15	0.36	0.72
P dose[4]	0.81	0.70	1.40

$A_{0,}$ is a scale parameter defining the height of the curve. (L/θ); θ, Mean retention time.

[1] n = 2 experimental units per treatment. An experimental unit was an aquarium containing 30 fish.

[2] Means did not differed between columns, $p < 0.05$.

[3] Standard error of the mean

[4] Probability of difference between at least two treatments.

Table 7.4. Recovery of *Bacillus stearothermophilus* excretion in the faeces (%)

Frequency of supplementation (days/days)	R_{data} [1] (%)	R_{model} [2] one pulse	R_{model} [2] all pulses
1/14	8.03	7.6	7.6
5/14	9.22	4.12	24.3
14/14	34.7	3.0	2.45
SEM[2]			
P dose[3]			

[1] Recovery calculated according to the equation 2.

[2] Recovery calculated according to the equation 6

Growth performance

Growth parameters of fish were not affected by *Lactobacillus* supplementation or by the absence of *Lactobacillus* in the feed (Table 7.5).

Table 7.5. Growth parameters of tilapia administered with *Lactobacillus* in various frequencies [1,2].

Growth parameters		Frequency of *Lactobacillus* supplementation (d/d)				
	Control	1/14	5/14	14/14	SEM[3]	P[4]
SGR (% · d^{-1})	3.32	3.27	3.30	3.30	0.041	0.85
Feed ration (% · d^{-1})	2.23	2.26	2.20	2.22	0.019	0.16
FCE (%)	156	152	158	156	2.31	0.12

[1]n = 2 experimental units per treatment. An experimental unit was an aquarium containing 30 fish.

[2] Means in a column without a common letter differ, $p < 0.05$.

[3] Standard error of the mean

[4] Probability of difference between at least two treatments.

Abbreviations: SGR, Specific growth rate; FCE, Feed conversion efficiency

Intestinal content and health performance

No health problems were detected in the supplemented groups. In the control group, 1 fish died during the experiment. Fish from control group and from 1/14 group were observed with intestinal lesions (bleeding). The other two groups appeared normal. From the group 14/14, one fish contained about $\approx 10^3$ lactobacilli and a similar number of total anaerobic bacteria in the second segment of the intestine. In other two fish from 14/14, all bacteria were below the detection level. Because in a previous research (Chapter 6), no bacteria was found in the intestinal content samples of tilapia, no more samples were analyzed in the present study.

7.4. DISCUSSION

The results show that the kinetic model of an ideal mixer with two compartments from a single dose is very useful to predict the behavior of the data of multiple dosage. Therefore, we have some information to start answering the questions how much probiotic should be consumed, how often, for how long; and what concentrations of probiotics should be present in the feed to reach the numerical levels of the total anaerobic flora (Marteau and Vesa, 1998).

Colonization can be defined as the potency for a probiotic to persist in the body for longer period than the inert marker (Marteau and Vesa, 1998). In this study, the excretion of spores of *B. stearothermophilus* had a longer retention time than *Lactobacillus*. A quicker fecal elimination than that of spores of *B. stearothermophilus* has also been observed for ingested *Lactococcus lactis* (Klijn et al.,1995) and *Saccharomyces boulardii* in the rat colon (Marteau and Vesa, 1998). In a review Marteau and Vesa (1998) stated that probiotics are usually excreted within a few days after their ingestion in faeces at the same rate or even quicker than a transit marker. Some studies have demonstrated that some spores of *Bacillus* can get adhered to hydrophobic materials (Faille et al., 2001). In general, hydrophobicity has been related to the capacity to adhere to organic surfaces like the mucosa. However, it seems that there is no specific information on the adherence of *Bacillus stearothermophilus*.

As *B. stearothermophilus* was used as biomarker its values were also useful as a reference in relation to the recovery of *Lactobacillus*. The spores of *B. stereathermophilus* used as biomarkers in terrestrial animals have been reported

as resistant to stomach acidity and its recovery from the faeces have been as high as 99% of the dose applied (Marteau and Vesa, 1998). We recovered only about 10% of the *Bacillus* spores. One possible cause of loss of spores in our experiment could be due to a washing effect in the faeces by its continuous exposure to the water, which was being exchanged at a rate of 5 liters per minute influencing, likely the bacterial load. The losses of bacterial spores suggest the possibility that some losses of *Lactobacillus* may also be attributable to washout. Therefore, it is possible that the survival rates of *Lactobacillus* may be higher than the values reported in Table 7.2

In our study, the detection of *Lactobacillus* or anaerobic facultative bacteria in the different segments of the intestine was not accomplished. Three samples were analyzed and only one contained numbers of both, *Lactobacillus* and total anaerobic bacteria in numbers very close to the detection level. It is possible that the anesthesia or other handling procedures had an effect on the microbial populations, a topic that deserves further research.

L. plantarum 44a taken orally in high numbers in four trials did not show any side effects. No health problems were detected in the supplemented groups. In the control group, and in the group 1/14 the presence of intestinal lesions suggest some harmful effects caused by the resident flora. The absence of those symptoms in the groups 5/14 and 14/14 suggest a beneficial effect of the consumption of the *L. plantarum* 44a.

Specific growth rate of fish and feed conversion efficiency remained without change in the treatments. Feed conversion efficiencies were very high in all treatments as compared to our previous feeding experiments (Chapter 6). It is possible that these high values would not allow any further room for improvement.

7.4. CONCLUSIONS

The mathematical model for a single dose predicted very well the data for multiple dosage. The predictive model was highly correlated with the data. The number of *Lactobacillus* in faeces during the period of supplementation did not differ significantly from the total anaerobic flora. After suspending the supplementation of *Lactobacillus*, its presence decreased, until complete disappearance. Therefore, we are closer to start answering the questions formulated by Marteau and Vesa (1998) in relation to how much probiotic should be

consumed, how often, for how long; and what concentrations of probiotics should be present in the commercial preparations; and also to correlate the effects with the concentration of the probiotic at the target site (Marteau and Vesa, 1998).

The low recovery of *B. stearothermophilus* spores suggest the possibility of a washing effect of both spores and *Lactobacillus* by the flow of water of the recirculation system, that was five liters per minute.

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8. General Discussion

8.1. INTRODUCTION

As *Lactobacillus* has many documented health effects (Ouwehand et al., 1999), and are naturally present in the gastrointestinal tract of man and animals (Fuller, 1989), we started studies aimed to investigate the intestinal *Lactobacillus* in fish; with the goal to select a strain to be used as a feed supplement for warm freshwater fish. Farmed fish are a considerable reservoir of pathogens (Austin and Austin, 1985; Austin and Gibb, 1993; Sugita et al., 1996; Wedemeyer, 1996; Apun et al., 1999) and little research has been generated in the development of probiotic products to improve health (Chapter 3). New alternatives are necessary to reduce the use of antibiotics. It has been stated that 25-50% of the antibiotic production goes to prophylactic use in livestock feed (Palumbi, 2001).

Alternatives to antibiotic use

The use of antibiotics as growth promoters is currently restricted or forbidden in many countries (Verstegen, 2001). A growing concern about the high consumption of antibiotics in aquaculture has initiated a search for alternative methods of disease control (Gildberg et al., 1997) and growth promotion (Byun et al., 1997). The resident microflora of the small intestine may not always be optimal and can depress the growth of the animals by: i) competing with the animal for nutrients, and ii) production of toxic metabolites which increase mucosal gut turnover. Possible solutions include probiotics, prebiotics, organic acids, enzymes, etc. (Verstegen, 2001).

8.2. SOME PROPERTIES OF THE *Lactobacillus* STRAINS

Wild fish

We quantified, isolated and characterized *Lactobacillus* from several fish species from a river and a farm to make a bank collection to be used for screening and selection of the strains. Most of the *Lactobacillus* strains were numerically important in wild fish and had the capacity to inhibit pathogens *in vitro* mainly by acid production. Although acid production is not an important mechanism of antibiosis in the intestine due to the buffering effect of the bicarbonate secretions; acid production may affect health as it is a major source of energy for the colonic mucosa (Rowe and Bayless, 1992) and likely stimulate mucus production (Kaunitz and Akiba, 2001). Mucus protects epithelial cells from pathogens by providing a physical barrier between the lumen and the epithelium. In addition immune response to bacterial antigens is stimulated in the mucosa by the presence of acids (Chapman, 2003). It seems that in nature, the natural diet with fiber and fermenting bacteria will maintain the bacterial ecology of the intestine, particularly of the colon, and induce growth of the intestinal mucosa (Bengmark, 1995) and the secretion of mucus (Kaunitz and Akiba, 2001). It has been suggested that the physiological effects of short chain fatty acids on gastrointestinal organs in teleosts are similar to those seen in mammals (Kihara and Sakata, 1997). Although we did not conduct mucosa and mucus assessment on an histological basis, in wild fish it was macroscopically observed as normally developed.

Farmed fish

Lactobacillus populations were scarcely detected in farmed fish. It has been shown that when the intestinal microflora is disrupted changes may occur over the entire length of the gastrointestinal tract from the mouth to the anus and across the lumen to the epithelial surface (Mathus-Vliegen, 1996). Hosts deprived of *Lactobacillus*, like farmed fish may have less well developed intestinal mucosa and a thin mucus gel, and may be devoid of some important mechanisms to maintain health. This may, at least in part, explain why *A. hydrophila*, present in numerous species of freshwater fish causes disease most significantly in cultured freshater fish (Aoki, 1999).

8.3. SOME CRITERIA FOR SELECTION OF *Lactobacillus* STRAINS

Probiotics are "live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance" (Fuller, 1989). The theoretical basis for the selection of probiotic microorganisms includes safety, functional and technological aspects (Table 8.1).

8.4. *LACTOBACILLUS PLANTARUM* 44A

We selected *Lactobacillus plantarum* 44a *in vitro* by most of the criteria in Table 8.1. This strain possesses inhibitory activities toward pathogens, resistance to gastric and intestinal fluids, viability during storage conditions for long periods of time and viability on inoculated feed after gastrointestinal transit (Chapter 4 and 5).

Table 8.1. Three major group of criteria for selection of probiotic bacteria (adapted from Saarela et al., 2000).

Safety aspects	Functional aspects	Technological aspects
• Strains for fish use are preferably from fish origin	• Acid tolerance and tolerance to fish gastric juice	• Capable of being prepared on an industrial scale
• They have been isolated from a healthy individual	• Antagonistic activity against pathogens	• Good palatability for the fish
• They have not been related with diseases in fish or humans	• Improve microbial balance, and is excreted in numbers as high as total anaerobic flora	• Viability during processing and storage
• No amine production	•Potential growth enhancer for the host	• High viability in water

Studies of supplementation of L. plantarum 44a to tilapia in a recirculation system.

Feed supplemented with *L. plantarum* 44a was consumed as much as the normal feed, and excreted as pulses dependent on the dosage ingested and periodicity dependent on the frequency of *Lactobacillus* consumption.

L. plantarum 44a and total anaerobic flora , healthy flora?

In our studies, a single dose of approximately 10^{11} of *L. plantarum* 44a was necessary to reach similar numbers of the counts of the total anaerobic flora in the peaks of excretion of *L. plantarum* 44a in the faeces of tilapia. These peaks of excretion likely coincides with the peaks of excretion of faeces resulting from the feed ration. Under these conditions, the numbers of *Lactobacillus* were not significantly different from the numbers of total anaerobic flora.

There is some evidence that a flora dominated by *Lactobacillus* correlates with prevention of infection (Reid and Burton, 2002). Therefore, the kinetic model describing the numerical dominance of *L. plantarum* 44a in the faeces of tilapia in response to the dose and the frequency of *L. plantarum* 44a supplementation (Chapter 6 and 7) can help to estimate how much probiotic should be consumed by tilapia, how often, for how long; and what concentrations of probiotics should be present in the feed to reach the numerical levels of the total anaerobic flora in the faeces produced (after Marteau and Vesa, 1998).

Biomarker

Bacillus stearothermophilus spores used as a biomarker were recovered for less than 10 %; The recovery of spores of *B. stereathermophilus* from terrestrial animals can be up to 99% (Marteau and Vesa, 1998). One possible cause of loss of spores in our experiment could be the washing effect in the faeces by its continuous exposure to the water. The loss of spores suggest also loss of *Lactobacillus* by effect of sampling procedures; therefore the survival within the gastrointestinal tract of *L. plantarum* 44a should be higher than the values reported in Table 7.2.

Colonization?

In nature, it seems that the retention of *Lactobacillus* within the host involves a certain level of specificity (Savage, 1980). It has been suggested that during periods of limiting food supply, the lactic acid bacteria, in order to withstand washout, rely on mechanisms such as co-aggreagation and attachment to the gut wall (Vandevoorde et al., 1992). This aspect should be taken in to consideration for further studies, as the tilapia in our experiments were fed almost to satiety.

Adhesion seems to be mediated by carbohydrates, proteins, lipoteichoic acids and other polymers associated with the cell wall. The adhesion of lactic acid

bacteria to animal tissue depends on the host's tissue surface characteristics (Vandevoorde et al., 1992). In a study it was shown that the presence of mucin (the glycoprotein of the mucus) in a culture medium of lactobacilli, triggered the mucus-binding activity in many *Lactobacillus reuteri* strains (Jonsson et al., 2001). Therefore a host well provided with mucin could have an advantage to be colonized by *Lactobacillus* .

Adhesion of probiotic strains to the intestinal mucosa is a desirable characteristic for colonization since it may prevent wash-out (Ouwehand et al., 1999). Although some studies have demonstrated differences in adhesiviness among several probiotic strains, and non specificity to the kind of host (Rinkinen et al., 2003); adherence of probiotic strains *in vivo* is rarely reported (Ouwehand et al., 1999).

Health effects

L. plantarum 44a taken orally in high numbers in four trials did not show any side effects. No health problems were detected in the supplemented groups. In the control group, there were some intestinal lessions that seems to be prevented by the use of *L. plantarum* 44a. However, a more detailed study is necessary to confirm this finding.

Growth performance

Some probiotic strains show enhanced growth performance in several hosts, including fish. In our studies we found non-significantly enhanced growth of fish (Chapter 6 and 7). A possible explanation for our results is that the duration of the trials were set up primarily to make studies on kinetics of *Lactobacillus* excretion. Other studies have shown that longer trials than we did are required to demonstrate significant effects. Pham et al (2003) studied the effect of two probiotic strains on the weight gains of chickens for intervals of 10 days up to 40 days, and found significance after 30 days. Weight was increased by 10.7%. Lara-Flores et al (2003) found that the ingestion of *Lactobacillus* by tilapia larvae for 9 weeks improved growth rate and feed conversion (Lara-Flores et al., 2003).

Another reason for not finding a growth promotion may be that our studies were carried out under extremely optimal rearing conditions for the fish in terms of water quality, diet and management. It is feasible that probiotics are mainly effective as growth enhancers when stressful conditions are prevalent as was demonstrated

many years ago with the use of antibiotics as growth promoters (McDonald et al., 1973).

8.12. CONCLUSIONS

Lactobacillus plantarum 44a may be a valuable probiotic strain for freshwater fish. While beneficial effects of probiotics may be less obvious when fish can be grown under optimal rearing conditions, they may still be used as a safeguard to infectious disease. New experiments are necessary in sub-optimal conditions.

Another approach might be to use a combination of *Lactobacillus* strains: some that produce hydrogen peroxide and may be effective in the upper part of the gut where oxygen is available and others that inhibit pathogen, e.g. by acid production, and survives to exposure to gastrointestinal fluids.

The benefits of probiotics as healthful ingredients could also be enhanced if used in combination with other health promoting dietary strategies, such as acids, vitamins, antioxidants and fibre.

8.13. RECOMMENDATIONS

Lactobacillus plantarum 44a seems to have good potential for probiotic applications (Table 8.1). Its use in farmed fish seems to be desirable, but further research in relation to beneficial effects to the host need to be studied.

Lactobacillus plantarum 44a may also be tested with other hosts, as it has a very high ability to withstand the bactericidal effect of the gastrointestinal fluids.

10. Summary

This thesis is the result of a work carried out within a four year project entitled "*Lactobacillus* as a probiotic for freshwater fish". This section is a summary of the research described in this thesis.

Chapter 1 is a general introduction, concluding with the specific aims of this thesis.

Chapter 2 is a literature review of the bacterial flora of freshwater fish and its environment. It describes the interaction between fish and bacteria in all the life-stages of fish, stressing some biotic and abiotic factors leading to bacterial overgrowth and disease. The current methods to prevent and control fish pathogens in fish farming, including the use of disinfectants, chemicals, antibiotics and vaccines is described. A literature review on the use of lactic acid bacteria as healthy inoculants in salmonids is also described.

The presence of *Lactobacillus* in the gastrointestinal tract is considered beneficial in a wide range of hosts. In **Chapter 3,** it is shown that various species of *Lactobacillus* are present in relatively high number in the intestines of fresh water fish from a river, especially in the warm season but in low numbers in the intestines of farmed fish reared in a recirculation system in warm water. *Lactobacillus* are reported for the first time from the intestines for several common fish species in Europe. They are *Anguilla anguila*, *Perca fluviatilis*, *Scardinius erithrophthalmus*, *Gymnocephalus cernuus*, *Alburnus alburnus*, *Blicca bjoerkna*, *Leuciscus cephalus*, *Silurus glanis* and *Clarias gariepinus*. The two first species, commonly named eel and perch and the last one are highly valuable species for aquaculture. Additionally, improved methods for storage and bacteriological analysis of fish intestinal content are described.

Probiotics are live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance (Fuller, 1989). **Chapter 4** describes a screening system to select a *Lactobacillus* with probiotic abilities suitable for *in vivo* studies in farmed freshwater fish. As a result, two strains were selected mainly on

basis of pathogens inhibition, and survival throughout the gastrointestinal tract fluids in a simulation model. Selected strains were *Lactobacillus plantarum* 44a and *L. brevis* 18f. *L. plantarum* 44a survived better to the exposure to gastrointestinal fluids than the other strains. *L. plantarum* 44a co-cultured with *A. hydrophila* in fish feed extract with an initial ratio ≈ 10^3 : 10^7 and 10^7: 10^3 respectively, started killing the pathogen when pH was around 5.5. *L. plantarum* 44a has potential applications as probiotic in freshwater fish. *L. brevis* 18f was identified as a H_2O_2 producer, active against *Aeromonas hydrophila* at pH 6.0. It may have application as a possible fish pathogen antagonist in the upper gastrointestinal tract, the skin, the gills and eggs where oxygen is available.

A good probiotic strain should be able to survive the conditions of handling and storage to be delivered in high concentration to the host. That is especially important when stressful conditions are prevalent in the carrier, for instance in low water content foods like animal feed. **Chapter 5** is a study to optimise the survival of the probiotic candidate *Lactobacillus plantarum* 44a after spraying and drying in feed, and during storage and exposure to gastrointestinal tract fluids *in vitro*. In addition, the viability of the strain during exposure to distilled water and 2% NaCl was studied. Feed was sprayed with a suspension of ≈ 2 x 10^{10} CFU of *L. plantarum* 44a in 10, 15, 20, 25 and 30 % v/w of the feed and dried to constant weight (6% moisture) in a convective oven at 25^{o}C. *L. plantarum* 44a survived 15, 36, 52, 79, and 100% respectively in relation to the original % v/w of the bacterial suspension applied to the feed. After three weeks of storage at 25^{o}C, survival was similarly low in all the treatments. *L. plantarum* 44a stored in feed containing 13% of moisture, vacuum-packaged and stored in refrigeration, maintained high viability (≈100%) after 1 year of storage. Survival was not affected after feed-containing *L. plantarum* 44a was exposed to gastrointestinal fluids in a simulation model. Viability of *L. plantarum* 44a as a cell suspension in buffer phosphate saline added directly to distilled water or distilled water with 2% NaCl was maintained up to 48 h; after 72 h, viability started to decline. As conclusions, *L. plantarum* 44a maintained high viability after dried and stored in feed even after exposure to gastric and intestinal fluids *in vitro*.

In **Chapter 6,** the aims were to investigate the ability of *L. plantarum* 44a to survive passage throughout the tilapia gastrointestinal tract when ingested as a feed supplement on the following single dosages: ≈10^{12} CFU; ≈10^9 CFU; ≈10^6 CFU; 0 CFU given to eight groups of fish, with 26 fish per group with an average of 70g per fish. Faeces were periodically collected in sedimentation tanks and analyzed for

its *Lactobacillus* and total anaerobic flora content. *L. plantarum* 44a appeared in faeces as mathematically defined pulses in response to the dosage. After some days, the *Lactobacillus* population in all the treatments followed an exponential decay pattern with detectable levels of organisms for several days. The highest dose of *L. plantarum* 44a ingested, reached the same level as the total anaerobic flora. All randomly picked isolates from the highest dilution plates gave a fermentation pattern typical of *L. plantarum* 44a. *L. plantarum* 44a has a high potential to pass the gastrointestinal tract alive and to be present in faeces in very high numbers with potentially growth promotion effects in tilapia.

Chapter 7 is a study on the effect of the frequency of *Lactobacillus plantarum* 44a supplementation on the kinetics passage of *Lactobacillus* within the gastrointestinal tract of tilapia. Feed containing 1.07×10^{11} *Lactobacillus* per ration were supplied as follows: once in 14 days; 5 times in 14 days and 14 times in 14 days. Faeces were periodically collected in sedimentation tanks and analyzed for *Lactobacillus* content. For the analysis of the kinetic data, a previously described model for single dosages was used. The kinetic parameters of *Lactobacillus* passage through the gastrointestinal tract of tilapia were not significantly affected by the frequency of *Lactobacillus* intake. The mathematical model for a single dose predicted well the data for multiple dosage. The estimated values were highly correlated with the data. The number of *Lactobacillus* in faeces during the period of supplementation did not differ significantly from the total anaerobic flora. After suspending the supplementation of *Lactobacillus*, its presence decreased, until complete disappearance. Growth parameters of the fish were not affected by the frequency of *Lactobacillus* supplementation.

Chapter 8, is a general discussion of the outcomes of this thesis, putting forward some hypothesis and concluding remarks.

In summary, *Lactobacillus plantarum* 44a seems to have a good potential for probiotic applications.

10. Samenvatting

Dit proefschrift is het resultaat van een vierjarig project met de titel "*Lactobacillus* as a probiotic for freshwater fish" (*Lactobacillus* als probiotica in zoetwater vis). Dit hoofdstuk geeft een samenvatting van het onderzoek zoals beschreven in dit proefschrift.

Hoofdstuk 1 is een algemene inleiding van het vakgebied, met als afsluiting de doelen van het onderzoek.

Hoofdstuk 2 is een literatuuronderzoek naar de bacterieflora van zoetwater vissen en hun omgeving. De interactie tussen de vis en bacterie in de verschillende levensstadia wordt beschreven. Hierbij worden diverse factoren, gezien vanuit de vis of vanuit de omgeving, betrokken die een rol spelen bij bacteriële overgroei en bacteriële infecties van de vis. De huidige methoden voor het voorkómen en beperken van pathogene bacteriën in intensieve viskweek worden besproken. Hieronder vallen desinfectantia, chemicaliën, antibiotica en vaccins. Ook bevat het hoofdstuk een literatuuronderzoek naar het gebruik van lactobacillen als gezondheidsbevorderende ingrediënten voor zalmachtigen.

De aanwezigheid van lactobacillen in het maagdarmkanaal wordt gezien als gunstig voor het organisme. In **Hoofdstuk 3** wordt beschreven dat verschillende soorten *Lactobacillus* kunnen worden aangetroffen in vrij hoge aantallen in de darm van diverse soorten riviervissen in de zomer, maar in lage aantallen in de winter of bij vis uit viskwekerijen. Er is voor het eerst aangetoond dat *Lactobacillus* soorten in de darmen van een aantal Europese soorten zoetwatervis voorkomen. Het gaat hierbij om de volgende soorten *Anguilla anguilla*, *Perca fluviatilis*, *Scardinius erithophtalmus*, *Gymnocephalus cernuus*, *Alburnus alburnus*, *Blicca bjoerkna*, *Leuciscus cephalus*, *Silurus glanis* en *Clarias gariepinus*. De eerste twee soorten, paling en baars, zijn twee soorten die van belang zijn als kweekvis.

Verder worden in het hoofdstuk nog verbeterde methoden voor het bewaren en analyseren van de darminhoud van vissen beschreven.

Probiotica zijn levende voedselsupplementen, die de gezondheid van de gastheer bevorderen door de darmflora te verbeteren (Fuller, 1989). **Hoofdstuk 4**

beschrijft een methode om lactobacillen te screenen op probiotische eigenschappen, voor gebruik als voedingssupplement in zoetwatervis. Twee stammen werden na de screening geselecteerd op basis van werking tegen pathogenen en overleving in een model van het maagdarmkanaal. De geselecteerde stammen werden geïdentificeerd als *Lactobacillus plantarum* L44a en *L. brevis* 18f. *L. plantarum* 44a vertoonde de beste overleving in het maagdarm-model van alle geteste stammen. In experimenten waarbij deze stam samen opgekweekt werd met de pathogeen *Aeromonas hydrophila* met een beginverhouding van 10^3:10^7 of 10^7:10^3 in een vismeelmedium, werd de pathogeen afgedood wanneer de pH een waarde van 5.5 of lager bereikte. *L. plantarum* 44a heeft hierdoor potentie als een mogelijk probioticum voor zoetwatervis.

L.brevis 18f had als eigenschap de productie van waterstofperoxide, waardoor ook deze stam actief was tegen *A. hydrophila*, bij pH 6.0 of lager. Omdat de aanwezigheid van zuurstof vereist is voor deze werking, heeft de stam mogelijk toepassing in het bovenste deel van het maagdarmkanaal of op de huid of kieuwen van vissen, of bij de oppervlaktebehandeling van larven en eieren.

Een goed probioticum moet, om commercieel toegepast te worden, goed kunnen overleven tijdens productie, opslag en transport van het product waarin het wordt toegepast. Dit is met name van belang in producten waarvan de eigenschappen slecht zijn voor bacteriële overleving, zoals producten met lage wateractiviteit, waartoe ook visvoer behoort. **Hoofdstuk 5** beschrijft een studie naar de overleving van de mogelijk probiotische stam *L.plantarum* 44a in gedroogd voer gedurende productie en opslag, en vervolgens naar de overleving van de bacteriën in dit voer in het maardarm simulatie model. Als modelsysteem werd ook de overleving in gedestilleerd water en water met 2% zout bestudeerd.

De overleving werd bepaald door gedroogd voer te mengen met een suspensie van $2x10^{10}$ kve *L. plantarum* 44a tot een initieel vochtgehalte van 10, 15, 20, 25 en 30% v/w, wat daarna gedroogd werd in een oven bij 25°C tot een constant gewicht werd bereikt (vochtgehalte 6%). De overleving van de stam was respectievelijk 15, 36, 52, 79 en 100% en dus sterk afhankelijk van het initiële vochtgehalte. Na 3 weken bewaren bij 25°C in los visvoer, was de overleving voor alle behandelingen erg laag. Wanneer de stam echter werd opgeslagen in visvoer met 13% vocht onder vacuüm in de koelkast, was er geen afname te zien in kiemgetal na 1 jaar opslag. Na opslag was de overleving van de stam in het maagdarm simulatie model identiek aan de stam vóór opslag.

Uit de experimenten met gedestilleerd water of 2% zout, kan geconcludeerd worden dat de stam 48 uur overleeft, pas na 72 uur wordt een duidelijke afname in kiemgetal gevonden.

De conclusie van dit hoofdstuk is dat *L.plantarum* 44a zeer goed overleeft gedurende drogen en opslag en dat opslag geen effect heeft op de overleving in het maagdarmkanaal.

Hoofdstuk 6 beschrijft een proef, waarin de overleving van *L. plantarum* 44a in tilapia wordt bestudeerd. De stam werd aan het voer voor de tilapia toegevoegd in een éénmalige dosis met volgende concentraties: 10^{12}, 10^{9}, 10^{6} of 0 kve/g in duplo. Voor iedere behandeling werden 26 tilapia gebruikt met een gemiddeld gewicht van 70 gram per vis. De feces werd opgevangen in sedimentatietanks en op regelmatige tijdstippen onderzocht op de aanwezigheid van lactobacillen en totaal aantal bacteriën. *L. plantarum* 44a werd aangetoond als een, wiskundig te beschrijven, puls, die in relatie stond met de toegevoegde dosis. De uitspoelcurve na enkele dagen volgde een exponentieel verloop en lactobacillen waren na een aantal dagen na de éénmalige dosis niet meer aantoonbaar in de feces. De hoogste aantallen gevonden lactobacillen benaderden de totale aantallen bacteriën aangetroffen in de feces. Alle willekeurig geïsoleerde stammen uit de hoogste verdunningen, vertoonden hetzelfde fermentatiepatroon als *L. plantarum* 44a. Uit het experiment kan geconcludeerd worden dat de stam goed overleeft in tilapia en dat aantallen vergelijkbaar met het totaal kiemgetal bereikt kunnen worden. Bovendien werden potentiële groeibevorderende eigenschappen gevonden.

Hoofdstuk 7 beschrijft een studie naar het effect van meermalige doses *L.plantarum* 44a op de passagesnelheid van deze stam in het maagdarmkanaal van tilapia. Hiertoe werd een dosis van 1.07×10^{11} kve/gram visvoer toegediend aan tilapia volgens het volgende schema: éénmaal per 14 dagen, 5x per 14 dagen en dagelijks gedurende 14 dagen. Feces werd opgevangen in sedimentatietanks en periodiek geanalyseerd op aanwezigheid van lactobacillen. Voor de analyse van de data werd het model uit Hoofdstuk 6 gebruikt. De kinetische parameters van de passage van *Lactobacillus* door het darmkanaal werden niet significant beïnvloed door de frequentie van *Lactobacillus* opname. Het mathematische model voor de eenmalige opname voorspelde goed de data voor meermalige opname.

Bij de dagelijkse toediening van lactobacillen waren de bereikte aantallen in de darm vergelijkbaar met het totaal aantal bacteriën in de darm. Na het stoppen van de toediening nam het aantal lactobacillen exponentieel af en waren na enkele dagen niet meer aantoonbaar.

De toediening van lactobacillen aan het voer had geen effect op de groeiparameters van de tilapia.

Hoofdstuk 8 is een algemene discussie over de resultaten van dit proefschrift, met hypotheses en aanbevelingen voor verder onderzoek.

Als algehele conclusie kan gesteld worden dan *Lactobacillus plantarum* 44a een bacteriestam is met potentieel probiotische eigenschappen.

11. Resumen

Esta tesis es el resultado de una investigación de 4 años titulada "*Lactobacillus* como suplemento alimenticio probiótico para peces de agua dulce". Esta sección es un resumen de la tesis.

El **Capitulo 1** es una introducción general, concluyendo con los objetivos específicos de la tesis.

El **Capitulo 2** es una revisión de literatura de la flora bacteriana de los peces de agua dulce y su ambiente. Esta sección describe la interacción entre pez y bacterias en todos los estadios de vida de los peces, haciendo énfasis en algunos factores bióticos y abióticos que llevan a una sobrepoblación de bacterias que puede llevar a enfermedades. También se describen los métodos actuales de prevención y control de los patógenos de peces en las granjas, incluyendo el uso de desinfectantes, agentes químicos, antibióticos y vacunas. Se hace una revisión de la literatura acerca del uso de bacterias ácido lácticas como inoculantes saludables en salmónidos.

La presencia de *Lactobacillus* en el tracto gastrointestinal es considerada benefica en una gran variedad de huéspedes. In el **Capitulo 3**, es mostrado que varias especies de *Lactobacillus* están presentes en relativamente alto numero en los intestinos de los peces de agua dulce de un rió, pero en bajos números en peces en un sistema de recirculación de agua. *Lactobacillus* es reportado por vez primera en los intestinos de varias especies comunes de peces de agua dulce en Europa. Ellas son *Anguilla anguilla*, *Perca fluviatilis*, *Scardinius erithophtalmus*, *Gymnocephalus cernuus*, *Alburnus alburnus*, *Blicca bjoerkna*, *Leuciscus cephalus*, *Silurus glanis* y *Clarias gariepinus*. Las dos primeras especies, comúnmente conocidas como Anguilla y Perca, y la ultima conocida como pez gato africano son altamente valuadas en acuacultura. Adicionalmente, se describen métodos mejorados para el almacenamiento y análisis bacteriológico de muestras intestinales.

Probióticos son suplementos microbianos que al ser ingeridos benefician al animal mejorando su balance intestinal (Fuller, 1989). En el **Capitulo 4** se describe un sistema de análisis para seleccionar *Lactobacillus* en laboratorio con capacidades probióticas para estudios posteriores en peces de las granjas. Como resultado, dos cepas fueron seleccionadas principalmente en base a inhibición de patógenos, y sobreviviencia a través de fluidos del tracto gastrointestinal en un modelo de simulación. Las cepas seleccionadas fueron *Lactobacillus plantarum* 44a y *Lactobacillus brevis* 18f. *L. plantarum* 44a sobrevivió mejor a la exposición de fluidos gastrointestinales que las otras cepas. *L. plantarum* 44a en cultivo mixto con *Aeromonas hydrophila* en extracto de alimento de pescado con una proporción inicial $\approx 10^3$:10^7 año 10^7:10^3 respectivamente, inicio la matanza del patógeno cuando el pH era alrededor de 5.5. *L. plantarum* 44a tiene potencial para ser aplicado como probiótico en peces de agua dulce. *L. brevis* 18f fue identificado como un productor de H_2O_2 (peroxido de hidrogeno) un bactericida que mata a *A. hydrophila* a pH 6.0. Puede tener aplicación como posible antagonista de patógenos en la parte superior del tracto gastrointestinal, la piel, las branquias y los huevos donde hay disponibilidad de oxigeno.

Un buen microorganismo probiótico debe ser capaz de sobrevivir las condiciones de manejo y almacenamiento para tener alta viabilidad antes de ser comercializado y consumido. Esto es especialmente importante cuando algunas condiciones estresantes prevalecen en el vehículo, por ejemplo en alimentos que contienen una baja actividad de agua como el alimento animal. El **Capitulo 5** es un estudio para optimizar la sobreviviencia del candidato a probiótico *Lactobacillus plantarum* 44a después de aplicarlo en spray y secarlo en el alimento, y durante el almacenamiento y exposición a fluidos gastrointestinales *in vitro*. En adición, la viabilidad de la cepa durante la exposición a agua destilada y 2% de NaCl fue estudiada. El alimento fue aplicado en spray con una suspensión de $\approx 2x10^{10}$ CFU de *L. plantarum* 44a en 10, 15, 20, 25 and 30% v/p de el alimento y secado hasta peso constante (6% de humedad) en un horno convectivo a 25^0C. *L. plantarum* 44a sobrevivió 15, 36, 52, 79 y 100% respectivamente en relación al volumen / peso de la suspensión bacteriana aplicada al alimento. Después de tres semanas de almacenamiento a 25^0C, la sobreviviencia fue similarmente baja en todos los tratamientos. *L. plantarum* 44a almacenado en alimento conteniendo 13% de humedad, empacado al vació y almacenado en refrigeración, mantuvo alta viabilidad (100%) después de un ano de almacenamiento. La sobrevivencia no fue afectada después de que el alimento conteniendo *L. plantarum* 44a fue expuesto a

fluidos gastrointestinales en un modelo de simulación. La viabilidad de *L. plantarum* 44a s como una suspensión celular en solución de amortiguadores de fosfato salino agregado directamente a agua destilada o agua destilada con 2% NaCl fue mantenida hasta 48 h; después de 72h, la viabilidad comenzó a declinar. Como conclusiones, *L. plantarum* 44a mantuvo alta viabilidad después del secado y almacenaje en alimento aun después de exposición a fluidos gástricos e intestinales *in vitro*.

En el **Capitulo 6** los objetivos fueron investigar la capacidad de *L. plantarum* 44a para sobrevivir el transito a través del transito gastrointestinal de tilapia cuando ingerido como suplemento alimenticio en las siguientes dosis individuales: 10^{12}, 10^{9}, 10^{6} y 0 CFU suministrado a ocho grupos de peces, con 26 peces por grupo con un promedio de 70g por pez. Las heces fueron periódicamente colectadas en tanques de sedimentación y analizadas por su contenido de *Lactobacilos* y flora anaeróbica total. *L. plantarum* 44a apareció en las heces como pulsos definidos matemáticamente en respuesta la dosificación. Después de algunos días, el registro de *Lactobacillus* en todos los tratamientos siguió una trayectoria exponencialmente decreciente con niveles detectables de organismos por varios días. La mas alta dosis de *L. plantarum* 44a ingerida, alcanzo números similares a los de la flora anaeróbica total. Todos los *Lactobacilos* aislados aleatoriamente a partir de los platos de mayor dilución presentaron un patrón de fermentación típico al de *L. plantarum* 44a. *L. plantarum* 44a tienen un alto potencial para pasar vivo el tracto gastrointestinal y estar presente en las heces en muy altos números con efectos de promoción del crecimiento en tilapia.

El **Capitulo 7** es un estudio del efecto de la frecuencia de suplementacion de *L. plantarum* 44a en la cinética de paso de *Lactobacillus* dentro del tracto gastrointestinal. Alimento conteniendo 1.07×10^{11} *L. plantarum* 44a por ración fue suministrada como sigue: una vez cada 14 días; 5 veces en 14 días y 14 veces en 14 días. Las heces fueron periódicamente colectadas en tanques de sedimentación y analizadas por su numero de *Lactobacillus*. Para el análisis de los datos de la cinética, se utilizo el modelo utilizado para dosis individuales. Los parametros de cinetica del pasaje de *Lactobacillus* a traves del tracto gastrointestinal no fueron significativamente afectados por la frecuencia de consumo de *Lactobacillus.* El modelo matematico para dosis de un dia fue util para predecir dosis en varios dias. Los valores estimados estuvieron altamente correlacionados con los datos.

Durante el tratamiento con *L. plantarum* 44a, el numero de *Lactobacillus* en las heces no fue diferente que el de la flora anaeróbica total. Después de suspender la suplementación de *L. plantarum* 44a, su presencia decreció, hasta completa desaparición. Los parámetros de crecimiento en los peces no fueron afectados por la frecuencia de suplementacion de *Lactobacilos.*

El **Capitulo 8** es una discusión general de la información generada en esta tesis, estableciendo algunas hipótesis y notas concluyentes.

En resumen, *Lactobacillus plantarum* 44a parece tener un buen potencial para aplicaciones probióticas.

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Curriculum vitae

Adolfo Bucio Galindo was born on July, 25, 1962 in México City. Between 1984-1989 he studied Biology in the Faculty of Sciences, at the Universidad Nacional Autonoma de México. He carried out his bachelors research in the area of aquatic sciences in Colegio de Postgraduados, Campus Tabasco (CP-Tabasco). From 1995-1997 he studied Food Sciences, at the Universidad Iberoamericana, México and made research in the Universidad Autonoma Metropolitana, on *Lactobacillus* as biopreservative. In 1998 he worked for CP-Tabasco and planned PhD studies in the area of *Lactobacillus* and feeds for aquatic organisms. From 1999-2003 he made PhD studies described this thesis in Wageningen University. From 2004 he will work as a researcher for the CP.