Non specific immune stimulation of Bokashi in head kidney cells of carp

Feed Innovation Services (F.I.S.) bv

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In order of:
Agriton
Noordwolde

August 2005

CONTENTS

1 INTRODUCTION

There is a clear increase of intensive aquaculture in the future as a result of the demand for fish as food and the restriction of fisheries. At present the production of salmon for example, is an important industry in Europe and South America. However, in Europe salmon culture deals with more difficulties, since in Europe antibiotics are no longer allowed for animal production. At the moment, alternatives in health promotion, disease prevention and treatment are investigated. Immune stimulants like ß-glucans (MarcroGard) and nucleotides seem to be good candidates. As expected, the use of antibiotics will be restricted or banned also in other areas of the world, which will lead to an increasing demand for immune stimulants. In addition, an increased demand for immune stimulants is caused by the recent focus on other cultured species, like for example cod. In addition to aquaculture, the effect of immune stimulation is important for other animal species for animal production and for pets.

ß-glucans have a stimulatory effect on macrophages and on macrophages of Salmon (*Salmo salar* L.) ß-glucan receptors were found (Engstand & Robertson, 1994).
The working mechanism is based on a chain reaction. Glucans from a broad spectrum of yeast cell walls stimulate both the specific and the a-specific immune system. They attach to the mucus of the gut, where ß-glucanes selectively cooperates with specific receptors on the macrophages, granulocytes, NK cells, causing a direct activation of these defenses. The cells start producing cytokines that in turn activate T-lymphocytes, which activate ß-lymphocytes that will turn more active in producing antibodies against bacteria and viruses. In this way, ß-glucans are, besides their positive effect on the a-specific immune system, are also involved in the specific resistance against infectious diseases, increasing the power of antibiotics and vaccines

For an immune stimulation, it is known that an optimum dosage should be important.
The objective of the present study is to get more information about the potential immune stimulation of Bokashi and the optimal dosage.

The material and methods of the assays are given in Chapter 2. In Chapter 3, the results are presented. Chapter 4 contains the conclusions.

2 MATERIAL AND METHODS

The specific assay work has (as is written in 2.2) been carried out by the Cell Biology and Immunology Group of the Wageningen Institute of Animal Sciences.

2.1 Substrates

The negative control in each experiment is no addition of substrates. The positive control in the experiments is a supplementation of a substrate (MacroGard®) with a known stimulation of the cells. MacroGard® consists of ß-glucans which has been used in practise as immunostimulant. It is used in three dosages as a commercial control.

The test product is Bokashi (with Effective Micro-organisms fermented wheat bran) delivered by Agriton. Four dosages of Bokashi are tested.

2.2 Reactive oxygen species / oxidative burst assay

Introduction

The effective functions of phagocytes are considerable, but perhaps one of the most important functions is the ability to kill pathogens. The (microbicidal) killing activity of mammalian phagocytes, such as macrophages and polymorphonuclear granulocytes, has been conventionally divided into aerobic and anaerobic defences. Aerobic defences require oxygen which is converted into a number of oxygen free-radicals. This process is termed the oxygen burst or the respiratory burst. The microbicidal activity of the **reactive oxygen species (ROS)** is commonly increased after interaction with phagocyte enzymes such as peroxidases, and their production by fish phagocytes is well established. ROS generation is an evolutionary conserved mechanism that can even be found in plants.
Oxygen burst causes a marked increase in oxygen uptake, which is mainly channelled to a pathway initiated by the reduction of oxygen to superoxide anion (O_2^-), using NADPH or NADH as the electron donor. The O_2^- is subsequently converted to hydrogen peroxide (H_2O_2) with the aid of the enzyme superoxide dismutase (SOD).

$$2O_2 + NADPH \text{ ----------> } 2O_2^- + NADP^+ + H^+$$

$$2O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

Objective

The modulation of the oxygen burst activity in macrophages and neutrophilic granulocytes of the carp pronephros, or head-kidney, by Beta-glucans will be studied in two ways. One method used is based on the reduction of a salt: **NITRO BLUE TETRAZOLIUM (NBT)** by O_2^- to a blue colour, which is measured spectrophotometrically.

Materials

- Phorbol 12-myristate 13-acetate (PMA) as a positive control: handle with care: 0,1 ? g/ml final concentration
- Beta-glucan solutions and yeast products
- RPMI without phenol red: washing and culture media (the red indicator dye disturbs the final reading on the spectrophotometer)
- Nitro Blue Tetrazolium in RPMI without phenol red. (NBT; grade III) 1 mg/ml final concentration
- 2N KOH (Potassiumhydroxid)
- 100% and 70% methanol

- Dimethylsulfoxide (DMSO)
- flat bottomed 96-well microtiterplate (sterile) coated with adherent cells
- spectrophotometer with filter at 690 nm

Methods

- Leukocytes were collected from the head kidney and placed at 4 degrees Celsius overnight. Plate out 1.2 ml as follows: 100 ? l in each well of the first three rows, starting in column b ending in column e (b1..e3)
- Put the plate at 27 ^{o}C (5 % CO_2, humidity 100 %) and allow the cells to adhere for 30 mins. After the incubation: study the cells under the inverted microscope to examine the monolayer.
- Wash away the non-adherent cells by carefully rinsing twice with 100 ? l of RPMI-phenol red culture medium. Rinse one row at a time in order to prevent cells from drying.
- Start the NBT assay by carefully removing the supernatant and by adding 150 µl of NBT (a1 ...e3)
- Subsequently add the following stimulators according to schedule

  Column 1: 10 µl RPMI medium without phenol red (negative control)
  Column 2:10 µl of PMA (toxic!) solution (1.7 ? g/ml) (positive control)
  Column 3 1000 µl of B-glucan solution (…….)
  Column 4:100 µl of B-glucan solution (…….) etc.

- Incubate the cells for 90-120 minutes at 27 ^{o}C (5 % CO_2, humidity 100 %)
- After the incubation, remove the NBT solutions in all columns (collect the NBT because it is toxic).
- Wash the cells once by rinsing with RPMI medium without phenol red.
- Fixate the remaining cells for 5 minutes with 100 µl 100 % methanol.
- Then the cells are washed twice with 100 µl 70 % methanol to remove possible NBT-rests. The plate is left to dry.
- The blue formazan inside the cells is solubilised in 100 µl 2N KOH to which 100 µl DMSO (toxic!) is added. The content of the well is mixed thoroughly.
- The optical density is measured at 690 nm (ref. filter at 414 nm) in a spectrophotometer.

2.3 Statistical analysis

Each treatment has been carried out with three fishes and with three or four replicates (n=9-12). The results are analysed with one-way analysis of variance with the statistical program SAS Jump version 4.0.2. (2000). Tukey HSD test has been used to determine if differences between treatments are presents. A p-value of 0.05 is determined as significant.

3 RESULTS

3.1 Oxidative burst assay

All dosages of Bokashi are compared with a negative control (no addition of substrate) and a positive control (PMA; a standard). In order to compare the results with the most common used immunostimulant (MacroGard), also a comparison has been made with MacroGard (MG). The comparison is made with three effective dosages of 1000, 100 and 10 µg/ml (based on former experiments).

The results of the addition of the products are presented in Table 3.1 (n=9; 3 replicates in 3 fish).

Table 3.1 Effect of substrate and dosage on carp head kidney cells (Oxidative Burst assay).

Substrate	Dosage (µg/ml)	Mean
Negative control	-	0.25 a
Positive control	0.1	0.68 d
MarcoGard (MG)	1000	0.35 abc
MG	100	0.36 bc
MG	10	0.28 ab
Bokashi	10.000	0.39 c
Bokashi	1.000	0.32 abc
Bokashi	100	0.34 abc
Bokashi	10	0.34 abc

a-d: $p<0.05$

The positive control did, as expected, significantly stimulate the cells.
Of all tested substrates, Bokashi at a dosage of 10.000 µg/ml showed the highest immune stimulation and was, like MG in the dosage of 100 µg/ml, significantly different from the negative control.

For each fish, the mean (red bar) and the standard error (green bar) is presented in the following three figures.

Figure 1 NBT results (red bars) and standard error (green bars) per additive ; Fish A

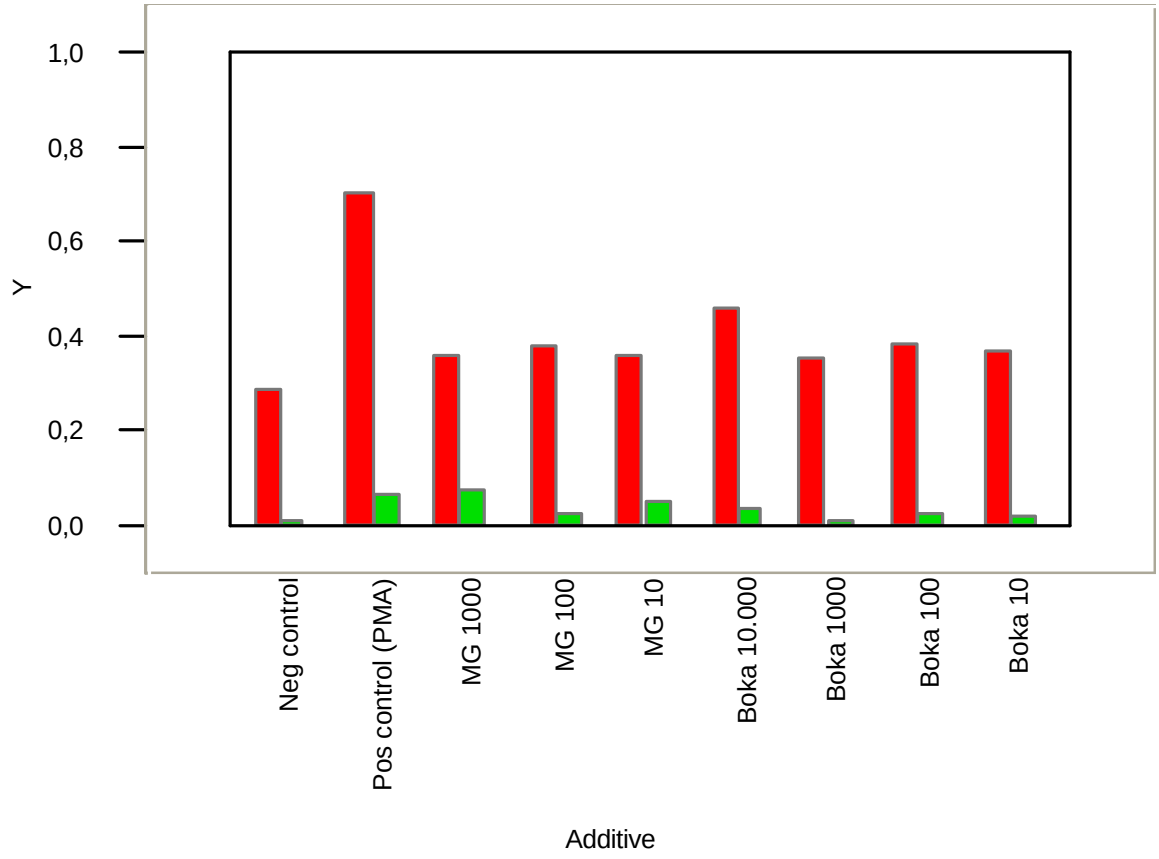


Figure 2 NBT results (red bars) and standard error (green bars) per additive ; Fish B

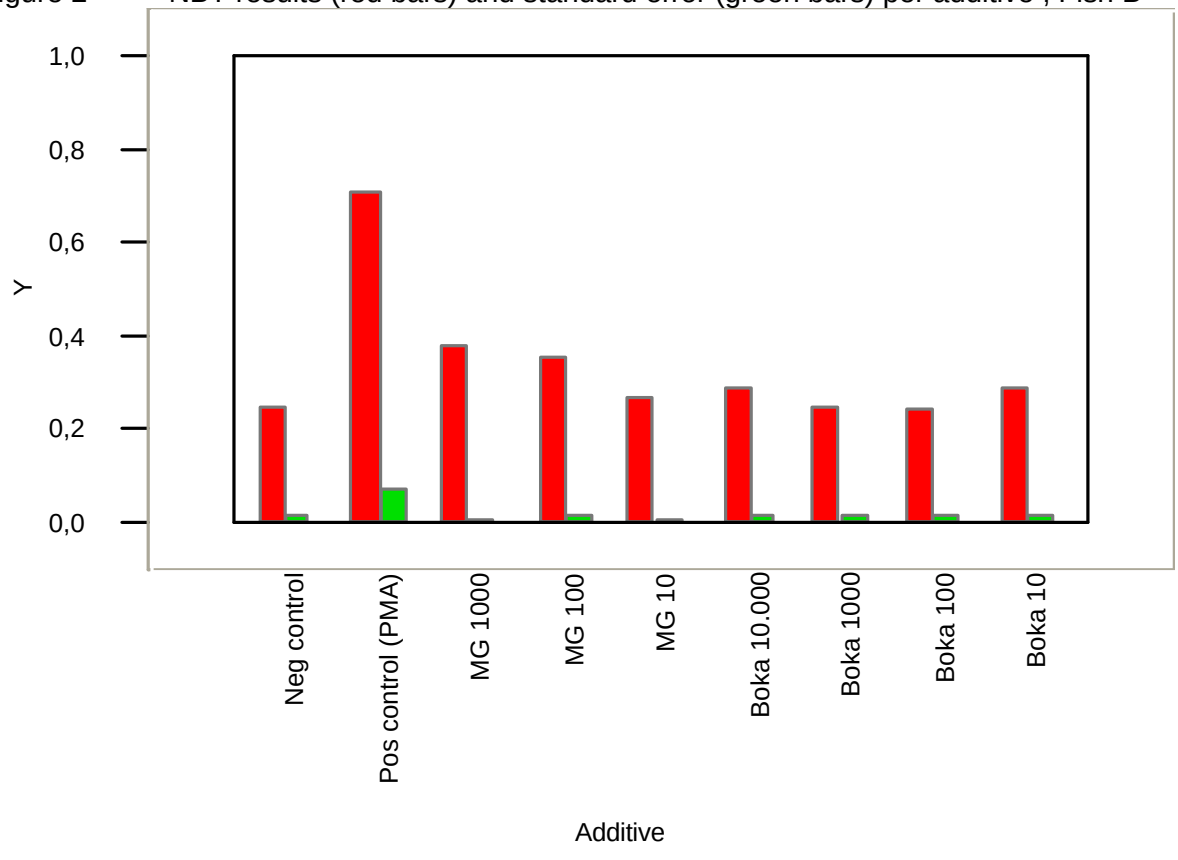
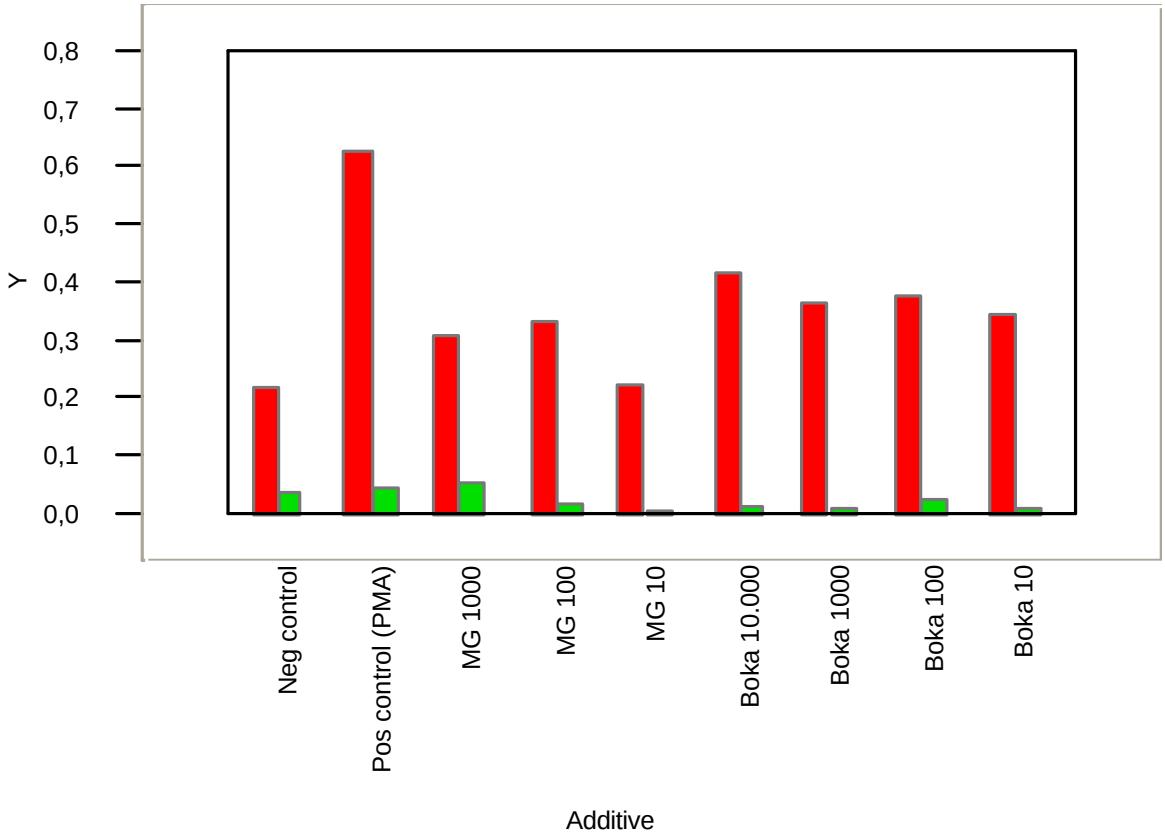


Figure 3 NBT results (red bars) and standard error (green bars) per additive; Fish C



4 CONCLUSIONS

Of the tested additives, Bokashi at a dosage of 10.000 µg/ml showed the highest immune response.

Both the commercial ß-glucan product MacroGard at a dosage of 100 µg/ml and Bokashi at a dosage of 10.000 µg/ml did statistically stimulate the cells.

From this trial it can be concluded that Bokashi is (like MacroGard) effective in stimulating the non-specific immune response in carp.