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FATE OF FOUR FISH PATHOGENS AFTER EXPOSURE TO AN ENSILING MIXTURE CONTAINING FISH FARM MORTALITIES

by

D A Smail, P J Huntly and A L S Munro

DAFS Marine Laboratory
Victoria Road
Aberdeen AB9 8DB
Scotland

SUMMARY

Exposing three bacterial fish pathogens (*Aeromonas salmonicida*, *Yersinia ruckeri* and *Renibacterium salmoninarum*) to a commercial fish silage caused their rapid destruction. However, it was found infectious pancreatic necrosis virus survived in silage for long periods at the prevailing ambient temperatures in Scotland. The virus would be inactivated by heating the silage preparation to a temperature of 60°C for five hours or by the addition of the virucidal agent VIRKON at a concentration of 1/100 w/v. The use of ensiling fish farm wastes to hygienically dispose of infected materials such as mortalities and infected offals is discussed.

INTRODUCTION

Mortality in fish farms is apparently a permanent feature of the rearing process and if not continuous frequently occurs. Disposal of the resulting carcasses can be a problem where fish have died from an infectious disease because unless they are disposed of in a manner which destroys or contains the infectious agent then there is a risk of transmitting infection. Where the disease is subject to legislative controls it may be compulsory to dispose of such carcasses in an approved manner. However the commonly used methods of disposal in terrestrial livestock farming eg burning or burying in quicklime, are not ideally suited for fish farms eg suitable terrain for burying may be remote and burning large quantities of fish difficult.

Ensiling fish in organic or mixtures of organic and mineral acids is an established process which is used to preserve the food value of fish without resorting to freezing or salting (Tatterson and Windsor, 1982). Commonly the product is used as an animal feedstuff. The low pH of the ensiled mixture and the bactericidal properties of the acids provide a milieu where the proteolytic enzymes of the fish digest the tissues without spoilage from bacterial growth. Used on a fish farm or a plant processing the carcasses of farmed fish (which may be carriers of infection) this process has been used as a means of the daily accumulation of carcasses and

offal in a form which allows preservation of their nutritional properties, their storage and subsequent disposal at a convenient time and causes none of problems of odour associated with decaying fish.

In this study we report on the fate of four fish pathogens either causing mortality or sometimes found in tissues of farmed fish when they were exposed to an ensiling mixture. They were *Aeromonas salmonicida*, *Yersinia ruckeri*, *Renibacterium salmoninarum* and infectious pancreatic necrosis virus.

METHODS

Silage

Three samples of fish silage from a fish farm using this process to treat mortalities and solid wastes from processing were obtained. The acid mixture contained only the organic acids, formic and propionic, from a proprietary brand called Bio-add (BP chemicals).

Bacteriology

The bacterial strains used were *A. salmonicida* (MT 004), *Y. ruckeri* (MT130) and *R. salmoninarum* (MT239). These species are subsequently referred to as AS, YR and RS respectively.

AS and YR were grown in agitated tryptic soya broth for 20 hours at 22°C. RS was grown on Muller-Hinton plus 0.1% L-cysteine agar for 28 days at 15°C. Cells were harvested by centrifugation, washed once in distilled water, adjusted to an optical density of 1.2 at 540 nm and 1 ml of this cell suspension centrifuged and the cells resuspended in 1 ml of fish silage or distilled water.

Viable counts were made of each cell preparation suspended in distilled water by making serial 10-fold dilutions and plating 0.1 ml onto TSA for AS and YR and MH+C for RS. Colony counts were made at three days for AS and YR and at eight weeks for YR. To assess survival in silage, samples (10 ul) were taken at intervals of 0, 15, 30, 90, 120, 180, and 240 minutes and agar plates and broths inoculated. Plates were examined for growth at 10 days and eight weeks respectively. The broths were incubated for 10 days before samples (10 ul) were inoculated onto respective agars and incubated for 10 days and eight weeks respectively.

Virology

The isolates used were the Marine Laboratory type strain for the Te serotype (SS/Sh80) and a recently isolated Sp strain Sp/WR/7/87 (SS is short for salmon strain, a collection of strains within the Te serotype). Both were originally isolated from salmon.

Sp and SS isolates were grown by infection of CHSE-214 cells at low multiplicity of infection (0.01 pfu/cell) and the cells maintained on Eagles MEM-Earles salts plus 2% foetal calf serum (Gibco). After five days the cultures were harvested, the cells separated from the supernatant and the virus precipitated from the supernatant using polyethylene glycol (PEG) by the method of Dixon and Hill (1983). For virus inactivation studies the PEG precipitate was resuspended in 2 ml of Hanks buffered saline solution (HBSS), stored at 4°C and an aliquot titrated on CHSE-214 cells.

Virus assay of silage. The silage samples were centrifuged at 1,500 g for 10 minutes to separate four different phases that were clearly visible: an uppermost oil phase, a lower solid phase, a lower liquid phase and a bottom solid phase.

Aliquots of each fraction were taken by needle and syringe. The oil fraction was extracted with an equal volume of tris glycine-HCl buffer pH 3.8 and the aqueous phase recovered after centrifugation. It was then diluted up to 10x in HBSS and filtered through a Millipore disposable 0.45 um filter. This aqueous extract of the oil fraction was then titrated on CHSE cells in 12 well plates using an inoculum volume of 0.5 ml and an agarose overlay. Plates were incubated at 15°C for 66 hours and then fixed and stained.

The solid and acid fractions were diluted 2x in sodium bicarbonate solution 7.5% and diluted further to 4x, 20x, 80x v/v in HBSS. The 4x dilutions of the solid fractions were discarded because they could not be filtered. All other dilutions were Millipore filtered and titrated as above.

Survival studies of virus in silage and in acid buffer. Sp and SS isolates were grown as above and the released virus in the supernatant titrated. Aliquots of the titred virus were diluted 1/20 in the silage preparation and samples stored at 4 and 20°C for up to five months. Aliquots were also stored in tris-glycine buffer at pH 3.8 for comparison at the same temperatures and for similar times. Residual infectivity was titrated at days 7, 14, 71 and 147 (five months) after start of the experiment. CHSE-214 cells were used for virus assay.

Inactivation studies in silage by use of heat or Virkon. Virus of both serotypes were titred as before and diluted in silage to approximately 10^6 pfu per ml. Aliquots of the virus/silage mixture were then heated to 45 or 60°C for 1-5 hours, the solid and acid phases allowed to separate on standing and the acid phase neutralised and titrated as above.

Virkon powder (Antec International - BDH Ltd) was dissolved to 1/50 w/v dilution and one vol added to one vol of the virus/silage mixture and shaken well. It was incubated for 30 minutes at room temperature. Sodium bicarbonate solution (7.5%) was added to an equal volume of the acid phase giving a dilution of 4x of the silage acid. The neutralised acid containing virus was then passed through a Millipore GV filter and titrated as above.

RESULTS

Bacteriology

Bacterial survival in silage. Viable counts showed there were 5 x 10^8 for AS, 2 x 10^9 for YR and >1 x 10^8 for RS per ml in the initial inocula. No AS or RS was isolated from the plates or broths after mixing in silage. Fewer than 10 colonies of YR were present on the zero time plate and the organism was isolated from the corresponding zero time broth but thereafter no isolates were made.

Virology

Pre-existing levels of IPNV in silage samples. In the three silage batches, A, B and C, it was found there was residual virus in batches A and B (Table 1). In batch A which had the highest titre in the acid phase virus was found in both solid phases but not those of batch B. Virus titre declined in time during the course of the experiment eg in batch A from 440 to 56 pfu/ml over four months.

Survival of IPNV added to silage or an acid buffer. Both Sp and SS survived well in silage A at 4°C (Table 2). SS was slightly more resistant than Sp. By contrast neither strain was detectable at 20°C by 71 days. Survival in tris-glycine buffer was similar but slightly better (Table 3).

Survival of IPNV in heated silage (batch C). Strain Sp showed a surprising resistance to heating at both 45 and 60°C when a high virus dose was used (Table 4). The titre reduction over five hours at 45 and 60°C was 1.24 and 2.58 $\log_{10}$ respectively. However at the typical values of virus found in the native silage (2-2.5 $\log_{10}$) no virus was detectable after five hours heating. SS was inactivated much more readily (Table 5).

Survival of IPNV in silage (batch A) with added virucidal agent Virkon. At a final dilution of Virkon of 1/100 w/v and an exposure of 30 minutes virus titres were reduced from high levels to below the point of detection (Table 6).

DISCUSSION

Under the laboratory conditions reported here all three species of bacteria were destroyed in fish silage. In the field situation it is probable that dissolution of the fish or offals would be necessary to ensure that the bacteria were exposed to the ensiling acids but once that stage was reached bacterial destruction would be rapid. To ensure effective disinfection against these species it is probable that a period of some days between the last addition of solids and disposal of the silage would be required. Alternatively mincing the solid wastes before addition to the ensiling mixture would ensure the more rapid exposure of the bacteria and cut down the time period between the last addition of wastes and their safe disposal.

Both strains of virus can survive for periods of many days in silage especially at low temperatures. This is not surprising as Jorgensen (1973) has reported on its resistance to inactivation in acid conditions. The survival of native virus in the samples supplied and the slower than linear decay in virus added to the silage may indicate a subpopulation more resistant to acid conditions. However increasing temperature does increase the susceptibility of virus to inactivation. Our current results indicate that 71 days would be sufficient at temperatures of 20°C or more or that 60°C for five hours or more would be sufficient for virus inactivation at native silage levels. Such methods employing heat would allow the silage to be used for feedstuff. The addition of Virkon rapidly inactivates virus but would render the silage unsuitable as an animal feedstuff although it could be safely disposed as a fertiliser or in waste disposal areas.

It is therefore possible to use the ensiling process to safely dispose of infected carcass and offals containing these three bacterial fish pathogens but additional treatment by heat or Virkon would be necessary to remove IPN virus.

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TABLE 1

Titres of IPN virus in three centrifuge fractionated silage batches

Fraction	A Date tested		B	C
	Feb 1990	June 1990		
Oil	-	<10	<8	<8
Solid (upper)	-	40	<40	<40
Acid	440*	56	60	<8
Solid (lower)	-	40	<40	<40

*Titre is pfu/ml

TABLE 2

Survival of Sp and Te strains of IPN virus in fish silage at 4 and 20°

	Temp °C	Day				
		0	7	15	71	147
Sp	4	8.30*	6.95	7.30	6.60	4.20
	20	8.30	6.32	5.84	<2.3	<2.3
SS	4	6.48	5.30	5.94	5.24	3.87
	20	6.48	4.87	4.90	<2.3	<2.3

*Titre is $\log_{10}$ pfu/ml in silage acid

TABLE 3

Survival of Sp and Te strains of IPN virus in tris-glycine acid buffer at pH 3.8 at 4 and 20°C

	Temp °C	Day				
		0	7	15	71	147
Sp	4	8.30*	6.81	7.67	6.40	5.99
	20	8.30	6.30	5.62	<2.0	<2.0
SS	4	6.48	5.58	5.88	5.62	5.91
	20	6.48	5.30	5.77	<2.0	<2.0

*Titre is $\log_{10}$ pfu/ml

TABLE 4

The effect of heat on the infectivity of IPN virus Sp in fish silage at high and low doses (reduction of titre* as $\log_{10}$ pfu/ml)

Temp	Dose	Heating time (hours)					
		0	1	2	3	4	5
60°	High	5.06*	3.89	2.98	2.70	2.65	2.48
	Low	2.78	<1.0	<1.0	<1.0	<1.0	<1.0
45°C	High	5.17	4.20	4.17	4.08	4.08	3.93
	Low	2.47	1.70	1.60	1.0	1.0	1.30

TABLE 5

The effect of heat on the infectivity of IPN virus SS in fish silage (reduction of titre* as $\log_{10}$ pfu/ml)

Temp	Heating time (hours)					
	0	1	2	3	4	5
60°C	4.17*	2.46	2.23	1.78	<1.0	<1.0
45°C	4.40	3.48	(<3.0)	3.48	3.25	2.30

TABLE 6

Exposure of IPN virus in fish silage to the virucidal agent Virkon at a concentration of 1/100 w/v final dilution (titre is pfu/ml)

	Titre	Titre after Virkon
Sp	4×10^6	<400
SS	1.8×10^5	<400