LACTIC ACID BACTERIA AS ANTAGONISTS OF PHYTOPATHOGENIC BACTERIA

by

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To the memory of my parents

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SUMMARY

Lactic acid bacteria were isolated from plants and plant-associated products. These isolates were identified and tested as possible biocontrol agents of certain phytopathogenic bacteria, namely: 2 strains of *Xanthomonas campestris*, 2 strains of *Pseudomonas syringae* and 1 strain of *Erwinia carotovora*.

In agar diffusion experiments, *in vitro* antagonism occurred and the majority of the lactic acid bacterial isolates formed clear inhibition zones. In associative broth cultures of lactic acid bacteria and phytopathogenic bacteria, the phytopathogens were completely killed within 12-36h.

In vivo experiments were carried out with bean plants grown in the greenhouse. The application of a suspension of lactic acid bacteria, 24h before treating the plants with the halo blight pathogen, *P. syringae*, led to a significant reduction in disease incidence.

The mechanism of antagonism was investigated and it was concluded that the lactic acid produced by the lactic acid bacteria was the most important inhibitory substance. Slight inhibition still occurred in broth with $CaCO_3$ added as buffer, but the phytopathogenic bacteria were not killed as in the case of unbuffered media. Hydrogen peroxide production by the lactic acid bacteria did not have an important effect on their antagonistic activity.

Preliminary trials were carried out to determine the survival rates of certain antagonistic lactic acid bacteria on the phylloplane. The lactic acid bacterial numbers decreased by approximately 1 log colony forming units/g per week. Thus, it seems as if repeated applications of the antagonists might be necessary, unless the environment or antagonists can be manipulated to ensure better survival rates. Electron microscopy of the leaves of bean plants indicated that the lactic acid bacteria occur in the grooves between epidermal cells.

Field trials were not included in this study. Therefore it cannot yet be claimed that the biological control of phytopathogenic bacteria by means of lactic acid bacteria is possible.

OPSOMMING

Melksuurbakterieë is geïsoleer vanaf plante en plantaardige produkte. Hierdie isolate is geïdentifiseer en getoets as moontlike biologiese beheer agente van sekere plantpatogene bakterieë, naamlik: 2 stamme van *Xanthomonas campestris*, 2 stamme van *Pseudomonas syringae* en 1 stam van *Erwinia carotovora*.

Antagonisme is *in vitro* aangetoon deur middel van agardiffusie eksperimente waarin die meerderheid van die melksuurbakterie-isolate helder inhibisiesones gevorm het. Vloeibare media is geïnokuleer met gemengde kulture van melksuurbakterieë en plantpatogene bakterieë. Plaattellings van hierdie media het getoon dat die melksuurbakterieë die plantpatogene bakterieë totaal gedood het binne 12-36h.

In vivo proewe is gedoen met behulp van boontjieplante wat in 'n glashuis gekweek is. Toediening van 'n melksuurbakterie-suspensie, 24h voor toediening van die "halo blight"-patogeen *P. syringae*, het gelei tot 'n betekenisvolle verlaging in simptoomontwikkeling.

Ondersoeke is gedoen na die meganisme van antagonimse en daar is tot die slotsom gekom dat melksuurproduksie deur die melksuurbakterieë die vernaamste bydrae lewer tot inhibisie. In vloeibare media waar $CaCO_3$ bygevoeg is as buffer, het 'n geringe mate van inhibisie steeds voorgekom maar die plantpatogene bakterieë is nie gedood soos in die geval van ongebufferde media nie. Waterstofperoksied-produksie deur die melksuurbakterieë het klaarblyklik nie 'n belangrike invloed op die inhibisievermoë van die antagoniste nie.

Daar is ook voorlopige proewe gedoen om die oorlewingstempo van sommige van die antagonistiese melksuurbakterieë op die oppervlak van bogrondse plantdele te bepaal. Die melksuurbakterie-getalle het afgeneem teen ongeveer 1 log kolonievormende eenhede/g per week. Dit blyk dus dat herhaaldelike toedienings van die antagoniste nodig sal wees, tensy die omgewing of antagoniste gemanipuleer kan word sodat beter oorlewingstempo's verkry kan word. Elektronmikroskopie van boontjieblare het getoon

dat die melksuurbakterieë voorkom in die groewe tussen epidermale selle.

Veldproewe is nie ingesluit in hierdie studie nie. Daar kan dus nie sonder twyfel aanvaar word dat melksuurbakterieë gebruik kan word in die biologiese beheer van plantpatogene bakterieë nie.

CHAPTER 1 : GENERAL INTRODUCTION

The surfaces of aerial plant parts provide a habitat for epiphytic microorganisms, many of which are capable of influencing the growth of foliar pathogens (Blakeman & Fokkema, 1982). In nature, numerous examples of spontaneous biological control can be found. The stabilisation of ecosystems such as plant surfaces through the antagonistic activity of saprophytic microorganisms against pathogens, results in the reduction of the incidence of plant diseases in the field (Cook & Baker, 1983). Antagonistic microorganisms active against foliar pathogens may be chosen either from the naturally occurring phylloplane population or from other habitats. The latter are, in general, less well adapted to the phylloplane environment (Blakeman, 1985).

Considering the variety of bacterial residents and their numbers (usually in the range of 10^3 - 10^7 colony forming units/cm^2) on the leaf surface, and realizing that pathogens seldom comprise more than 5% of this population, it is not surprising that many researchers are optimistic about the prospects for managing antagonists for biological control (Spurr & Knudsen, 1985). Biological control is preferable to pesticide treatment for various reasons, including the development of iatrogenic diseases due to the reduction in natural antagonists (Griffiths, 1981).

In the present study, lactic acid bacteria, isolated from a variety of plants and plant-associated products, were tested as biocontrol agents of certain bacterial plant diseases. Thus, naturally occurring antagonists were reapplied to the phylloplane. It has been stated that such attempts are more likely to be successful at times of the year when these or related microorganisms form a dominant component of the phylloplane microbes (Blakeman, 1985).

Examples of attempts to use bacteria to control bacterial plant diseases are not as numerous as those to control fungi (Blakeman & Fokkema, 1982). Possibly the best known example is that of the biological control of crown gall caused by *Agrobacterium tumefaciens* by means of the agrocin-producing *Agrobacterium radiobacter* strain 84 (Kerr, 1980).

Preinoculation of apple blossoms with *Erwinia herbicola* gave partial control of fire blight caused by *Erwinia amylovora*, both in the greenhouse and field (Riggle & Klos, 1972). An isolate of *Erwinia* and of *Pseudomonas* were able to prevent symptom development on rice by the bacterial leaf streak pathogen, *Xanthomonas translucens* ssp. *oryzicola* (Rao & Pavgi, 1976). Strains of *Pseudomonas syringae* and *E. herbicola* on potato leaves, known to induce ice nucleation and consequently to increase frost damage, could be inhibited by antagonistic fluorescent pseudomonads and an *E. herbicola* isolate (Lindow, 1979). Scherff (1973) has shown that the predatory bacterium *Bdellovibrio bacteriovorus* could protect soybeans against blight caused by *P. syringae* pv. *glycinea*. As well as producing antifungal antibiotics, isolates of *Pseudomonas fluorescens* were shown to produce an antibacterial substance which inhibited growth of *P. syringae* pv. *phaseolicola* on bean plants (Teliz-Ortiz & Burkholder, 1960).

Lactic acid bacteria were chosen to be tested as antagonists in this study because of their well-known ability to act as "preservatives" in food products such as sauerkraut (Stamer, 1975; Steinkraus, 1983), gherkins (Etchells *et al.*, 1975), fermented cereals and legumes (Steinkraus, 1983), silages (Langston & Bouma, 1960 a & b) as well as dairy products (Sharpe, 1981).

Investigations into biocontrol invariably include both *in vitro* and *in vivo* screening tests. There is, however often no significant correlation between antagonism demonstrable in culture and effectiveness in the field (Fokkema, 1976; Andrews *et al.*, 1983). On the other hand, *in vitro* screening tests do have the advantage that they could provide clues to the mode of antagonism as well as being far more rapid and less severe than *in vivo* screening tests (Andrews, 1985). The traditional approaches were followed in the present study namely *in vitro* screening tests to indicate whether the lactic acid bacteria were able to interfere with the pathogens and to investigate the mode of antagonism, as well as *in vivo* tests to evaluate the reduction in disease incidence and the survival rates of the lactic acid bacteria on the phylloplane.

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CHAPTER 2

ANTAGONISM OF LACTIC ACID BACTERIA AGAINST PHYTOPATHOGENIC BACTERIA

CHAPTER 2 : ANTAGONISM OF LACTIC ACID BACTERIA AGAINST PHYTOPATHOGENIC BACTERIA

2.1 ABSTRACT

A variety of lactic acid bacteria, isolated from plant surfaces and plant-associated products, were found to be antagonistic to test strains of the phytopathogens *Xanthomonas campestris*, *Erwinia carotovora* and *Pseudomonas syringae*. Effective "*in vitro*" inhibition was found both on agar plates and in broth cultures. In pot trials, treatment of bean plants with a *Lactobacillus plantarum*-strain before inoculation with *P. syringae*, caused a significant reduction of the disease incidence.

2.2 INTRODUCTION

Several members of the lactic acid bacteria are known to produce antibacterial substances. The antibacterial effect has been ascribed to the production of antibiotics or antibiotic-like substances such as acidophilin and lactocidin produced by *Lactobacillus acidophilus* (Vincent *et al*. 1959; Vakil & Shahani, 1965) or lactolin produced by *Lactobacillus plantarum* (Kodama, 1952) or nisin produced by *Streptococcus lactis* (Hurst, 1972). Wheater *et al*. (1951; 1952), Price and Lee (1970) and Gilliland and Speck (1975) ascribed the effect to hydrogen peroxide production while Kao and Frazier (1966) and Tramer (1966) reported lactic acid to be the antibacterial substance. In a heterogeneous population nutrient depletion and a decrease in the reduction-oxidation potential may cause competitive antagonism.

The interactions of lactic acid bacteria with other bacteria have been widely researched in food products and especially in fermented foods (Stamer, 1968; Carr, 1975; Stamer, 1975; Steinkraus, 1983) and silages (Keddie, 1959; Langston & Bouma, 1960; Stirling & Whittenbury, 1963). However, information on the occurrence of lactobacilli on living plants is scarce and no information is available on the interactions of plant-associated lactic acid bacteria with phytopathogenic bacteria.

Reports have been made on the isolation of atypical streptobacteria and betabacteria as well as the following *Lactobacillus* species from plants: *L. plantarum*, *L. fermentum* and small numbers of *L. brevis*, *L. casei*, *L. viridescens*, *L. cellobiosis* and *L. salivarius* (Stirling & Whittenbury, 1963; Mundt & Hammer, 1968; Sharpe, 1981). Although some authors (Mundt & Hammer, 1968) do not consider plants to be a natural reservoir of lactobacilli, this scarcity might rather be ascribed to the antibacterial effect of some extracts of higher plants, often due to 1,4-naphthaquinone derivates (Sharpe, 1981). On cut or bruised plant tissue, lactobacilli become more prevalent (Stirling & Whittenbury, 1963).

In the present study, preliminary tests were conducted to investigate possible antagonism between plant-associated lactic acid bacteria and some phytopathogenic bacteria. The ultimate aim would be the implementation of lactic acid bacteria for the biological control of bacterial plant diseases.

2.3 MATERIALS AND METHODS

2.3.1 ORGANISMS

i) LACTIC ACID BACTERIA

Authentic cultures of *Lactobacillus plantarum* were obtained from the German Culture Collection (DSM 20205) and the American Type Culture Collection (ATCC 8041). In our laboratories 41 isolates of lactic acid bacteria were obtained form a wide variety of plants including Haricot beans (*Phaseolus vulgaris*), gherkins (*Cucumis sativus*) and several plants and flowers indigenous to Southern Africa as well as plant-associated products such as Mageu (a drink produced from fermented maize) and coffee extract.

ii) PHYTOPATHOGENIC BACTERIA

Pseudomonas mangiferaeindicae (Ps 1) (Culture Collection: Department of Microbiology and Plant Pathology, University of Pretoria) was originally isolated from mangoes. The name was subsequently changed to *Xanthomonas campestris* (Lelliot, 1972; Robbs *et al.*, 1974). *P. syringae* (Ps 2), pathogenic to bean plants, was obtained from the S.A. National Institute for Plant Protection and *P. syringae* var *capsici* (Ps 3) was from the German Culture Collection (DSM 50336). *Erwinia carotovora* (Erw) was isolated in our Department and *Xanthomonas campestris* pv. *mangiferae-indicae* (Xan) was obtained from the British National Collection of Plant Pathogenic Bacteria (NCPPB 490).

2.3.2 MEDIA

Lactic acid bacteria were isolated on Rogosa agar (E. Merck AG) (Rogosa *et al.*, 1951) containing 0,1% (wt/vol) cycloheximide (Calbiochem-Behring) and were maintained in MRS broth (Merck) (De Man *et al.*, 1960). Plant pathogenic bacteria were grown at 25°C on nutrient broth-yeast extract agar (NBY) described by Schaad (1980).

2.3.3 ISOLATION AND IDENTIFICATION OF LACTIC ACID BACTERIA

One gram of plant material was vortexed for *ca.* 30 sec. in 9 mℓ of quarter strength Ringer solution (Merck) containing glass beads (diameter = 2mm). A dilution series was made in Ringer solution and plated onto Rogosa agar containing 0,1% (wt/vol) cycloheximide to inhibit possible fungal contamination. Colonies which developed within 48h at 30°C under anaerobic conditions, were tested for catalase activity with 4% H_2O_2 (Harrigan & McCance, 1966) and catalase-negative colonies were transferred to MRS agar. The colony and cell morphology of the pure cultures were examined and the following tests were done in order to identify the isolates: pseudo-catalase activity (Whittenbury, 1964), lactic acid configuration (Bergmeyer, 1965) using D- and L-lactate dehydrogenases (Boehringer Mannheim Biochemicals), presence of *meso*-diamino-

pimelic acid (m-A_2pm) in cell walls (Harper & Davis, 1979), growth at 4°C, 15°C, 19°C and 45°C, growth in the presence of 10% (wt/vol) NaCl and at pH 3,9. Arginine hydrolysis was tested according to the Nessler-method (Harrigan & McCance, 1966). The formation of gas from glucose and slime from 10% sucrose (Sharpe, 1962), served as additional criteria for classification. Motility, gelatinase activity and nitrate reduction was tested in a semi-solid medium described by Reuter (1970) and the sugar fermentation pattern of the isolates was determined (Sharpe, 1962; Sharpe & Fryer, 1965; Sharpe, 1981).

In order to verify some of the identifications, DNA- and cell wall analyses were carried out on 7 and 5 isolates respectively. The DNA of isolates L1084, L2503, L2508C, L2522, L2523, L2604 and L2606 was extracted according to the method of Marmur (1961). The cells were disrupted by means of lysozyme treatment (S.A. Egg Board) as well as sodium lauryl sulphate. The following modifications were made on Marmur's method: all reagent volumes were approximately halved, the DNA precipitate was collected by centrifugation (Sorvall Superspeed centrifuge, SS34, 4°C, 10 000 r.p.m. for 10 min.) instead of spooling the DNA strands around a glass rod, Marmur's acetate-EDTA step was omitted and the final DNA suspension was dialysed against saline sodium citrate (1XSSC). The melting temperature (Tm) of the purified DNA was measured (Beckman DU-8 Spectrophotometer, 260 nm) and the following equation was used to calculate the mol percent guanine plus cytosine (mol % G + C) of the DNA dissolved in 1 X SSC: mol % G + C = (Tm - 69,4) X 2,44.

The cell walls of isolates L2503, L2507B, L2508C, L2604 and L2606 were obtained by disrupting the cells in a French Pressure Cell Press (American Instrument Co.) at 8 000 - 16 000 psi. The cell walls were treated with trypsin, washed, freeze dried and completely hydrolyzed (4N HCl at 100°C for 18h) according to the method of Schleifer and Kandler (1972). The hydrolyzed samples were analysed by high pressure liquid chromatography (Waters chromatograph, National Chemical Research Laboratory, Council for Scientific and Industrial Research, Pretoria, S.A.).

2.3.4 DETERMINATION OF "*IN VITRO*" INHIBITION

An agar disc technique was used to determine whether the lactic acid bacteria were capable of inhibiting the plant pathogens *in vitro*. Pour plates were made of the lactic acid bacteria by mixing 1mℓ of a 36h broth culture in *ca*. 15 mℓ MRS agar. After incubation at 30°C for 48h, discs with a diameter of 7 mm were stabbed from the agar. The discs were placed on NBY agar covered with suspensions of 48h cultures of the plant pathogens in Ringer's solution. Sterile MRS agar discs were used as control. After an incubation period of 36h at 25°C the diameter of clear zones surrounding the discs was measured. The experiment was done in triplicate to ensure reproducibility.

In a separate experiment, the growth curves of the plant pathogens were compared with their growth curves in the presence of lactic acid bacteria. The five plant pathogens were each inoculated into two flasks containing 100 mℓ NBY broth. Into one of each pair of flasks, a strain of *L. plantarum* was inoculated before incubation at 25°C. Samples were taken from the cultures at 12h-intervals for 84h. Serial dilutions of the samples were plated onto NBY agar and incubated at 25°C under aerobic conditions and the colonies of phytopathogenic bacteria were counted after 48h.

2.3.5 DETERMINATION OF ANTAGONISM IN POT TRIALS

The effect of a *Lactobacillus plantarum*-isolate (L 292) on the pathogenicity of *Pseudomonas syringae* (Ps 2) was tested by spraying suspensions of the organisms onto the leaves of young Haricot beans. The suspensions were prepared of 48h cultures in sterile distilled water containing 1mℓ Tween 80 (Merck) per litre. The plants were germinated in the greenhouse (25-30°C) and as soon as the first two primary leaves were fully grown, 50 plants were sprayed with strain L292 only, 50 with Ps 2 only and 50 with Ps2 24h after being sprayed with the L292-suspension. The symptoms of halo blight (black watersoaked lesions with yellow haloes and the curling of infected leaves) developed *ca*. 14 days after inoculation with the plant pathogen. The number of lesions per

plant, total number of leaves per plant and the number of dead leaves per plant were counted and the dry mass of each plant determined.

2.3.6 STATISTICAL ANALYSIS

Results were analysed by Variance and Covariance analysis using the SAS statistical package (Ray, 1982). The LS means test was done and involves the comparison of the least squares means of the various parables in order to determine whether they differ for the different treatments.

2.4 RESULTS AND DISCUSSION

2.4.1 IDENTIFICATION OF LACTIC ACID BACTERIA

The lactic acid bacteria isolated from the plants and plant-associated products are shown in Table 2.1 as well as their antagonistic activity which was determined as described below. The results of the identification tests, mol % G + C-determinations and cell wall analyses are shown in the appendix (Tables 6.1 - 6.3).

2.4.2 DETERMINATION OF THE ANTAGONISTIC EFFECT ON VEGETATIVE CELLS

"*In vitro*" tests showed agar disc inhibition zones on all five plant pathogenic bacteria produced by 37 of the 43 lactic acid bacteria as in Figure 2.1. One isolate from gherkins, *L. hilgardii* (L2501), inhibited 4 of the 5 pathogens and isolate L2525 (*L. sake*) from beans inhibited only 3 of the pathogens. The 4 isolates which were not inhibitory (2 strains of *Leuconostoc mesenteroides* and 1 of *Lc. amelibiosum* and a heterofermentative *Lactobacillus*), grew very weakly in MRS agar pour-plates. However, most of the lactic acid bacteria had a wide range of inhibition against the pathogens. The average inhibition zones of the 15 most antagonistic lactic acid bacteria are presented in Table 2.2. Information on the other strains is presented in the appendix (Table 6.4).

TABLE 2.1 Lactic acid bacterial species isolated from plants with indication of the degree of "*in vitro*" antagonism found within each group of species, against five strains of phytopathogenic bacteria.

Species of lactic acid bacteria	Origin	No. of isolates	Degree of antagonism
Lactobacillus plantarum	Indigenous plants	10	++ to +++ *
	Coffee extract	3	++ to +++
	DSM 20205	1	+++
	ATCC 8041	1	++
L. brevis	Indigenous plants	3	++ to +++
	Gherkins	6	++
L. vaccinostercus	Indigenous plants	2	++ to +++
L. bavaricus	Indigenous plants	1	+++
L. hilgardii	Gherkins	2	+ to ++
L. sake	Beans	4	+ to +++
L. casei spp. *rhamnosus*	Gherkins	1	+++
Heterofermentative lactobacilli	Gherkins	1	++
	Mageu	1	-
Leuconostoc mesenteroides	Indigenous plants	1	++
	Beans	1	-
	Mageu	4	- to ++
Lc. paramesenteroides	Gherkins	1	++
Lc. amelibiosum	Mageu	1	-

* Determined by measuring the average diameter of clear zones surrounding agar discs cut from lactic acid bacterial cultures; - = no inhibition, + = weak, ++ = mild, +++ = strong inhibition

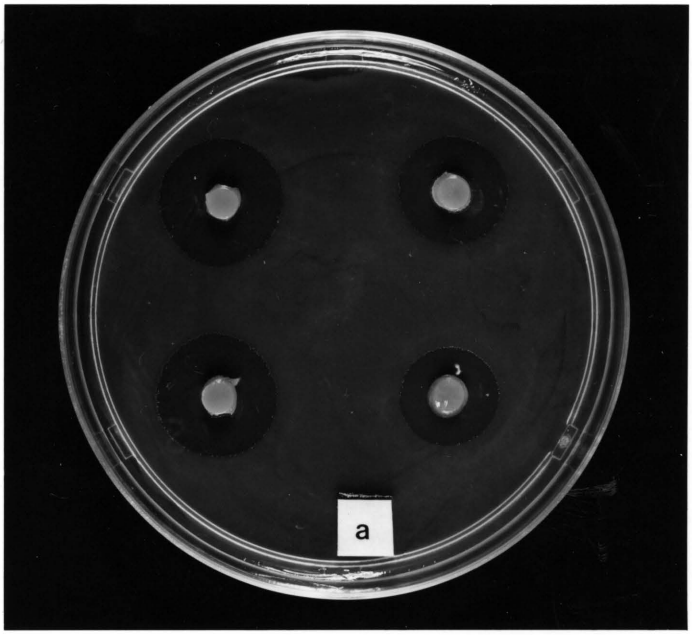

FIGURE 2.1a Inhibition zones surrounding discs of lactic acid bacterial cultures placed on NBY agar seeded with *Erwinia carotovora* (Erw)

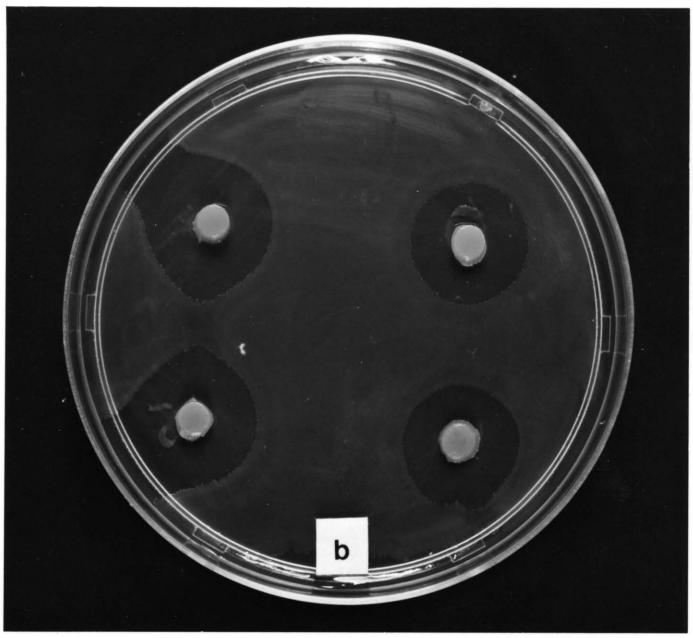

FIGURE 2.1b Inhibition zones surrounding discs of lactic acid bacterial cultures placed on NBY agar seeded with *Pseudomonas syringae* var *capsici* (Ps3).

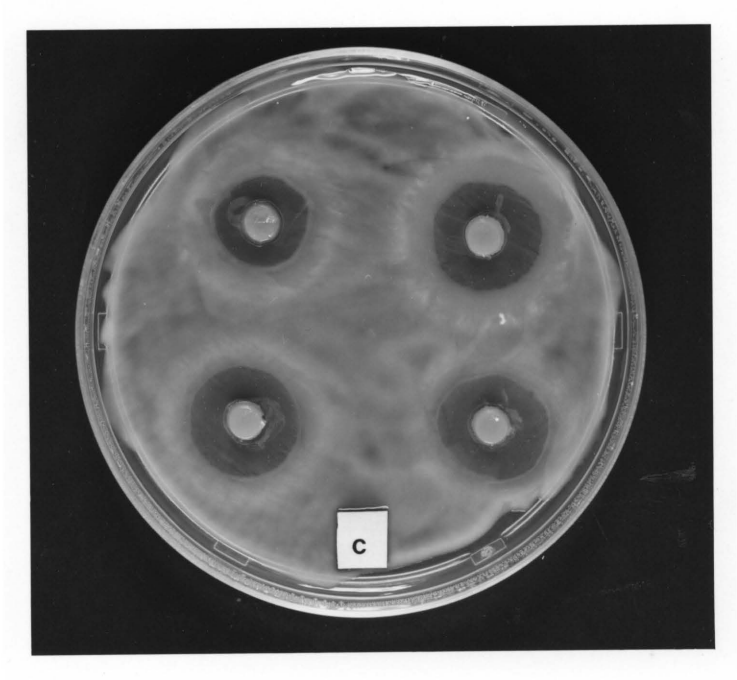

FIGURE 2.1c Inhibition and stimulation zones surrounding discs of lactic acid bacterial cultures placed on NBY agar seeded with *Xanthomonas campestris* (Psl).

TABLE 2.2 Diameter of zones formed on NBY-agar by the 15 most antagonistic lactic bacterial isolates against test strains of phytopathogenic bacteria (data on the other strains are presented in the Appendix Table 6.4)

		Zone diameter in mm				
Lactic acid bacteria	Isolate number	Ps 1[+]	Ps 2	Ps 3	Erw	Xan
L. plantarum	L 1515A	20.2*	25.5	22.0	21.3	33.1
L. plantarum	L 379	18.2	26.5	22.4	21.1	32.9
L. plantarum	L 1518	17.5	24.9	22.6	21.7	33.8
L. plantarum	L 392A	18.2	27.0	21.7	23.5	30.2
L. vaccinostercus	L 1506	20.5	26.4	21.5	19.3	31.0
L. sake	L 2522	18.7	22.8	20.7	20.4	33.9
L. sake	L 2521	19.9	25.0	18.7	20.1	32.2
L. plantarum	L 1056	18.6	22.4	21.7	20.9	31.9
L. plantarum	L 292	17.6	26.2	20.4	20.3	29.6
L. casei spp *rhamnosus*	L 2506	17.1	24.5	19.7	21.0	29.9
L. bavaricus	L 1553	17.4	25.8	20.7	17.7	28.0
L. plantarum	L 491	18.3	23.9	19.7	17.4	29.8
L. plantarum	L 2602	17.5	22.4	19.2	18.5	28.8
L. plantarum	DSM 20205	16.4	22.4	20.0	18.7	27.1
L. brevis	L 1084	17.5	19.8	18.0	17.8	29.7

* average of triplicate readings

\+ Ps 1 : *Xanthomonas campestris* (mango isolate)
Ps 2 : *Pseudomonas syringae* (bean pathogen)
Ps 3 : *P. syringae* var *capsici* (DSM 50336)
Erw : *Erwinia carotovora* (isolate)
Xan : *X. campestris* pv. *mangiferaeindicae* (NCPPB 490)

Growth of Ps1 was stimulated around the inhibition zones (Figure 2.1c), possibly because of an increase in available nutrients from the zone of no growth, or as a result of growth factors released by the lactobacilli. No inhibition or stimulation zones could be detected around the sterile MRS-agar discs used as control.

2.4.3 EFFECT OF LACTOBACILLI ON PLANT PATHOGENS IN BROTH CULTURES

Broth cultures of *Xanthomonas campestris* and *Erwinia carotovora* were completely killed by isolates of *Lactobacillus plantarum* within 36h, *Pseudomonas syringae* and *P. syringae* var *capsici* within 24h and *X. campestris* pv. *mangiferaeindicae* within 12h (Figure 2.2 a-e). Thus, even in competition with the pathogens, under conditions favourable to the pathogens, effective inhibition occurred.

2.4.4 EFFECT OF LACTIC ACID BACTERIA ON THE PATHOGENICITY OF *P. SYRINGAE* (PS 2) TO BEAN PLANTS

Plants treated with lactic acid bacteria (isolate L292) before inoculation with *P. syringae*, showed significantly fewer symptoms than those treated only with the pathogen (Figure 2.3). Plants treated only with lactobacilli, showed none of these symptoms. The following parameters differed significantly ($p < 0,05$) between the treatments: (a) average number of lesions per leaf (b) average number of lesions on leaves with lesions and (c) percentage of dead leaves (Table 2.3). The average dry mass of the plants in the three different treatments did not differ significantly ($p > 0,05$).

Pot trials showed an isolate of *Lactobacillus plantarum* (L292) to be effectively antagonistic against the bean pathogen *Pseudomonas syringae*. However, the effectiveness of the interaction has yet to be proved under field conditions where factors such as rain, fluctuations in temperature and relative humidity and a greater variety of competitive micro-organisms will play a role.

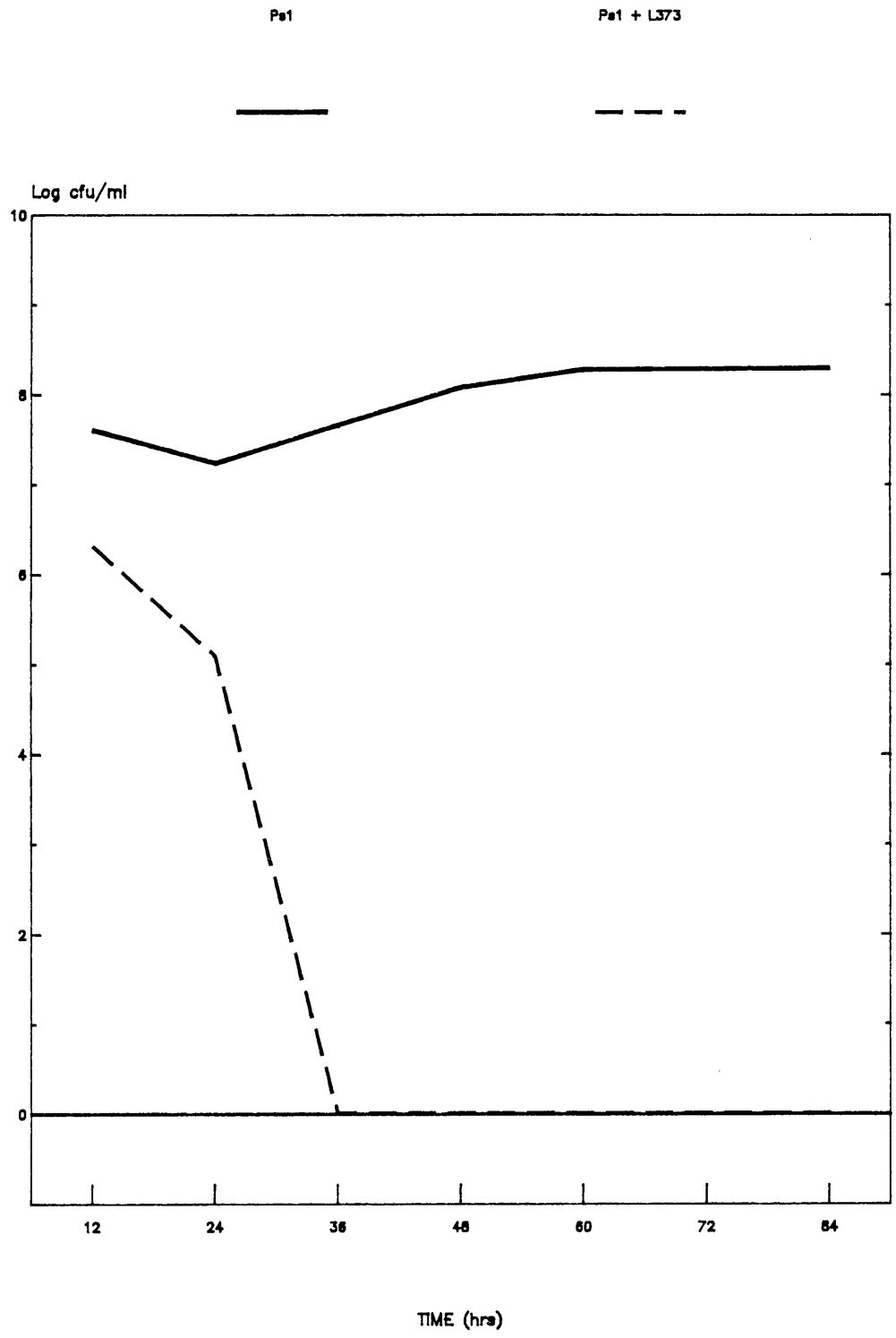

FIGURE 2.2(a) Effect of *L. plantarum* (L373) on *X. campestris* (Ps1) in NBY broth cultures.

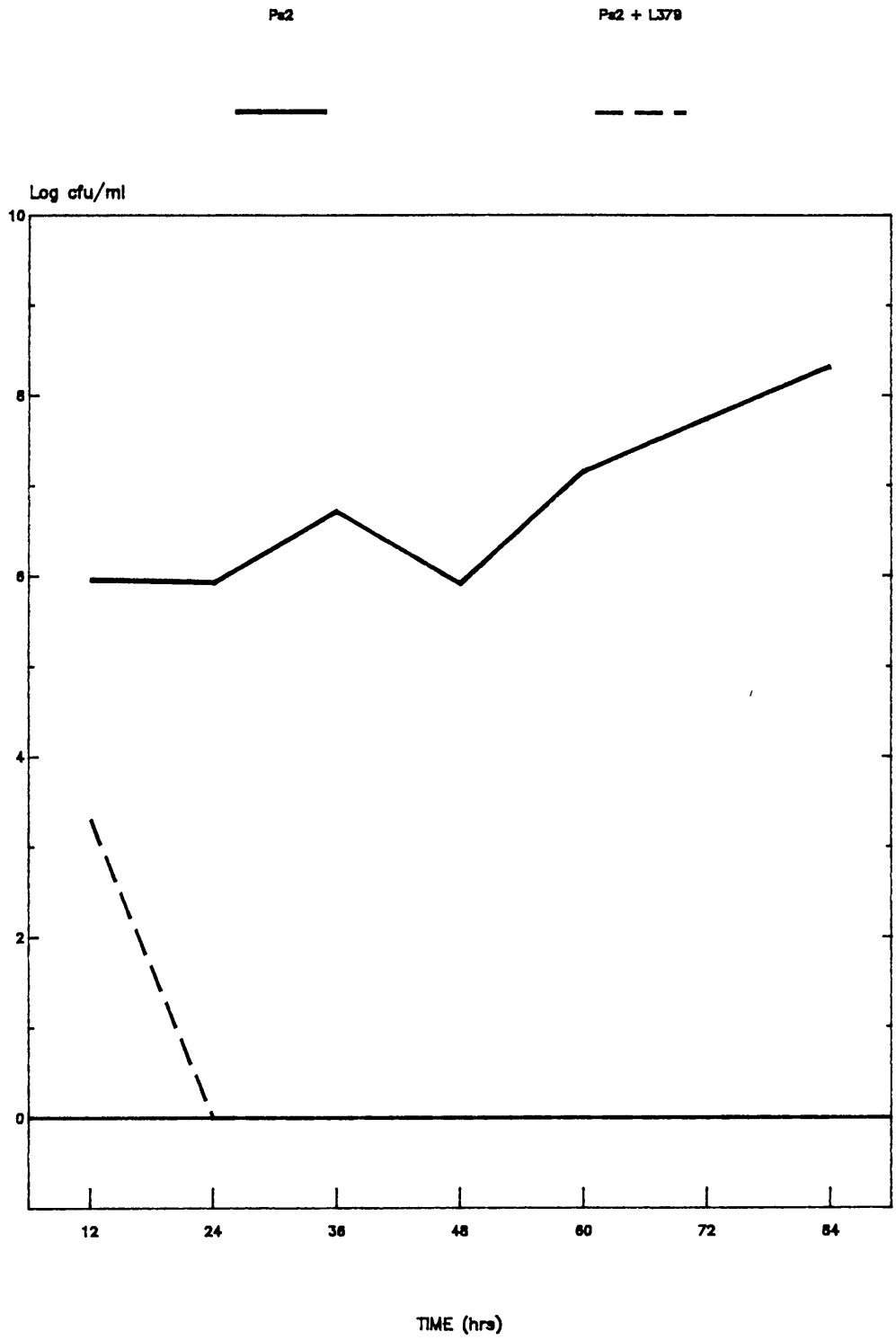

FIGURE 2.2(b) Effect of *L. plantarum* (L379) on *P. syringae* (Ps2) in NBY broth cultures.

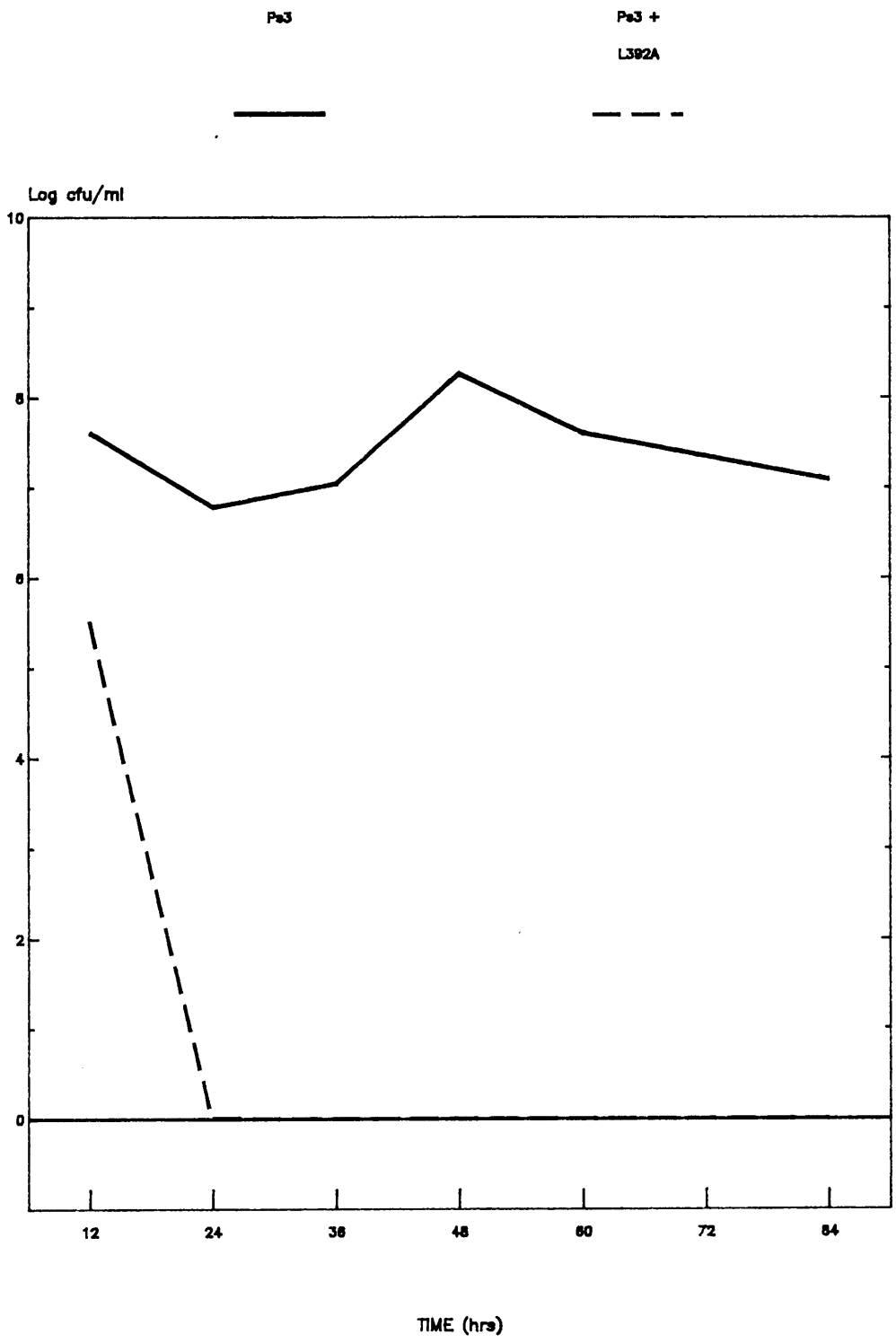

FIGURE 2.2(c) Effect of *L. plantarum* (L392A) on *P. syringae* var *capsici* (Ps3) in NBY broth cultures.

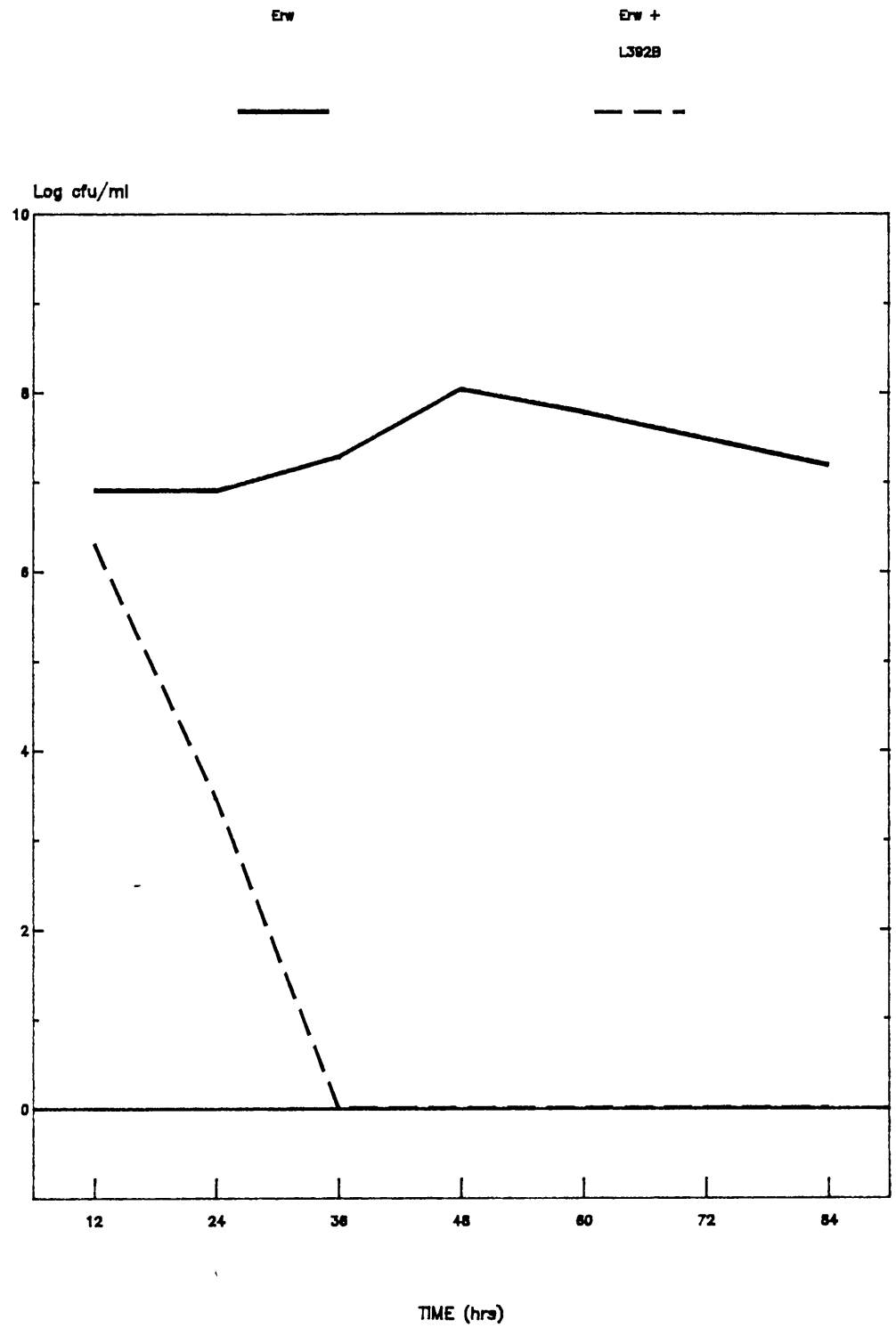

FIGURE 2.2(d) Effect of *L. plantarum* (L392B) on *E. carotovora* (Erw) in NBY broth cultures.

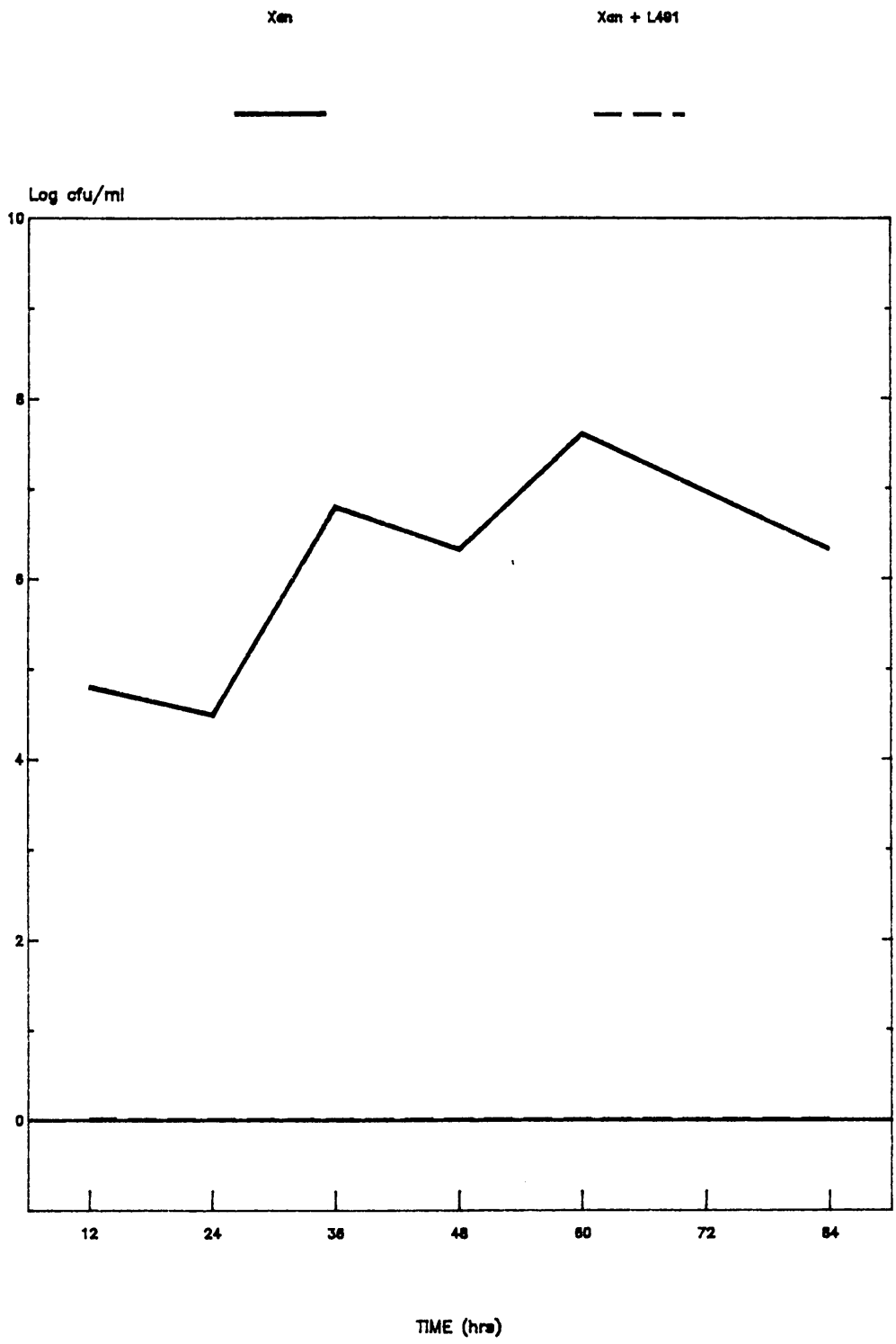

FIGURE 2.2(e) Effect of *L. plantarum* (L491) on *X. campestris* pv. *mangiferaeindicae* (Xan) in NBY broth cultures.

FIGURE 2.3 (a) Bean plant treated with *P. syringae* PS2 (right) versus one treated with lactobacilli prior to *P. syringae* treatment (left).

FIGURE 2.3(b) The symptoms of halo blight (caused by *P. syringae*) on Haricot beans (*Phaseolus vulgaris*).

TABLE 2.3 Effect of lactic acid bacteria on the pathogenicity of *P. syringae* (Ps 2) to Haricot bean plants

TREATMENT	Average number of lesions per leaf	Average number of lesions on leaves with lesions	% dead leaves	Dry mass in gram
Pathogen	2.0 a)*	4,7 c)	20,7 f)	0,9 i)
Pathogen + lactic acid bacteria	0,4 b)	1,6 d)	16,1 g)	0,8 i)
Lactic acid bacteria	0,0 b)	0,0 e)	1,6 h)	0,8 i)

* Means followed by the same letter do not differ significantly ($p > 0,05$) according to the LS Means test

Biocontrol of postharvest plant diseases of fruit and vegetables by lactic acid bacteria also seems an exceptionally exciting area to be explored. Limitations such as environmental conditions in the field, the targeting of biocontrol agents to the effective site and the economical feasibility of control procedures under field conditions may be overcome under storage conditions (Colyer & Mount, 1984; Wilson & Pusey, 1985). Leben and Daft (1965) reported that an epiphytic bacterium (isolate A 180) from cucumber leaves reduced cucumber anthracnose, early blight of tomato and northern leaf blight of corn when cultures or washed cells of the bacterium were applied as protectant sprays to seedlings in the greenhouse. Subsequently, Leben *et al*. (1965) have demonstrated that isolate A 180 was not effective under field conditions, probably as a result of its sensitivity to drying and ultraviolet rays.

Genetic manipulation could also be applied to produce effective antagonists that are ecologically adapted to the infection site. The incorporation of genes involved in the mode of action of an antagonist into the host plant itself is another possibility that deserves special attention (Wilson & Pusey, 1985).

The survival pattern of the lactic acid bacteria on the phylloplane and the mechanism of antagonism is at present being investigated in our laboratories.

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CHAPTER 3

INVESTIGATIONS INTO THE MECHANISM OF LACTIC ACID BACTERIAL ANTAGONISM AGAINST PHYTOPATHOGENIC BACTERIA

3.1 ABSTRACT

Preliminary experiments were conducted in order to determine the mechanism by which plant-associated lactic acid bacteria inhibit test strains of phytopathogenic bacteria (Chapter 2). Both lactic acid and acetic acid in *ca.* 1% concentrations were inhibitory to the plant pathogens. Acetic acid was slightly more inhibitory than lactic acid. Neither of these acids were quite as inhibitory to the phytopathogens as the highly antagonistic lactic acid bacteria. The pH reduction caused by lactic acid bacteria in associative broth cultures, was buffered with $CaCO_3$ and this resulted in the phytopathogens being slightly inhibited by the lactic acid bacteria but not killed as in the case of unbuffered broth. Hydrogen peroxide production by the lactic acid bacteria seemed to be unrelated to their antagonistic effect. Cell free filtrates of the lactic acid bacteria were not in the least inhibitory to the phytopathogenic strains.

3.2 INTRODUCTION

Test strains of plant-associated lactic acid bacteria were found to be antagonistic to phytopathogenic strains of *Xanthomonas campestris*, *Erwinia carotovora* and *Pseudomonas syringae* on solid media and in broth cultures. The application of *Lactobacillus plantarum*-suspensions to the leaves of young bean plants before inoculation with *P. syringae*, reduced the incidence of halo blight in pot trials (Chapter 2; Visser *et al.*, 1986). In the present study, preliminary tests were conducted to investigate lactic acid and hydrogen peroxide production as possible mechanisms of the inhibitory effect lactic acid bacteria have on the phytopathogenic bacteria.

A number of different antibacterial substances are produced by lactic acid bacteria. Lactic acid produced by *Lactobacillus acidophilus* has been reported to be strongly germicidal and possibly largely responsible for the inhibitory effect of these cultures on *Escherichia coli* (Tramer, 1966). Kao and Frazier (1966) tested lactic acid bacteria isolated from foods for their effect on the growth of *Staphylococcus aureus* in broth

cultures. It was found that most of the lactic acid bacterial cultures were inhibitory and some were lethal to the staphylococci. Inhibition was reported to be more effective at increased inocula and at lower temperatures (10-15°C) the lactic acid bacteria were more inhibitory than at 30-37°C. The killing of *S. aureus* was ascribed to pH reduction as a result of lactic acid production. In media buffered at pH 6,3, inhibition occurred without killing.

Haines and Harmon (1973) investigated the influence of lactic acid bacteria on the growth and enterotoxin production of *S. aureus* in associative cultures. The inhibitory effect which was observed, was ascribed to competition for vital nutrients as well as the production of hydrogen peroxide and lactic acid. Lactic acid inhibited the growth of *S. aureus* in the early stages of incubation but not in the later stages. Shillinglaw and Levine (1943) compared the germicidal efficiencies of a number of edible acids against *E. coli* and reported that the order of efficiency was different at different temperatures. At 30°C the order was tartaric > phosphoric > lactic > citric acid and at 0,6°C it became phosphoric = lactic > tartaric > citric acid. The germicidal efficiency of lactic acid was also increased by the addition of CO_2 or sucrose.

The antibacterial effect of lactic acid bacteria has also been ascribed to hydrogen peroxide production by a number of researchers. McLeod and Gordon (1922) were the first to report on the bacterial production of peroxide and they studied the inhibition of heterogeneous organisms by the peroxide formed by pneumococci. Whittenbury (1964) investigated hydrogen peroxide production and catalase activity in lactic acid bacteria. Some lactic acid bacterial cultures formed detectable hydrogen peroxide and some did not, regardless of their preference or requirement for aerobic or anaerobic conditions. Hydrogen peroxide production depended in some instances on the substrate used as energy source. Dahiya and Speck (1968) observed that the hydrogen peroxide contained in culture filtrates of *Lactobacillus lactis* and *Lactobacillus bulgaricus* was inhibitory to *Pseudomonas*, *Bacillus* and *Proteus* species. The active substance which accumulated in the culture media was dialyzable, heat labile and inactivated by catalase. Its production paralleled hydrogen peroxide production in the *Lactobacillus* cultures indicating that inhi-

bition resulted from hydrogen peroxide. Hydrogen peroxide production by *L. acidophilus* and its effect on *Pseudomonas fragi* was studied by Collins and Aramaki (1980) and it was found that larger amounts of peroxide were formed when the cultures were continuously agitated during growth at 37°C or storage at 4°C. Indications were found that the reduced nicotinamide adenine dinucleotide oxidases of these *L. acidophilus* strains mediated the formation of hydrogen peroxide as an end product. Baldry (1983) reported hydrogen peroxide to be more effective as a sporicide than as a bactericide. Bactericidal action against *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Streptococcus faecalis* and *Staphylococcus aureus* was poor, but hydrogen peroxide was bacteriostatic at concentrations above 0,15 mmol/ℓ.

Antibacterial substances other than organic acids and hydrogen peroxide are produced by various lactic acid bacteria. These substances include antibiotics, antibiotic-like substances and bacteriocins. Antibiotics produced by lactic acid bacteria were first observed by Rogers (1928) in strains of *Streptococcus lactis* which produced nisin and by Oxford (1944) in *Streptococcus cremoris* which produced diplococcin. Several species of lactic acid bacteria have since been found to produce a variety of antibiotics. *L. acidophilus* produced lactocidin (Vincent *et al.*, 1959) a weak and labile antibiotic-like substance (Sabine, 1963); acidophilin (Vakil & Shahani, 1965; Shahani *et al.*, 1976; 1977); acidolin (Hamdan & Mikolajcik, 1974; Mikolajcik & Hamdan, 1975 a & b) and a broad spectrum antibiotic protein which was isolated from strain AC_1 by Mehta *et al.* (1983). *Lactobacillus brevis* produced lactobrevin (Kavasnikov & Sudenko, 1967) and *L. bulgaricus* produced bulgarican (Reddy & Shahani, 1971). Ritter (1951) found that *Lactobacillus helveticus* and *L. lactis* produce antibacterial substances active against bacteria belonging to the coli-aerogenes group. *L. plantarum* produces lactolin (Kodama, 1952) and an extracellular protein-containing aggregate inhibitory to Gram-positive bacteria and spheroplasts of Gram-negative bacteria (Andersson, 1986). Low molecular weight peptides with broad inhibition spectra were isolated from *Streptococcus diacetylactis* and *Leuconostoc citrovorum* (Branen *et al.*, 1975). Lactic acid bacteria isolated from silages and starter cultures possessed macromolecular antibacterial activity (Lindgren & Clevström, 1978 a & b).

Reddy and Shahani (1971); Branen *et al*. (1975), Lindgren and Clevström (1978b) and Andersson (1986) reported that the antibacterial substances which they studied showed increased activity at low pH values. Pulusani and Rao (1984) observed that formate produced by *Streptococcus thermophilus* stimulated the antimicrobial activity of *L. bulgaricus* in milk. Some of the antimicrobial proteins were found to be heat resistant (Pinheiro *et al*., 1968; Branen *et al*., 1975; Pulusani *et al*., 1979) whereas some were labile (Sabine, 1963; Lindgren & Clevström, 1978 a & b). The proteinic inhibitors from lactic streptococci (Whitehead, 1933) and from *L. acidophilus* AC_1 (Mehta *et al*., 1983) were sensitive to trypsin but stable towards pepsin.

Several lactic acid bacteria produce bacteriocins i.e. antibacterial substances with inhibitory activity restricted to closely related species, a bactericidal mode of action and a proteinaceous nature (Reeves, 1965; Tagg *et al*., 1976). *L. acidophilus* produces lactacin B (Barefoot & Klaenhammer, 1983). A *Lactobacillus fermenti* bacteriocin was studied by De Klerk and Coetzee (1961), De Klerk (1967) and De Klerk and Smit (1967). Upreti and Hinsdill (1973) isolated a bacteriocin from a homofermentative *Lactobacillus* isolate. Kozak *et al*. (1978) and Geis *et al*. (1983) examined the potential of lactic streptococci to produce bacteriocins.

Bacteriocin production is not applicable in the present study where lactic acid bacteria were antagonistic to phytopathogenic bacteria and the production of antibiotics has not been investigated. Although some strains may produce antibiotics or antibiotic-like substances, it seems unlikely that all 37 antagonistic strains would. Therefore the common products such as lactic acid, acetic acid and hydrogen peroxide were investigated for bactericidal and/or bacteriostatic action against the test strains of phytopathogenic bacteria.

3.3 MATERIALS AND METHODS

3.3.1 ORGANISMS

The plant-associated *Lactobacillus* and *Leuconostoc* species as well as the phytopathogenic bacteria used in this study, were as described in section 2.3.1.

3.3.2 MEDIA

Lactic acid bacteria were grown on MRS agar (E. Merck AG) (De Man *et al.*, 1960) at 30°C for 24-48h under anaerobic conditions. The isolates were maintained in MRS broth (Merk or Biolab Chemicals). Heated blood o-dianisidine (HBD) agar was prepared according to the method described by Whittenbury (1964) with the exception that defibrinated human blood was used instead of ox blood. *o*-Dianisidine was obtained from Sigma. ABTS-medium consisted of Rogosa agar (Merck) (Rogosa *et al.*, 1951), 2,2'-azino-di (3-ethyl benzthiazoline-6-sulphonic acid) obtained from Boehringer Mannheim and horseradish peroxidase from Sigma. The ABTS-medium was prepared according to the method described by Marshall (1979). Phytopathogenic bacteria were grown for 48h at 25°C on nutrient broth-yeast extract (NBY) agar (Schaad, 1980). Ringer tablets were obtained from Merck and made up to quarter strength with distilled water for suspensions and dilutions.

3.3.3 ACID PRODUCTION BY LACTIC ACID BACTERIA AND DETERMINATION OF ITS EFFECT ON PLANT PATHOGENS

a. DETERMINATION ON SOLID MEDIA

15 mℓ of sterile MRS agar was poured per Petri dish and allowed to set and dry (*ca.* 3h at 37°C). 1,5 mℓ of a 10% (vol/vol) lactic acid (Saarchem) aqueous solution was added to some plates and 1,5 mℓ of 10%

(vol/vol) glacial acetic acid (Saarchem) to others. Discs with a diameter of 7 mm were cut from the acidified agar and placed on NBY agar plates seeded with the test strains of phytopathogenic bacteria. Sterile MRS agar without added acid was used as control. After incubation at 25°C/24h the plates were examined for inhibition zones surrounding the discs.

Discs of lactic- and acetic acid containing agar as well as discs cut from MRS pour plates of strains L1506, L1515A and L1553 (incubated at 30°C/48h) were also placed on sterile NBY agar plates which had been dried at 37°C/*ca*. 3h. The pH of the surface of the NBY plates with the different discs was determined by means of a contact electrode (Orion) at 6h-intervals for 24h and at increasing distances from the discs. The surface pH of the MRS agar, acidified MRS agar, lactic acid bacteria containing MRS agar and NBY agar was also measured.

b. DETERMINATION IN BROTH CULTURES

Each of the 5 test strains of phytopathogenic bacteria were inoculated into four 250 mℓ flasks containing 100 mℓ of the following four solutions:

i) NBY broth

ii) NBY broth inoculated with *L. plantarum* (L292)

iii) NBY broth containing 1% (wt/vol) $CaCO_3$

iv) NBY broth containing 1% (wt/vol) $CaCO_3$ and inoculated with *L. plantarum* (L292).

The flasks were incubated at 25°C and 10 mℓ samples were aseptically removed at 24h-intervals for 3 days. Serial dilutions of the samples were made in Ringer solution and plated onto NBY agar. The pH of each of the undiluted samples was then measured. The NBY plates were incubated at 25°C/24h and the number of colony forming units (cfu) of plant pathogenic bacteria per mℓ broth determined.

3.3.4 HYDROGEN PEROXIDE PRODUCTION BY THE LACTIC ACID BACTERIA

a. DETERMINATION ON HBD AGAR

The lactic acid bacteria were inoculated onto HBD agar (Whittenbury, 1964), incubated at 30°C under aerobic conditions and examined 1, 2, 5 and 7 days after inoculation for the development of a dark brown or black colour around the bacterial growth.

b. DETERMINATION ON ABTS MEDIUM

The lactic acid bacteria were inoculated onto ABTS medium (Marshall, 1979) and incubated at 30°C in an atmosphere of 80% H_2 : 20% CO_2 for 48h. The cultures were then removed from this atmosphere and kept at room temperature. Results were recorded after periods of 5h and 8h and the development of green or purple colour in the agar surrounding the bacterial growth indicated hydrogen peroxide production.

As a positive control, *ca.* 0,1 mℓ of 3% (vol/vol) H_2O_2 (Saarchem) was applied to both HBD and ABTS media.

3.3.5 PREPARATION OF CULTURE FILTRATES AND DETERMINATION OF INHIBITORY EFFECT

Broth cultures of the 15 isolates showing strongest antagonism "*in vitro*" (section 2.4.2) were filtered in order to determine the inhibitory effect of cell free suspensions (i.e. exogeneous products) on the test strains of phytopathogenic bacteria (Tramer, 1966). The lactobacilli were grown in 50 mℓ MRS broth (30°C/48h) and the pH of each culture was determined. The broths were divided in 5 X 10 mℓ volumes per culture. For each culture the pH of one of these volumes was then adjusted to 5,5 with NaOH and another was adjusted to pH 7,0. All the cultures were filtered through Millex GV 0,22 µm filter units (Millipore Corp.) into sterile McCartney bottles. One of the unadjusted filtrates

of each isolate was subjected to a heat treatment of 15 min at 100°C and another was frozen to examine the lability of the inhibitory substance (Sabine, 1963).

a. FILTRATES IN WELLS

Plates of NBY agar were seeded with the 5 plant pathogenic bacteria. Wells were cut into the agar using a sterile steel borer with a diameter of 7 mm. The bottom of each well was sealed with a few drops of molten NBY agar. The filtrates were placed into the wells in triplicate and the plates were incubated at 25°C for 48h before being examined for inhibition zones surrounding the wells.

b. FILTRATES IN FILTER PAPER DISCS

In order to verify the results obtained in the above experiment, Whatman no 1 filter paper was cut into discs using a punch with a diameter of 5mm. The discs were sterilized, dipped into the filtrates used in (a) and placed - in triplicate - onto NBY agar plates seeded with the phyto-pathogens. After incubation (25°C/48h) they were examined for inhibition zones surrounding the discs.

c. CONCENTRATED FILTRATES IN FILTER PAPER DISCS

Four of the untreated cell free filtrates namely L1515A, L379, L392A and L1518 were concentrated ten-fold by freeze drying 5mℓ of the filtrates and then suspending the products in 0,5mℓ Ringer solution. Sterile filter paper discs were dipped into the concentrates and placed onto the NBY plates seeded with the plant pathogens. These plates were examined for inhibition zones after incubation (25°C/48h).

3.4 RESULTS AND DISCUSSION

3.4.1 EFFECT OF LACTIC ACID AND ACETIC ACID ON THE PLANT PATHOGENS

This experiment was carried out in order to compare the effect of lactic and acetic acids with the antagonistic effect of plant-associated lactic acid bacteria against the test strains of phytopathogenic bacteria (section 2.4.2). The diameter of the clear zones surrounding the acid- and bacteria-containing discs are shown in Table 3.1. Most of the inhibition zones caused by the acids were approximately in the same order as those caused by the lactic acid bacteria, but some of the zones differed considerably. Directly around the acid containing discs placed on *P. syringae* (Ps2) and *P. syringae* var *capsici* (Ps3), small zones of growth of the pseudomonads developed, surrounded by the clear zones. This possibly indicates the development of resistance to the acids by these two strains. This did not occur with lactic acid bacteria in the discs. Lactic acid was not inhibitory to *X. campestris* pv *mangiferae-indicae* (Xan) at all, whereas lactic cultures were highly antagonistic. The zones formed by acetic acid on Xan were much smaller and not as clear as the bacterial inhibition zones. Around the inhibition zones against *X. campestris* (Ps1), zones of growth stimulation occurred on the periphery. Similar stimulation zones formed with the bacteria-containing discs (section 2.4.2; Fig. 2.1 (c)). the inhibition zones caused by the acids against Ps1 were however not as clear as those caused by the bacteria. Acetic acid was on average more inhibitory to the plant pathogens than lactic acid.

TABLE 3.1 Diameter (in mm) of inhibition zones on certain phytopathogenic bacteria (NBY agar/24-48h/25°C), surrounding MRS agar discs containing lactic acid, acetic acid or plant-associated lactic acid bacteria.

Phytopathogenic bacteria tested	Discs containing 1% concentrations of:		Lactic acid bacteria-containing discs (section 2.4.2)			
	Lactic acid (a)	Acetic acid (a)	Average of all 43 isolates (b)	Variation in zones Minimum	Maximum	Average of 15 most antagonistic strains
X. campestris (Ps1)	12,6	13,5	14,057	No zone (6 strains)	20,50 (L1506)	18,229
P. syringae (Ps2)	18,8	20,2	20,265	No zone (4 strains)	27,00 (L392A)	24,357
P. syringae var capsici (Ps3)	16,5	22,6	16,543	No zone (4 strains)	22,57 (L1518)	20,606
E. carotovora (Erw)	14,3	21,9	15,347	No zone (5 strains)	23,47 (L392A)	18,909
X. campestris pv mangiferaeindicae (Xan)	No zone	17,2	25,671	No zone (4 strains)	33,87 (L2522)	30,793

a) average of duplicate readings on duplicate NBY plates.

b) average of triplicate readings using all 43 isolates of lactic acid bacteria

c) average of triplicate readings using the 15 most antagonistic strains, namely L1515A, L379, L1518, L392A, L1506, L2522, L2521, L1056, L292, L2506, L1553, L491, L2602, DSM 20205 and L1084.

According to Tramer (1966), the size of inhibition zones caused by acids, is governed by the ability of the acid to diffuse into the medium. The diffusion of lactic acid and acetic acid as well as the acids produced by some strains of lactic acid bacteria into NBY agar was measured by means of a contact electrode and these results are represented in Table 3.2. The readings were taken from duplicate plates each in triplicate but still discrepancies can be seen. In each case all readings were taken firstly using a dry electrode and then using the same electrode wet with distilled water. The pH readings taken with the wet electrode varied more than those using the dry electrode. The pH values of the untreated NBY agar plates were between 6,8 and 6,9 (dry electrode) and 6,5 and 6,9 (wet electrode). The pH values of the untreated MRS agar was *ca*. 6,0 (dry electrode) and *ca*. 6,1 (wet electrode). The 3 lactic acid bacterial strains were chosen because their media had very similar pH values after incubation but their inhibitory abilities differed in the "*in vitro*" agar disc experiments (section 2.4.2.; Table 2.2).

TABLE 3.2 Diffusion as a function of pH of acids into NBY agar from MRS agar discs containing either lactic acid, acetic acid or a 48h-culture of lactic acid bacteria

Type of disc	pH of disc		Time (h) after placement	pH values on surface of NBY agar at various distances from discs								(d)
				0 mm		10 mm		20 mm		30 mm		
	dry	wet		dry	wet	dry	wet	dry	wet	dry	wet	
Lactic acid	4,1 (b)	4,1	0	6,8 (c)	6,6	6,8	6,7	6,8	6,8	6,9	6,7	
			6	6,4	6,2	6,8	6,6	6,8	6,5	6,8	6,6	
			12	6,4	6,2	6,8	6,4	6,8	6,5	6,8	6,5	
			18	6,5	6,4	6,7	6,6	6,8	6,6	6,8	6,7	
			24	6,5	6,4	6,7	6,6	6,8	6,6	6,8	6,7	
Acetic acid	4,5	4,3	0	6,7	6,5	6,7	6,7	6,8	6,7	6,9	6,7	
			6	6,2	6,2	6,7	6,5	6,8	6,7	6,8	6,8	
			12	6,2	6,2	6,6	6,4	6,8	6,6	6,8	6,7	
			18	6,3	6,4	6,6	6,5	6,8	6,7	6,8	6,8	
			24	6,4	6,3	6,6	6,6	6,7	6,7	6,8	6,8	
Strain L1506 (a)	3,7	3,8	0	6,8	6,6	6,8	6,7	6,8	6,6	6,8	6,8	
			6	5,8	5,9	6,7	6,6	6,8	6,8	6,8	6,8	
			12	6,0	5,8	6,7	6,6	6,8	6,7	6,8	6,8	
			18	5,9	6,2	6,6	6,6	6,8	6,8	6,8	6,6	
			24	5,9	5,7	6,5	6,5	6,7	6,7	6,8	6,7	
Strain L1515A	3,7	3,6	0	6,8	6,6	6,8	6,6	6,8	6,6	6,8	6,7	
			6	5,8	6,1	6,8	6,6	6,9	6,7	6,8	6,8	
			12	5,8	5,7	6,7	6,6	6,8	6,6	6,8	6,8	
			18	5,8	6,0	6,5	6,5	6,8	6,7	6,8	6,8	
			24	6,0	6,1	6,5	6,4	6,7	6,6	6,8	6,8	
Strain L1553	3,7	3,7	0	6,8	6,5	6,8	6,6	6,8	6,6	6,8	6,7	
			6	6,5	6,4	6,7	6,7	6,8	6,8	6,8	6,8	
			12	6,4	5,2	6,8	6,6	6,8	6,7	6,8	6,8	
			18	6,4	6,3	6,7	6,6	6,8	6,8	6,8	6,7	
			24	6,4	6,2	6,6	6,6	6,7	6,6	6,8	6,9	

a) 48h cultures (30°C) in MRS agar
b) average of duplicate readings on duplicate plates
c) average of triplicate readings on duplicate plates
d) dry/wet indicates whether the contact electrode was wiped dry or used wet with distilled water

In spite of the fact that the contact electrode seemed slightly inaccurate, these results generally correspond with the findings of Tramer (1966) namely that the diffusion of the acids determine the size of the inhibition zones formed. Acetic acid was more inhibitory than lactic acid (Table 3.1) and although its initial pH was higher than lactic acid, acetic acid reduced the pH of the NBY agar to a greater extent. Strain L1515A was the most antagonistic of the 43 strains tested and it lowered the pH of the NBY agar more than L1506 (the 5th best antagonist) which in turn caused a greater pH reduction than L1553 (the 11th best antagonist). The diffusion of the acids into agar differ and this has an effect on the inhibition zones which further indicate that acid production is a major mechanism of antagonism. This also indicates discrepancies which can be expected between laboratory and field experiments i.e. substances which diffuse best into agar might not necessarily react in a similar fashion on leaf surfaces.

3.4.2 EFFECT OF BUFFERING pH REDUCTION BY LACTIC ACID BACTERIA ON BROTH CULTURES OF PLANT PATHOGENS

The effect of adding $CaCO_3$ to broth cultures of the phytopathogenic bacteria in pure culture and in associative culture with *L. plantarum* (L292), is shown in Fig. 3.1 - 3.5. The plant pathogens grew well in pure culture either with or without $CaCO_3$ added to the NBY broth (a and c in Fig. 3.1 - 3.5). An exception was *P. syringae* var *capsici* (Ps3) which died in the presence of $CaCO_3$ (Fig. 3.3c) but not in the presence of $CaCO_3$ plus L292 (Fig. 3.3d). This may be due to neutralisation of the effect of $CaCO_3$ through lactic acid production by the growing lactobacilli. L292 killed all the plant pathogens within 48h in the absence of $CaCO_3$ (b in Fig. 3.1 - 3.5). The lactobacilli lowered the pH of the NBY broth from *ca.* 7,2 to 5,9 - 3,6 during the 3 day period. The addition of $CaCO_3$ plus L292 to the broth cultures did not result in the killing of the plant pathogens but they did not grow as well as in the absence of L292. Although this observation suggests an antagonistic effect additional to that caused by lactic acid production, the latter definitely seems to be the most important factor in the antagonism against phytopathogenic bacteria. These results correspond to those

reported by Tramer (1966) namely that the lactic acid produced by *L. acidophilus* was highly bactericidal to *Escherichia coli* and that this effect was alleviated by increasing the pH. Kao and Frazier (1966) also reported lactic acid to be the antibacterial substance produced by lactic acid bacteria tested against *Staphylococcus aureus*. In buffered medium (pH 6,3), *S. aureus* was inhibited but not killed which also corresponds to the results obtained in this study. These results could indicate the production of an inhibitory substance(s) other than lactic acid but also shows the important effect of reduced pH on antagonism.

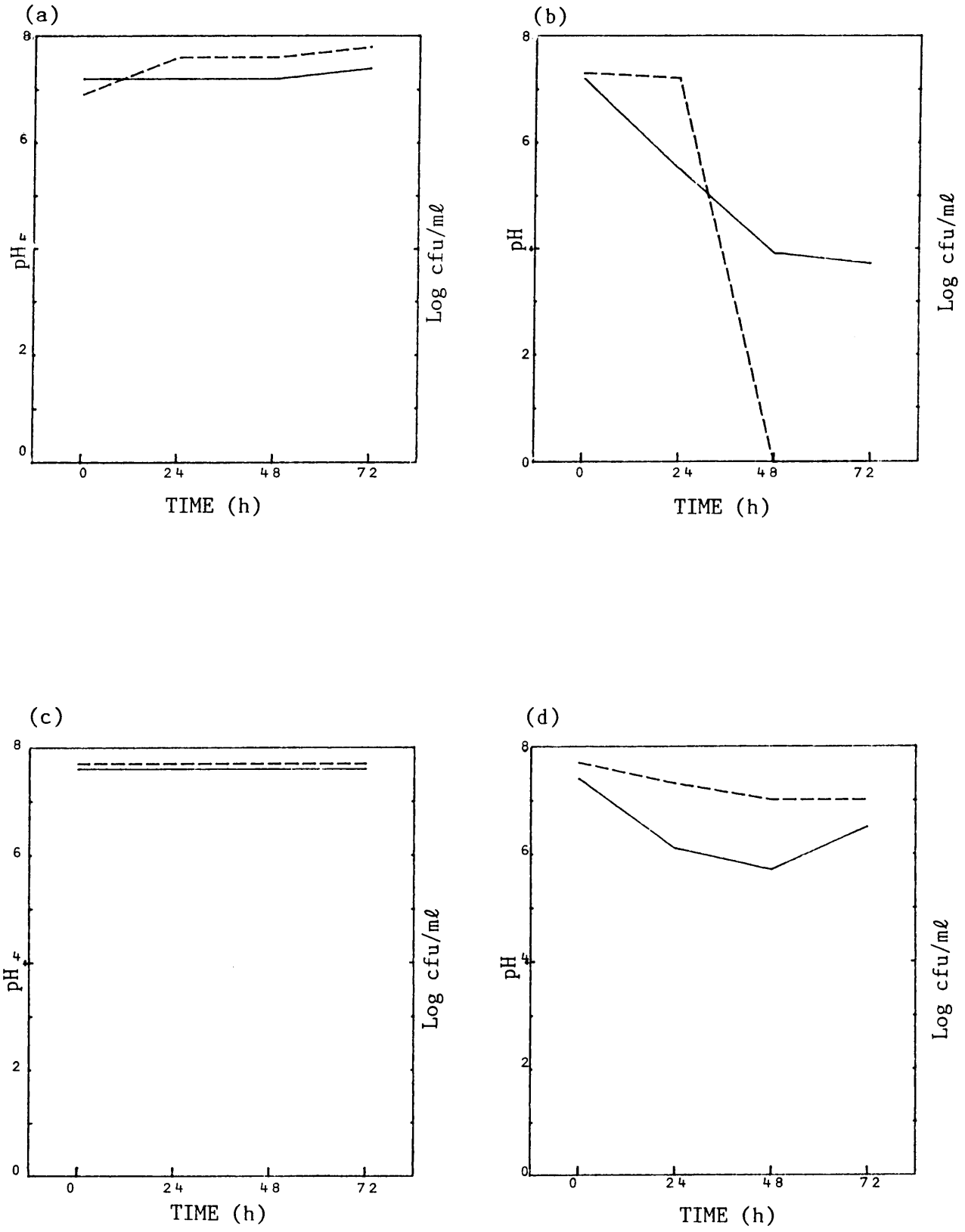

FIGURE 3.1 Growth curves of *X. campestris* (Ps1) in NBY broth (25°C) and pH changes over 72h. (a) Pure culture of Ps1; (b) mixed culture of Ps1 and *L. plantarum* (L292); (c) Pure culture of Ps1 in NBY broth containing 1% $CaCO_3$; (d) mixed culture of Ps1 and L292 in NBY broth containing 1% $CaCO_3$.

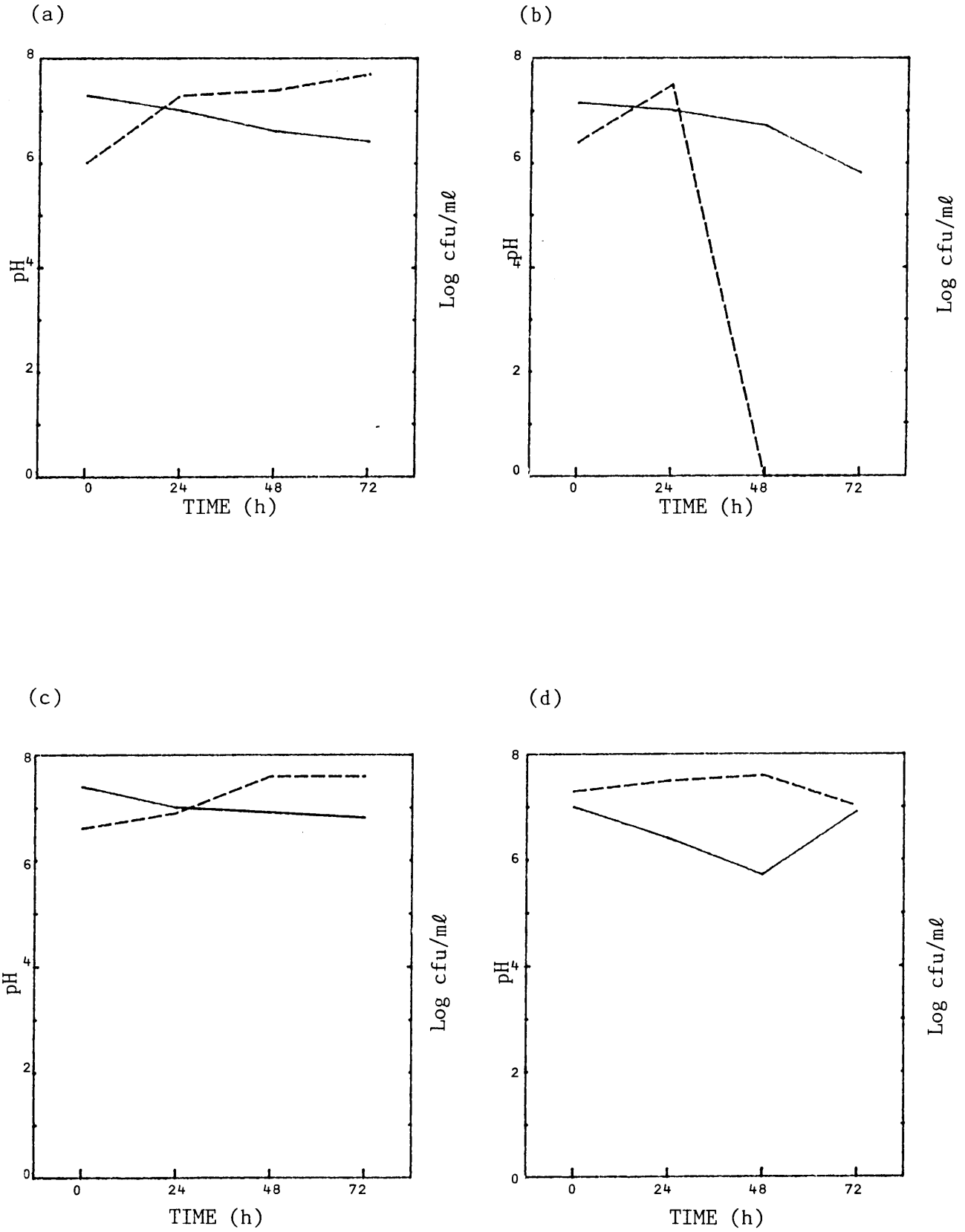

FIGURE 3.2 Growth curves of *P. syringae* (Ps2) in NBY broth (25°C) and pH changes over 72h. (a) Pure culture of Ps2; (b) mixed culture of Ps2 and *L. plantarum* (L292); (c) Pure culture of Ps2 in NBY broth containing 1% $CaCO_3$; (d) mixed culture of Ps2 and L292 in NBY broth containing 1% $CaCO_3$.

– – – – Ps2 ——— pH

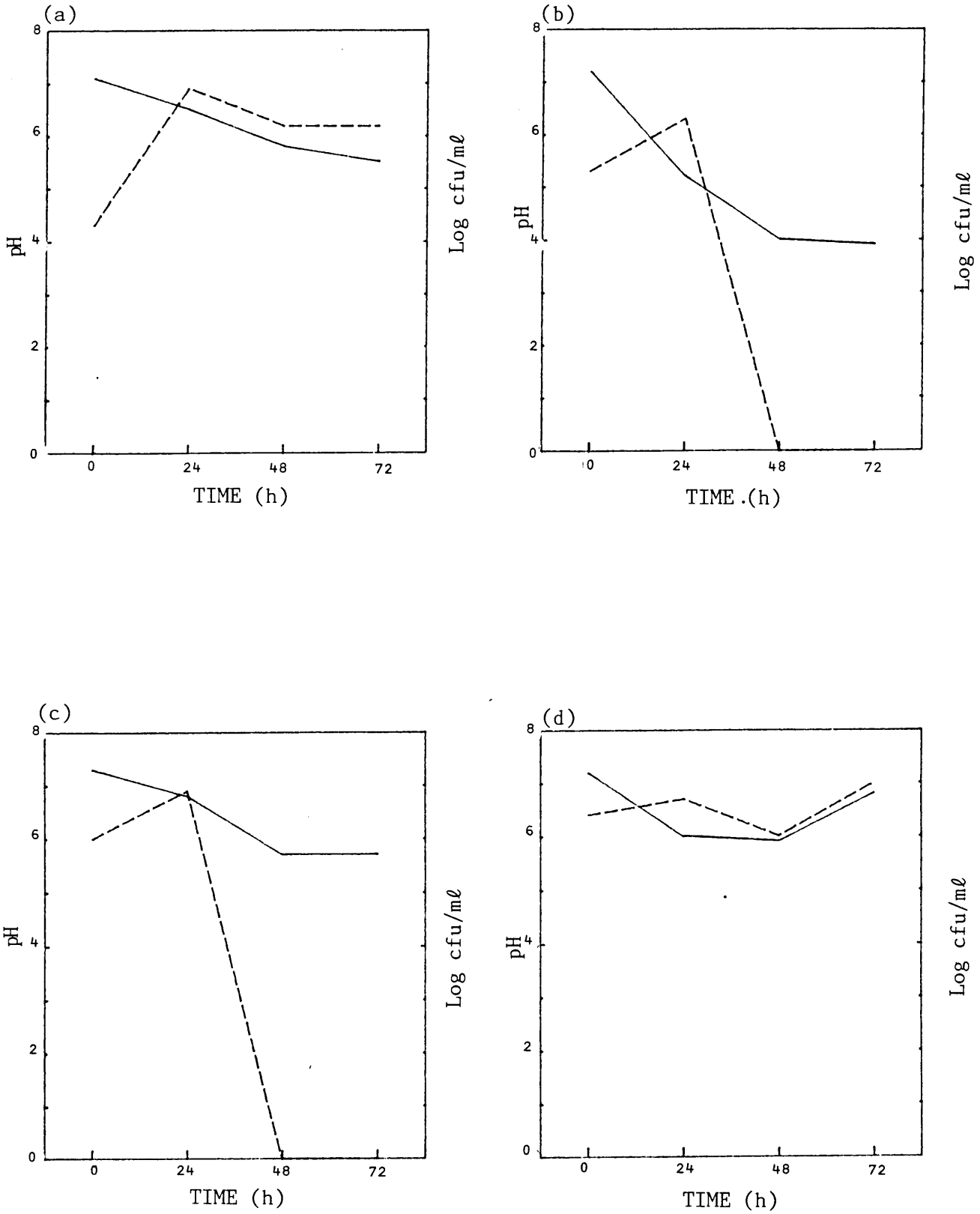

FIGURE 3.3 Growth curves of *P. syringae* var *capsici* (Ps3) in NBY broth (25°C) and pH changes over 72h. (a) Pure culture of Ps3; (b) mixed culture of Ps3 and *L. plantarum* (L292); (c) Pure culture of Ps3 in NBY broth containing 1% $CaCO_3$; (d) mixed culture of Ps3 and L292 in NBY broth containing 1% $CaCO_3$.

– – – –Ps3 ——— pH

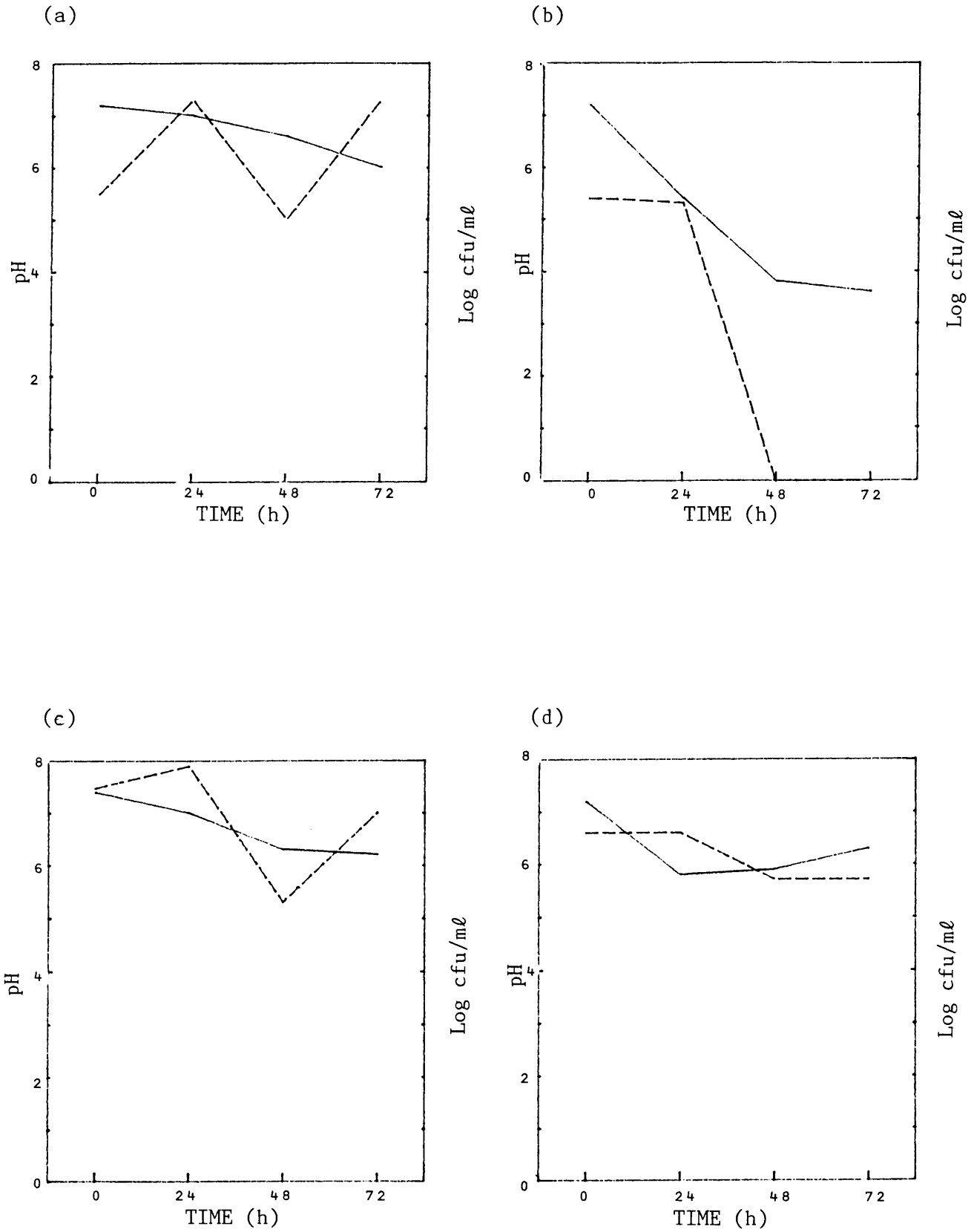

FIGURE 3.4 Growth curves of *E. carotovora* (Erw) in NBY broth (25°C) and pH changes over 72h. (a) Pure culture of Erw; (b) mixed culture of Erw and *L. plantarum* (L292); (c) Pure culture of Erw in NBY broth containing 1% $CaCO_3$; (d) mixed culture of Erw and L292 in NBY broth containing 1% $CaCO_3$.

– – – – Erw ——— pH

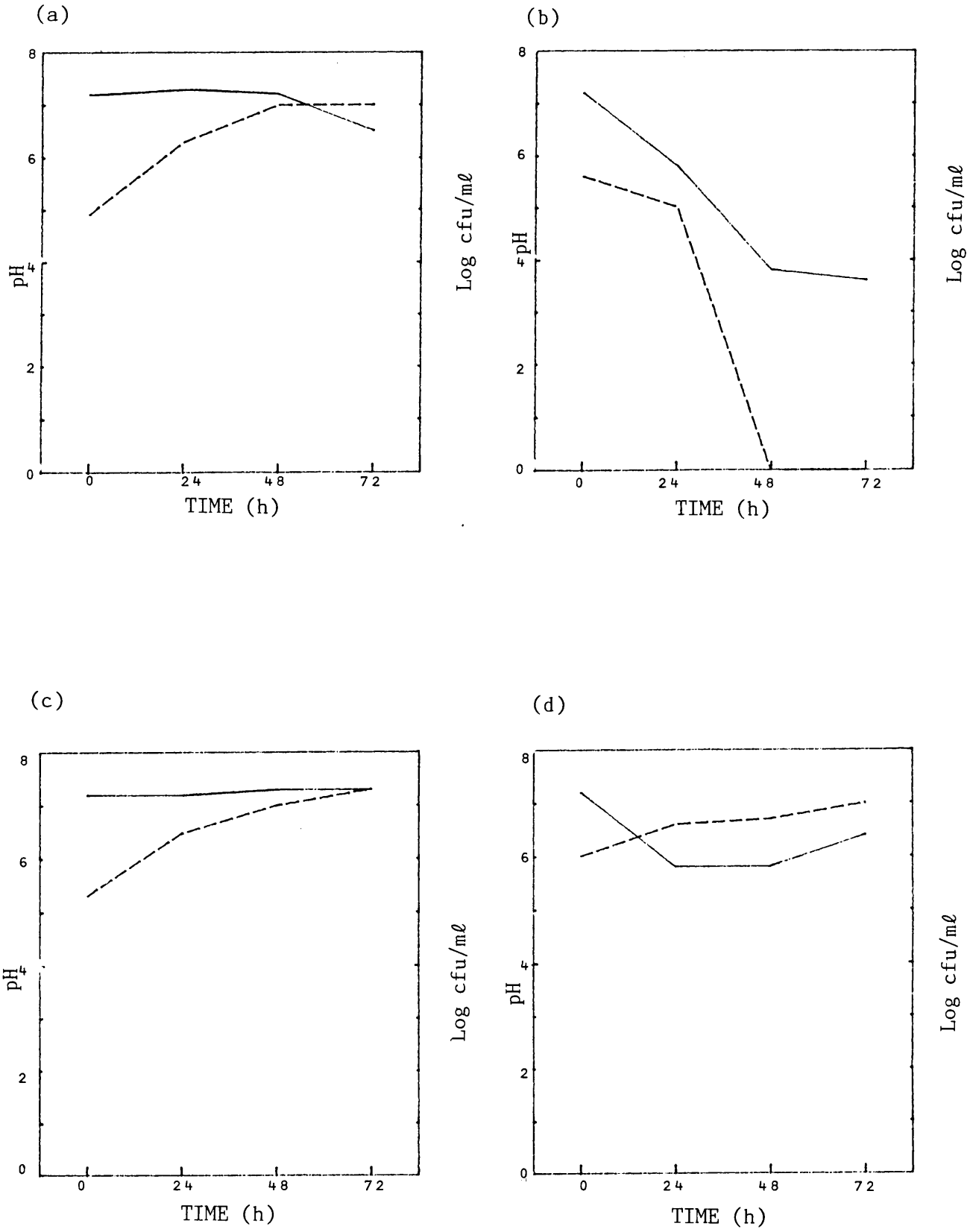

FIGURE 3.5 Growth curves of *X. campestris* pv *mangiferaeindicae* (Xan) in NBY broth (25°C) and pH changes over 72h. (a) Pure culture of Xan; (b) mixed culture of Xan and *L. plantarum* (L292); (c) Pure culture of Xan in NBY broth containing 1% $CaCO_3$; (d) mixed culture of Xan and L292 in NBY broth containing 1% $CaCO_3$.

– – – –Xan ——pH

3.4.3 HYDROGEN PEROXIDE PRODUCTION BY LACTIC ACID BACTERIA

a. DETERMINATION ON HBD AGAR

15 of the plant-associated lactic acid bacterial strains were tested for hydrogen peroxide production on HBD agar. The results are represented in Table 3.3 with indication of the amount of colour development around the growth of the lactic acid bacteria. According to Whittenbury (1964) 5 of these strains did not produce hydrogen peroxide and of the 10 positive strains, 2 coloured the agar slightly, 4 mildly and 4 strongly. The strains which produced hydrogen peroxide according to this determination, were not necessarily the most antagonistic to the phytopathogenic bacteria "*in vitro*" (Section 2.4.2). For example strain L1515A was the most antagonistic strain but only coloured the HBD agar *ca*. 5 days after inoculation and then only sligthly. L379 was the second most antagonistic strain but did not produce hydrogen peroxide on HBD agar. On the other hand, L1084 produced peroxide almost immediately and a very intense colour developed, however, this strain was not a strong antagonist. Whittenbury (1964) did not state whether the amount of colour developed gave an indication of the amount of hydrogen peroxide produced, but Gilliland (1969) developed a method for the enzymatic determination of hydrogen peroxide in milk using horseradish peroxidase and *o*-dianisidine as chromogenic hydrogen donor. The amount of colour developed as a result of this enzymatic reaction gave a measurement of the amount of peroxide. According to Whittenbury (1964) the haem compounds in HBD agar have a peroxidase-like reaction in the oxidation of *o*-dianisidine by peroxide.

b) DETERMINATION ON ABTS MEDIUM

14 of the 43 lactic strains could not be sustained on the ABTS-containing agar in an atmosphere of 80% H_2:20% CO_2. Of the 29 remaining strains, 22 did not change the colour of the medium and thus did not produce hydrogen peroxide according to Marshall (1979). Mild green or purple colour developed around 4 of the strains and strong colour deve-

lopment was observed around 3 strains (Table 3.3). These results are vastly different to the results obtained on HBD agar and a far lower percentage of strains reacted positively. Whittenbury (1964) reported that the production of hydrogen peroxide depended in some instances on the substrate used as energy source. This might account for some of the discrepancies found on the 2 different media.

The strains which produced hydrogen peroxide on the ABTS medium were - as in the case of HBD agar - not necessarily found to be more antagonistic to plant pathogenic bacteria than the strains which were negative (Section 2.4.2). Both HBD and ABTS agar showed strong positive reactions to the 3% H_2O_2 used as control, further complicating the decision on which of the two methods is more accurate.

The chromogens used as hydrogen donors in these methods namely *o*-dianisidine (Whittenbury, 964) and ABTS (Marshall, 1979; Müller, 1984) as well as benzidine (Penfold, 1922; Kraus *et al*., 1957) or *o*-tolidine (Martin and Gilliland, 1978), are usually colourless compounds which change colour when oxidised in the following reaction:

$$H_2O_2 + AH_2 \text{ (donor)} \xrightarrow{\text{peroxidase}} H_2O + A \text{ (oxidised donor)}$$

(Gilliland, 1969).

A quantitative hydrogen peroxide determination by means of a biometer using 9,9'-bis-(N-methyl acridinium nitrate) ("lucigenin") might be more accurate than these enzymatic methods (Methods of Bioluminescence Analysis, Boehringer Mannheim).

TABLE 3.3 Hydrogen peroxide production by plant-associated lactic acid bacteria on ABTS medium (48h/30°C/80% H_2 : 20% CO_2) and on HBD agar (to 7 days/30°C/O_2)

Strain number	ABTS medium		HBD agar			
	5h at room temperature	8h at room temperature	Days after inoculation 1	2	5	7
L292	+	++	++	++	++	++
L373	-	-	-	(+)	+	+
L379	-	-	-	-	-	-
L392A	-	-	+	+	+	+
L392B	-	-	-	-	-	-
L491	-	-	(+)	(+)	(+)	+
L1056	-	-	+	+	++	++
L1064	•	•	-	-	-	-
L1079	-	-	(+)	(+)	+	+
L1084	-	-	++	++	++	++
L1131	++	++	-	-	-	-
L1506	-	+	-	-	-	(+)
L1515A	-	+	-	-	(+)	(+)
L1518	-	-	++	++	++	++
L1553	-	-	-	-	-	-
L2501	-	-				
L2502	-	-				
L2503	-	-				
L2504	-	-				
L2507A	-	-				
L2507B	-	-				
L2508A	-	-				
L2508B	-	-				
L2508C	-	-				
L2510	-	-				
L2521	-	-				
L2522	-	+				
L2525	-	+				
DSM20205	-	-				
ATCC8014	++	++				

Legend:

- : no colouration
(+) : slight colouration
++ : strong colouration

The following strains did not grow on ABTS medium:

L1064, L2506, L2523, L2524, L2601, L2602, L2603, L2604, L2605, L2606, L2607, L2608, L136.

3.4.4 EFFECT OF CELL FREE CULTURE FILTRATES OF LACTIC ACID BACTERIA

The pH values of the cultures (48h/30°C) before filtration, varied between 3,66 and 5,12. None of the filtrates at any of the pH values (original pH, pH 5,5 or pH 7,0) or the heat treated or frozen filtrates were in the least inhibitory to the plant pathogenic bacteria whether applied in wells or in filter paper discs. The concentrated filtrates were not inhibitory either. This phenomenon can not be ascribed to interaction with the filters since Millex GV filters are non-reactive, low binding and manufactured for the filtration of aqueous solutions.

3.5 LITERATURE CITED

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CHAPTER 4 : SURVIVAL OF PLANT-ASSOCIATED LACTIC ACID BACTERIA ON THE PHYLLOPLANE

4.1 ABSTRACT

Strains of plant-associated lactic acid bacteria found to be antagonistic to test strains of phytopathogenic bacteria, were administered to young bean plants in the greenhouse and fibre glass tunnel. The survival of these lactic acid bacteria on the phylloplane was followed over a period of 3 weeks (as colony forming units/g of plant material). The viable lactic acid bacterial numbers decreased by *ca.* 1 log per week on plants treated with *Lactobacillus plantarum* L1515A. Five strains of lactic acid bacteria were subsequently administered to the leaves of bean plants: *Leuconostoc mesenteroides* 1079, *Lactobacillus brevis* L1084, *Lactobacillus sake* L2522, *L. plantarum* L373 and *L. plantarum* L1515A. The *L. plantarum* strains had the highest survival rates on the phylloplane. The lactic acid bacterial treatments had no significant effect on the number of fungi and yeasts on the bean leaves and was strongly correlated to the total number of bacteria. Electron microscopy of treated leaves showed the lactic acid bacteria concentrated in the grooves on both the abaxial and adaxial leaf surfaces.

4.2 INTRODUCTION

Plant-associated lactic acid bacterial strains were found to be antagonistic to test strains of phytopathogenic bacteria in agar diffusion tests, associative broth cultures and on the phylloplane in pot trials (Chapter 2; Visser *et al.*, 1986).

Before attempting to apply the lactic acid bacteria as biocontrol agents against bacterial plant diseases, the survival of the antagonists on the phylloplane needs to be investigated. The biocontrol potential of an organism depends on both its antagonistic activity and its survival on the phylloplane. Although survival as such does not imply antagonism, the ability to survive, and preferably to grow and spread on the phylloplane, are pre-requisites for effective, sustained biological control. An outstanding "*in vitro*" antagonist which dies rapidly on the leaf surface, has little potential unless it, or the restrictive environmental conditions can be modified, that is the colonizing ability of the

microbe promoted (Andrews, 1985). Alternatively, the lack of persistence of antagonists less adapted for growth and survival on plant surfaces, may be overcome by repeated applications (Blakeman, 1985).

In spite of the complex nutritional requirements of lactic acid bacteria, they are able to grow in a wide variety of habitats, including on the phylloplane (Sharpe, 1981). Sources of nutrients for phylloplane microorganisms include leaf exudates, organic debris (for example dust), pollen and aphid honeydew (Blakeman, 1985). On young plants leaf exudates are the only nutrient source which results in low nutrient levels (Tukey, 1971; Fokkema, 1981; Rodger & Blakeman, 1984). Pollen has a profound influence on microbial diversity and activity (Leben, 1985) and after the first few weeks of the growing season, bacterial populations on the phylloplane decrease as competition for nutrients increases (Blakeman, 1985). Numerous organic and inorganic substances leached from the leaf tissue are found in free moisture on the leaf surfaces (Morris & Rouse, 1985). The quantity and quality of nutrients provided as leachate to epiphytic microorganisms by a plant host vary with plant age (Tukey, 1970; Blakeman, 1972; Wildman & Parkinson, 1981), light intensity (Tukey *et al.*, 1957), temperature (Tukey, 1970), type of nitrogen fertilizer (Weissman, 1964; Sol, 1967), pH of leaching medium (Lepp & Fairfax, 1976) and plant injury (Tukey & Morgan, 1963). Substances leached from fungal spores and extracellular microbial metabolites are also sources of nutrients for epiphytic microorganisms (Morris & Rouse, 1985). There are several possible ways nutrients can influence populations of epiphytic microorganisms. The nutrient level may be low enough to limit the growth of some, or all, of the epiphytic microorganisms. Limited nutrient availability can be further accentuated by competition among epiphytes. Nutrients may affect the metabolism of the host plant or of the epiphytes as stimulants in the synthesis of antimicrobial substances or as catabolite repressors, respectively (Morris & Rouse, 1985). The effects of nutrients on microorganisms associated with plants may be difficult to distinguish from the direct effects of nutrients alone on the host plant, for example amino acids can stimulate the host to synthesize enzymes indirectly involved in increased resistance to pathogens (Van Andel, 1966 a & b).

Morris and Rouse (1985) examined the effect of artificially increased levels of naturally occurring organic nutrients on the composition of the epiphytic bacterial community on bean (*Phaseolus vulgaris*) leaflets and pods. The application of simple organic compounds could alter the population size of fluorescent pseudomonads and thus reduce disease (bacterial brown spot) caused by *Pseudomonas syringae*. This could be due to antagonism or the direct or indirect effects of the chemical on *P. syringae*.

Relative humidity (rH) at the plant surface is likely to be the single most important factor influencing the growth and survival of microorganisms in this habitat. Leaves are continually transpiring, therefore, the surface humidity is higher than the surrounding air (Blakeman, 1985). Many foliar pathogens require the presence of a drop or film of water to germinate and/or penetrate the host. The survival of bacterial antagonists may be primarily determined by the presence or absence of such a water film (Spurr & Knudsen, 1985). Other factors influencing the relationship between a foliar pathogen, the host and associated microbes, includes the microclimate at the plant surface, seasonal changes, the duration of leaf wetness, leaf temperature, the succession of microorganisms, stage of leaf maturity and degree of weathering of surface layers (Blakeman, 1985), as well as the susceptibility of the host breeding line to the pathogen (Daub & Hagedorn, 1981).

Lactic acid bacterial strains can utilize the nutrients on the phylloplane, but they occur only in small numbers on intact plant material (Keddie, 1959). Lactobacilli become more prevalent on cut or bruised plant tissue. Leuconostocs constitute 80% of the lactic acid bacteria naturally occurring on plants and lactobacilli only 10% (Stirling & Whittenbury, 1963). The antibacterial effect of extracts of some plants might contribute to this sparsity on the phylloplane although saponin-containing plants were not found to be inhibitory (Schcherbanovsky *et al.*, 1975). The following *Lactobacillus* species have been isolated from plants: *L. plantarum*, *L. fermentum*, *L. brevis*, *L. casei*, *L. salivarius*, *L. viridescens* and *L. cellobiosus* as well as atypical streptobacteria and betabacteria (Sharpe, 1981).

Parbery *et al*. (1981) and Hirano and Upper (1983) have reviewed some of the difficulties inherent to sampling phylloplane bacteria. Problems include the nonuniform distribution of the organism and the tenacity with which certain bacteria adhere to the leaf surface.

In the present study, pot trials were used to investigate the survival rates of antagonistic lactic acid bacterial strains on the leaf surfaces of young bean plants over a period of 3 weeks. These were merely preliminary tests for survival. Conclusive evidence that the biological control of foliar phytopathogenic bacteria by surface sprays would be effective in the field, could not be provided yet.

4.3 MATERIALS AND METHODS

4.3.1 ORGANISMS

The lactic acid bacterial strains used in these experiments were obtained from plants and plant-associated products as described in section 2.3.1 and by Visser *et al*. (1986).

4.3.2 MEDIA

Lactic acid bacteria were grown on MRS agar (E. Merck AG) (De Man *et al*., 1960) at 30°C/48h under anaerobic conditions (Anaerocult; Merck), and maintained in MRS broth. Bacterial suspensions and dilutions were made in quarter-strength Ringer solution (Merck). Total aerobic bacterial numbers were determined on Standard 1 (Std 1) agar (Biolab Chemicals) and were incubated at 30°C/48h. Yeast and fungal numbers were determined on potato dextrose agar (PDA) (Biolab) with the pH adjusted to 3,7 after sterilization, by adding 14 mℓ of sterile 10% tartaric acid per liter to eliminate bacterial contamination. PDA plates were incubated at 25°C/72h. The lactic acid bacterial population was enumerated on modified MRS agar (MRS-SA agar). The pH of MRS-SA agar was adjusted to 5,6 and it contained 0,2% (wt/vol) potassium sorbate (Merck) and 0,1%

(wt/vol) cycloheximide (Calbiochem Behring) to eliminate fungi, yeasts and bacteria other than lactic acid bacteria. MRS-SA plates were incubated at 30°C/48-60h under anaerobic conditions (Anaerocult; Merck).

4.3.3 PLANT TREATMENTS AND SAMPLING METHODS

Certified seeds of Haricot beans were obtained from the National Institute of Plant Protection, Pretoria, S.A. These were germinated in sterile Vermiculite (no. 8) at 25-30°C/*ca*. 1 week. The seedlings were planted in unsterilized soil in pots with a diameter of 15 cm with 2 seedlings in each pot. These were grown in a greenhouse (25-30°C) or glass fibre tunnel (15-30°C). Each pot (2 plants) represented one repetition and at every sampling time, 6 repetitions were sampled and analysed per treatment. Treatments consisted of surface sprays of selected lactic acid bacterial strains. Cultures (48h) of the lactic acid bacteria were washed from MRS agar using sterile quarter strength Ringer solution containing 0,1% (wt/vol) Tween 80 (Merck). Plastic spray bottles were used to administer approximately 10g of these bacterial suspensions to each of the young bean plants as soon as the first two primary leaves were fully grown. Equal amounts were sprayed onto the abaxial and adaxial leaf surfaces.

The leaves were allowed to dry (*ca*. 30 min.) before day 0-samples were taken and there-after samples were taken once or twice weekly, depending on the experiment. One primary leaf of each of the two plants per repetition was aseptically severed and placed into a sterile plastic Petri dish where it was then aseptically cut into pieces of *ca*. $5mm^2$. One gram of this plant material was weighed out and vortexed for 30s in 9 mℓ of Ringer solution in large test tubes containing *ca*. 1 cm^3 of glass beads (diameter = 2mm). Dilution series were made in quarter strength Ringer solution and plated out in duplicate onto the surface of the various media used for plate counts. All plates yielding less than 300 colonies after incubation were counted and the average number of colony forming units (cfu) per gram of plant material calculated.

4.3.4 DETERMINATION OF THE SURVIVAL PATTERN OF *LACTOBACILLUS PLANTARUM* (L1515A) ON BEAN PLANTS IN THE GREENHOUSE

Samples were taken from untreated plants germinated and grown in the greenhouse. The dilutions were plated onto MRS-SA agar to determine the initial number of cfu of lactic acid bacteria per gram. The plants were treated with a suspension of *L. plantarum* strain L1515A which was the most antagonistic strain "*in vitro*" (section 2.4.2). Samples were taken on days 0, 3, 7, 10, 14, 17 and 21 and plated onto MRS-SA agar. The number of typical colonies which developed within 48h at 30°C were recorded and the cfu/g calculated.

4.3.5 SCANNING ELECTRON MICROSCOPY OF LEAVES TREATED WITH *L. PLANTARUM*

Leaf samples were taken 3 days after treating the bean plants with *L. plantarum* (L1515A) (section 4.3.4) and prepared for electron microscopy. Samples of *ca.* 1 cm^2 were cut from the middle of the leaves. The bacteria were fixed to the leaf surfaces by placing each sample in 6% glutaraldehyde in 0,1 M sodium cacodylate buffer (pH 7,35) under low vacuum for 24h. The samples were washed in 0,1M sodium cacodylate buffer (20 min), placed in 2% OsO_4 in cacodylate buffer (60 min), washed in cacodylate buffer (20 min) and dehydrated in an ethanol series (20 min each in 50, 70, 90 and (3x) 100% ethanol). The samples were dried in a critical point drier (Hitachi HCP-2) using CO_2 before they were mounted onto brass stubs and sputter-coated with gold (Eiko IB-3). The abaxial and adaxial leaf surfaces were viewed using an Hitachi S-450 scanning electron microscope.

4.3.6 DETERMINATION OF THE SURVIVAL OF 5 STRAINS OF LACTIC ACID BACTERIA AND INTERACTIONS WITH OTHER MICROBIAL POPULATIONS ON THE PHYLLOPLANE

Altogether 288 young plants (144 repetitions) were randomly divided into 6 groups of 24 repetitions each. These groups were placed *ca.* 3 m apart in a fibre glass tunnel. Each group of plants was sprayed with one of the following treatments:

1. Control (sterile Ringer solution with Tween 80)
2. *Leuconostoc mesenteroides* L1079 suspension
3. *Lactobacillus brevis* L1084 suspension
4. *Lactobacillus sake* L2522 suspension
5. *Lactobacillus plantarum* L373 suspension
6. *L. plantarum* L1515A suspension.

When the treatments were administered, care was taken not to contaminate other groups. Samples were taken on days 0, 7, 14 and 31 and plated onto Std. 1 agar, MRS-SA agar and PDA. The number of cfu/g of total aerobic bacteria, lactic acid bacteria, yeasts and fungi were calculated after incubation.

4.3.7 STATISTICAL ANALYSIS

Results were analysed by variance and covariance analysis as well as linear regression, using the SAS statistical package (Ray, 1982).

4.4 RESULTS AND DISCUSSION

4.4.1 SURVIVAL OF *L. PLANTARUM* ON BEAN PLANTS

A low average initial number of 83 viable lactic acid bacteria per gram, was found on the young bean leaves just prior to treatment. An average of $3,9 \times 10^7$ lactobacilli/g were present on the leaves directly after treatment and this number decreased by *ca.* 1 log per week to an average of $3,3 \times 10^4$ cfu/g at the end of the 21-day period (Figure 4.1). Statistical analysis of the results indicated that there was a strong negative correlation ($r = 0,933$; $P = 0,0001$) between log cfu of lactobacilli/g and time. The time elapsed since treatment, was responsible for 87,18% of the variation in the log cfu/g. Linear regression was significant and the line had a slope of $b = 0,126$ ($P = 0,0001$) which indicates that log cfu/g decreased by 0,126 per day (Figure 4.1).

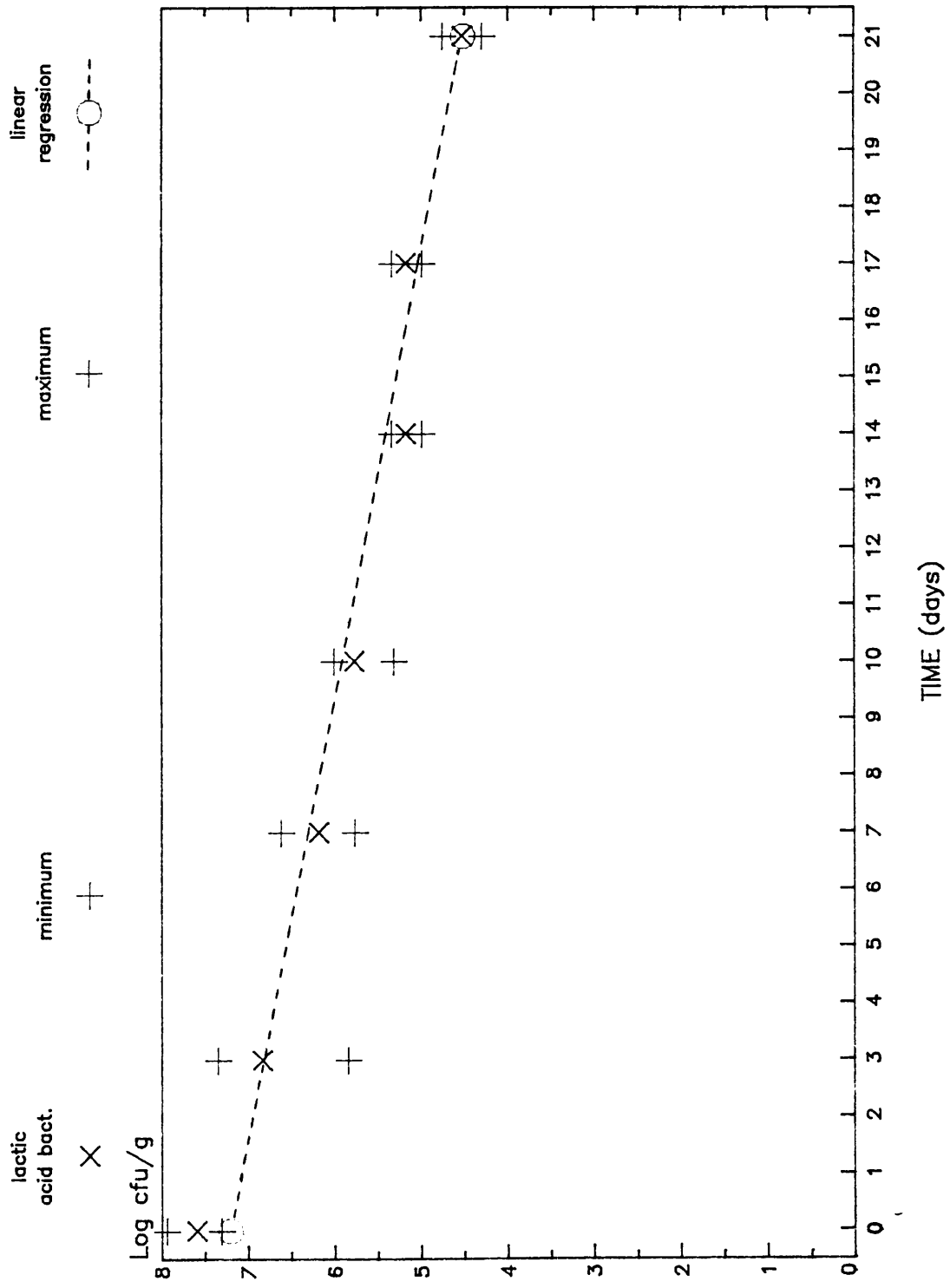


FIGURE 4.1 Survival of *Lactobacillus plantarum* L1515A on the leaves of bean plants in the greenhouse.

Thus, the lactobacilli did not multiply but survived reasonably well on the phylloplane in the greenhouse. This suggests that the survival of these and other strains of lactic acid bacteria on the phylloplane, warrants further investigation.

4.4.2 SCANNING ELECTRON MICROGRAPHS OF *L. PLANTARUM* ON THE PHYLLOPLANE

As shown in Figure 4.2, the lactic acid bacteria were present on both the abaxial and adaxial surfaces of the bean leaves 3 days after being administered. There were no great differences in the amounts or locations of the bacteria on the 2 surfaces. The bacteria were mainly concentrated in the grooves on the leaves at the lines of junction between epidermal cells. Some lactic acid bacteria were found in the stomas. No attachment structures could be discerned.

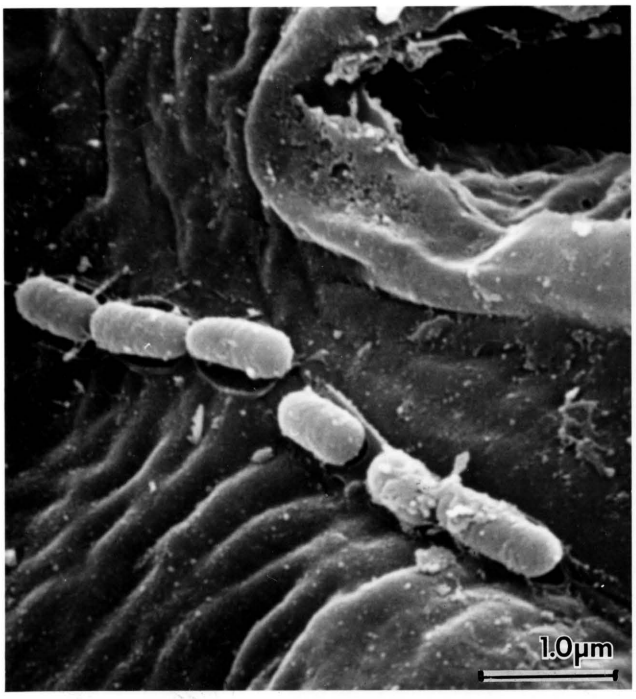
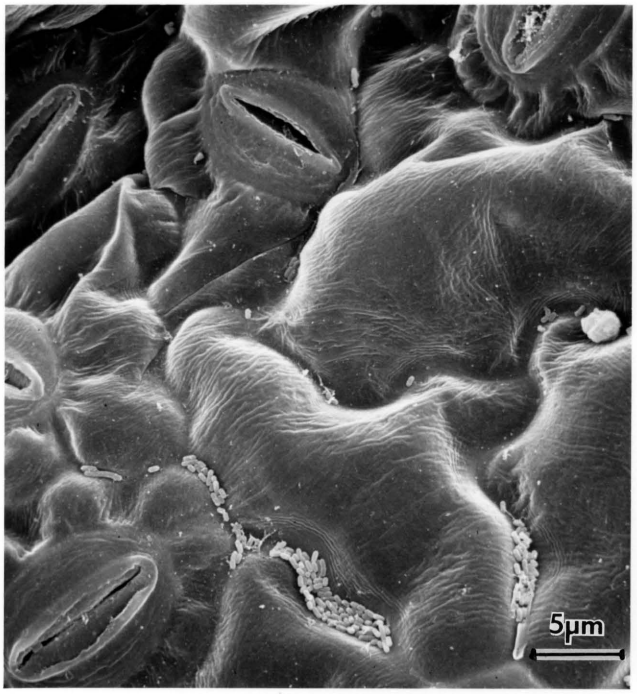


FIGURE 4.2a Electron micrograph of the adaxial leaf surface of a bean plant 3 days after being treated with *L. plantarum* L1515A.



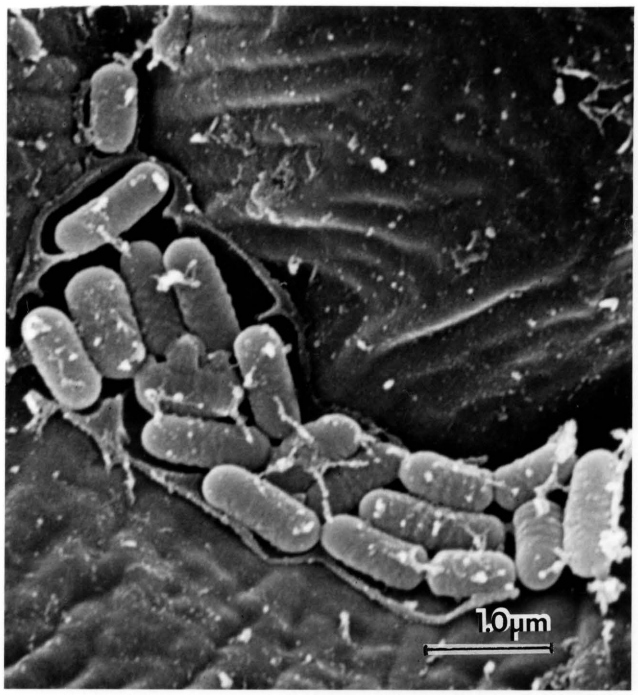


FIGURE 4.2b Electron micrograph of the abaxial leaf surface of a bean plant 3 days after being treated with *L. plantarum* L1515A.

4.4.3 SURVIVAL PATTERNS OF 5 DIFFERENT STRAINS OF LACTIC ACID BACTERIA AND OTHER MICROBIAL POPULATIONS ON BEAN PLANTS

In order to verify the results obtained in section 4.4.1, the pot trials were repeated in a fibre glass tunnel. Four additional strains of lactic acid bacteria were administered to the young bean plants as surface sprays. In addition to lactic acid bacterial counts, the total number of aerobic bacteria, yeasts and fungi respectively, were determined per gram of plant material.

These results are represented in Figures 4.3 - 4.8. The cfu/g of yeasts and of fungi remained approximately constant during the 3-week sampling period for all 6 treatments, that is, their numbers were not influenced by either treatment or the time which had elapsed since treatment. The cfu/g of lactic acid bacteria were low (*ca*. 10 - 100 cfu/g) on the control plants and these numbers were not influenced by the time which had elapsed since treatment. The log total number of aerobic bacteria/g on these controls decreased by 0,046 per day (b = -0,046) (Table 4.1).

On the plants treated with lactic acid bacteria (treatments 2-6), there was a strong positive correlation (r = 0,832; P = 0,0001) between the total number of aerobic bacteria/g and the lactic acid bacteria/g, indicating that the lactic acid bacteria grew on the Std 1 agar under aerobic conditions. Both the total aerobic bacterial numbers and the lactic acid bacterial numbers decreased during the 3-week sampling period (Table 4.1). The number of lactic acid bacteria/g decreased slightly faster than the total number of aerobic bacteria/g. The greatest difference in these two ratios was found in the case of treatment 6 (*L. plantarum* L1515).

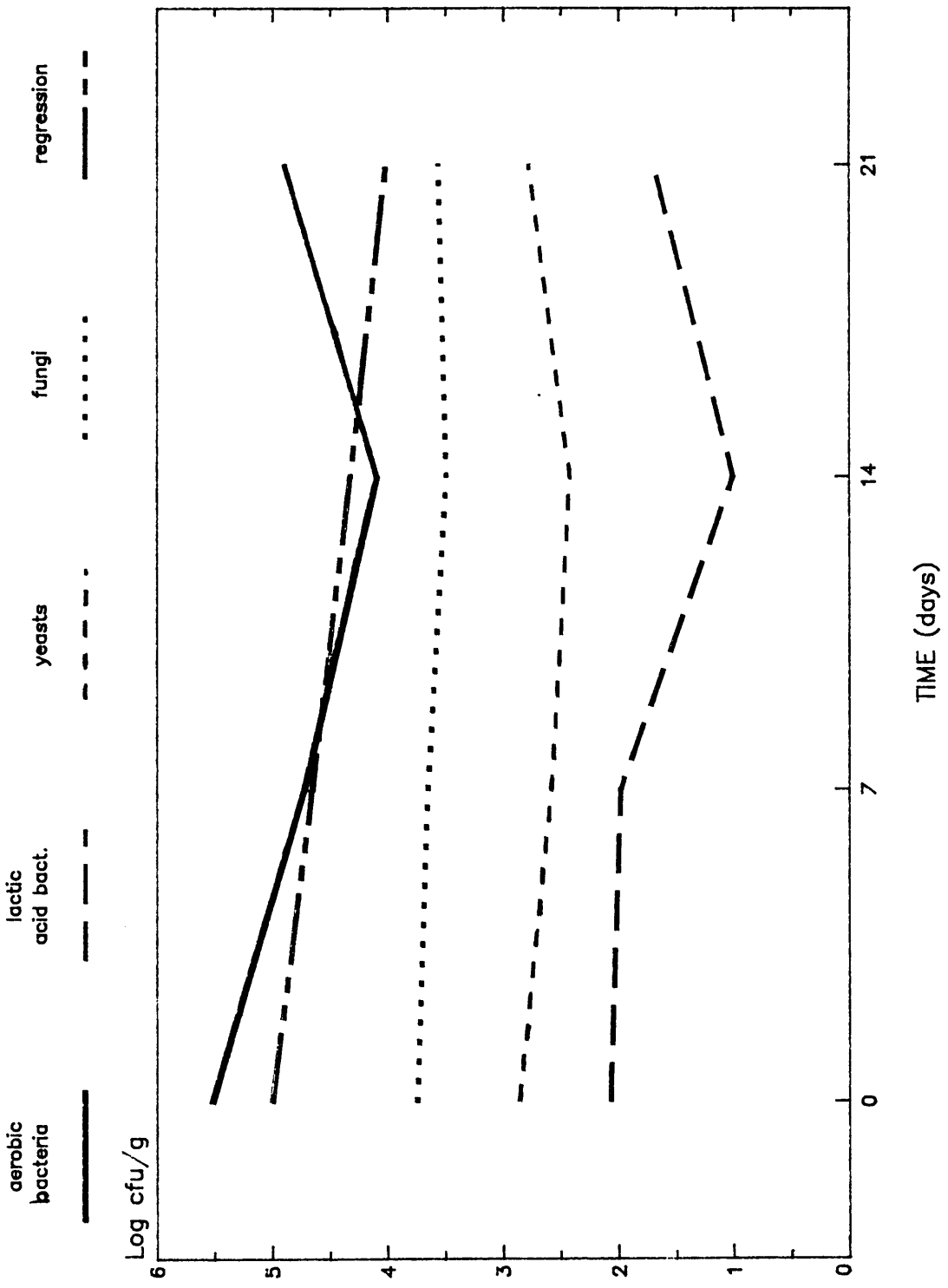


FIGURE 4.3 The number of lactic acid bacteria, total number of aerobic bacteria, yeasts and fungi (as Log cfu/g) on leaves of control plants, that is, plants not treated with lactic acid bacteria.

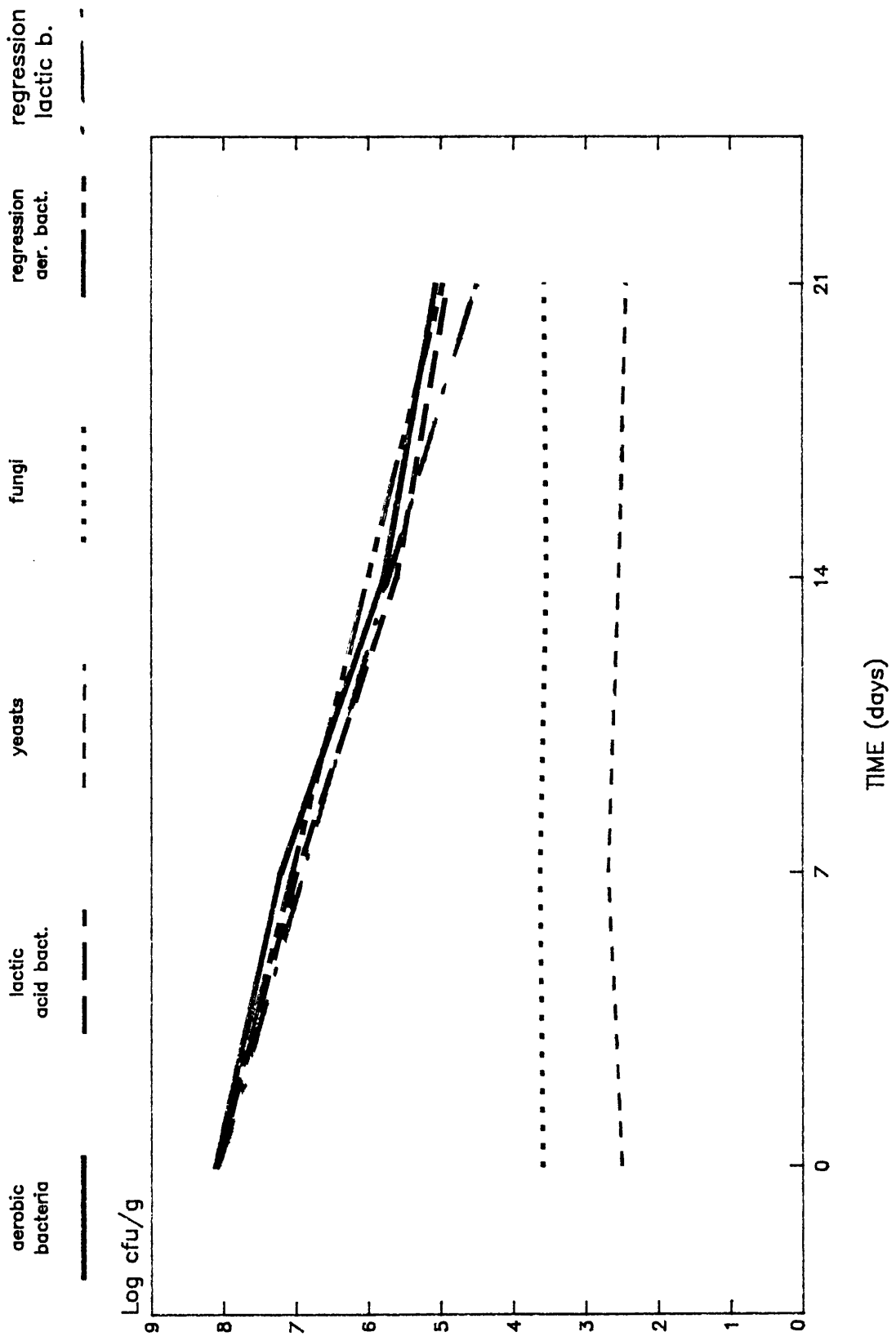


FIGURE 4.4 The number of lactic acid bacteria, total number of aerobic bacteria, yeasts and fungi (as Log cfu/g) on leaves of plants treated with *Leuconostoc mesenteriodes* L1079.

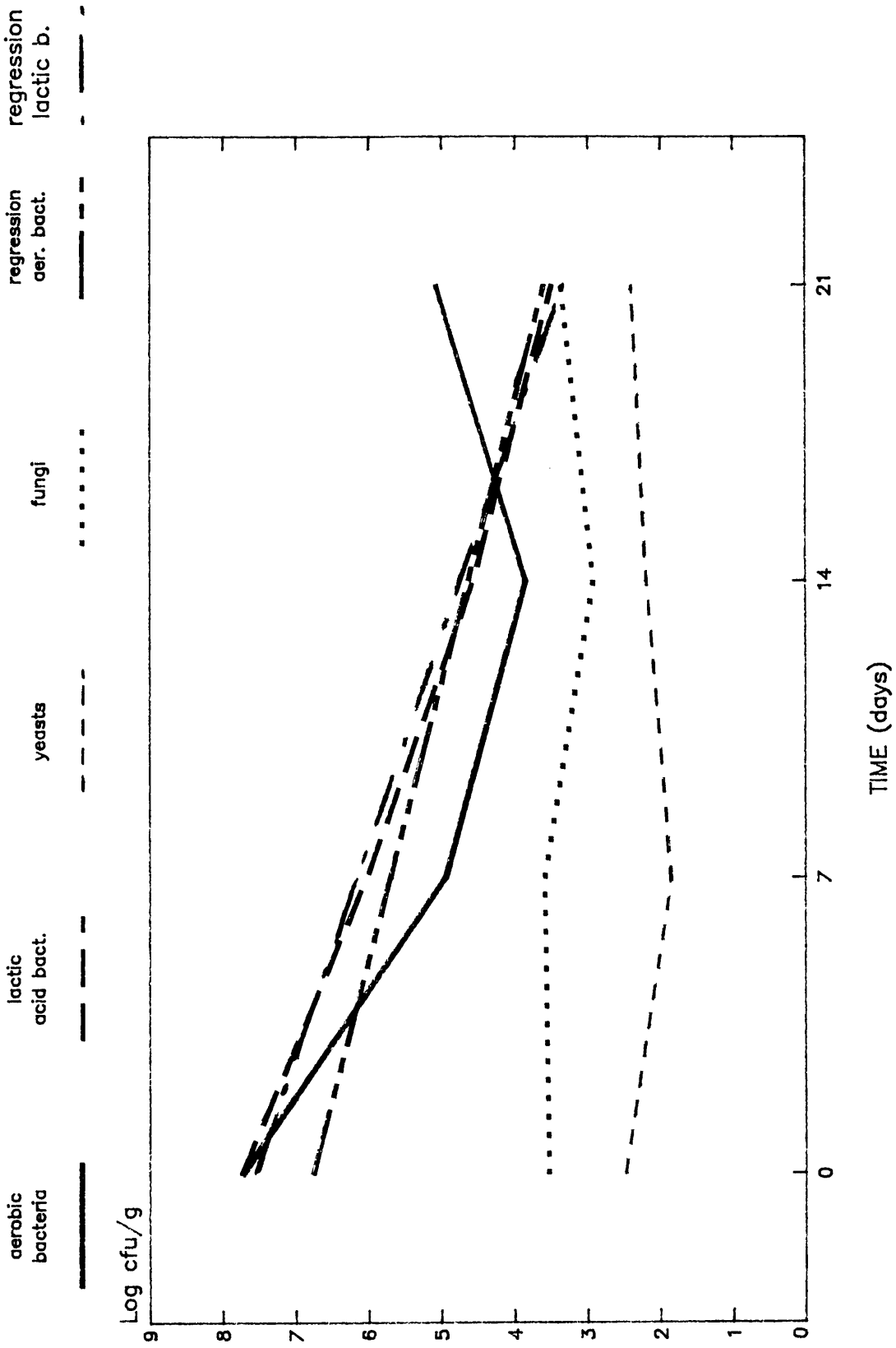


FIGURE 4.5 The number of lactic acid bacteria, total number of aerobic bacteria, yeasts and fungi (as Log cfu/g) on leaves of plants treated with *Lactobacillus brevis* L1084.

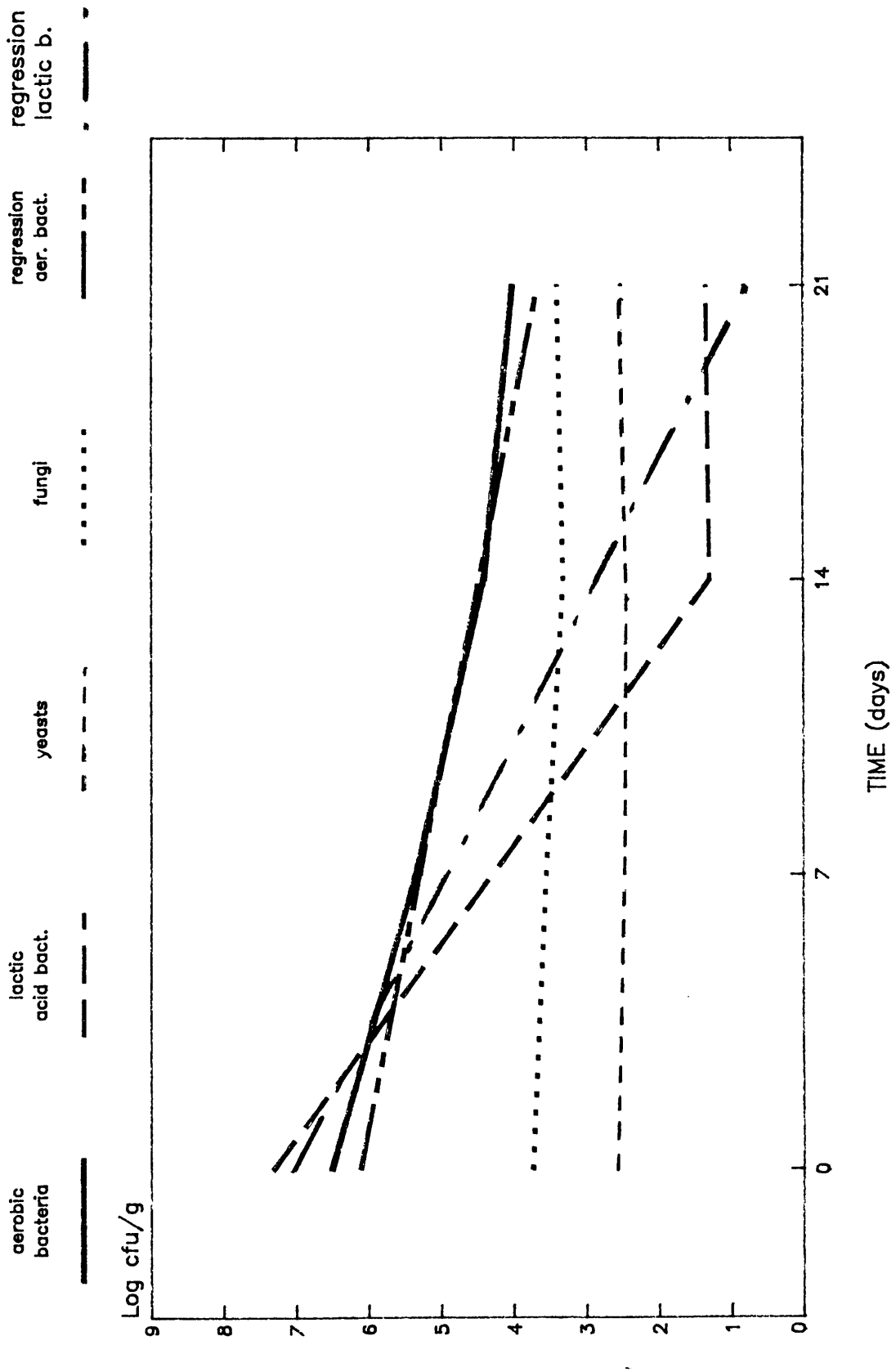


FIGURE 4.6 The number of lactic acid bacteria, total number of aerobic bacteria, yeasts and fungi (as Log cfu/g) on leaves of plants treated with *Lactobacillus sake* L2522.

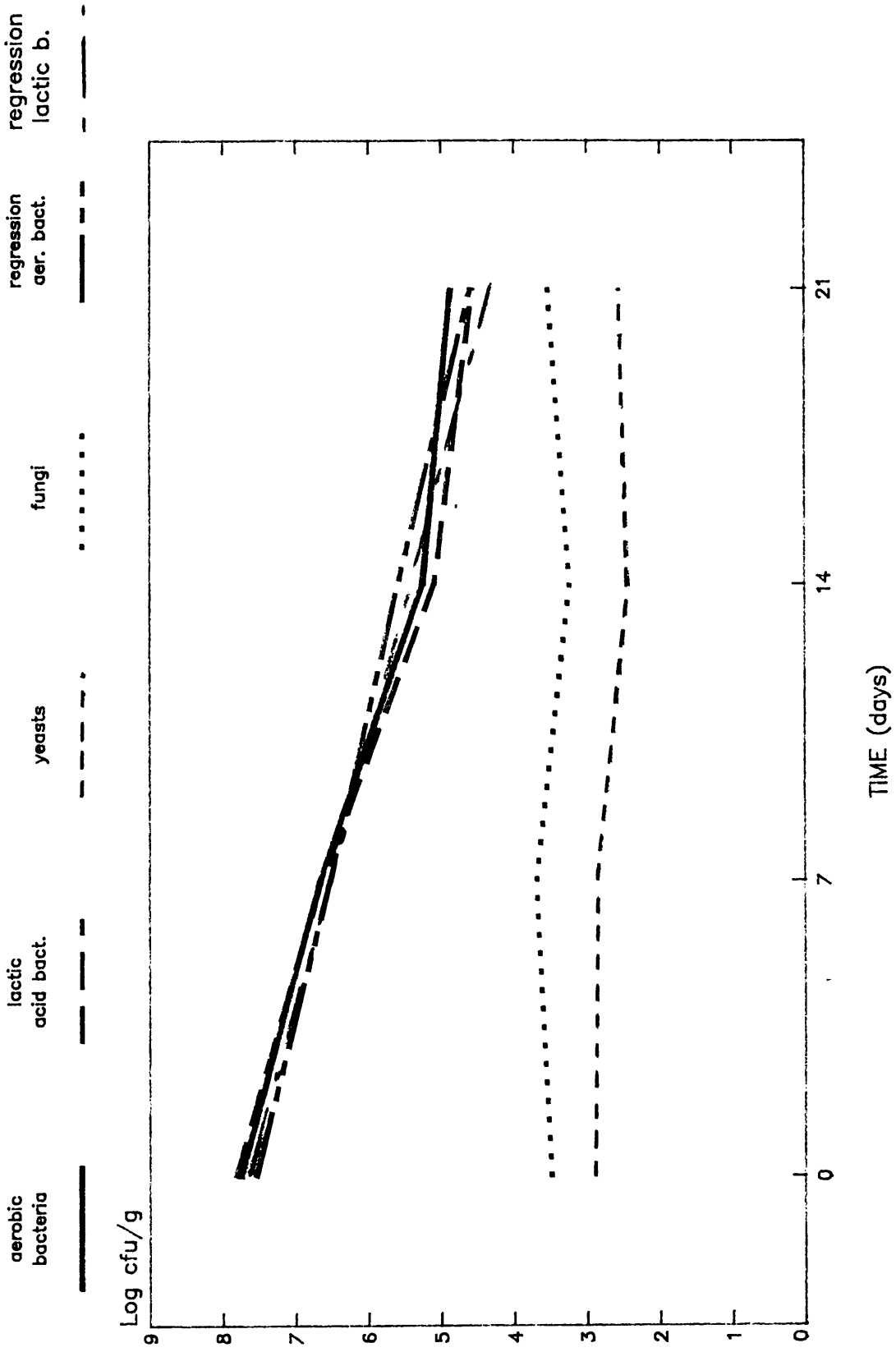


FIGURE 4.7 The number of lactic acid bacteria, total number of aerobic bacteria, yeasts and fungi (as Log cfu/g) on leaves of plants treated with *Lactobacillus plantarum* L373.

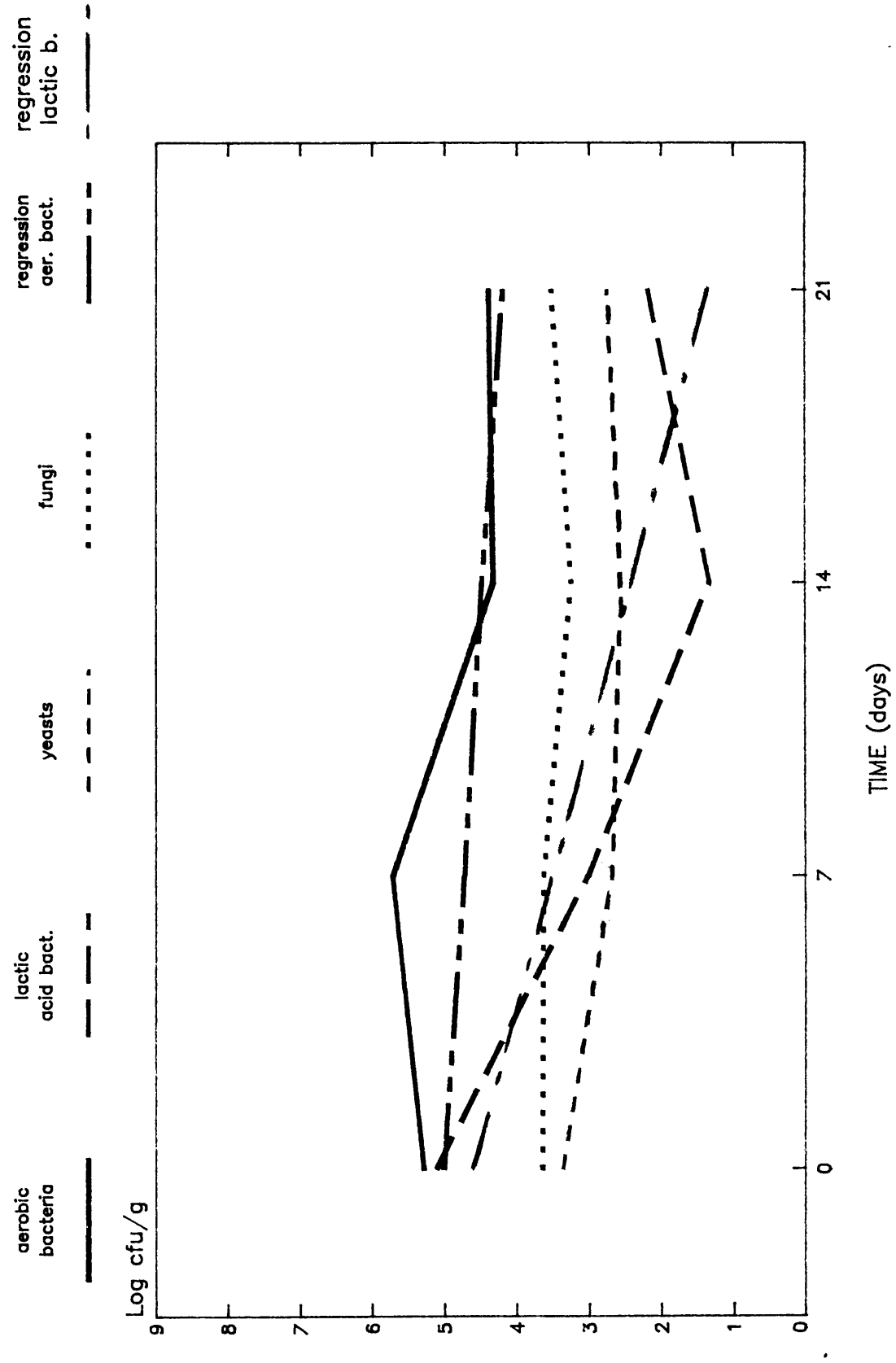


FIGURE 4.8 The number of lactic acid bacteria, total number of aerobic bacteria, yeasts and fungi (as Log cfu/g) on leaves of plants treated with *Lactobacillus plantarum* L1515A.

TABLE 4.1 Daily changes in the log cfu/g of total aerobic bacteria and of lactic acid bacteria on bean plants over a 3-week sampling period.

Treatment	Daily changes in log cfu/g of: Total aerobic bacteria	lactic acid bacteria
(1) Control	-0,046[a]	-[b]
(2) *Lc. mesenteroides* (L1079)	-0,160	-0,173[c]
(3) *L. brevis* (L1084)	-0,155	-0,233
(4) *L. sake* (L2522)	-0,115	-0,287
(5) *L. plantarum* (L373)	-0,141	-0,165
(6) *L. plantarum* (L1515A)	-0,043	-0,170

(a) Slope (b-value) of linear regression curve, that is change in log cfu/g per day.

(b) Regression was not significant, that is, log cfu/g was not influenced by time.

(c) The differences in lactic acid bacterial counts for the different treatments were not significant (p = 0,95).

Of the 5 lactic acid bacterial strains tested, *L. plantarum* L373 had the best survival rate, (b = 0,165) on the leaves of bean plants in the fibre glass tunnel. Strain L1515A survived almost as well (b = 0,170) and was a much stronger antagonist *in vitro* (section 2.4.2). The b-value of *L. plantarum* L1515A in the greenhouse was -0,126 (section 4.4.1) and in the tunnel it was -0,170 (Table 4.1). This difference, although not great, could indicate that the more controlled temperature in the greenhouse (25-30°C) was less detrimental to the survival of the lactic acid bacteria on the phylloplane (in the fibre glass tunnel, the temperature varied between 15°C and 30°C).

The relative survival of the lactic acid bacterial strains on the phylloplane, is shown in Figure 4.9 as a function of the log percentage surviving lactic acid bacteria. *L. plantarum* L1515A showed the highest survival rate (-0,95 log %) after 21 days although there was a lower log % of these bacteria present on day 14.

The adequate survival of the antagonist on the phylloplane is absolutely essential to the kind of biological control investigated here. Thus, before lactic acid bacteria could be applied to plant surfaces as bio-control agents of bacterial plant diseases, further isolations, screening tests, pot trials as well as field experiments would have to be conducted in order to find the most successful biocontrol agent. Alternatively, the antagonist or the environment would have to be altered so as to support a higher survival rate of the antagonist. The antagonist could be altered by genetic manipulation (Lindeman, 1985). The environment could be altered by, for example, adding nutrients to surface sprays which leads to improved disease control (Leben, 1985). Another alternative would be the repeated application of lactic acid bacterial surface sprays in order to keep their numbers high enough for effective biological control.

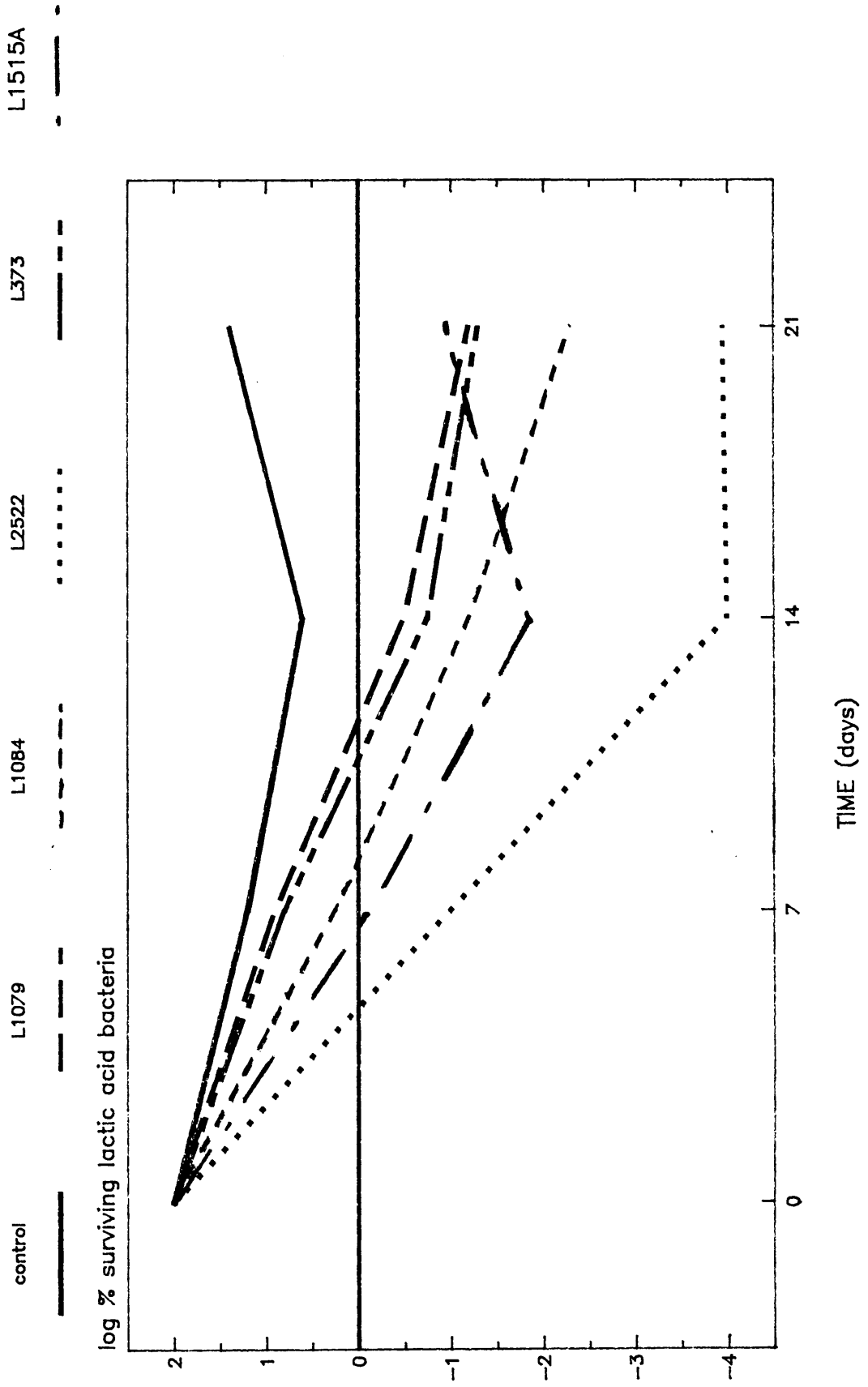


FIGURE 4.9 Relative survival of lactic acid bacteria as a function of the log % survivors.

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CHAPTER 5 : CONCLUSIONS

1. Various strains of lactic acid bacteria isolated from plants and plant-associated products as well as authentic strains of *Lactobacillus plantarum*, were found to be antagonistic to 5 test strains of foliar phytopathogenic bacteria of the following species: *Xanthomonas campestris*, *Erwinia carotovora* and *Pseudomonas syringae*.

2. Effective antagonism was observed in agar diffusion tests where 37 of the 43 test strains of lactic acid bacteria caused varying degrees of inhibition, as indicated by the diameter of the inhibition zones.

3. The antagonistic effect of the plant-associated lactic acid bacteria was found to be even more dramatic in associative broth cultures where the phytopathogenic bacteria were completely killed within 12 - 36h.

4. Pot trials indicated that the application of an *L. plantarum* strain to young bean plants as a surface spray prior to treatment with *P. syringae*, significantly reduced the incidence of halo blight in the greenhouse.

5. The production of lactic acid by the lactic acid bacteria was found to be the major mechanism of antagonism. Lactic acid and acetic acid were both inhibitory to the plant pathogens. In associative broth cultures where the pH reduction by the lactic acid bacteria was buffered, slight inhibition still occurred but the phytopathogenic bacteria were not killed as in the case of unbuffered cultures.

6. Hydrogen peroxide production by the lactic acid bacteria seemed insignificant as a mechanism of antagonism and cell free filtrates of the lactic acid bacteria were not in the least inhibitory to the phytopathogenic bacteria.

7. The survival of 5 strains of lactic acid bacteria on bean plants was investigated in the greenhouse and fibre glass tunnel. The number of viable lactic acid bacteria on the phylloplane decreased by *ca*. 1 log cfu/g per week. This rate was closely related to that of the total number of aerobic bacteria on the phylloplane but unrelated to the survival of yeasts and fungi.

8. Electron microscopy of the phylloplane after the application of lactic acid bacteria to bean plants, showed the lactic acid bacteria to be concentrated in the grooves on both the adaxial and abaxial leaf surfaces. No attachment structures or penetration could be discerned.

9. Field trials investigating the survival and effectiveness of lactic acid bacteria as biocontrol agents need yet to be carried out before possible biological control of foliar bacterial pathogens by lactic acid bacteria could be claimed.

TABLE 6.1 CHARACTERISTICS OF LACTIC ACID BACTERIA

ISOLATE NO.	IDENTIFICATION	Habitat*	Lactic acid configuration	M-A_2pm	Gas from glucose	Growth at						End pH	Slime from glucose	Arginine hydrolysis	Lithmus Milk	
						4°C	15°C	19°C	45°C	pH 3,9	10% NaCl				2 days	6 days
L292	L. plantarum	a	DL	+	-	-	+	+	-	+	(+)	3,42	(+)	-	AR	ACR
L373	L. plantarum	b	DL	+	-	-	+	+	(+)	+	+	3,47	-	-	ACR	ACR
L379	L. plantarum	b	DL	+	-	-	+	+	(+)	+	+	3,47	-	-	ACR	ACR
L392A	L. plantarum	b	DL	+	-	-	+	+	(+)	+	+	3,47	-	-	ACR	ACR
L392B	L. plantarum	b	DL	+	-	-	+	+	(+)	+	+	3,46	-	-	ACR	ACR
L491	L. plantarum	c	DL	+	-	-	+	+	-	+	(+)	3,45	-	-	ACR	ACR
L1056	L. vaccinostercus	d	DL	+	+	-	+	+	+	+	(+)	3,87	-	-	-	-
L1064A	L. vaccinostercus	d	DL	+	+	-	+	+	(+)	+	-	3,98	+	(+)	-	AR
L1064B	L. brevis	d	DL	-	+	-	+	+	(+)	+	-	4,14	+	-	-	AR
L1079	Lc. mesenteroides	d	D(-)	-	+	-	+	+	-	+	-	4,10	(+)	-	-	AC
L1084	L. brevis	d	DL	-	+	-	+	+	-	+	(+)	4,13	-	(+)	-	R
L1131	L. brevis	c	DL	-	+	-	+	+	-	+	(+)	4,12	-	-	-	R
L1506	L. plantarum	e	DL	+	-	-	+	+	-	+	(+)	3,60	-	-	ACR	ACR
L1515A	L. plantarum	e	DL	+	-	-	+	+	-	+	(+)	3,61	-	-	ACR	ACR
L1518	L. plantarum	e	DL	+	-	-	+	+	-	+	(+)	3,57	-	-	ACR	ACR
L1553	L. bavaricus	e	L(+)	-	-	-	+	+	(+)	+	+	3,59	-	-	R	R
L2501	L. hilgardii	e	DL	-	(+)	-	-	-	-	+	-	4,70	-	-	-	R
L2502	L. hilgardii	e	DL	-	(+)	-	+	+	-	+	-	4,71	-	(+)	AR	AR
L2503	heterofermentative Lactobacillus sp.	e	DL	-	(+)	-	+	+	-	+	-	4,13	-	-	-	R
L2504	L. brevis	e	DL	-	+	-	+	+	-	+	-	4,14	-	-	-	-
L2506	L. casei spp rhamnosus	e	L(+)	-	-	-	+	+	-	+	(+)	3,83	-	-	-	-
L2507A	L. brevis	e	DL	-	+	-	+	+	-	+	-	4,22	-	-	AR	AR
L2507B	Lc. paramesenteroides	e	D(-)	-	+	-	+	+	(+)	+	-	4,13	-	-	AR	AR
L2508A	L. brevis	e	DL	-	+	-	+	+	(+)	+	-	4,10	-	-	AR	AR
L2508B	L. brevis	e	DL	-	+	-	+	+	(+)	+	(+)	4,10	-	-	AR	AR
L2508C	L. brevis	e	DL	-	+	-	+	+	(+)	+	-	4,10	-	-	-	AR
L2510	L. brevis	e	DL	-	+	-	+	+	-	+	-	4,18	-	-	-	-
L2521	L. sake	f	DL	-	-	-	(+)	(+)	-	(+)	-	5,31	-	-	-	-
L2522	L. sake	f	DL	-	-	-	(+)	+	-	+	(+)	5,32	-	-	-	-
L2523	L. sake	f	DL	-	-	-	-	-	-	(+)	-	5,32	-	-	-	R
L2524	Lc. mesenteroides	f	D(-)	-	+	-	+	+	-	+	-	4,03	+	-	A	ACR
L2525	L. sake	f	DL	-	-	-	(+)	(+)	-	+	-	5,17	-	-	-	-
L2601	L. plantarum	g	DL	+	-	-	+	+	+	+	(+)	3,57	-	-	ACR	ACR
L2602	L. plantarum	g	DL	+	-	-	+	+	+	+	(+)	3,54	-	-	ACR	ACR
L2603	L. plantarum	g	DL	+	-	-	+	+	+	+	(+)	3,58	-	-	ACR	ACR
L2604	heterofermentative Lactobacillus sp.	h	DL	-	+	-	+	+	-	+	-	3,99	+	(+)	-	R
L2605	Lc. mesenteroides	h	D(-)	-	+	-	+	+	-	+	-	4,02	+	-	-	R
L2606	Lc. amelibiosum	h	D(-)	-	+	-	+	+	-	+	-	3,99	+	-	-	R
L2607	Lc. mesenteroides	h	D(-)	-	+	-	+	+	-	+	-	4,02	+	-	-	R
L2608	Lc. mesenteroides	h	D(-)	-	+	-	+	+	-	+	-	4,01	+	-	-	R
DSM20205	L. plantarum	Auth	DL	+	-	-	+	+	(+)	+	(+)	3,59	-	-	ACR	ACR
ATCC8014	L. plantarum	Auth	DL	+	-	-	+	+	(+)	+	+	3,59	-	-	ACR	ACR
L136	L. plantarum	Meat	DL	+	.	-	+	+	(+)	+	+	.	.	.	ACR	ACR

Fermentation of : (5 days/25°C)

ISOLATE NO.	arabinose	cellobiose	esculin	fructose	galactose	K-D-gluconate	lactose	gas from malate	maltose	mannitol	melezitose	melibiose	raffinose	rhamnose	ribose	salisin	sorbitol	sorbose	starch	sucrose	trehalose	xilose
L292	(+)	+	(+)	+	+	+	(+)	+	+	(+)	+	(+)	+	(+)	+	+	+	(+)	(+)	(+)	+	(+)
L373	(+)	+	(+)	+	+	+	(+)	+	+	(+)	+	(+)	+	(+)	+	+	+	(+)	(+)	(+)	+	(+)
L379	(+)	+	(+)	+	+	+	(+)	+	+	(+)	+	(+)	+	(+)	+	+	+	(+)	(+)	(+)	+	(+)
L392A	(+)	+	(+)	+	+	+	(+)	+	+	(+)	+	(+)	+	(+)	+	+	+	(+)	+	(+)	+	(+)
L392B	+	+	(+)	+	+	+	(+)	+	+	(+)	+	(+)	+	(+)	+	+	+	(+)	(+)	(+)	+	(+)
L491	(+)	+	(+)	+	+	+	+	(+)	+	(+)	(+)	(+)	+	(+)	+	+	+	(+)	(+)	(+)	+	(+)
L1056	(+)	-	-	(+)	(+)	-	-	-	+	(+)	-	-	(+)	-	+	-	-	-	-	-	-	+
L1064A	(+)	(+)	+	(+)	(+)	-	(+)	-	+	(+)	-	(+)	-	-	+	(+)	-	-	-	(+)	(+)	+
L1064B	-	-	+	(+)	(+)	-	-	-	+	-	-	-	-	-	(+)	(+)	-	-	-	-	-	+
L1079	+	(+)	+	(+)	(+)	(+)	(+)	-	+	(+)	-	(+)	+	(+)	(+)	(+)	(+)	(+)	(+)	(+)	+	+
L1084	+	-	(+)	(+)	(+)	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	+
L1131	-	-	+	(+)	(+)	-	-	-	(+)	-	-	-	-	-	+	-	-	-	-	-	-	+
L1506	(+)	+	(+)	+	+	+	+	+	+	(+)	+	(+)	+	(+)	+	+	+	(+)	(+)	(+)	+	(+)
L1515A	(+)	+	(+)	+	+	+	(+)	+	+	(+)	(+)	(+)	+	(+)	+	+	+	(+)	(+)	+	+	(+)
L1518	(+)	+	+	+	+	+	+	+	+	(+)	+	(+)	+	(+)	+	+	(+)	(+)	(+)	(+)	+	(+)
L1553	(+)	+	+	+	+	+	(+)	-	+	+	+	+	-	(+)	+	+	+	+	+	+	+	(+)
L2501	(+)	(+)	-	-	-	-	-	-	(+)	-	-	-	-	-	+	-	-	-	-	-	-	+
L2502	(+)	-	-	-	(+)	-	-	-	(+)	-	-	-	-	(+)	+	-	(+)	-	-	-	-	+
L2503	-	-	-	(+)	(+)	-	-	-	(+)	-	(+)	-	(+)	-	(+)	-	-	-	-	-	-	-
L2504	-	-	+	(+)	(+)	-	-	-	(+)	-	-	-	-	-	(+)	-	-	-	-	-	-	+
L2506	(+)	(+)	-	+	-	+	-	+	+	+	-	(+)	-	+	(+)	-	-	-	(+)	(+)	(+)	-
L2507A	(+)	(+)	-	-	+	(+)	-	-	(+)	-	-	(+)	-	-	+	-	-	-	-	-	(+)	-
L2507B	(+)	-	(+)	-	+	(+)	-	-	+	-	-	(+)	-	(+)	+	-	-	(+)	-	-	-	+
L2508A	(+)	(+)	+	-	+	(+)	-	-	(+)	-	-	(+)	-	-	+	-	-	-	-	-	-	+
L2508B	(+)	-	-	-	+	(+)	-	-	(+)	-	-	(+)	-	-	+	-	-	-	-	-	-	+
L2508C	(+)	-	(+)	-	+	(+)	-	-	(+)	-	-	(+)	-	-	-	-	-	-	-	-	-	+
L2510	-	-	-	(+)	(+)	-	-	-	(+)	-	-	-	-	-	(+)	-	-	-	-	-	-	+
L2521	-	-	-	(+)	-	-	-	-	-	-	-	(+)	-	(+)	-	-	-	-	(+)	-	(+)	-
L2522	-	-	-	(+)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L2523	-	-	-	(+)	-	-	-	-	-	-	-	(+)	-	-	-	-	-	-	-	(+)	-	-
L2524	(+)	-	+	+	+	-	-	+	(+)	-	-	-	+	-	(+)	+	-	-	-	(+)	-	+
L2525	(+)	(+)	-	(+)	-	(+)	(+)	-	(+)	(+)	-	(+)	-	-	(+)	-	-	-	(+)	(+)	(+)	(+)
L2601	+	+	(+)	+	+	+	(+)	+	+	(+)	+	(+)	+	+	+	+	+	+	(+)	(+)	+	+
L2602	+	+	(+)	+	+	+	(+)	(+)	+	(+)	+	(+)	+	+	+	+	+	+	(+)	(+)	+	+
L2603	+	+	(+)	+	+	+	(+)	+	+	(+)	+	+	+	+	+	+	+	+	(+)	+	(+)	+
L2604	+	(+)	+	+	(+)	(+)	-	-	+	-	-	(+)	-	-	-	(+)	-	-	-	-	-	-
L2605	+	-	+	+	(+)	-	-	+	(+)	-	-	-	-	-	-	(+)	-	-	-	-	(+)	-
L2606	+	-	+	+	(+)	-	-	(+)	(+)	-	-	-	-	-	-	(+)	-	-	-	-	-	-
L2607	+	-	+	+	(+)	-	-	+	(+)	-	-	-	-	-	-	(+)	-	-	-	-	(+)	-
L2608	+	-	+	+	(+)	-	(+)	+	(+)	-	-	-	-	-	-	(+)	-	-	-	-	-	-
DSM20205	+	+	(+)	+	+	+	(+)	(+)	+	(+)	+	(+)	+	+	+	+	+	(+)	(+)	(+)	+	(+)
ATCC8014	+	+	(+)	+	+	+	(+)	+	+	(+)	+	(+)	+	+	+	+	+	(+)	(+)	(+)	+	(+)
L136	.	.	.	.	+	.	.	+	.	.	+	.	(+)	(+)	.	+	+	(+)	.	.	.	.

* a = sour-dough; b = indigenous South African plants; c = sauerkraut; d = blossoms; e = ins; f = haricot beans; g = coffee extract; h = mageu;
Symbols: + = good growth/acid production (yellow); (+) = mild growth/acid (orange); - = owth/acid (red); . = not determined
All cultures negative for: gas from K-D-gluconate, motility, gelatinase activity and alase activity. Nitrate reduction only one positive strain: L2501;
Haem-requiring catalase activity only one positive strain: L2601, Lithmus milk reacti acid (pink); C = coagulated; R = reduction (loss of colour).

TABLE 6.2 DNA analyses of certain lactic acid bacterial isolates

Isolate No.	Tm value (°C)	mol % G + C
L1084	88,8	47,30
L2503	87,0	42,94
L2508C	88,3	46,12
L2522	87,0	42,94
L2523	86,9	42,70
L2604	85,5	39,30
L2606	87,8	44,89

TABLE 6.3 Molar ratios of the key amino acids in the cell walls of certain lactic acid bacterial isolates

	Molar ratios of amino acids				
Isolate No.	Glu*	Lys	Ala	Asp	Peptidoglycan type
L2503	1	1	2	1	Lys-Asp
L2507B	1	1	2	1	Lys-Asp
L2508C	1	1	2	1	Lys-Asp
L2604	1	1	2	1	Lys-Asp
L2606	1	1	4	0	Lys-$(Ala)_2$

* Glu = glutamic acid
Lys = lysine
Ala = alanine
Asp = aspartic acid

TABLE 6.4 Average inhibition zone - diameter (in mm) of the lactic acid bacteria against the 5 test strains of phytopathogenic bacteria

Lactic acid bacterial isolate no.	Diameter of inhibition zones (mm)				
	Ps 1[a]	Ps 2	Ps 3	Erw	Xan
L292	17,60[b]	26,17	20,43	20,33	29,57
L373	15,47	22,03	16,83	15,23	25,77
L379	18,17	26,47	22,40	21,10	32,90
L392A	18,17	27,00	21,67	23,47	30,17
L392B	14,33	23,93	15,97	16,37	27,60
L491	18,28	23,90	19,67	17,40	29,77
L1056	18,55	22,43	21,73	20,87	31,90
L1064A	16,07	23,50	17,13	17,23	26,80
L1064B	15,27	24,37	16,30	14,83	29,53
L1079	14,77	21,43	16,43	14,87	26,93
L1084	17,50	19,77	18,00	17,83	29,70
L1131	17,10	26,83	17,77	18,20	29,37
L1506	20,50	26,40	21,53	19,27	31,07
L1515A	20,20	25,47	22,03	21,27	33,10
L1518	17,47	24,90	22,57	21,73	33,83
L1553	17,43	25,80	20,70	17,67	27,97
L2501	-[c]	18,10	12,97	12,29	20,03
L2502	11,50	20,23	14,10	12,76	20,20
L2503	18,43	22,17	18,87	15,20	29,77
L2504	14,67	22,90	18,67	18,07	26,93
L2506	17,10	24,47	19,73	21,00	29,93

TABLE 6.4 / Continued

Lactic acid bacterial isolate no.	Diameter of inhibition zones (mm)				
	Ps 1[a]	Ps 2	Ps 3	Erw	Xan
L2507A	12,83	16,97	14,87	14,77	23,80
L2507B	13,83	18,47	17,90	15,63	30,47
L2508A	18,03	25,83	21,20	20,63	30,63
L2508B	14,50	19,03	16,20	14,60	27,23
L2508C	15,27	21,87	16,53	14,13	28,20
L2510	17,13	22,17	17,33	16,93	28,63
L2521	19,90	24,97	18,73	20,10	32,17
L2522	18,73	22,77	20,70	20,37	33,87
L2523	13,67	21,77	16,57	15,67	26,87
L2524	-	-	-	-	-
L2525	-	10,83	12,77	-	18,20
L2601	16,50	22,97	18,03	18,10	28,13
L2602	17,47	22,43	19,23	18,53	28,83
L2603	17,13	23,47	21,37	18,37	29,30
L2604	-	-	-	-	-
L2605	-	-	-	-	-
L2606	-	-	-	-	-
L2607	12,10	17,43	13,47	11,97	24,70
L2608	12,63	15,37	13,13	11,00	24,10
DSM 20205	16,37	22,40	19,97	18,70	27,07
ATCC 8014	14,73	21,67	18,00	16,47	29,00
L136	14,83	22,73	19,90	17,07	30,03

a Ps 1 = *Xanthomonas campestris*
Ps 2 = *Pseudomonas syringae*
Ps 3 = *P. syringae* var *capsici*
Erw = *Erwinia carotovora*
Xan = *X. campestris* pv. *mangiferaeindicae*

b Average of triplicate readings on separate agar plates

c No inhibition zone surrounded the lactic acid bacterial disc