

Chapter Six

6. Characterisation of lactic acid bacteria with antifungal capabilities: physiological studies and the interaction with yeast

6.1 Introduction

The work presented in this chapter was conducted to obtain a broader understanding of the antifungal activity expressed by the organisms identified in Section 5. The organisms were characterised metabolically, their activity towards a larger range of spoilage organisms investigated, and their ability to inhibit yeast under different conditions was studied.

6.2 Metabolic profile

The 12 strains found in Section 5.3 to be active in the initial screen were assessed for acidifying capacity by growing them in replicate anaerobic cultures of SGM (Section 3.4). The results are shown in Table 6.1. As expected, the control strain MTD/1 was very effective at lowering the pH, but not significantly better than other strains which had a positive antifungal effect, such as LPC-22. This suggests that the antifungal effect caused by the test organisms is not due to their ability to acidify the medium.

In the previous chapter, three of these twelve strains were identified as warranting closer scrutiny. The metabolic profiles of these three strains, and the control organism MTD/1, were determined using an API 50-CHL kit (API-bioMeriuex). This kit consists of a series of cuvettes containing the metabolite of interest and a pH sensitive dye. Growth

on the metabolite is marked by a decrease in pH. The manufacturer's recommended protocol was followed, and the results are shown in Table 6.2.

A comparison of the results with the manufacturer's estimations of the metabolic profiles of type strains suggested that the strains were all either *Lactobacillus plantarum* or *Lactobacillus pentosus*, although no strain completely matched the profile for any species. For example, the ability of LPC-22, M0050 and MTD/1 to metabolise β Methyl-xyloside is puzzling, since neither *L. plantarum* nor *L. pentosus* should have this ability.

Table 6.1. pH of test cultures after a 24 hour incubation in SGM. Bars connect means which are not significantly different (Students t-test).

Strain	Final pH; mean (SEM), $n=6$
55-1	4.04 (0.01)
<i>Lactobacillus plantarum</i> MTD/1	4.18 (0.05)
LPC-22	4.20 (0.05)
L18	4.21 (0.01)
U392	4.22 (0.01)
LPE-11	4.26 (0.01)
M0042	4.45 (0.01)
M0050	4.47 (0.04)
SI 15	4.51 (0.05)
L17	4.71 (0.01)
L10B	4.72 (0.01)
SI 8a	4.85 (0.03)

Table 6.2. Metabolic profile of selected antifungal strains and the control strain MTD/1. (An “X” indicates a positive result. Numbers are the percentage of type strains found positive, according to the manufacturer. A positive result indicates an ability to metabolise the substrate).

Metabolite	<i>L. plantarum</i>	<i>L. pentosus</i>	LPC-22	M0042	M0050	MTD/1
Control	0	0	-	-	-	-
Glycerol	1	75	X	-	-	-
Erythritol	0	0	-	-	-	-
D-Arabinose	0	0	-	-	-	-
L-Arabinose	74	100	X	X	X	X
Ribose	92	100	X	X	X	X
D-Xylose	2	100	-	X	X	X
L-Xylose	0	0	-	-	-	-
Adonitol	0	0	-	-	-	-
β Methyl-xyloside	0	0	X	-	X	X
Galactose	92	100	X	X	X	X
D-Glucose	100	100	X	X	X	X
D-Fructose	100	100	X	X	X	X
D-Mannose	100	100	X	X	X	X
L-Sorbose	2	0	-	-	-	-
Rhamnose	33	25	-	X	-	X
Dulcitol	0	0	-	-	-	-
Inositol	0	0	-	-	-	-
Mannitol	99	100	X	X	X	X
Sorbitol	78	100	X	X	X	X
α Methyl-D-mannoside	55	1	-	X	-	X
α Methyl-D-glucoside	33	50	X	X	X	X
N Acetyl glucosamine	100	100	X	X	X	X
Amygdaline	94	100	X	X	X	X
Arbutine	99	99	X	X	X	X
Esculine	99	100	X	X	X	X
Salicine	99	100	X	X	X	X
Cellobiose	99	100	X	X	X	X
Maltose	100	100	X	X	X	X
Lactose	99	100	X	X	X	X
Melibiose	94	100	X	X	X	X
Saccharose	88	100	X	X	X	X
Trehalose	96	100	X	X	X	X
Inuline	0	0	-	-	-	-
Melezitose	92	25	X	X	X	X
D-Raffinose	74	75	X	X	-	X
Amidon	7	0	-	-	-	-
Glycogene	7	0	-	-	-	-
Xylitol	0	0	-	-	-	-
β Gentiobiose	98	99	X	X	X	X
D-Turanose	62	50	-	X	-	-
D-Lyxose	0	0	-	-	-	-
D-Tagatose	7	1	-	-	-	-
D-Fucose	0	0	-	-	-	-
L-Fucose	0	0	-	-	-	-
D-Arabitol	36	0	X	-	-	-
L-Arabitol	0	0	-	-	-	-
Gluconate	62	50	X	X	X	-
2 ceto-gluconate	0	0	-	-	-	-
5 ceto-gluconate	0	0	-	X	-	-

6.3 Activity against representative yeast species and *Acetobacter* spp.

The strain which had been shown to possess the most effective antifungal action, as demonstrated both by qualitative and quantitative judgement of the agar plate screening, and in liquid co-culture (Section 6.5), was LPC-22. To examine its ability to inhibit yeast strains other than the strains which had been tested in the initial screen, the standard screening protocol (Section 3.5.2) was followed, but with different indicator organisms. One other modification was made. This was that the initial, anaerobic, phase during which the LAB are grown was extended to 48 hours, because it had previously been observed that an extended anaerobic phase markedly improved LPC-22's antifungal effect towards *S. exiguus*. The indicator strains were used at two inoculum levels, 4 μ l and 40 μ l per plate (in 15 ml SSM). Growth of the indicator species was observed relative to the growth over *Lactobacillus plantarum* MTD/1. The results are shown in Table 6.3.

For most strains there was no difference between LPC-22 and MTD/1, but LPC-22 was found to effectively inhibit a strain of *Candida holmii* (Y8) and a strain of *Saccharomyces cerevisiae* (Y14), as well as *S. exiguus*. No effect was seen against *Candida famata* (Y20), against which LPC-22 had previously observed to exert an antifungal effect (Section 5.2), a result which must cast some doubt upon the reliability of this test protocol. It was already known that wheat germ lipase (lipase I) was able to abolish the antifungal effect expressed by LPC-22 against *S. exiguus* (Section 7.1), and this proved to also be the case with *Candida holmii* Y13 and *S. cerevisiae* Y14.

An interesting footnote is that it was observed that, with some yeast strains, MTD/1 was able to exert an inhibitory effect, but only when frozen stocks were used to prepare the starter cultures (Section 3.1.5). When this effect was observed, care was taken to ensure that all tests were repeated with starter cultures taken from agar. The mechanism by which MTD/1 exerts this effect is unknown, but it is not sensitive to lipase I.

Table 6.3. The effect of test lactobacilli on the growth of potential silage spoilage organisms, mainly yeast species. The relative inhibition caused by test lactobacilli MTD/1 and LPC-22 is shown.

Yeast Strain	<i>Lactobacillus</i> strain which had the greatest inhibitory effect	Effect of Lipase I
<i>Acetobacter</i> sp.	Neither	-
<i>Candida famata</i> Y20	Neither	-
<i>Candida famata</i> Y20 (from frozen stock)	MTD/1	None
<i>Candida guilliermondii</i> Y23	MTD/1	-
<i>Candida holmii</i> Y8	LPC-22	Abolished effect
<i>Candida holmii</i> Y11	Neither	-
<i>Candida holmii</i> Y11 (from frozen stock)	MTD/1	None
<i>Candida lambica</i> Y16	Neither	-
<i>Candida lambica</i> Y18	Neither	-
<i>Candida lambica</i> Y18 (from frozen stock)	MTD/1	None
<i>Candida milleri</i> Y3	Neither	-
<i>Candida</i> sp. Y13	Neither	-
<i>Geotrichum candidum</i> Y1	Neither	-
<i>Geotrichum candidum</i> Y1 (from frozen)	Neither	-
<i>Hansenula anomola</i> Y19	Neither	-
<i>Hansenula anomola</i> Y19 (from frozen)	Neither	-
<i>Saccharomyces cerevisiae</i> Y14	LPC-22	Abolished effect
<i>Saccharomyces cerevisiae</i> Y22	Neither	-
<i>Saccharomyces exiguus</i>	LPC-22	Abolished effect
<i>Saccharomyces dairensis</i> Y17	Neither	-
<i>Trichosporon adeninovorius</i> Y4	Neither	-
<i>Verticillium psalliotae</i> Y6	Neither	-

Key: - Effect of lipase I not determined.

6.4 Antifungal activity using alternative screening techniques

6.4.1 Well diffusion test of live cultures

A starter culture of the test strains (MTD/1, LPC-22, M0042, M0050) was prepared. The well diffusion protocol (Section 3.5.3) was followed; the medium was SGM or SSM and the indicator organism was *S. exiguus*. Cultures of the test strains were added to each well (one well per plate, four plates per test organism), and the plates incubated. No inhibition was observed at any volume of test culture.

6.4.2 Killed plate test

The procedure was as described previously in the screening protocol (Section 3.5.2), except that the anaerobic incubation period was 24, 48 or 72 hours, and that before the SSM layer containing indicator yeast was overlaid, the test strains were killed. This was done by inverting the plates over a disc of filter paper soaked in 1 ml of chloroform for 30 minutes, by which time the chloroform had evaporated. The test strains used were MTD/1, LPC-22, M0042 and M0050. A very faint inhibition was observed with LPC-22 and M0042 after 72 hours anaerobic growth, but no inhibition was observed with either M0050 or MTD/1.

6.4.3 Agar plug test

The method used is detailed in Section 3.5.4. Briefly, a section of agar with cultures of the test strains (MTD/1, LPC-22, M0042, M0050) was placed onto a Petri dish containing SSM seeded with the indicator yeast (*S. exiguus*). These dishes were incubated (30°C), and observed for inhibition of yeast growth around the agar plugs. No inhibition was observed on any of the plates.

6.5 Liquid co-culture of LPC-22 and MTD/1 with target organisms

The protocol used was as described previously (Section 3.5.6). It was found that, in these conditions, MTD/1 produced a dense flocculate when grown in co-culture with *S. exiguus*. Flocculation of yeast is a phenomenon which has previously been observed both in mono-culture and in co-culture with LAB. This flocculation requires the presence of divalent cations, and is inhibited by sugars, especially mannose (Johnson *et al.*, 1988, Stratford, 1992, Santos & Yokoya, 1993). pH may also play a role, probably due to dissimilar isoelectric point of yeast and LAB (Momose *et al.*, 1969, Stratford, 1996). The flocculation of MTD/1 with *S. exiguus* makes it difficult to compare this co-culture with that of LPC-22 and *S. exiguus*, as it is likely to interfere with both bacterial and yeast growth independently of any specific antimicrobial effects.

Flocculation will also cause the CFU count of the co-culture to be underestimated, because aggregates of microbes will manifest as a single CFU. Fortunately, when serial dilutions (in 1/4-strength Ringers solution) of these cultures were made for CFU determination, the flocculation was less evident. The possibility remains, however, that the flocculation caused the CFU count in cultures containing MTD/1 to be underestimated. The CFU in co-cultures of MTD/1 or LPC-22 and *S. exiguus* were nevertheless determined. The results (Figure 6.1) show that, in the co-culture containing LPC-22, there are more bacteria and fewer yeast – suggesting that LPC-22 is a better competitor. Because of the uncertainties in CFU estimation, the effect was not investigated intensively.

In an attempt to minimise the flocculation, MTD/1 was grown with *S. exiguus* in the presence of the divalent cation chelator, EDTA. Unfortunately, the addition of EDTA was not able to prevent flocculation, but it did have an extremely inhibitory effect on both yeast and LAB growth, even at the lowest concentration tested (0.002 M). As a result it was decided not to use EDTA in the co-culture experiments.

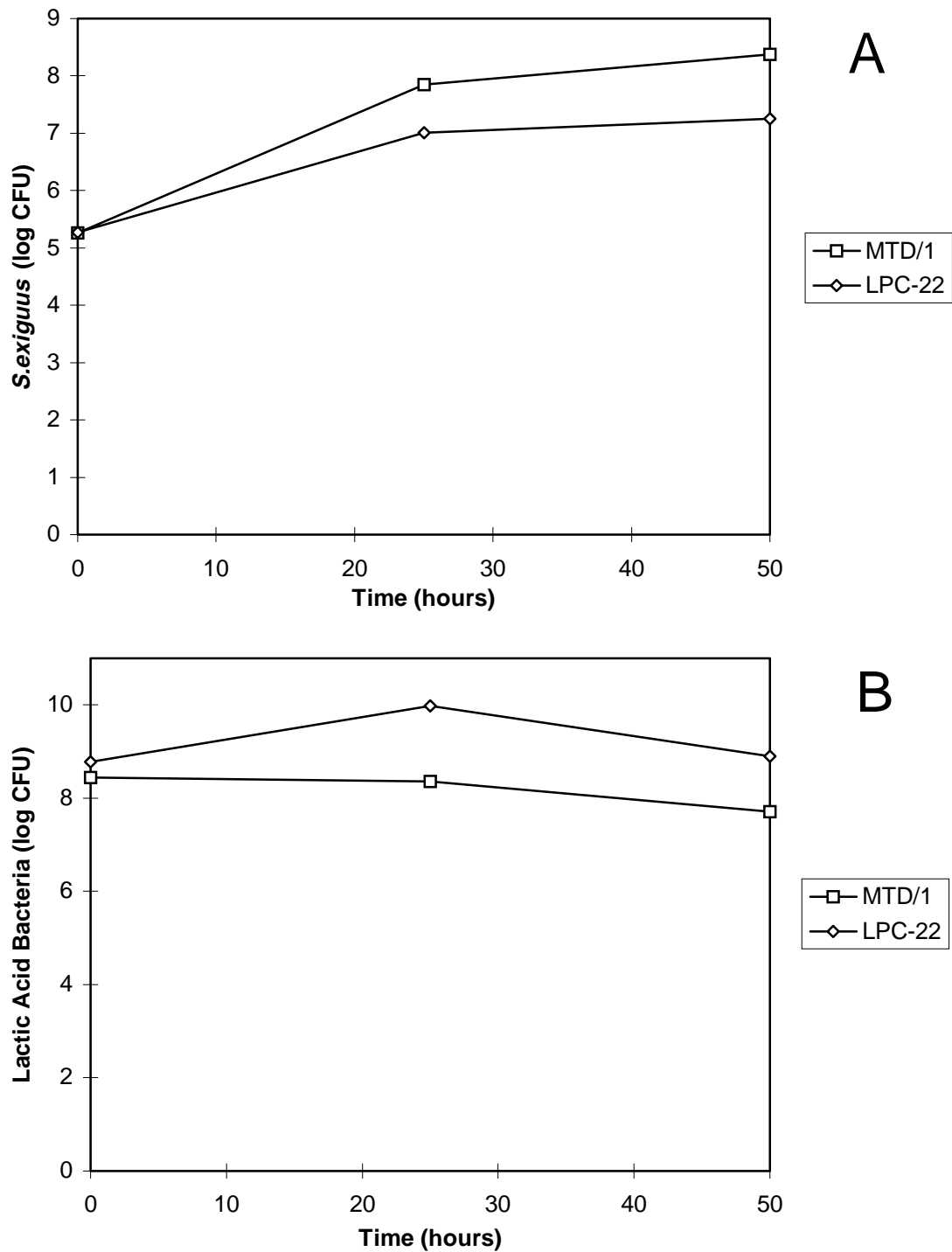


Figure 6.1. Co-culture of *S. exiguus* with the test LAB. LAB cultures were grown in SGM, before addition to an equal volume of SSM inoculated with *S. exiguus*. The co-cultures were incubated aerobically (30°C). (A) Change in yeast CFU, $n=1$. (B) Change in LAB CFU, $n=1$.

The principal aim of an antifungal inoculant is to maintain a low pH in the silage. It is also more convenient to assess the fermentation by following its pH, rather than by more laborious CFU counts. The pH of co-cultures of the test strains with the indicator organisms *S. exiguus*, *S. cerevisiae*, *C. lambica* and *Acetobacter* sp was followed and, in the case of *S. exiguus*, the effect of lipase I (which had previously shown to diminish the antifungal effect of LPC-22 and M0050, see Section 7.2) on the changing pH was studied, and the results are shown in Figure 6.2. Although several replicates were studied, no statistical analysis was attempted, firstly because it was anticipated (and observed) that the results would be non-parametric, making analysis problematic. Secondly, this work was necessarily a pilot study, with a large number of variables, a small number of replicates, and no predefined endpoint.

Figure 6.2(A) shows that, in mono-culture, the test LAB themselves maintain the pH at a low value – it drops slightly as more lactic acid is produced from sugars in the SSM. A representative spoilage organism, *S. exiguus*, causes a marked increase in pH as it metabolises the lactic acid. When the test LAB and spoilage organism are combined (Figure 6.2(B)), there is a marked difference between the cultures containing the different test organisms. MTD/1 and M0042 have almost no effect on the rise in pH caused by *S. exiguus*, whereas the rise in pH is delayed by at least 24 hours in cultures containing LPC-22 and M0050. This effect is also seen when the starting pH is made a little higher by the addition of 2M NaOH (Figure 6.2(C)). The addition of lipase I to these co-cultures (Figure 6.2(D)) was expected to alleviate the inhibition caused by LPC-22 and M0050, and this has occurred to some extent, but it also appears to have increased the inhibition caused by MTD/1.

When this test is repeated using different spoilage organisms, the results partially reflect the results found when testing on agar (Table 6.3). *C. lambica* Y18 is inhibited principally by MTD/1 (although LPC-22 is quite effective), but *S. cerevisiae* Y14 is inhibited mainly by LPC-22. When using *Acetobacter* sp, the result did not support the agar tests (where no difference could be seen between LPC-22 and MTD/1). In liquid

co-culture, the three putative antifungal organisms (LPC-22, M0042 and M0050) all had a similar effect, and were all more inhibitory than the control organism MTD/1.

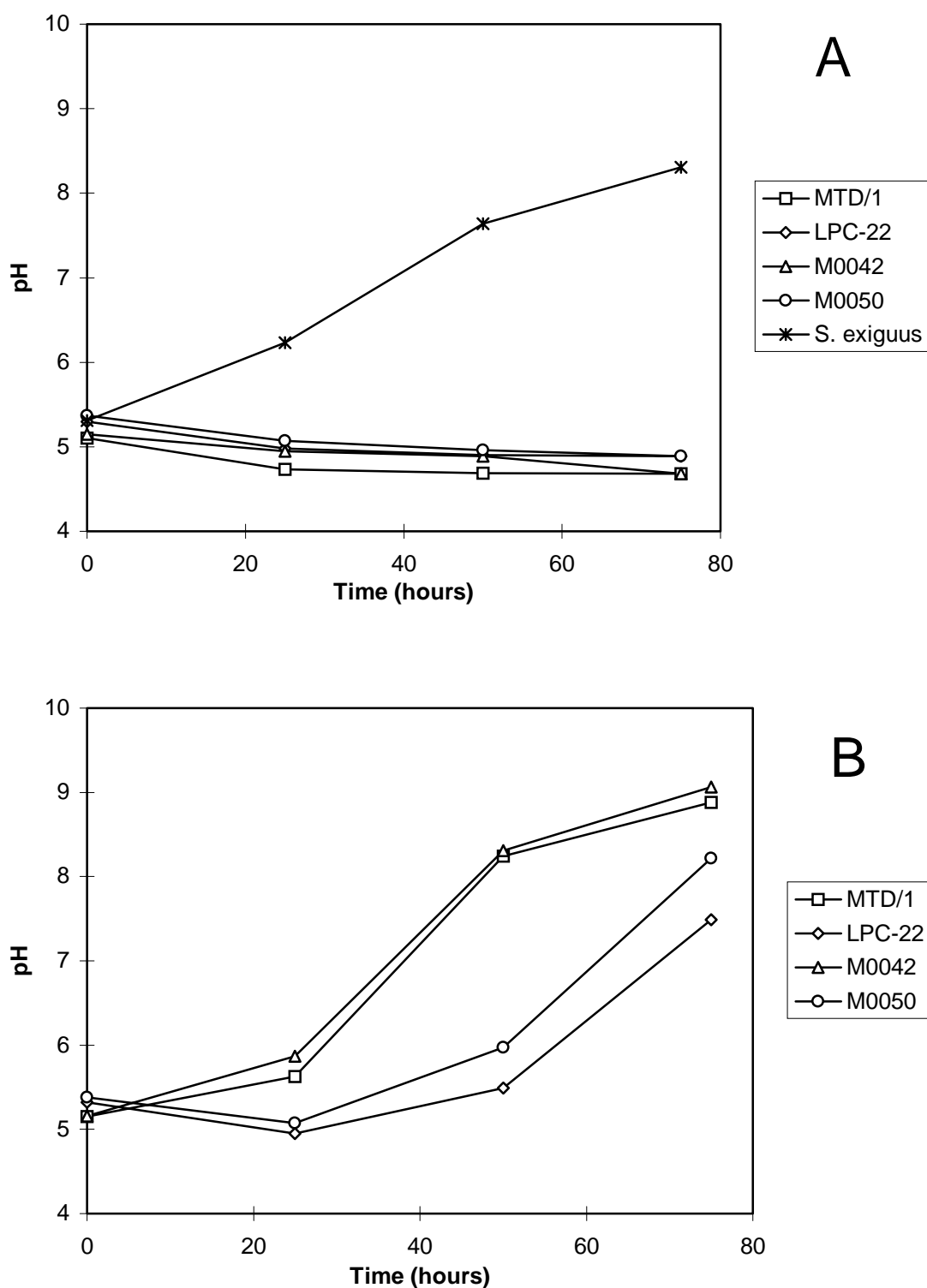


Figure 6.2. Effect on pH of the co-culture of indicator organisms with test LAB. LAB cultures were grown in SGM (30°C). before addition to an equal volume of SSM inoculated with the test organism. The cultures were incubated aerobically (30°C). **(A)** Control mono-cultures, free from indicator organism or free from test LAB, $n=1$. **(B)** Indicator organism = *S. exiguus*, initial pH 5.2, $n=4$.

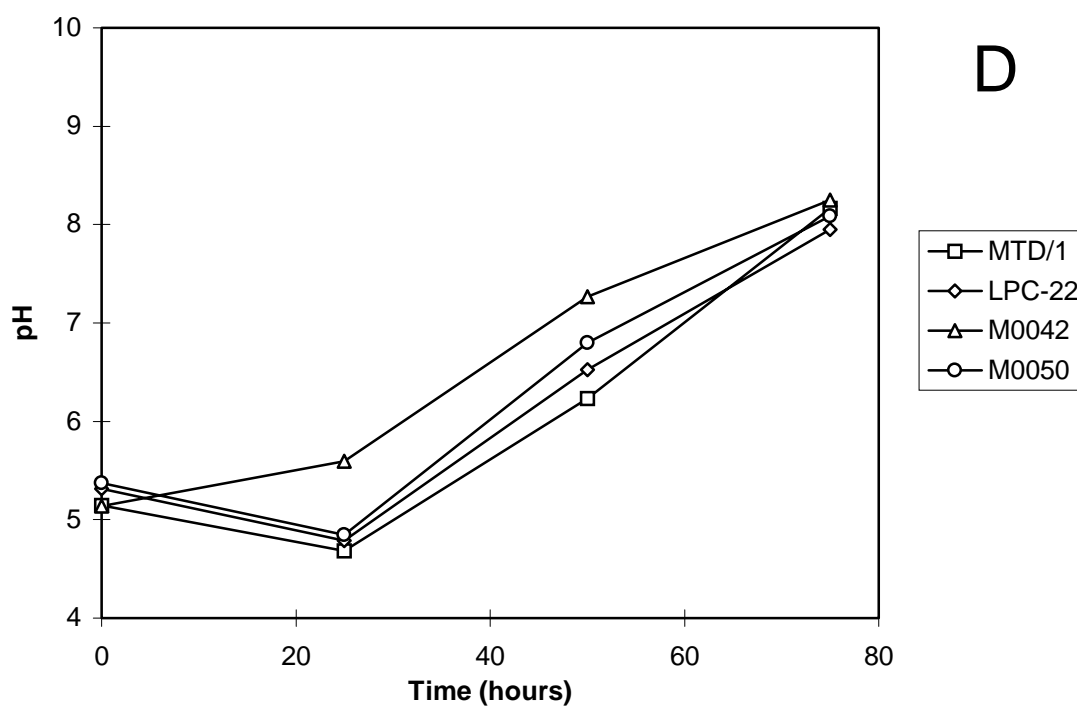
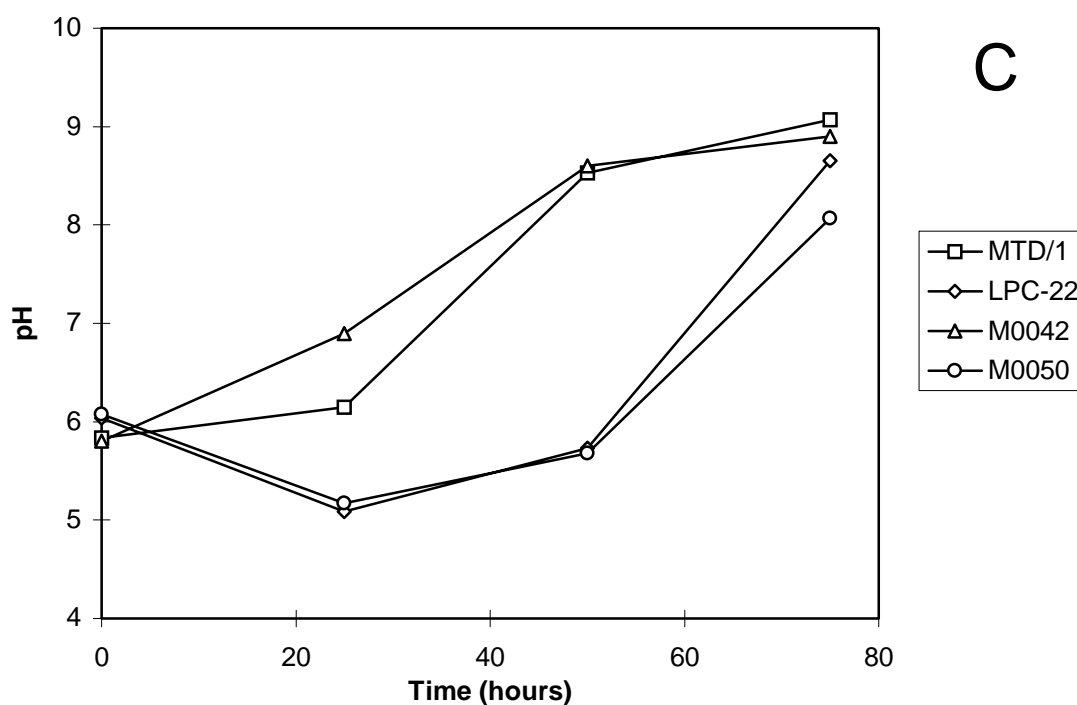


Figure 6.2 (Cont.). Effect on pH of the co-culture of indicator organisms with test LAB. LAB cultures were grown in SGM (30°C), before addition to an equal volume of SSM inoculated with the test organism. The co-cultures were incubated aerobically (30°C). (C) Indicator organism = *S. exiguus*, initial pH 6.0, *n*=2. (D) Indicator organism = *S. exiguus*, initial pH 5.2. Lipase was added to SSM at 5 mg/ml. *n*=4

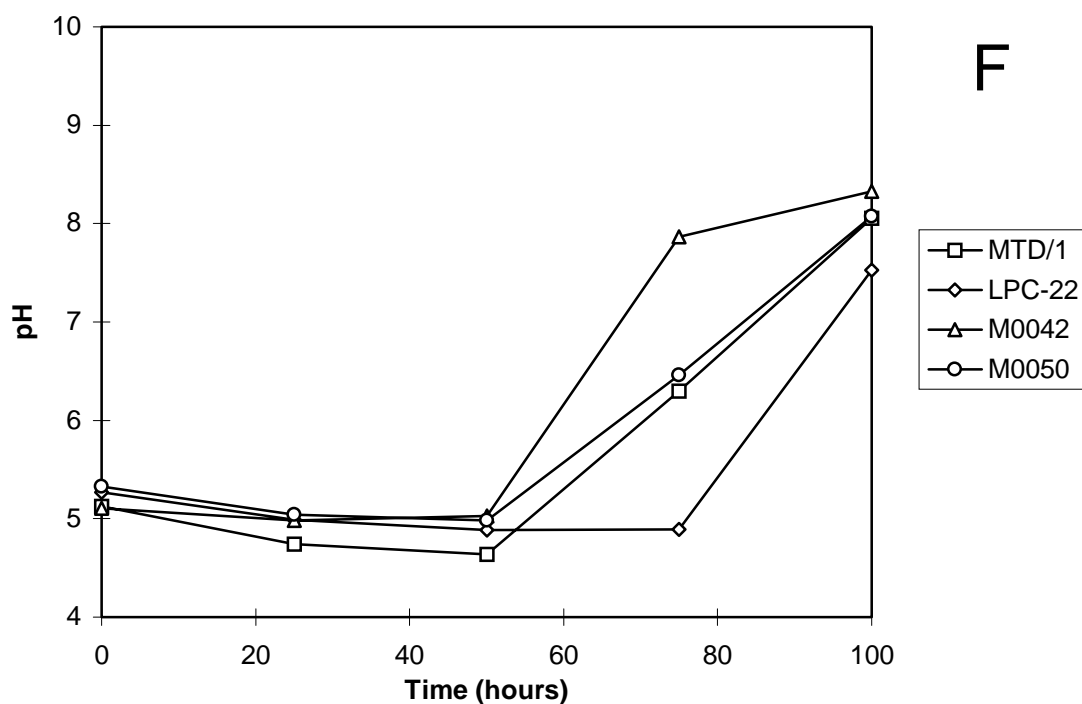
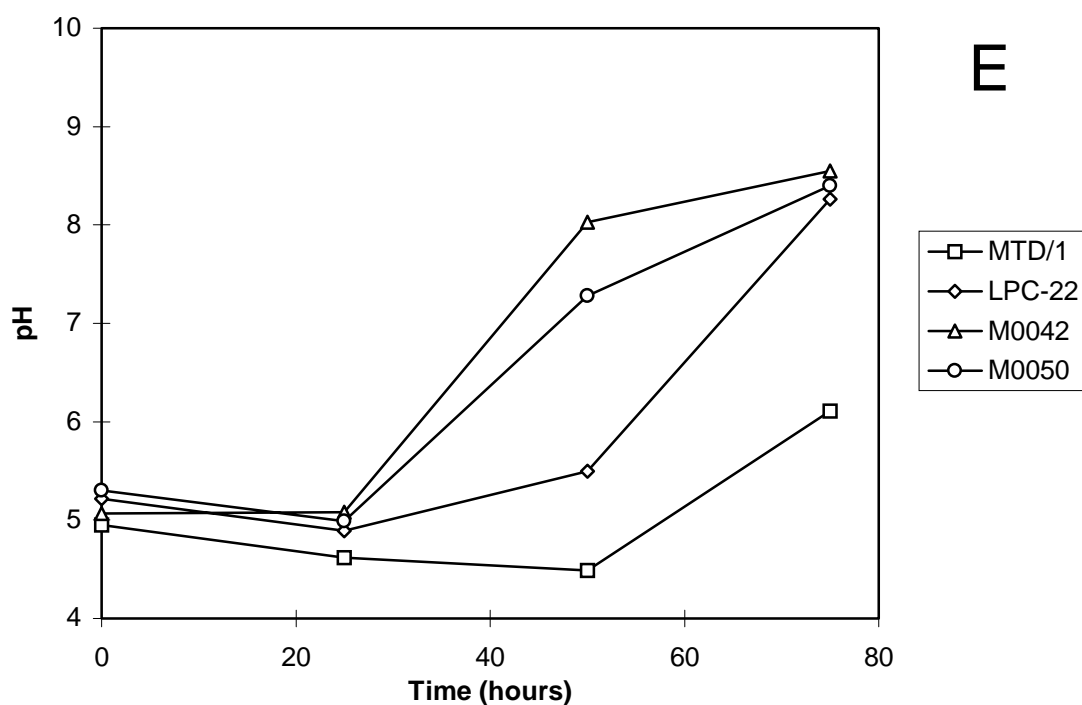


Figure 6.2 (Cont.). Effect on pH of the co-culture of indicator organisms with test LAB. LAB cultures were grown in SGM (30°C), before addition to an equal volume of SSM inoculated with the test organism. The co-cultures were incubated aerobically (30°C). **(E)** Test organism = *C. lambica* Y18, $n=1$. **(F)** Test organism = *S. cerevisiae* Y14, $n=2$.

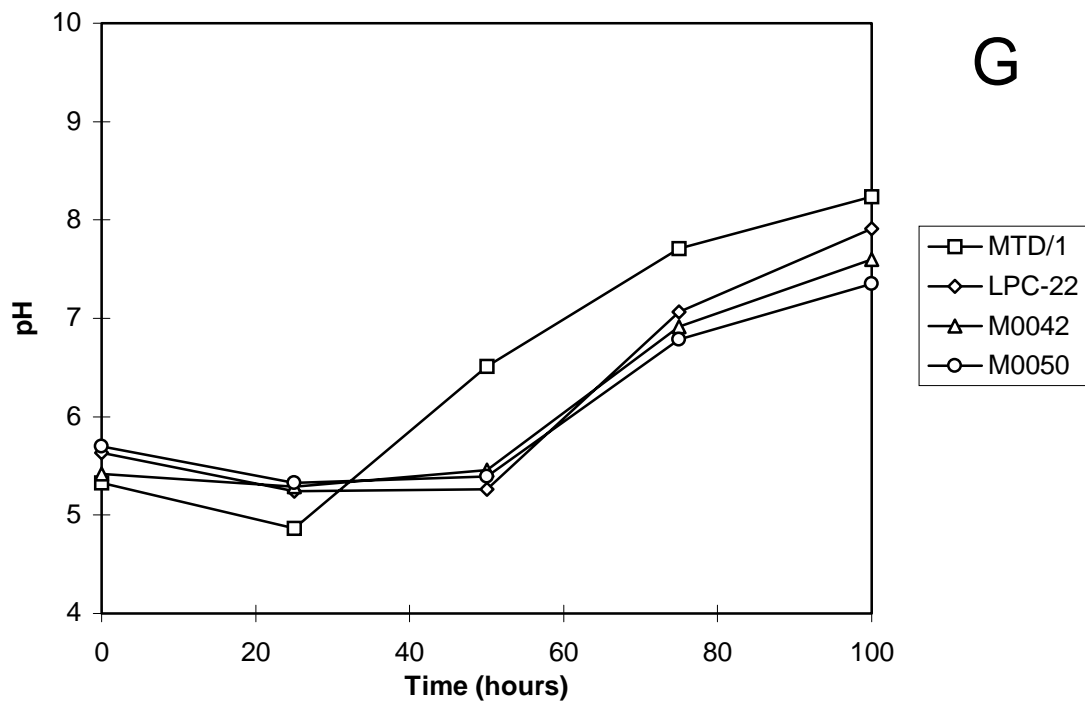


Figure 6.2 (Cont.). Effect on pH of the co-culture of indicator organisms with test LAB. LAB cultures were grown in SGM (30°C), before addition to an equal volume of SSM inoculated with the test organism. The co-cultures were incubated aerobically (30°C). (**G**) Test organism = *Acetobacter* sp., $n=2$