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Degradation of Organochlorine Contaminants by Yogurt Culture Organisms and the Effect on Their Growth

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Organochlorine pesticides (OCPs) and polychlorinated biphenyls (PCBs) are persistent environmental contaminants that can enter the food chain due to their high stability and tendency to concentrate in the fat. The diet is the main way of human exposure to these organochlorine compounds (OCs). Residues of these components in milk are of special concern because the general population consumes milk in relatively large quantities. The fermented dairy products such as yogurt are also important, since their beneficial effects make them a valuable component in the diet of vulnerable populations (infants, children and elderly). It can be generally stated that the contamination of milk by OCPs and PCBs has declined in developed countries as a result of the restrictive measures in pesticide usage applied by Governments following the recommendations of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO). However, the occurrence of these compounds at low concentrations has been recently reported in milk and dairy products by different authors in Spain (Hernández et al. 1994; Martínez et al. 1997) and other countries (Atuma et al. 1996; Mallatou et al. 1997). Therefore, their control continues to be of great importance, as well as the study of the factors that could reduce such contamination to ensure public health.

Previous research has demonstrated that organochlorine concentrations become considerably reduced in the course of food processing. The removal of residues by processing is influenced by the type of food, location of pesticide and especially by the type of processing operation (Abou-Arab 1997). In some cases, lowering of pesticide residues has occurred during the preparation of fermented milk products or ripening of cheese. Thus, in milk processing technology it is important the role that could play the microbial starter cultures as processing aids for degrading these contaminants and supplying food less harmful to human health than raw materials. In this way, some studies confirm that lactic acid bacteria are able to degrade the OCPs in milk (Abou-Arab 1997; El Hoshy 1997) or in synthetic culture media (Shaker et al. 1988; Abou-Arab 1997), although some results are contradictory (Kim and Harmon 1970). However, there is scarce scientific information available related with the degradation of polychlorinated biphenyls by microorganisms of interest in food technology. Our laboratory began to study the microbial degradation of organochlorine compounds by starter cultures from the meat industry, and a significant reduction in PCB 153 levels in liquid media by the action of these microorganisms and was reported (Bayarri et al. 1997a). But the xenobiotic chemicals may have in turn an adverse effect on the microbial population; that is, they may act as antimicrobials, as it has been demonstrated in several studies (Hantke and Bradley 1972; Sandford and Langlois 1976; Magdoub

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et al. 1993; Bayarri et al. 1997a; Dhanalakshmi et al. 1997). For this reason, extra care should be exercised when determining the xenobiotic concentration to which microorganism should be exposed in *in vitro* assays since any antimicrobial activity can reduce the possibility of their degradative mechanisms. The aim of the present research is to study the microbial degradation of three organochlorine pesticides γ-hexachlorocyclohexane (HCH), hexachlorobenzene (HCB) and pp'DDE, and two polychlorinated biphenyls (PCB 28 and PCB 153) by the yogurt culture organisms. The effect of these chlorinated compounds on the growth of the two microbial species was studied as well.

MATERIAL AND METHODS

All glassware was cleaned with water and soap, rinsed with distilled water and dried, and was rinsed with organic solvent prior to use. Reagents were HPLC grade or better. All solvents (Lab Scan, Ireland) were checked for the absence of components that would produce interfering peaks in the GC analysis.

Trials were conducted with yogurt culture organisms obtained from the Spanish type culture collection (CECT): *Lactobacillus delbrueckii* subsp *bulgaricus* CECT-4005 and *Streptococcus salivarius* subsp *thermophilus* CECT-801. Stock cultures were stored at -18°C in De Man, Rogosa & Sharpe (MRS) + glycerol and skim milk (Difco), respectively. De Man, Rogosa & Sharpe (MRS; Merck) and Terzaghi (M17; Merck) broths were used for growing *L delbrueckii* subsp *bulgaricus* and *S salivarius* subsp *thermophilus*, respectively. Water was distilled using a MilliQ water purification system and extracted with hexane prior to preparation of the liquid media.

Evaluation of organochlorine residues on growth of yogurt culture organisms was performed inoculating each bacterial species at a concentration of 10^5 cells/mL from an 18-hr actively growing culture into their respective broths, spiked at 0.1, 0.5, or 1.0 μg/mL of the organochlorines. Thus test tubes had a final volume of 10 mL; two replicates were made for each microorganism under study. The mixture of organochlorines used were HCB, γ-HCH, pp'DDE, PCB 28 and PCB 153 (> 99.0% purity; Dr Ehrenstofer, Germany) prepared in acetone. Broth tubes without organochlorines (non-spiked, controls) and with 0.1 mL of added acetone in order to check the effect of this solvent on the growth of the microorganisms, were inoculated under similar conditions. All cultures were incubated on a rotary shaker at 42°C for 48 hours.

The effect of organochlorines on bacterial growth was determined by plate counts. Counts were made by inoculating 0.1 mL of sample every 3 hours during the first 12 hours of incubation, and again at 24 hours and 48 hours thereafter. MRS and M17 agars were used for plate counts and peptone water (Difco; 1 g/liter) for decimal dilutions as needed.

pH was measured with a pHmeter (Crison) at the time of inoculation and after 48-hr of incubation.

Degradation of OCPs and PCBs by yogurt culture organisms was determined inoculating tubes containing MRS or M17 broth with *L delbrueckii* subsp *bulgaricus* or *S salivarius* subsp *thermophilus* at a concentration of 10^5 cells/mL from an 18-hr actively growing culture. An appropriate volume of a working OCP and PCB standard solution was added in order to have a final concentration of 0.1 μg/mL in the test tubes. Final volume in tubes was 10 mL, and four assays

with three replicates each were made for each microorganism under study. Tubes containing only organochlorine residues and liquid media were run as controls. All tubes were incubated on a rotary shaker at 42°C for 48 hours.

Following incubation, the procedure described elsewhere by Bayarri et al. (1997b) was used to prepare samples for gas chromatographic determination. Determination of OCPs and PCBs was carried out on a Hewlett-Packard HP 5890 gas chromatograph equipped with an automatic injector HP 7673A and a ^{63}Ni electron-capture detector. A fused silica capillary column coated with 5% phenyl methyl polysiloxane (Quadrex 007-2, New Haven, CT 50 m x 0.25 mm id x 0.25 μm film thickness) was used. The column temperature program was as follows: 125°C (2 min), 10°C/min to 204°C, 2°C/min to 290°C (hold 11 min). The injector and detector temperatures were set at 210°C and 300°C, respectively. Nitrogen was used as carrier gas at a linear velocity of 33.5 cm/sec.

The chromatograms of the extracts from the inoculated and non-inoculated samples were compared, to determine if the microorganisms had degraded any amount of OCPs and PCBs. For every *in vitro* assay, blank analyses were performed with each medium to check for interferences due to the reagents or the matrix.

To check the analytical precision of this method, recoveries of OCPs and PCBs were determined by fortification of MRS and M17 broths with standard solution of the mixture of organochlorines in acetone at 0.1 μg/mL; percentage of recuperation ranged from 95 to 110%, in agreement with FDA (1994) recommendations. The repeatability was very good with relative standard deviations in the range of 4-7%.

The quality of analytical data was assured by participation in the Intercalibration Exercise for Chlorobiphenyl and Organochlorine Pesticides organized by the SOAFD Marine Laboratory, Aberdeen (UK) and sponsored by the EEC, Community Bureau of Reference.

RESULTS AND DISCUSSION

Two different types of growth pattern were observed when yogurt culture organisms grew in liquid media containing 0.1, 0.5, and 1.0 μg/mL of the mixture of organochlorines under study.

Figure 1 shows the growth of *L delbrueckii* subsp *bulgaricus*, which grew equally well in MRS broth containing acetone, 0 (control), 0.1, 0.5, and 1.0 μg/mL of the mixture of organochlorines. After the initial exponential phase of growth, the number of viable cells per mL decreased in all tubes from 10^7-10^8 at 12-hr to less than 1 colony forming unit (cfu)/mL at 48 hours. The only difference observed in the growth of *L delbrueckii* subsp *bulgaricus* was a faster decrease in growth rate between 12 and 48-hr of incubation in non-spiked tubes than in spiked ones (See Fig 1A).

The pattern of growth of *S salivarius* subsp *thermophilus* was different. In M17 broth containing acetone, 0 (non-spiked) and pesticide standard solution at 0.1 μg/mL *S salivarius* subsp *thermophilus* grew similarly. However, this microorganism was inhibited at concentrations of 0.5 and 1.0 μg/mL of the

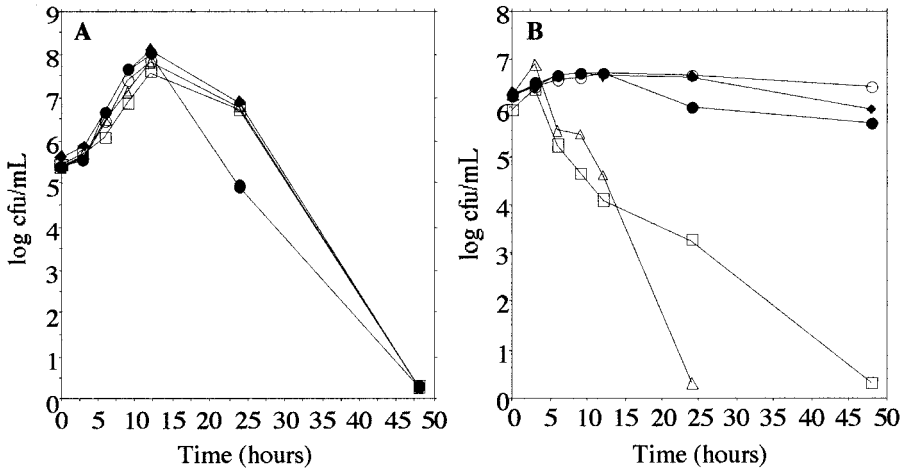

Figure 1. Effect of the mixture of organochlorine residues on growth of (A) *Lactobacillus delbrueckii* subsp *bulgaricus* and (B) *Streptococcus salivarius* subsp *thermophilus*. Results are the mean of two replicates (●) non-spiked medium; (o) medium with acetone; (Δ) medium spiked at 1 μg/mL; (□) medium spiked at 0.5 μg /mL; (◆) medium spiked at 0.1 μg /mL.

mixture of organochlorines, showing a clear decrease in viable cell numbers. This effect is illustrated by growth curves shown in Fig 1B. Tubes spiked at 1.0 μg/mL showed counts of <1 cfu/mL after 24-hr incubation; those spiked at 0.5 μg/mL attained <1 cfu/mL after 48-hr incubation. The number of viable cells per mL amounted to 10^5-10^6 cfu/mL in tubes spiked at 0.1 μg/mL, tubes with acetone and controls (non-spiked).

pH values decreased from 5.8 to 4.7 in MRS broth in all cases. In M17 broth the pH decreased from 7.3 to 6.8-6.9 in non-spiked controls, tubes containing acetone, and tubes spiked at 0.1 μg/mL, and pH remained unchanged in tubes containing 0.5 and 1.0 μg/mL.

Observing the growth curves of *L delbrueckii* subsp *bulgaricus* and *S salivarius* subsp *thermophilus*, it can be said that acetone showed no negative effect on growth of both microorganisms. This is in agreement with Kim and Harmon (1968) who also reported that acetone used at concentrations up to 1% in the culture medium had no effect on the growth of several lactic culture organisms. Therefore, extra care should be exercised so that the volume of acetone added to culture medium never exceeds 0.1 mL in a 10-mL test tube.

Our results are in agreement with those of Sandford and Langlois (1976) who reported that the potential effects of organochlorine residues on microbial growth depend on different factors such as microorganism species and type and concentration of pesticide. Thus, the two microorganisms employed in this study behaved in a distinct manner to the presence of xenobiotics. The growth of *L delbrueckii* subsp *bulgaricus* was not affected at any of the three levels of spiking, while the growth of *S salivarius* subsp *thermophilus* was markedly

inhibited at increasing concentrations of the xenobiotics. However, the inhibition in the growth of *S salivarius* subsp *thermophilus* at the higher concentrations was not surprising because this microorganism is highly sensitive to the presence of inhibitory substances in milk.

In other studies with yogurt culture organisms, Dhanalakshmi et al. (1997) also observed that *S salivarius* subsp *thermophilus* was most sensitive to lindane when compared with other lactic cultures, while the rate of multiplication of *L delbrueckii* subsp *bulgaricus* was lower, maybe due to the extended lag phase of the organism. Magdoub et al. (1993) reported that these microorganisms were not influenced by the presence of β-HCH, γ-HCH and pp'DDT in reconstituted dried skim milk. However, Sandford and Langlois (1976) obtained that the growth of *L delbrueckii* subsp *bulgaricus* was completely inhibited in broth containing 10 ppm chlordane and heptachlor.

In a previous study carried out in our laboratory, Bayarri et al. (1997a) found that the addition of a mixture of OCPs (HCB, α-HCH, β-HCH, γ-HCH, pp'DDE and dieldrin) and PCB 153 resulted in decreased viable counts of *Micrococcus varians* –a starter microorganism used in the meat industry– during the initial 24-hr of incubation in mineral salt medium. Then, the microorganism recovered and began to grow logarithmically, though at a lower rate than in a normal situation. Viable counts in the nutrient medium Tryptic Soy broth (TSB) spiked with the organochlorine residues were below those found in the same medium in the absence of added compounds.

Sandford and Langlois (1976) reported that 10 ppm of chlordane or heptachlor inhibited the growth of Gram positive bacteria in nutrient broth, but not in skim milk. This may be explained by the protective effect of protein milk reported by several researchers (Hantke and Bradley 1972).

The degradative assay was only carried out at a spiking level of 0.1 μg/mL of pesticide and PCB mixture as greater concentrations inhibited the growth of *S salivarius* subsp *thermophilus*.

The effect of *L delbrueckii* subsp *bulgaricus* and *S salivarius* subsp *thermophilus* on the degradation of HCB, γ-HCH, pp'DDE, PCB 28 and PCB 153 is presented in Table 1. Data show that none of the strains was able to degrade these compounds after 48 hours of incubation at 42°C in their respective broths, except for a significant reduction ($p< 0.02$ by Mann-Whitney's non-parametric test) of PCB 153 (7.4% decrease) attributable to methodological differences.

Our results are in disagreement with Shaker et al. (1988) who concluded that γ-HCH was significantly degraded by 65% after 48 hours of incubation (APT broth at 37°C) in the presence of either *L delbrueckii* subsp *bulgaricus* or *S salivarius* subsp *thermophilus*, and initial lindane concentrations decreased to 33% by the mixture of both strains. These authors also studied the effect on decamethrin, chlorpyrifos and aldicarb, and concluded that *S salivarius* subsp *thermophilus* was more active than *L delbrueckii* subsp *bulgaricus*, and the mixture of both strains was more degradative than each strain individually. Moreover, lindane was the most stable compound among the tested compounds.

Abou-Arab (1997) observed reduced levels of total DDT in Ras cheese made from contaminated milk with different levels of DDT (0.1, 1.0 and 10.0 mg/kg fat); average percentage reductions were 40.6, 33.9 and 25.5%, respectively, at the end

of storage period. These reductions were attributed to a possible effect of ripening microorganisms during storage. The *in vitro* study confirmed that the microorganisms isolated from Ras cheese could reduce the total DDT residues by 10.8 and 11.8% for Streptococci and Lactobacilli, respectively, in liquid media after 10 days of incubation. El Hoshy (1997) observed a reduction of 65.8, 56.1 and 8.2% of pp'DDT, lindane and dieldrin, respectively, during the manufacture of yogurt.

Table 1. Residual amounts of xenobiotics remaining in the culture media after incubation with yogurt culture organisms.

	L. delbrueckii subsp *bulgaricus*	*S. salivarius* subsp *thermophilus*
Compound	Percentage [a] ± RSD (%)	
HCB	102.6 ± 7.2	105.5 ± 2.7
γ-HCH	101.0 ± 6.1	101.7 ± 2.5
pp'DDE	119.1 ± 15.6	99.9 ± 7.0
PCB 28	107.6 ± 10.5	108.0 ± 4.2
PCB 153	115.7 ± 16.1	92.6 ± 7.9[b]

[a] Related to the concentration of chemical remaining in non-inoculated media. All data are means of four *in-vitro* assays ± the relative standard deviation.

[b] Significant reduction ($p < 0.02$; Mann-Whitney's non-parametric test).

In our laboratory, Bayarri et al. (1997a, 1998) reported that *Micrococcus varians* was able to degrade 12.7% HCB, 17.7% pp'DDE, 16.7% dieldrin and 15.5% PCB 153 in a minimum nutrient broth (mineral salt medium), whereas the HCH isomers were practically unaltered. However, the same compounds were not degraded in nutrient broth.

In consequence, the results of our research indicate that yogurt culture organisms were not able to degrade a mixture of organochlorines in nutrient broth. This fact may be due to several factors as cited elsewhere (Bayarri et al. 1997a). One factor may be the xenobiotic concentration, that was appropriate to prevent the cellular death (Somasundaram and Coats 1990), but was not high enough to induce enzymes necessaries for biodegradation (Matsumura 1985). Other factor may be that the microorganisms were not previously exposed to xenobiotics for a period of acclimation (Boethling 1993).

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