Effect of White Mustard Essential Oil on the Growth of Foodborne Pathogens and Spoilage Microorganisms and the Effect of Food Components on its Efficacy

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ABSTRACT

Antimicrobial preservative compounds are added to foods to target specific pathogens and spoilage organisms. White mustard essential oil (WMOE) is an extract that contains 4-hydroxybenzyl isothiocyanate, a compound which has been demonstrated to have antimicrobial activity in limited studies. The objective of this research was to determine the in vitro antimicrobial activity of WMOE against gram-positive and gram-negative spoilage and pathogenic bacteria and determine the effect of food components on the antimicrobial activity. The bacteria Escherichia coli, Salmonella enterica serovar Enteritidis, Enterobacter aerogenes, Staphylococcus aureus, Listeria monocytogenes, Bacillus cereus, and Lactobacillus fermentum, as well as the acetic- and preservative-resistant yeast Schizosaccharomyces pombe, were evaluated. All microorganisms were inhibited by WMOE at 8.3 g/liter (equivalent to 1,000 mg/liter 4-hydroxybenzyl isothiocyanate). In general, WMOE was more effective against gram-negative than against gram-positive bacteria. Salmonella Enteritidis and S. pombe were the most sensitive, with inhibition at as low as 2.1 g/liter. The effects on growth profiles varied but included increased lag phases and lethality, indicating both bacteriostatic and bactericidal activity. Soybean oil had a negative effect on the efficacy of WMOE against L. monocytogenes, and at 5% soybean oil, the antimicrobial activity against Salmonella Enteritidis was eliminated after 48 h. Sodium caseinate at 1% also negated the antimicrobial effect of WMOE against Salmonella Enteritidis and decreased its effectiveness against L. monocytogenes. The presence of starch had no significant effect on the antimicrobial activity of WMOE against L. monocytogenes and Salmonella Enteritidis. Thus, WMOE is effective against a wide range of microorganisms and has potential to be used in foods, depending upon the target microorganism and food components present.

The preservation of foods against pathogenic and spoilage microorganisms is an ongoing challenge to the food industry. In terms of processed food safety, Escherichia coli, Salmonella enterica subsp. enterica serovar Enteritidis, Staphylococcus aureus, and Listeria monocytogenes are all microorganisms of concern to consumers, the industry, and regulators. In the United States, there are an estimated 9.4 million foodborne illnesses, 56,000 hospitalizations, and 1,350 deaths each year caused by the major foodborne pathogens, and the expenses associated with these illnesses are approximately $51 billion (24, 25). Further, Scallan et al. (13) estimated that there were approximately 175,000 cases of O157 and non-O157 Shiga toxin–producing E. coli illness, 1.02 million cases of salmonellosis, 240,000 cases of foodborne S. aureus intoxication, and 1,590 cases of listeriosis among foodborne illnesses annually in the United States.

There are several organisms that can cause spoilage due to by-products produced during growth. Enterobacter aerogenes has been known to cause spoilage due to its gas production (26). Additionally, its production of biogenic amines, such as histamine in meat products, as well as cadaverine and putrescine, can cause off flavors and odors in food (33). Lactobacilli can cause spoilage due to acetic and lactic acid production, as well as gas formation via CO2. Lactobacillus bacteria have been known to cause souring, sliminess, and discoloration in refrigerated meats (3) and off-flavors and odors described by consumers as sour and smelling like hydrogen sulfide in smoked fish (16). These bacteria are some of the most difficult to inhibit using traditional antimicrobials. In wine, for example, the addition of sorbic acid inhibits spoilage yeast but not lactic acid bacteria causing spoilage (28). Schizosaccharomyces pombe is a yeast that has been known to cause spoilage of wine, causing sedimentation and off-flavors, as well as spoilage of foods high in sugar, benzoic acid, or acetic acid, such as ketchup (29).

To combat all of these microorganisms, the food industry is turning increasingly to natural antimicrobials to develop control measures in products for which there traditionally have been no antimicrobial solutions. These
compounds can be incorporated during food manufacturing as an extra hurdle to microbial growth (6). Possible promising sources of natural antimicrobials are essential oils and extracts derived from plants (7).

Mustard extracts have shown potential as antimicrobials against bacteria including Salmonella and E. coli (31), and the isothiocyanates they contain are also known to have antimicrobial activity when isolated (8, 13, 19). These compounds are also present in other cruciferous plants, such as horseradish (9). There are several varieties of mustard used in the food industry, including Brassica nigra L., known as black mustard, Brassica juncea, which is known as Indian or oriental (also sometimes called black) mustard, and Sinapis alba, which is known interchangeably as white or yellow mustard. The different varieties also contain different glucosinolates; sinigrin (2-propenyl or allyl glucosinolate) can be found in black mustard seed, while sinalbin (4-hydroxybenzyl glucosinolate) can be found in white mustard seed (12). Glucosinolates are present in most plants belonging to the family Cruciferae, with leaves containing 0.1% and seeds containing up to 5% (23). One purpose of these compounds in plants may be their insecticidal properties (1). Glucosinolates are degraded by the enzyme myrosinase, which is released by the plant when the tissues are damaged. Several products may result, the composition of which depends on factors including pH and the metal ions present (2, 12). One group of resulting products is the isothiocyanates. Sinigrin is degraded to allyl isothiocyanate and sinalbin to 4-hydroxybenzyl isothiocyanate (4-HBITC).

Both sinalbin and myrosinase are present in white mustard flour and, upon wetting of the flour, the myrosinase degrades sinalbin to 4-HBITC. This compound can produce a hot sensation in the mouth, which is not always desirable to consumers. A “deheated” mustard flour can be created by heating the ground mustard to deactivate myrosinase, thereby ensuring that the sinalbin remains intact and is not reduced to 4-HBITC (11). Much of the literature on the antimicrobial activity of isothiocyanates has focused on allyl isothiocyanate rather than 4-HBITC due to the greater instability of the latter, namely, its faster decomposition in aqueous solution (17, 18). Ekanayake et al. (10) attempted to address this issue by developing a stable white mustard essential oil (WMEO), produced by wetting ground white mustard seed and extracting the resulting 4-HBITC by supercritical fluid extraction. The resulting product contains 25 to 35% 4-HBITC and was commercialized as Isogard (10). This product exhibited antimicrobial activity in a sauce containing particulates of chicken, carrots, peas, and potatoes (5).

Although the antimicrobial activity of WMEO has been studied in a single food product, it is important to evaluate the activity of any antimicrobial against multiple target microorganisms that are of concern to the food industry and evaluate the influence of food or food components on the antimicrobial activity. Factors such as additives, minerals, fat, protein, and starch can have an effect on the efficacy of an antimicrobial (15, 22). Therefore, the purpose of this study was to test the in vitro effectiveness of WMEO on the growth of pathogenic and spoilage gram-positive (L. monocytogenes, Bacillus cereus, S. aureus, and Lactobacillus fermentum) and gram-negative (E. coli, Salmonella Enteritidis, and E. aerogenes) bacteria, as well as an acid- and preservative-resistant yeast (S. pombe), all of which are currently of concern in the food industry. The evaluation of these compounds was done using time-kill curves (32), which give additional information about the effect on target organisms that is not available from antimicrobial endpoint tests, such as agar or broth dilution assays. The effects of a protein, starch, and lipid on the antimicrobial efficacy of WMEO were also determined. To accomplish this, a gram-negative and a gram-positive bacterium, along with the acid- and preservative-resistant yeast were tested, and the effects of sodium caseinate, soybean oil, and potato starch on the antimicrobial efficacy of WMEO were evaluated.

MATERIALS AND METHODS

Preparation of bacterial and yeast cultures. Frozen stock cultures of each organism were propagated after transferring and growing them for 24 h twice. E. coli BA-1882, Salmonella Enteritidis BAA 1045, and E. aerogenes ATCC 13048 were grown in tryptic soy broth (TSB; BD Sparks, MD) with a pH of 7.2 at 37°C, S. aureus ATCC 25923 in brain heart infusion (BHI) broth (BD) with a pH of 7.3 at 37°C, L. monocytogenes ATCC 19111 in BHI broth with a pH of 7.3 at 32°C, vegetative cells of B. cereus F4433/73 in BHI broth with 0.1% glucose and a pH of 7.3 at 37°C, Lactobacillus fermentum ATCC 14932 in de Man Rogosa Sharpe (MRS) broth (BD) with a pH of 6.5 at 32°C, and an S. pombe ketchup isolate in yeast extract–glucose (YEG) broth containing 30 g/liter dextrose (Fisher Scientific, Fair Lawn, NJ) and 5 g/liter yeast extract (BD) with a pH of 7.2 at 32°C. After growth, cultures were diluted in the appropriate broth to ensure a concentration of ca. 5 × 10⁷ CFU/ml. Aliquots of 20 μl of the diluted cultures were added to 9.8-ml broth tubes to give final concentrations of ca. 10⁵ CFU/ml.

Preparation of WMEO. Frozen stocks of WMEO (Procter & Gamble, Mason, OH) containing 120 g/liter 4-HBITC were thawed and dispersed by vortexing in dimethyl sulfoxide (DMSO) (Fisher Scientific) to give concentrations of 50, 25, and 12.5 g/liter 4-HBITC. Dispersal in DMSO allowed for the solubilization of WMEO in broth. Aliquots of 0.2 ml of these stock solutions were added to the inoculated broth samples and dispersed by vortexing to give final concentrations of 0.41, 0.83, 1.7, 2.1, 4.2, and 8.3 g/liter WMEO, which correspond to concentrations of 50, 100, 200, 250, 500, and 1,000 mg/liter 4-HBITC, respectively. The final concentrations of DMSO in the samples were 19.6, 19.2, 18.3, 17.9, 15.8, and 11.6 g/liter, for 0.41, 0.83, 1.7, 2.1, 4.2, and 8.3 g/liter WMEO, respectively. The positive controls contained only the microbial culture and 20 g/liter DMSO.

Preparation of model media with food ingredients. To the microbiological media (TSB, YEG, and MRS) was added sodium caseinate (Acros Organics/Fisher Scientific) or soluble potato starch (Fisher Scientific) at 1 or 5%, followed by autoclaving. Soybean oil (Fisher Scientific, Pittsburgh, PA) was sterilized by autoclaving, added to 10 ml of TSB, YEG, or MRS containing 0.05% Tween 80 (Acros Organics/Fisher Scientific), and vortexed to aid in creating an emulsion. Once a stable emulsion was created, the mixture was added to sterile broth to achieve a final concentration of 1 or 5% soybean oil.
Monitoring of microbial growth. Samples were incubated at 22°C for 48 to 72 h, and growth was monitored by periodically removing samples and plating on TSA, YEG agar, or MRS agar after serial dilution in sterile 0.1% peptone. In addition, to confirm the absence of cells in samples in which lethality reduced cell numbers below the detection limit, 1-ml aliquots of each sample were removed at various time points, enriched in 9 ml of Dey Engley neutralizing broth (BD), and incubated at 37°C or 32°C for 48 h. The presence of viable cells was indicated by a color change in the broth from purple to yellow, which was confirmed by plating. All trials were conducted in triplicate.

Statistical analysis. Bacterial counts were transformed to log values, and the data were analyzed by analysis of variance using the PROC GLM procedure in SAS, version 9.3 (SAS Institute, Inc., Cary, NC), as well as the Student-Newman-Keuls test for comparison of treatments. An α value of 0.05 was used as the level of significance.

RESULTS

The effects of WMEO on bacterial and yeast growth are shown in Figures 1 to 8. The growth of all organisms was inhibited at a WMEO concentration of 8.3 g/liter (equivalent to 1,000 mg/liter 4-HBITC). Among the gram-negative bacteria, the lag phase of *E. coli* BA-1882 was extended to 8 and 24 h, respectively, by 2.1 g/liter (equivalent to 250 mg/liter 4-HBITC) and 4.2 g/liter (equivalent to 500 mg/liter 4-HBITC) WMEO. This was followed by growth in the case of 2.1 g/liter and lethality to undetectable levels at 4.2 g/liter after 48 h. At 8.3 g/liter WMEO, a 4-log reduction occurred by 8 h compared with the count in the control, and the bacterium was not detectable after 48 h (Fig. 1). The absence of viable cells was verified by incubation in Dey Engley broth, which both neutralizes the antimicrobial agent...
present in the sample and contains yeast extract and glucose to facilitate the growth of survivors (30). Against Salmonella Enteritidis BAA 1045, WMEO at 2.1 and 4.2 g/liter caused an increased lag phase up to 8 h and ca. 24 h, respectively, followed by growth (Fig. 2). With 8.3 g/liter WMEO, lethality occurred immediately and no survivors were detected up to 48 h. The results with E. aerogenes ATCC 13048 in the presence of WMEO were similar to the results for Salmonella, with increased lag phases at 2.1 and 4.2 g/liter and lethality at 8.3 g/liter, although survivors were detected at the latter concentration through 48 h (Fig. 3).

For the gram-positive bacteria, growth inhibition and lethality were more varied. With S. aureus ATCC 25923, all concentrations of WMEO extended the lag phase for 24 h, and 4.2 and 8.4 g/liter caused a reduction in numbers of 4 to 5 log compared with the count of the control at 48 h (Fig. 4). For B. cereus F4433/73, all concentrations of WMEO caused lethality to undetectable or nearly undetectable levels (1 log CFU/ml) (Fig. 5). The results with L. monocytogenes ATCC 19111 were similar to the results for S. aureus in that 2.1 and 4.2 g/liter WMEO caused increased lag phases and demonstrated bacteriostasis, while 8.3 g/liter caused an initial 3- to 4-log lethality followed no decrease in numbers after 8 h (Fig. 6). In contrast to the other gram-positive bacteria, Lactobacillus fermentum ATCC 14932 was more resistant to WMEO at 2.1 and 4.2 g/liter (Fig. 7). The use of 2.1 g/liter WMEO had no significant effect on the growth of the bacterium, and while there was an initial 1- to 2-log decrease in counts after 24 h, the counts increased thereafter. WMEO at 8.3 g/liter caused a decrease in cell numbers to nearly undetectable levels (1 CFU/ml) after 6 h with no subsequent increase up to 72 h.

![Figure 5](http://meridian.allenpress.com/jfp/article-pdf/77/12/2062/1684098/0362-028x_jfp-14-257.pdf)

**FIGURE 5.** Effects of white mustard essential oil at concentrations of 0 (○), 2.1 (■), 4.2 (▲), and 8.3 (×) g/liter on growth of vegetative cells of Bacillus cereus F4433/73 in BHI broth plus 0.1% glucose (pH 7.3) at 22°C. Data points along the limit of detection (—) indicate samples in which viable cells were detected only after enrichment, unless followed by an asterisk, which indicates no viable cells were detected after enrichment.

![Figure 6](http://meridian.allenpress.com/jfp/article-pdf/77/12/2062/1684098/0362-028x_jfp-14-257.pdf)

**FIGURE 6.** Effects of white mustard essential oil at concentrations of 0 (○), 2.1 (■), 4.2 (▲), and 8.3 (×) g/liter on growth of Listeria monocytogenes ATCC 19111 in BHI broth (pH 7.3) at 22°C. Data points along the limit of detection (—) indicate samples in which viable cells were detected only after enrichment, unless followed by an asterisk, which indicates no viable cells were detected after enrichment.

![Figure 7](http://meridian.allenpress.com/jfp/article-pdf/77/12/2062/1684098/0362-028x_jfp-14-257.pdf)

**FIGURE 7.** Effects of white mustard essential oil at concentrations of 0 (○), 2.1 (■), 4.2 (▲), and 8.3 (×) g/liter on growth of Lactobacillus fermentum ATCC 14932 in MRS broth (pH 6.5) at 22°C. Data points along the limit of detection (—) indicate samples in which viable cells were detected only after enrichment, unless followed by an asterisk, which indicates no viable cells were detected after enrichment.
The addition of WMEO at 1.7 g/liter starch % Enteritidis BAA was significantly inhibited by the without the presence of WMEO or food components. Data points along the limit of detection (−) indicate samples in which viable cells were detected only after enrichment, unless followed by an asterisk, which indicates no viable cells were detected after enrichment.

The greatest antimicrobial effect was observed with the ketchup isolate of the acid- and preservative-resistant yeast S. pombe (Fig. 8). The addition of WMEO at 1.7 g/liter (equivalent to 200 mg/liter 4-HBITC) caused immediate lethality. With 0.83 g/liter WMEO (equivalent to 100 mg/liter 4-HBITC), the cell counts dropped below the detection limit after 8 h. However, the cells began to recover after 48 h, resulting in final counts ca. 5 log lower than in the control. The same trend was observed with 0.41 g/liter WMEO (equivalent to 50 mg/liter 4-HBITC). The cells recovered faster (after 24 h), resulting in final counts ca. 4 log lower than in the control at 72 h.

The effects of food components on the antimicrobial efficacy of WMEO against Salmonella Enteritidis and L. monocytogenes are shown in Figures 9 and 10, respectively. Soybean oil and sodium caseinate had a negative effect on the antimicrobial activity of WMEO. In the case of L. monocytogenes, the lag phase of growth was still extended in the presence of 1 and 5% soybean oil and sodium caseinate, but the cell counts did not decrease as in the samples that contained 8.3 g/liter WMEO alone. Against Salmonella Enteritidis, the initial decrease in population immediately following the addition of 8.3 g/liter WMEO was not apparent in media containing soybean oil or sodium caseinate. Samples with 5% casein and 1 and 5% oil had significantly decreased antimicrobial efficacy, with 5% oil and 5% casein having the greatest effect on antimicrobial activity. At the end of 48 h, the cell counts in samples with 5% oil and 5% casein were not significantly different from the counts in the positive control. The final cell counts were ca. 7 log lower than in the control in samples with 1% oil, but lethality was not achieved.

The addition of starch had no significant effect on the antimicrobial efficacy of WMEO. Against L. monocytogenes in the presence of 1% starch, there was no decrease in the efficacy of WMEO, with the population declining steadily throughout storage. With the addition of 5% starch, there was a slight decrease in activity, with the lag phase extended to 24 h, followed by a decrease in population after 48 h. Against Salmonella Enteritidis, 1 and 5% starch had no significant effect on the antimicrobial efficacy of WMEO; lethality was still achieved after 8 h of incubation.

Against S. pombe, initial cell death occurred in all samples containing 4.2 g/liter WMEO with or without the addition of any food components (data not shown).

**DISCUSSION**

WMEO had antimicrobial activity at 22 °C against all of the organisms tested in this study. However, the effects on the different phases of growth varied between organisms. The growth of the gram-positive organisms L. monocytogenes ATCC 19111, L. fermentum ATCC 14932, B. cereus F4433/73, and S. aureus was significantly inhibited by the addition of 8.3 g/liter WMEO; in the case of B. cereus, a decrease in cell numbers occurred after the initial addition of WMEO, and even 4.2 g/liter WMEO caused a decrease of ca. 4 log CFU/ml after 12 h, indicating bactericidal activity. However, there were still surviving bacterial cells present. Bactericidal activity at 8.3 g/liter WMEO was also observed in L. fermentum, with a decrease of ca. 4 log CFU/ml 3 h after the initial exposure. In the case of the gram-negative organisms E. coli BA-1882 and Salmonella Enteritidis BAA 1045, 8.3 g/liter WMEO eliminated the organisms. The effects on these organisms were dose dependent; concentrations of 2.1 and 4.2 g/liter extended the lag phase but still allowed for bacterial growth. When the concentration was increased to 8.3 g/liter, bactericidal activity was exhibited. In Salmonella Enteritidis, this occurred only 3 h after initial exposure, while in E. coli, the cell numbers decreased less rapidly but all cells were eliminated after 48 h. At 4.2 g/liter,
however, WMEO appeared to have a greater effect on *E. coli*, inhibiting growth for 24 h and eliminating the organism after 48 h, while in *Salmonella* Enteritidis, the lag phase of growth was increased, but after 48 h, there was no significant difference from the control sample. These results match those of David et al. (5), where an initial decrease in *Salmonella* cell counts was observed when WMEO at 4-HBITC concentrations of 562.5 and 750 mg/liter was added to a sauce with particulates that was incubated at 21°C for 5 h. *E. aerogenes* ATCC 13048, also a gram-negative organism, had the same response as *Salmonella* Enteritidis to WMEO at concentrations of 2.1 and 4.2 g/liter; however, when exposed to 8.3 g/liter, there was an initial decrease of ca. 3 log CFU/ml, after which growth remained static.

Although WMEO was more bactericidal against gram-negative organisms at 8.3 g/liter, in regards to bacteriostatic activity, the gram-positive organisms were more sensitive; even the lowest concentration of 2.1 g/liter significantly extended the lag phase of growth, and this concentration even exhibited bactericidal activity against *B. cereus*. This corresponds to research conducted by Kurepina et al. (19), who investigated the antimicrobial activity of several natural and synthetic isothiocyanates against a range of bacteria and fungi. They found that the compounds were more active against gram-positive than against gram-negative bacteria. Some of the compounds simply exhibited a lower MIC against gram-positive organisms (2 to 16 μg/ml) than against gram-negative ones (32 to 64 μg/ml), while others were only effective against gram-positive organisms.

The most sensitive organism was the ketchup isolate of the yeast *S. pombe*. The addition of 1.7 g/liter WMEO caused immediate lethality, and 0.41 g/liter caused a significant decrease in cell counts. Isothiocyanates in general have exhibited greater antimicrobial activity against yeasts and molds than against bacteria (8, 19). Other researchers have found similar results, with WMEO at 4-HBITC concentrations of 150 mg/liter exhibiting a bactericidal effect against the preservative-resistant yeast *Zygosaccharomyces bailii* in ketchup (Denise Becker, personal communication), and Isogard, containing 25 mg/ml 4-HBITC, inhibiting the growth of *Z. bailii* for 28 days in fruit drink stored at room temperature (10). Kurepina et al. (19) also found that the MICs of several isothiocyanates against several *Candida* spp. were low, ranging from 0.5 to 8 μg/ml.

Generally, the lower concentrations of WMEO increased the lag phase of the organisms and did not cause bactericidal activity, but they may still be useful in food products. When incorporated as an additional hurdle along with other control methods, the increase in lag phase may lengthen the shelf life and improve food safety (6), since slower growth increases the time it takes for the number of organisms to reach the level required to cause spoilage or, in some cases, affect human health. Other control methods may include other antimicrobial additives, decreased pH, refrigeration, or thermal treatment of the food product. For example, allyl isothiocyanate has a MIC of 500 μl/liter against *E. coli* O157:H7 at pH 8.5, but that decreases to 25 μl/liter at pHs of 4.5 and 5.5 (21). Additionally, Ekanayake et al. (10) showed that 4-HBITC is more stable at 10°C than at 21°C in pH 3.6 buffer. It is therefore possible that the antimicrobial activity of WMEO could be increased in a refrigerated, low-pH food product.

The composition of a food product is an important factor to consider when determining the necessary concentration of any compound for achieving antimicrobial efficacy. Several researchers have shown that the antimicrobial activity of essential oils can be adversely affected in food systems (15, 20, 27). Fat in particular appears to have a protective effect for bacteria (corroborated by the results of this study), which may be due to the fact that the essential oil dissolves in the lipid phase and is therefore not able to act against the bacteria present in the aqueous phase. Gutierrez et al. (15) also found that in essential oils from lemon balm, marjoram, oregano, rosemary, thyme, sage, and basil, the addition of sunflower oil at concentrations of 1 and 10% decreased the lag phase of growth in *L. monocytogenes*. Similar effects can also be seen in other types of antimicrobials in dairy products (4, 27). The theory that the attraction of essential oil to the lipid phase causes this decrease in activity is supported by data from a study by Rico-Muñoz and Davidson (22), where the addition of corn oil at 3 and 4.1% decreased the efficacy of the lipophilic antioxidant butylated hydroxyanisole against *Saccharomyces cerevisiae*, *S. aureus*, and *Pseudomonas fluorescens*. Tertiary butylhydroquinone had a lower affinity for the lipid phase and, thus, its antimicrobial efficacy was less affected by the presence of corn oil (22). In the same study, casein decreased antimicrobial activity against some organisms but did not eliminate it, similar to what was found against *L. monocytogenes* in our study. The increased protein content in the medium could have promoted bacterial growth (14), reducing the apparent antimicrobial effect. Starch had the least effect among the food components tested.

In conclusion, the results of this study show that WMEO had broad-spectrum antimicrobial activity against all of the microorganisms tested, but the effects on the growth profiles of bacteria and yeast cells varied widely and were species specific. One limitation of the study is that single strains were used, and thus, the efficacy may differ somewhat for other strains of the same microorganism. The inclusion of fat or protein decreased the antimicrobial activity of WMEO, while starch had no effect. These data are valuable, as they show that the amount of the compound required will depend on the target organism in the food, as well as the components present and the storage life of the product.

REFERENCES


