Thermal Resistance of *Salmonella* spp. and *Listeria monocytogenes* in Liquid Egg Yolk and Egg White

JAMES D. SCHUMAN and BRIAN W. SHELDON*

Department of Food Science, North Carolina State University, Raleigh, North Carolina 27695-7624, USA

(Received 30 July 1996/Accepted 25 October 1996)

**ABSTRACT**

Decimal reduction times (D values) were determined for *Salmonella* spp. and *Listeria monocytogenes* (five pooled strains per pathogen) in raw liquid egg yolk (pH 6.3) and liquid egg white (pH 8.2) by using a low-volume (0.05 ml per sample) immersed sealed-glass capillary-tube procedure. For *Salmonella*, D values ranged from 0.087 min (at 62.2°C) to 0.28 min (at 60°C) in yolk, and from 1.00 min (at 58.3°C) to 7.99 min (at 55.1°C) in egg white (pH 8.2). For *Listeria*, D values ranged from 0.58 min (at 62.2°C) to 1.34 min (at 60°C) in yolk, and from 2.41 min (at 58.3°C) to 7.59 min (at 55.1°C) in egg white (pH 9.1). Mean D values for *Salmonella* ranged from 3.54 to 4.33°C; for *Listeria*, D values ranged from 6.06 to 9.43°C. In egg white, the heat sensitivity of both pathogens was enhanced at pH 9.1, although this trend was more evident for *Salmonella* spp. than for *L. monocytogenes* over the temperature range tested. The results indicate that USDA-prescribed minimal pasteurization requirements for liquid egg yolk (equivalent to 3.9- to 22.1-D processes, on the basis of the present study) would be far more lethal to the *Salmonella* and *L. monocytogenes* strains tested than would the corresponding thermal processes for liquid egg white (equivalent to 0.7- to 2.2-D processes).

Key words: Egg white, egg yolk, *Salmonella*, Listeria, pasteurization

The U.S. egg industry is currently in a state of transition. At the expense of shell eggs, a variety of processed egg products (i.e., refrigerated liquid, frozen, dried, and specialty value-added products) have emerged as growing segments of the total egg market. The proportion of eggs sent to commercial breaking operations for the production of egg products increased from 13.8% (8.7 billion eggs) in 1980 to 24.6% (15 billion eggs) in 1992 (18). Among the advantages cited for the use of refrigerated liquid egg over shell eggs or frozen egg blends are consistent quality, convenience, storage-space savings, elimination of freezing costs, labor savings at the point of use, and improved shelf stability and microbiological safety.

The primary means of ensuring the microbiological safety of liquid egg products is the use of appropriate egg pasteurization processes. Current U.S. Department of Agriculture regulations stipulate that liquid, frozen, and dried whole egg, yolk, and white be pasteurized or otherwise treated to inactivate viable *salmonellae* (4). U.S. Department of Agriculture-mandated egg-pasteurization specifications, listed in the Code of Federal Regulations (Title 7, Section 59.570), require that every particle of egg be held for at least a specified time and temperature to “assure complete pasteurization” (subsection b) and to produce “a *Salmonella*-negative product” (subsection c) (1). *Salmonella* is the only bacterial pathogen specifically addressed within the context of these regulations (29).

In recent years, research efforts aimed at more accurately defining the microbiological safety of current USDA-mandated egg pasteurization processes have been initiated. Although the implementation of uniform egg pasteurization requirements for liquid egg products has clearly improved consumer safety, questions remain regarding the adequacy of such thermal processes in terms of the inactivation of egg-associated bacterial pathogens. *Salmonella* species have had a long historical association with contaminated eggs and egg products. Over the last decade, the egg-associated serotype *S. enteritidis* has emerged as one of the leading causes of foodborne salmonellosis in the U.S., Canada, the United Kingdom, and other nations (28). In addition, the bacterial pathogen *L. monocytogenes* has been isolated from commercially processed liquid whole egg in the U.S. (17) and Northern Ireland (20). This pathogen poses a particular concern if present in chilled liquid egg due to its ability to multiply in foods at proper refrigerated storage temperatures (i.e., ≤5°C) (11). Although several studies have documented the thermal resistance of *Salmonella* spp. and *L. monocytogenes* in liquid whole egg (5, 7, 11, 25), there is a comparative lack of kinetic data documenting the heat resistance of these important bacterial pathogens in unsupplemented liquid egg yolk and egg white.
The objectives of this study were (i) to determine the heat resistance of *Salmonella* spp. and *Listeria monocytogenes* in raw liquid egg yolk and liquid egg white (at pH 8.2 and 9.1) using a low-volume (0.05 ml per tube) immersed sealed-glass capillary-tube procedure, and (ii) to evaluate the resultant thermal inactivation data relative to current USDA minimum egg-pasteurization requirements (1).

**MATERIALS AND METHODS**

**Cultures**

The *Salmonella* strains used in this study (and their sources) included *S. enteritidis* strains ME-15 (shell egg processing-belt isolate) and ME-18 (five poultry isolate), obtained from Dr. H. M. Optiz (University of Maine, Orono, ME); *S. enteritidis* ATCC 4931 (clinical isolate), and *S. typhimurium* ATCC 14028 (type strain), obtained from the American Type Culture Collection (Rockville, MD); and *S. typhimurium* 2564-2 (egg layer house isolate), obtained from our own culture collection. *L. monocytogenes* strains Scott A (clinical isolate), NCF-P1K4 and NCF-U2K3 (raw liquid whole egg isolates), and F5069 (raw milk isolate) were supplied by Dr. P. M. Foegeding (North Carolina State University, Raleigh, NC); and strain ATCC 19115 (clinical isolate) was obtained from the American Type Culture Collection. Stock cultures were maintained in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) plus 20% (vol/vol) glycerol and stored at −20°C.

For each pathogen and strain, individual 10-ml BHI cultures were prepared (22 to 24 h, 37°C) and a 0.1-ml subsample of each was transferred to 15 ml of fresh BHI broth (22 to 24 h, 37°C). The cells were pelleted by centrifugation (12 min at 5°C at 90,000 x g), resuspended in sterile 0.1% peptone water (PW), pooled into a common tube (five strains per pathogen), and centrifuged again as previously described. The cell pellet from 75 ml of BHI broth culture was then held on ice (<1 h) before inoculation of liquid egg.

**Preparation of liquid egg**

Nest-run white shell eggs (mean mass, 54 to 60 g per egg) from a single flock were obtained within 24 h of laying from the North Carolina State University Department of Poultry Science. The eggs were washed in warm soapy water, and the shell surfaces were sanitized in a 50 ppm sodium hypochlorite solution (5 min) and rinsed in sterile water. The contents of 5 shell eggs per trial were broken out, and the yolks and whites were aseptically separated and collected in sterile blender jars. Liquid yolks or whites were blended for 2.5 to 3 min at low speed at 30% power in a preheated circulating water bath equipped with a calibrated temperature control module accurate to ±0.5°C (Model DC1, Haake, Inc., Karlsruhe, Germany). Preliminary experiments demonstrated that come-up times over the temperature range tested averaged 2 to 4 s at the approximate center of each capillary tube. At eight evenly spaced timing intervals, a single tube was removed from the heating bath, rapidly cooled by immersion in an ice-water slurry, and held for 5 to 10 min. The exterior of each tube was briefly sanitized in a 200 ppm sodium hypochlorite solution and rinsed in sterile water. Individual capillary tubes were then transferred to test tubes containing 5 ml of sterile PW, crushed using a flame-sterilized glass rod (a 10−2 dilution), serially diluted in sterile PW, and surface-plated onto BHI agar. The detection limit of this procedure was 2.0 log CFU/ml. After incubation for 48 h at 37°C, typical isolated colonies of *Salmonella* or *Listeria* were enumerated. Suspect colonies were further evaluated using wet mounts, Gram stains, and catalase tests.

**Determination of heat resistance**

Inoculated liquid egg (0.05 ml) was dispensed into individual sterile glass capillary tubes (0.8 to 1.1 mm i.d. by 90 mm long (no. 34502, Kimble, Vineland, NJ) using a sterile syringe fitted with a 100-mm blunt stainless steel needle. Filled tubes (with a headspace of approx. 4 mm) were held in an ice-water slurry (<20 min), flame sealed, and stored at 5°C for 18 to 20 h (to simulate holding in a liquid egg bulk tank) before determining decimal reduction times (D values). Initial inoculum levels averaged 9.3 to 9.8 log CFU per ml of liquid egg. Capillary tubes were placed upright in a mesh screen-covered test tube rack and completely immersed in a preheated circulating water bath equipped with a calibrated temperature control module accurate to ±0.5°C (Model DC1, Haake, Inc., Karlsruhe, Germany). Preliminary experiments demonstrated that come-up times over the temperature range tested averaged 2 to 4 s at the approximate center of each capillary tube. At eight evenly spaced timing intervals, a single tube was removed from the heating bath, rapidly cooled by immersion in an ice-water slurry, and held for 5 to 10 min. The exterior of each tube was briefly sanitized in a 200 ppm sodium hypochlorite solution and rinsed in sterile water. Individual capillary tubes were then transferred to test tubes containing 5 ml of sterile PW, crushed using a flame-sterilized glass rod (a 10−2 dilution), serially diluted in sterile PW, and surface-plated onto BHI agar. The detection limit of this procedure was 2.0 log CFU/ml. After incubation for 48 h at 37°C, typical isolated colonies of *Salmonella* or *Listeria* were enumerated. Suspect colonies were further evaluated using wet mounts, Gram stains, and catalase tests.

**Liquid egg inoculation procedures**

Triplicate thermal inactivation experiments were conducted for each pathogen at 60, 61.1, and 62.2°C (for egg yolk), and at 55.1, 56.7, and 58.3°C (for albumen at both pH values). D values (min) were calculated as the negative reciprocal of the survivor curve (log viable CFU/ml versus time) slope obtained by linear regression analysis using a personal computer and a graphics-statistics program (CA-Cricket Graph III, version 1.5.1, Computer Associates International, Islandia, NY). Decimal reduction time curves (mean log D value versus temperature) were also constructed for each pathogen, and ZD values were calculated, where ZD = slope−1 (i.e., the temperature change in °C necessary to effect a 10-fold change in the D value). Activation energies of inactivation were determined from Arrhenius plots as described previously (11).
RESULTS AND DISCUSSION

Thermal resistance in liquid egg yolk

Table 1 documents the mean decimal reduction time data for *Salmonella* spp. and *L. monocytogenes* in liquid egg yolk heated over a temperature range of 60 to 62.2°C. Survivor curves were linear in each case with correlation coefficients of ≥0.92 and ≥0.90 for *Salmonella* and *Listeria* strains, respectively. The temperature dependence factors (\(Z_D\) and \(E_a\) values) are also presented for each pathogen. For *Salmonella*, \(D\) values in yolk ranged from 0.087 min (at 62.2°C) to 0.28 min (at 60°C). The thermal resistance data for *Salmonella* spp. in yolk were similar to the results of Garibaldi et al. (13), who reported a \(D\) value at 60°C of 0.4 min and a \(Z_D\) value of 4.4°C for *S. typhimurium* TM-1 heated in yolk. In contrast, Humphrey and coworkers (15) documented \(D\) values at 60°C of 0.8 min and 1.1 min for *S. typhimurium* PT141 and *S. enteritidis* PT4, respectively, heated in yolk. Similarly, Palumbo et al. (21) reported \(D\) values for six *Salmonella* strains at 60 to 62.2°C which were (on average) 1.8- to 2.6-fold larger than those listed in Table 1.

*L. monocytogenes* is among the most heat-resistant vegetative bacterial pathogens associated with foods (19). In liquid yolk, the *Listeria* strains evaluated were 4.8- to 6.7-times more heat resistant than *Salmonella* over the temperature range tested (Table 1). \(D\) values for *Listeria* in yolk ranged from 0.58 min (at 62.2°C) to 1.34 min (at 60°C). The \(Z_D\) value of 6.06°C was within the range cited for most non-spore-forming bacteria (14). In the only published study on the thermal resistance of this pathogen in unaltered yolk, Palumbo et al. (21) reported a mean \(D\) value at 61.1°C of 1.28 min and a \(Z_D\) value of 7.76°C for five individual strains of *L. monocytogenes*.

Direct comparisons between studies are often not possible due to differences in the bacterial strains tested. However, the bacterial thermal inactivation literature indicates that the geometry of the heating vessel (e.g., test tubes, reaction vials, capillary tubes, or flasks) and its headspace volume and orientation within the heating bath may bias the resultant kinetic data (6, 9, 16). While there remains no general consensus on the best experimental methodology for bacterial thermal-resistance testing, the advantages of using small, sealed, and fully-immersed heating vessels have been documented (9, 23, 27). For example, Donnelly et al. (9) demonstrated that *L. monocytogenes* appeared to be significantly more heat resistant in fluid milk heated in 10-ml capped test tubes than in 2-ml sealed and immersed reaction vials. The capped test tube procedure also yielded nonlinear (tailing) survivor curves, which suggested that the observed tails represented artifacts of the methodology used. A similar phenomenon was recently demonstrated for cells of *Aeromonas hydrophila* suspended in liquid whole egg and heated in capped test tubes versus immersed sealed capillary tubes (24). Unlike the present study, several previous investigators reported the use of inoculation procedures which involved the addition of non-egg ingredients (e.g., broth culture at levels up to 10%, vol/vol) to the liquid egg heating menstruum. The effects of such inoculation procedures on bacterial heat resistance and the pH of the heating menstruum have yet to be thoroughly investigated.

In the context of current USDA minimum pasteurization requirements for liquid yolk (1), a holding time of 3.5 min at 61.1°C would yield a 21.9-log-unit reduction of viable *Salmonella* spp. and a 3.9-log-unit reduction of viable *L. monocytogenes* (Table 1). The alternative pasteurization schedule of 6.2 min at 60°C would represent a 22.1-\(D\) process for *Salmonella* spp. and a 4.6-\(D\) process for *Listeria* strains. Thus, current minimal pasteurization requirements for liquid yolk provide a large margin of safety with respect to the inactivation of *Salmonella* spp. There is currently no general agreement as to what constitutes an adequate level of *Listeria* inactivation in heat-processed, refrigerated foods. In a review of the published literature, Mackey and Bratchell (19) reported that the high-temperature, short-time pasteurization requirements for fluid milk (15 s at 71.1°C) achieve a 5.2-log-unit reduction of viable listeriae; the results of the present study indicate that a 6.2 min-60°C process for liquid yolk provides a comparable margin of *Listeria* safety.

It should be noted that an additional safety factor existed in that blended yolk from fresh (<24-h-old) shell eggs with a solids content of 53.2% was used as the heating menstruum in the present studies. In the egg industry, plain liquid yolk (no added non-egg ingredients) is typically diluted with liquid egg white to a solids content of 43 to 44% for improved rheological properties (7). The moisture content of natural egg white is nearly twice that of egg yolk; thus, the industry practice of adding egg white (or allowing a portion to adhere) to yolk would be predicted to slightly reduce the heat resistance of microorganisms present in liquid yolk during pasteurization (16).

**Table 1.** \(D\) values, \(Z_D\) values, and activation energies of inactivation for *Salmonella* spp. and *Listeria monocytogenes* in raw liquid egg yolk

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Egg pH</th>
<th>60</th>
<th>61.1</th>
<th>62.2</th>
<th>(Z_D) value (°C)</th>
<th>(E_a) (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella</em> spp.</td>
<td>6.3</td>
<td>0.28 (0.00)</td>
<td>0.16 (0.02)</td>
<td>0.087 (0.012)</td>
<td>4.33</td>
<td>492</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>6.3</td>
<td>1.34 (0.19)</td>
<td>0.89 (0.09)</td>
<td>0.58 (0.07)</td>
<td>6.06</td>
<td>353</td>
</tr>
</tbody>
</table>

\(a\) Mean of 3 trials (\(n = 3\)).
**Thermal resistance in liquid egg white**

Table 2 summarizes the thermal resistance data for each pathogen in inoculated, pH-adjusted egg white. Survivor curves were again linear, with correlation coefficients of $\geq 0.76$ (and typically $\geq 0.93$) for *Salmonella* and $\geq 0.94$ for *Listeria*. As expected, $D$ values for *Salmonella* in pH 8.2 albumen were larger than those observed at pH 9.1. $D$ values for *Salmonella* in egg white ranged from 1.00 min (at 58.3°C) to 7.99 min (at 55.1°C) at pH 8.2, and from 0.52 min (at 58.3°C) to 1.17 min (at 55.1°C) at pH 9.1. It is well documented that a reduction of the pH of egg white from 9.0 to 6.5 to 7.0 markedly increases the stability of salmonellae to heat (10, 29). Over the temperature range evaluated, $D$ values for *Salmonella* spp. were 1.9- to 2.5-fold larger in albumen at pH 8.2 than at pH 9.1 (Table 2). These results correspond closely with the results of a recent study by Palumbo et al. (22). In that report, a six-strain *Salmonella* inoculum yielded a $D$ value at 56.7°C ($D_{56.7}$) of 1.44 min in egg white at pH 8.6 to 8.8. In contrast, Garibaldi et al. (13) and Humphrey et al. (15) reported smaller $D$ values for *S. typhimurium* TM-1 ($D_{56.7} = 0.22$ min; pH 9.2) and *S. enteritidis* PT4 ($D_{56.7} = 0.76$ min; unspecified pH), respectively, heated in liquid egg white. An inoculum pool consisting of three *S. enteritidis* strains and two *S. typhimurium* strains was used in the present study. The results of the most comprehensive studies indicate that *S. enteritidis* is not an unusually heat-resistant serotype, on the basis of the $D$ values determined in raw liquid whole egg (3, 25). The $z_D$ and $E_a$ values determined in the present study (Table 2) are in the range previously reported for salmonellae heated in either liquid egg white (pH 9.0) or liquid whole egg (10).

In comparison to *Salmonella*, *L. monocytogenes* was less affected by the pH of the heating menstruum over the temperature range evaluated (Table 2). $D$ values for *Listeria* strain's in egg white ranged from 3.47 min (at 58.3°C) to 7.58 min (at 55.1°C) at pH 8.2, and from 2.41 min (at 58.3°C) to 7.59 min (at 55.1°C) at pH 9.1. This observation may reflect compositional differences in the cell wall structures of the two organisms. However, the smaller $z_D$ value (and larger $E_a$ value) obtained with pH 9.1 albumen indicates a greater temperature dependence for *Listeria* inactivation in more alkaline egg white (Table 2). In pH 9.1 albumen, $D$ values for the *L. monocytogenes* strains tested were 2.4 to 4.6 times larger than those determined for *Salmonella* spp. In the only other publication on the thermal resistance of *Listeria* spp. in liquid egg white, Palumbo et al. (22) reported $D$ values in pH 8.6 to 8.8 albumen which were 2 to 3 times larger than those determined in the present study for pH 9.1 albumen. For the strains tested, minimum conventional pasteurization parameters for liquid egg white (3.5 min at 56.7°C) would represent a 1.2-$D$ (pH 8.2) to 2.2-$D$ (pH 9.1) process for *Salmonella* spp., and a 0.7-$D$ (pH 8.2) to 0.8-$D$ (pH 9.1) process for *Listeria* strains (Table 2). Current U.S. egg pasteurization requirements do not specifically address the issue of process lethality as a function of albumen pH (1).

Because of the limited heat tolerance of unsupplemented liquid egg white proteins, currently recommended conventional pasteurization temperatures are $\leq 56.7°C$ (134.1°F) in the holding tube. The minimum pasteurization time-temperature curves prescribed by the USDA (29) were designed to yield a 9-$D$ process for *Salmonella* spp. in liquid whole egg and to assure "approximately equal degrees of pasteurization effectiveness" in other liquid egg products (29). While the use of relatively low temperatures helps to limit protein denaturation and the loss of functional properties, the current time-temperature combinations prescribed by the USDA for unsupplemented egg white also appear to be far less lethal to *Salmonella* and *Listeria* strains than are the higher-temperature processes used for liquid yolk and liquid whole egg.

Clearly, thermal treatments which represent 1- to 2-$D$ processes for *Salmonella* and *Listeria* strains in liquid egg white do not provide a large safety factor, nor a large margin for error in terms of processing-plant sanitation. These risks must be evaluated in light of actual pathogen incidence-population level data from microbiological surveillance studies, and relative to growth potential risks during the refrigerated storage of liquid egg. Relatively little published quantitative data on pathogen levels in liquid egg is currently available. Garibaldi et al. (12) reported that 30% of raw liquid egg white samples ($n = 98$) tested positive for the presence of *Salmonella* spp., with a maximum population of 110 salmonellae per ml. In a second study, 60 of 160 (37%) samples of raw liquid whole egg and egg white collected over a 1-year period were *Salmonella* positive, with a

---

### TABLE 2. D values, $z_D$ values, and activation energies of inactivation for *Salmonella* spp. and *Listeria* monocytogenes in raw liquid egg white

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Egg pH</th>
<th>55.1 (min) (SD)</th>
<th>56.7 (min) (SD)</th>
<th>58.3 (min) (SD)</th>
<th>$z_D$ value (°C)</th>
<th>$E_a$ (kJ/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella</em> spp.</td>
<td>8.2</td>
<td>7.99 (0.65)</td>
<td>2.96 (0.18)</td>
<td>1.00 (0.05)</td>
<td>3.54</td>
<td>586</td>
</tr>
<tr>
<td></td>
<td>9.1</td>
<td>3.17 (1.75)</td>
<td>1.58 (1.14)</td>
<td>0.52 (0.34)</td>
<td>4.08</td>
<td>510</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>8.2</td>
<td>7.58 (0.26)</td>
<td>4.76 (0.27)</td>
<td>3.47 (0.31)</td>
<td>9.43</td>
<td>221</td>
</tr>
<tr>
<td></td>
<td>9.1</td>
<td>7.59 (0.64)</td>
<td>4.35 (0.44)</td>
<td>2.41 (0.22)</td>
<td>6.41</td>
<td>324</td>
</tr>
</tbody>
</table>

$^a$ Mean of 3 trials ($n = 3$).
maximum population of 5 salmonellae per ml. The results of an unpublished 1991-92 U.S. national survey conducted by the USDA (Agricultural Marketing Service, Poultry Division, Egg Products Inspection Branch) demonstrated that 2.5% of raw liquid yolk products (n = 203) and 2.3% of conventionally-pasteurized liquid yolk products (n = 86) tested positive for the presence of L. monocytogenes. In the same survey, 41 raw and 26 pasteurized samples of unsupplemented liquid egg white were free of detectable L. monocytogenes, although 2.4% of raw samples and 7.7% of pasteurized samples tested positive for L. innocua. Moore and Madden (20) determined the numbers of Listeria spp. in samples of raw liquid whole egg collected on 9 consecutive days from a commercial processing plant. The mean Listeria population was 1.0 CFU/ml, with a maximum of 40 CFU/ml.

Prior published research has demonstrated that the growth and survival potential of Salmonella and Listeria strain’s in egg white is reduced at more alkaline pH values (i.e., near pH 9) (8, 26). The present study confirms that higher albumen pH values also reduce the heat resistance of these two bacterial pathogens (Table 2). Therefore, in addition to strict plant sanitation, the use of slightly aged or chemically adjusted egg white with a pH near 9.0 could enhance the safety of conventionally pasteurized albumen, especially with regard to Salmonella spp. It is also noteworthy that while Listeria spp. are psychrotrophic pathogens (growth at ≥1°C), Salmonella spp. generally will not multiply at temperatures below 8 to 10°C (8). Therefore, proper refrigeration (or freezing) remains an important factor in ensuring the microbiological safety of pasteurized liquid egg products.

In recent years, a renewed interest in accurately defining the microbiological safety of current liquid-egg-processing parameters has emerged. The use of scientifically sound methodology and experimental designs (including confirmation using continuous-flow pasteurization systems) should provide the reliable thermal resistance data necessary for the public health and regulatory agencies to define desirable and reasonably-achievable pathogen inactivation requirements for pasteurized liquid egg products.

ACKNOWLEDGMENTS

This research was funded in part by both the North Carolina Agricultural Research Service and the North Carolina State University/ Michael Foods, Inc. Single Sponsor Laboratory. The authors thank S. A. Hale and R. Qureshi for technical assistance in conducting these studies. The authors also thank Dr. H. R. Ball and Dr. P. M. Foegeding for valuable input regarding the experimental design.

REFERENCES