International Journal of Food Microbiology 252 (2017) 10–17

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

# Modelling the effect of combined antimicrobials: A base model for multiple-hurdles☆,☆☆

M. Anastasiadi[a], R.J.W. Lambert[b,*]

[a] Cranfield University, Cranfield, Bedfordshire, UK
[b] Dept. Life Sciences Imperial College London, South Kensington Campus, London, UK

ARTICLE INFO

*Keywords:*
Combination index
Synergism
Isobologram
Modelling
Gamma model

ABSTRACT

Combining antimicrobials to reduce microbial growth and to combat the potential impact of antimicrobial resistance is an important subject both in foods and in pharmaceutics. Modelling of combined treatments designed to reduce or eliminate microbial contamination in foods (microbiological predictive modelling) has become commonplace. Two main reference models are used to analyse mixtures: the Bliss Independence and the Loewe reference models (LRM).

By using optical density to analyse the growth of *Aeromonas hydrophila*, *Cronobacter sakazakii* and *Escherichia coli* in combined NaCl/NaCl (a mock combination experiment) and combined NaCl/KCl experiments, previous models for combined antimicrobials in foods, based on the Bliss approach, were shown to be inconsistent and that models based on the LRM more applicable.

The LRM was shown, however, to be valid only in the specific cases where the concentration exponents of all components in a mixture were identical. This is assured for a mock combination experiment but not for a true mixture. This, essentially, invalidates the LRM as a general reference model. A new model, based on the LRM but allowing for mixed exponents, was used to analyse the combined inhibition data, and concluded that the NaCl/KCl system gave the additive effect expected from literature studies. This study suggests the need to revise current models used to analyse combined effects.

## 1. Introduction

Combining appropriate antimicrobials whether in foods or in pharmaceutics is a strategy to reduce the total loading of the combined preservatives or drugs, potentially reduce drug toxicity, increase the spectral range of the mixture beyond that of any one adjunct, and of increasing importance - to help combat the emergence of antimicrobial resistance (CDC, 2013; Krueger et al., 2014). In foods the combination of several preservation methods can be used to reduce organoleptically deleterious effects of using a single or a few factors to preserve food products. This approach, known as combined hurdle technology, although distinct from combined antimicrobials in pharmaceutics has the same goal – to reduce a negative effect through combination (Leistner and Gorris, 1995).

Much effort has gone into developing and advancing mathematical models for the prediction of growth of food borne pathogens in foods preserved by combinations of hurdles such as thermal processing, holding temperature, acidity, water activity, multiple preservatives, initial inoculum size, the shelf-life and the impact of transportation. These models have become an integral part of modern-day food microbiology, e.g. in HACCP and microbiological risk analysis (Dominguez and Schaffner, 2009; Membré and Lambert, 2008; Nychas et al., 2008).

One particular approach to modelling microbial growth in foods is the Gamma approach in which individual effects are combined multiplicatively and is based on Leistner's Hurdle idea (Zwietering et al., 1992). For each inhibitory effect a growth factor is calculated based on the ratio of the applied level to the optimum level for microbial growth. Multiplication of these gamma factors (γ) gives the overall growth factor which alters, for example, the growth rate from its optimum value.

$$\gamma_{total} = \frac{\mu}{\mu_{opt}} = \gamma(T).\ \gamma(pH).\ \gamma(Aw).\ \gamma(Pres) \quad (1)$$

Eq. (1) shows the Gamma model combining the gamma factors (γ) for temperature (T), pH, water activity (Aw) and applied preservatives

☆ Financial support from the EPSRC, grant ref. EP/K039342/1.
☆☆ The authors declare no potential conflicts of interest.
* Corresponding author at: 5 Station Rd, Sharnbrook, Beds, UK.
*E-mail address:* rjwlambert@gmail.com (R.J.W. Lambert).

http://dx.doi.org/10.1016/j.ijfoodmicro.2017.04.004
Received 20 January 2017; Received in revised form 27 March 2017; Accepted 13 April 2017
Available online 14 April 2017

(Pres) to predict the microbial growth rate (μ), relative to the optimal growth rate ($\mu_{opt}$).

As presented the Gamma hypothesis collates the applied factors as independent entities. This is an oversimplification, and Eq. (1) can only be considered a first approximation. The reason being that temperature affects pH, water activity and also the efficacy of preservatives – especially those that have partition abilities and furthermore weak acid preservatives are affected by temperature, pH and water activity. Some of these effects can be incorporated into a modelling scheme (e.g. pH and weak acids through the use of the pKa), whilst others have to be modelled on a case-by case basis (e.g., Arroyo-Lopez et al., 2012; Coroller et al., 2012; Lambert and Bidlas, 2007). Combinations of hurdles which appear to give a greater effect than that described by the Gamma model may claim to show synergy: the magnitude of the synergy is claimed relative to the expected effect (Eq. (1)) (Augustin and Carlier, 2000a, 2000b).

Previously, the effect of individual preservatives against spoilage and pathogenic bacteria had been successfully modelled using a monotonic exponential decay function (Lambert and Pearson, 2000). Later studies of inhibition using multiple inhibitory factors assumed that the gamma factor for an individual preservative could be expanded for combinations, giving a model, based upon the Gamma hypothesis, which simply combined the contribution from each component (Eq. (2)).

$$\gamma(Pres)_{total} = \gamma(Pres_1).\ \gamma(Pres_2).\ \gamma(Pres_3)\ldots \quad (2)$$

For example the combined effect of pH, acetic and propionic acids against *Aeromonas hydrophila* was given as

$$\gamma(Pres) = exp\left\{-\left[\left(\frac{10^{-pH}}{P_1}\right)^{m_1} + \left(\frac{Acetic}{P_2}\right)^{m_2} + \left(\frac{Propionic}{P_3}\right)^{m_3}\right]\right\} \quad (3)$$

Eq. (3) shows a Gamma model used for the prediction of the effect of combined acetic and propionic acids at a given pH. $P_i$ are concentration parameters and $m_i$ are the concentration exponents.

This model gave a very good fit to the observed data and gave us confidence in describing the combination as additive (in the sense of independent action (Lambert and Bidlas, 2007)).

Within pharmaceutics the basis of much of the literature on drug combinations is based on one of two reference models, the Bliss independence model, of which the Gamma model (Eq. (1)) is an example, and the Loewe reference model (LRM, Eq. (4)) (Chou, 2006; Greco et al., 1995).

$$\sum_{i=1}^{n} \frac{x_i}{X_i} = 1 \quad (4)$$

Eq. (4) shows the Loewe Reference Model (LRM): an n-component mixture has a given effect, which is elicited individually at concentrations Xi; in the mixture the fractional amount of each component, $x_i/X_i$, sums to give the same effect.

Eq. (4) is the equation of a (n-1)-dimensional hyperplane and it defines the expected additive behaviour of a mixture and "deviation from expectation unequivocally indicates an interaction and its type" (Berenbaum, 1985). A mixture, which satisfies the LRM, is labelled as Loewe additive; if the combination achieved the effect, but with a value less than 1 then the mixture is labelled as synergistic, and antagonistic if it is greater than 1. For binary combinations a linear line (an isobole) joining $x_1$ and $x_2$ indicates additive behaviour, a concave line describes the presence of synergy and a convex one the presence of antagonism (Berenbaum, 1978).

One of the most used methods for analysing synergy in pharmaceutical combinations is that of Chou and Talalay (CT), (Chou, 2006). This uses the Hill model to describe the action of individual drugs (Goutelle et al., 2008). The CT method, however, does not model an overall effect, but calculates a measure of the interaction - the Combination Index (CI) for each observed combination of drugs, based on the LRM. The CI is therefore identical to the sum of the fractional inhibitory concentrations (ΣFIC) much used in the analysis of antimicrobial combinations (Hall et al., 1983).

Herein we present a more general model for combined antimicrobials, through a revision of the LRM, which gives a more consistent framework for producing more complex models – both in foods and with pharmaceutics. To achieve this we have examined the effect of NaCl and/or KCl on the growth of 3 organisms: *Aeromonas hydrophila*, *Cronobacter sakazakii* and *Escherichia coli*.

## 2. Methods

### 2.1. Microbes and experimental set up

*Cronobacter sakazakii* (FSM263, isolated from a factory producing infant formula), *Aeromonas hydrophila* (ATCC 7966) or *Escherichia coli* (ATCC 11229) were grown overnight in a flask containing 80 ml tryptone soya broth (TSB; Oxoid CM 129) shaking at 30 °C. The cells were harvested, centrifuged to a pellet, washed and re-suspended in peptone water. A standard inoculum was produced by diluting the culture to an optical density (OD) of 0.5 at 600 nm. This standardized culture was then further diluted to produce the starting inoculum of approximately $1 \times 10^5$ cfu $ml^{-1}$.

All analyses were performed in Bioscreen Microbiological Analysers (Bioscreens), Labsystems Helsinki, Finland.

The analysis of NaCl or KCl on the organisms used twenty linear dilutions of a stock solution (10% (wt/vol) to 0.5% in 0.5% intervals) of sodium chloride or potassium chloride (Sigma Aldrich, UK) prepared in TSB. Each dilution (200 μl) was placed in a column of the Bioscreen plate, giving 10 replicates per concentration (2 plates per experiment). For each protocol diluted standard inoculum was added (50 μl) to all wells except the negative control wells (+ 50 μl of TSB). Plates were incubated for 7 days at 30 °C taking OD measurements automatically every 10 min at 600 nm.

For combined NaCl/NaCl and NaCl/KCl experiments a 20 × 20 grid over 4 Bioscreen plates was used. Linear dilutions of each test antimicrobial were made (10% (wt/vol) to 0.5% in 0.5% intervals) and each dilution (100 μl) placed in either a column or a row of the Bioscreen plates. Standard inoculum (100 μl) was then added to each well. Plates were incubated in two Bioscreens for 7 days at 30 °C taking OD measurements automatically every 10 min at 600 nm.

The time to detection (TTD) was defined as the time to produce an OD = 0.2, the time to detection was obtained through polynomial interpolation and has an accuracy of ± 1 min.

### 2.2. Theory and model development

For a single bioactive, with a monotonic response to concentration and which follows the Lambert-Pearson model (Lambert and Pearson, 2000, LPM), two parameters are required to describe its action (Eq. (5)). If a system of combined hurdles is purely additive, then observations should be predictable using the parameters derived from the fitting of the LPM to each of the individual bioactives used.

$$eff = \exp\left[-\left(\frac{X}{P}\right)^m\right] \quad (5)$$

Eq. (5) shows where eff is the effect measured, P is the concentration at the inflexion point and m is the concentration exponent and X is the concentration of the bioactive substance.

#### 2.2.1. Mock experiment

A standard method used in the development of combination models is the combination of self with self, known as the mock experiment; this cannot be synergistic only additive.

Consider an antimicrobial compound *a*, and another compound *b*,