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Factors that inhibit growth of *Listeria monocytogenes* in nature-ripened Gouda cheese: A major role for undissociated lactic acid

E. Wemmenhove [a, b, c], H.J.F. van Valenberg [b], A.C.M. van Hooijdonk [b], M.H.J. Wells-Bennik [a, *], M.H. Zwietering [c]

[a] NIZO Food Research, Ede, The Netherlands
[b] Food Quality and Design, Wageningen University, The Netherlands
[c] Laboratory of Food Microbiology, Wageningen University, The Netherlands

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A B S T R A C T

In this study, factors relevant to nature-ripened Gouda cheese were evaluated for their potential to inhibit growth of *Listeria monocytogenes*. Factors included water activity, pH, undissociated acetic and lactic acid, diacetyl, free fatty acids, lactoferrin, nitrate, nitrite and nisin. In addition, the effect of temperature was evaluated. For each factor, the actual concentrations and values relevant to Gouda cheese were obtained and the inhibitory effect of these individual factors on growth of *L. monocytogenes* was assessed. This evaluation revealed that undissociated lactic acid is the most important factor for growth inhibition of *L. monocytogenes* in Gouda cheese and that, additionally, low water activity as present in the cheese rind and after prolonged ripening times can also cause full growth inhibition. Gouda cheeses have a typical total lactic acid content of 1.47% w/w. In a 2-week old Gouda cheese, with a pH value of 5.25 and a moisture content of 42% w/w, the concentration of undissociated lactic acid in the water phase is 10.9 mM. Growth of *L. monocytogenes* is not supported when the undissociated lactic acid concentration is >6.35 mM. Concentrations of undissociated lactic acid in the water phase of Gouda cheese will be higher than this value when the total lactic acid content is >0.86% w/w at a pH < 5.25 (relevant to young Gouda cheese), or >1.26% w/w at a pH < 5.50 for mature Gouda cheese (moisture content of 35% w/w). This study underlines the importance of undissociated lactic acid as growth inhibitor for *L. monocytogenes* in Gouda cheese.

1. Introduction

Listeria monocytogenes is a foodborne pathogen and the causative agent of listeriosis (Lou & Yousef, 2000). *L. monocytogenes* is able to form biofilms (Pan, Breidt, & Kathariou, 2006), can grow at low temperatures and can adapt to highly acidic or saline conditions (Cole, Jones, & Holyoak, 1990). This pathogen is a concern to the food industry, in particular in ready-to-eat (RTE) products.

Nature-ripened Dutch-type Gouda cheese is cheese that is coated after production and then dried during ripening. This study focused on the vast majority of Dutch-type Gouda cheeses which are produced using mesophilic starter cultures that primarily consist of strains of *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*, but may also contain *Leuconostoc species* and *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* (Stadhouders, 1974). Nature-ripened Dutch-type Gouda cheese as produced on an industrial scale by the Dutch dairy industry is made from pasteurized cow's milk and is a RTE food. To avoid contamination of cheese with *L. monocytogenes*, raw milk is subjected to minimal pasteurization conditions of 15 s 72 °C as one of the options according to regulation (EC) No 1662/2006 (European Commission, 2006) amending regulation (EC) No 853/2004 laying down specific hygienic rules for food of animal origin (European Commission, 2004). The estimated concentration of *L. monocytogenes* in raw milk

* Corresponding author.
E-mail address: marjon.wells-bennik@nizo.com (M.H.J. Wells-Bennik).

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is low: Meyer-Broseta, Diot, Bastian, Rivière, & Cerf (2003) reported maximum concentrations of 0.1 cfu mL^{-1} in 7.7% of the raw milk samples (<0.04 cfu mL^{-1} for the remaining 92.3% of samples), and Ruusunen et al. (2013) reported maximum concentrations of 30 cfu mL^{-1} for 5.5% of the raw milk samples (<1 cfu mL^{-1} for the remaining 94.5% of samples). Minimum pasteurization of 15 s at 72 °C leads to a reduction of *L. monocytogenes* with 10.4 log units based on the average $D_{72^\circ C}$ (and a minimum inactivation of 2.7 logs based on the 95% prediction interval of $D_{72^\circ C}$ [Den Besten & Zwietering, 2012; ILSI, 2012]). Contamination of cheese during further processing and storage is furthermore limited by applying good manufacturing processing conditions, and sampling schemes are in place to verify absence of *L. monocytogenes* in processing areas, on equipment and on finished products.

Microbiological food safety criteria have been set in European Union regulation EC 2073/2005, which includes criteria for *L. monocytogenes* in RTE foods for three different RTE food categories (European Commission, 2005). For food category 1.1, "Ready-to-eat foods intended for infants and ready-to-eat foods for special medical purposes" this is absence in 25 g (n = 10) for products placed on the market during shelf life. For food category 1.2, "Ready-to-eat foods able to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes" the maximum limit is 100 cfu g^{-1} (n = 5) for products placed on the market during their shelf-life. This criterion shall apply if the manufacturer is able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit 100 cfu g^{-1} throughout the shelf-life. The operator may fix intermediate limits during the process that must be low enough to guarantee that the limit of 100 cfu g^{-1} is not exceeded at the end of shelf-life. For food category 1.2, an alternative criterion may apply, namely, absence in 25 g (n = 5) before the food has left the immediate control of the food business operator, who has produced it; this criterion shall apply to products before they have left the immediate control of the producing food business operator, when he is not able to demonstrate, to the satisfaction of the competent authority, that the product will not exceed the limit 100 cfu g^{-1} throughout the shelf-life. Lastly, for food category 1.3, "Ready-to-eat foods unable to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes" the maximum limit is 100 cfu g^{-1} (n = 5) for products placed on the market during their shelf-life.

When a RTE product intended for consumption by the general population has a pH ≤ 4.4 or $a_w \le 0.92$, a pH ≤ 5.0 and $a_w \le 0.94$, or a shelf life <5 days, the product falls into category 1.3. If this is not the case, evidence for no growth potential can be obtained by predictive mathematical modelling, durability tests and/or challenge tests (European Commission, 2005).

In the case of Dutch-type Gouda cheese, the pH is > 5.0 and a_w > 0.94. Nevertheless, various challenge studies have shown that *L. monocytogenes* does not grow in the core or on the surface of this cheese (Northolt et al., 1988; Wemmenhove, Beumer, Van Hooijdonk, Zwietering, & Wells-Bennik, 2014; Wemmenhove, Stampelou, Van Hooijdonk, Zwietering, & Wells-Bennik, 2013). In this study, the impact of growth-inhibiting factors that are present in Gouda cheese or relevant to storage of Gouda cheese was evaluated for growth inhibition of *L. monocytogenes*, and the predictions were compared with the fate of this bacterium in Gouda as determined in previous challenge studies. Factors with potential to inhibit growth in this evaluation included temperature (T), pH, water activity (a_w), undissociated acetic acid, undissociated lactic acid, diacetyl, free fatty acids, lactoferrin, nitrate, nitrite and nisin. Data were obtained from the literature, or experimentally obtained in the absence of data in the literature. This evaluation leads to a better understanding of the most important factors in Gouda cheese that result in growth inhibition of *L. monocytogenes.*

2. Materials and methods

2.1. Comparison of growth-inhibiting potentials

Components that are present in Gouda cheese were evaluated individually for their potential to inhibit growth of *L. monocytogenes* and ranked in order of importance for growth inhibition. The evaluation was based on the concentration present in cheese, the concentration needed for inhibition of growth of *L. monocytogenes* in culture medium and/or cheese, and the Gamma factor formula describing the relationship between the concentration in cheese and the concentration needed for inhibition of the pathogen.

2.2. Data search

For each component that had the potential to inhibit growth, the scientific literature was evaluated for data on concentrations in Gouda cheese, critical growth limits and Gamma factor formulas. Data on concentrations in Gouda cheese were obtained using a cheese handbook (Fox, McSweeney, Cogan, & Guinee, 2004) and various literature databases (Web of Science, Scopus, PubMed) by using the search terms [cheese] AND [temperature OR water activity OR pH OR diacetyl OR lactoferrin OR nitrate OR nitrite OR nisin OR acetic acid OR lactic acid]. Data on concentrations that were minimally needed to inhibit growth of *L. monocytogenes* were obtained from publications and by using the same search terms as for data on concentrations in Gouda cheese, but by replacing the term [cheese] with [*Listeria monocytogenes*]. In addition, data on concentrations needed for growth inhibition were obtained from Combase (www.combase.cc) using terms ([*Listeria monocytogenes*]; [cheese]; minimum pH OR minimum water activity OR minimum temperature]). The obtained results were sorted on relevance, and data were extracted from the first 500 hits. Critical growth limits for T, a_w and pH in culture medium were obtained from ICMSF (1996). Additional experimental data were generated (see sections 2.3 and 2.4) when no literature data were available.

Additional data on the pH of Gouda after two weeks of ripening and on the total lactic acid content of Gouda ripened during 3–26 weeks were supplied by four Dutch cheese-producing companies (BelLeerdammer, DOC kaas, FrieslandCampina and Rouveen Kaasspecialiteiten), resulting in n = 24502 data points for pH and n = 89 data points for the total lactic acid content. The data on pH as supplied by different companies were clustered, following a Post-hoc test (Duncan, alpha = 0.05, harmonized mean sample size) with SPSS Statistics (IBM, NY, US) showing alpha >0.05. All available data on the total lactic acid content obtained from cheeses of different ages were clustered, as it has previously been observed that the total lactic acid content did not change in Gouda cheese ripened for two weeks until six months (Wemmenhove et al., 2013).

Gamma factor formulas were available in the literature for T, a_w, pH, undissociated lactic acid and acetic acid (Supplemental Material S1), but not for diacetyl, free fatty acids, lactoferrin, nitrate, nitrite, and nisin. For these potentially inhibiting factors, we assumed a linear relationship between the concentration of the factor in the cheese and the degree of inhibition of growth of *L. monocytogenes*. The Gamma factors were calculated by dividing the concentration of the inhibiting factor in cheese by the maximum concentration needed to inhibit growth of *L. monocytogenes* and subtracting this fraction from 1 (as in Equation (5) from Supplemental Material S1).