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# Dynamic modelling of brewers' yeast and *Cyberlindnera fabianii* co-culture behaviour for steering fermentation performance

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A B S T R A C T

Co-cultivation of brewers' yeast (*Saccharomyces cerevisiae*) with *Cyberlindnera fabianii* makes it possible to steer aroma and alcohol levels by changing the inoculation ratio of the two yeasts. A dynamic model was developed based on mono-culture performance of brewers' yeast and *C. fabianii* in controlled bioreactors with aerated wort as medium, describing growth rate, carbohydrate utilization, ethanol production, maintenance, oxygen consumption and ergosterol biosynthesis/use for cell membrane synthesis (the last one only for brewers' yeast). The parameters were estimated by fitting models to experimental data of both mono-cultivations. To predict the fermentation outcome of brewers' yeast and *C. fabianii* in co-cultivation, the two models were combined and the same parameter settings were used. The co-cultivation model was experimentally validated for the inoculum ratios 1:10 and 1:100 brewers' yeast over *C. fabianii*. The use of predictive modelling supported the hypothesis that performance of brewers' yeast in co-cultivation is inhibited by oxygen depletion which is required for the biosynthesis of ergosterol. This dynamic modelling approach and the parameters involved may also be used to predict the performance of brewers' yeast in the co-cultivation with other yeast species and to give guidance to optimize the fermentation outcome.

## 1. Introduction

Spontaneously fermented foods generally have a complex aroma bouquet that is perceived as authentic and valuable compared to the same raw materials fermented with simple defined starter cultures. However, in order to reduce batch to batch variation and also as result of industrial innovations more use is made of defined starter cultures composed of only one or a limited number of selected well performing strains, providing consistent end-products with specific, but generally less complex aroma bouquets. To overcome this drawback of defined starter cultures, the cheese industry developed the so-called "adjunct cultures", which are lactic acid bacteria that do barely contribute to the acidification of the milk, but produce specific desired aroma compounds (El Soda et al., 2000; Spus et al., 2017). Recently, the wine industry also implemented this approach and developed multiple strain starter cultures to produce wines with consistent quality but also a complex aroma bouquet (Andorrà et al., 2012; Barbosa et al., 2015; Ciani et al., 2010; Erten and Tanguler, 2010; Kim et al., 2008; Medina et al., 2012; Renault et al., 2015; Sadoudi et al., 2012; Tristezza et al., 2016). So far, the beer industry has focussed on optimization of the performance of single strain starter cultures (including hybrids and non-*Saccharomyces* yeast) (Michel et al., 2016; Steensels et al., 2012).

Recently, van Rijswijck et al. (2017) showed that co-cultivation of brewers' yeast (*Saccharomyces cerevisiae*) with *Cyberlindnera fabianii* resulted in a beer with a more complex aroma bouquet. Interestingly, inoculation ratios with a higher dose of *C. fabianii* over brewers' yeast revealed specific esters to be formed in higher levels compared to mono-cultivations of the two yeasts, whereas ethanol concentrations were lower, indicating inhibition of brewers' yeast by *C. fabianii*. This pointed to metabolic interactions between the two yeasts and it was hypothesised that oxygen depletion by *C. fabianii* limits the growth performance of brewers' yeast (Smid and Lacroix, 2013; van Rijswijck et al., 2017).

Molecular oxygen is required for several pathways in brewers' yeast, such as biosynthesis of sterols and fatty acids (Daum et al., 1998; David and Kirsop, 1972; Snoek and Steensma, 2007). For the production of 1 mol ergosterol under aerobic conditions, brewers' yeast needs 12 mol oxygen. Excess produced ergosterol is stored intracellularly and can be used for membrane synthesis during growth under anaerobic conditions in the absence of added ergosterol (0.113 mmol of stored ergosterol is needed to produce 1 Cmol biomass) (Klug and Daum, 2014; Rosenfeld et al., 2003; Snoek and Steensma, 2007; Vanegas et al., 2012). Supplementation of the wort with ergosterol (sterol) and Tween 80 (medium chain fatty acids) eliminates oxygen requirements for brewers' yeast thus supporting growth in anaerobic conditions (Longley et al., 1978; van Rijswijck et al., 2017).

Here, we developed a dynamic model to describe the interactions between brewers' yeast and *C. fabianii* in co-cultivation and to predict

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**Abbreviations**

| | | | |
|---|---|---|---|
| N = | yeast cell (number/ml) | q = | specific consumption rate ($h^{-1}$) |
| X = | Biomass (Cmol) | m = | maintenance (mol $Cmol^{-1}$ $h^{-1}$) |
| G = | Glucose ((C)mol) | Y = | Yield |
| M = | Maltose ((C)mol) | MW = | molecular weight (g/mol) |
| R = | Maltotriose ((C)mol) | Km = | half-velocity constant (mol/L) |
| O = | Oxygen (mol) | Ki = | inhibition constant (mol/L) |
| E = | Ergosterol (mol) | ATP = | Adenosinetriphosphate |
| F = | Fraction | Resp = | respiration |
| t = | time (hours) | Ferm = | fermentation |
| | | R = | rate of degradation (mol $h^{-1}$ $L^{-1}$) |
| | | EtOH = | ethanol (mol) |

the fermentation outcome. The model is based on mono-culture performance of both yeasts and considers oxygen consumption, substrate utilization, ethanol production, growth and maintenance. Additionally, for brewers' yeast, ergosterol biosynthesis and use for cell membrane synthesis are accounted for. Finally, the aroma profiles of both mono-cultivations and co-cultivations are discussed.

## 2. Materials and Methods

### 2.1. Yeast strains

Two yeast species were used in this study: *Cyberlindnera fabianii* 65 isolated from the microbiota of fermented masau fruits (Nyanga et al., 2007) and brewers' yeast (*Saccharomyces cerevisiae*) from Lallemand danstar called "Nottingham". Both strains were stored at −80 °C in 15% (v/v) glycerol.

### 2.2. Media

For viable counts of mono-cultures and total viable counts of the co-cultures, Malt Extract Agar (MEA) plates (Oxoid, prepared according manufacturers' instructions) were used. In co-cultures, the viable counts per species were distinguished using selective plates.

Brewers' yeast was distinguished using MEA supplemented with ergosterol and Tween 80 (0.01 volume of 2.5 g/l ergosterol (SIGMA-Aldrich Co., St. Louis, USA) dissolved in a solution of 50% (v/v) ethanol (VWR Chemicals, Amsterdam, The Netherlands) and 50% (v/v) Tween 80 (Merck, KGaA, Darmstadt, Germany)). Plates were incubated anaerobically at 30 °C for 48 h. *C. fabianii* is not able to grow anaerobically even when ergosterol is supplemented.

*C. fabianii* was distinguished using Yeast Nitrogen Base (YNB)/sorbitol agar plates (1× YNB w/o amino acids and ammonium (Becton, Dickinson and Company Sparks, USA), 45.4 mM $(NH_4)_2SO_4$ (Merck, KGaA, Darmstadt, Germany), 20 g/l sorbitol (Merck, KGaA, Darmstadt, Germany), 20 g/l agar (Oxoid Limited, Basingstoke, UK)) incubated aerobically at 30 °C for 48 h. Brewers' yeast is not able to grow on sorbitol as sole carbon source.

Appropriate dilutions of the cultures were made in Peptone Physiological Salt (PPS) tubes (Tritium Microbiology BV, Eindhoven, the Netherlands).

### 2.3. Wort preparation

Pils wort extract (Brewferm®, Beverlo, Belgium) was heated in a water bath at 55 °C for 1 h to liquefy the extract. The extract was diluted with approximately 12 L of 55 °C water until 12 °Brix was reached (measured using a refractometer). The wort was sterilized at 105 °C for 15 min and stored at 4 °C until use.

### 2.4. Propagation

The stock of strains was streaked onto MEA and incubated for 24 h at 30 °C. A single colony was picked and inoculated in 10 ml sterile wort and incubated at 30 °C, 200 RPM for 24 h 1% of the overnight culture was inoculated in an Erlenmeyer flask containing 100 ml sterile wort and incubated static for 3 days at 30 °C.

### 2.5. Fermentation

Wort fermentations were performed in 500 ml Infors bioreactors (Multifors, Infors HT, Bottmingen, Switzerland). Temperature, pH (not regulated) and dissolved oxygen (DO) was continuously monitored with internal probes. The bioreactors were continuously stirred at 100 RPM and before inoculation the wort was flushed with sterile air (1 h) for complete saturation with oxygen (at 20 °C and 21% oxygen in air). At this point, the DO probe was set at 100%. The air inlet was closed, just before inoculation. The sample point of the bioreactor was 115 mm from the top. The volume at the start of the fermentations was 450 ml for the liquid phase and 200 ml for the gas phase.

The wort was inoculated with brewers' yeast and/or *C. fabianii*. Before inoculation the cells were counted using a Bürker-Türk counting chamber (Cell vision technologies, Heerhugowaard, The Netherlands) and the appropriate volume was centrifuged for 5 min at 10,620 × *g* to obtain the desired pitching rate. Samples were taken at appropriate time points. One part of the sample was immediately used for viable cell count, another 2 × 2 ml was transferred and stored at −20 °C in GC-MS vials and the remainder was separately frozen at −20 °C until analysis of the residual sugars and ethanol.

### 2.6. Residual sugars and ethanol analysis

High Performance Liquid Chromatography (HPLC) was performed to quantify ethanol, glucose, maltose and maltotriose on an Ultimate 3000 HPLC (Dionex) equipped with an RI-101 refractive index detector (Shodex, Kawasaki, Japan), an auto sampler and an ion-exclusion Aminex HPX – 87H column (7.8 × 300 mm) with a guard column (Bio-Rad, Hercules, CA). As mobile phase, 5 mM $H_2SO_4$ was used at a flow rate of 0.6 mL/min and the column was kept at 40 °C. Total run time was 30 min. The injection volume was 10 µl. Samples were deproteinated with 0.5 vol Carrez A (0.1 M potassium ferrocyanide trihydrate) and 0.5 vol Carrez B (0.2 M zinc sulfate heptahydrate) and 2 times diluted with MilliQ water. Diluted samples and standards (4–20 mM for glucose, 8–40 mM for maltose and 2–10 mM for maltotriose; 0.72–3.6% (v/v) for ethanol) were injected into the column.

### 2.7. Volatile organic compound analysis

The following method was used to determine the volatile organic compounds present in the sample using headspace solid phase micro extraction gas chromatography mass spectrometry (HS-SPME GC-MS) analysis. Samples were defrosted and incubated for 5 min at 60 °C, followed by extraction for 20 min at 60 °C using a SPME fiber (Car/DVB/PDMS, Supelco). The compounds were desorbed from the fiber for 10 min on a Stabilwax®- DA-Crossband®- Carbowax®-polyethylene-

glycol column (30 m length, 0.25 mmID, 0.5 μm df). The gas chromatograph settings were: PTV Split-less mode (5 min) at 250 °C. Helium was used as carrier gas at a constant flow of 1.5 ml/min. The GC oven temperature, initially at 40 °C for 2 min, raised to 240 °C (10 °C/min) and kept at 240 °C for 5 min (Gamero et al., 2013). Total run time was 28 min. Mass spectral data were collected over a range of m/z 33–250 in full scan mode with 3.0030 scans/seconds. Peaks were annotated using Chromeleon® 7.2. The ICIS algorithm was used for peak integration and the NIST main library to match the mass spectral profiles with the profiles of NIST. Peak areas were calculated using the MS quantitation peak (highest m/z peak per compound).

### 2.8. Statistical analysis

Statistical analysis on the data was performed using the student t-test ($p < 0.05$).

### 2.9. Modelling

Dynamic models were built based on experimental data of brewers' yeast and *C. fabianii* in mono-cultivation, which were combined to confirm and predict the behaviour of both yeast species in co-cultivation. The model for *C. fabianii* is a simplified version of the model for brewers' yeast. Therefore, we first explain the model for brewers' yeast in mono-cultivation. The time interval used was 0.2 h.

The cultivation of brewers' yeast consists of 5 different growth phases: i) a lag phase, ii) an aerobic growth phase, iii) an anaerobic growth phase, iv) a stationary phase, and v) a death phase (Fig. 1).

The following assumptions were made:

i) Lag phase:
   a Specific growth rate = 0 $h^{-1}$
   b Consumption of internal carbohydrates (glycogen and/or trehalose)
   c Consumption of oxygen to generate energy for synthesis of enzymes necessary for growth (implied to be maximum ($q_O^{max}$))
ii) Aerobic growth phase:
   a Oxygen concentration > 0
   b Growth rate depends on oxygen concentration (Monod kinetics)
   c Oxygen consumption depends on the amount of biomass formed
   d Carbohydrate consumption depends on the amount of biomass formed
   e Ergosterol is produced (1 mol ergosterol per 12 mol oxygen)
iii) Anaerobic growth phase:
   a Oxygen concentration = 0
   b Ergosterol > 0
   c Growth rate depends on substrate consumption rates, biomass yield and maintenance
   d Ergosterol depletion depends on the amount of biomass formed.
iv) Stationary phase:
   a Oxygen concentration = 0
   b Ergosterol depletion depends on the ergosterol consumption rate
   c Ergosterol is not yet depleted for more than 24 h
v) Death phase:
   a Oxygen concentration = 0
   b Substrate concentration = 0 and/or ergosterol is depleted for more than 24 h
   c Cell number decreases

All the above assumptions are made for brewers' yeast. For *C. fabianii* ii(e),iii(b), iii(d), iv(b), iv(c) and v are not assumed. Glucose, maltose and maltotriose are considered as substrates for brewers' yeast, whereas only glucose is considered as substrate for *C. fabianii*.

The above assumptions were combined in dynamic models using the equations given in Table I including the equations for growth rate, sugar consumption rate and differential equations for biomass, oxygen, ergosterol and sugars.

The models for growth, sugar and oxygen consumption were fitted using the experimental data of the mono-cultivations. The fraction of oxygen used for ergosterol production ($F_E$) and death rate ($K_d$) of brewers' yeast could only be fitted by the co-cultivations, since ergosterol was not limiting in the mono-cultivation of brewers' yeast. The parameters were fitted first by hand, then using the solver add-in in Microsoft excel 2010 by minimizing the sum of (LogN (exp) – logN (fit))$^2$, (sugar (exp) – sugar (fit))/minimum (sugar (exp,fit)))$^2$ and (oxygen (exp) – oxygen (fit))/minimum (oxygen (exp,fit)))$^2$.

## 3. Results

### 3.1. Brewers' yeast mono-cultivation on wort

The carbohydrate composition of wort consists of maltose (150 mmol/L), glucose (58 mmol/L) and maltotriose (35 mmol/L). Before fermentation, the wort and headspace are saturated with oxygen by flushing with air (21% oxygen). Bioreactors were used to monitor growth, sugar utilization, dissolved oxygen (DO %), ethanol formation and volatile organic compounds (VOCs) profiles. The wort was pitched with $10^5$ colony-forming units (CFU)/mL brewers' yeast and fermented for 7 days at ambient temperature (20 °C). Under these circumstances, brewers' yeast reached $10^8$ CFU/mL and was able to ferment all carbohydrates in wort to carbon dioxide, ethanol (5.2% v/v) and biomass (Fig. 2). As a result of catabolite repression glucose was utilized first,

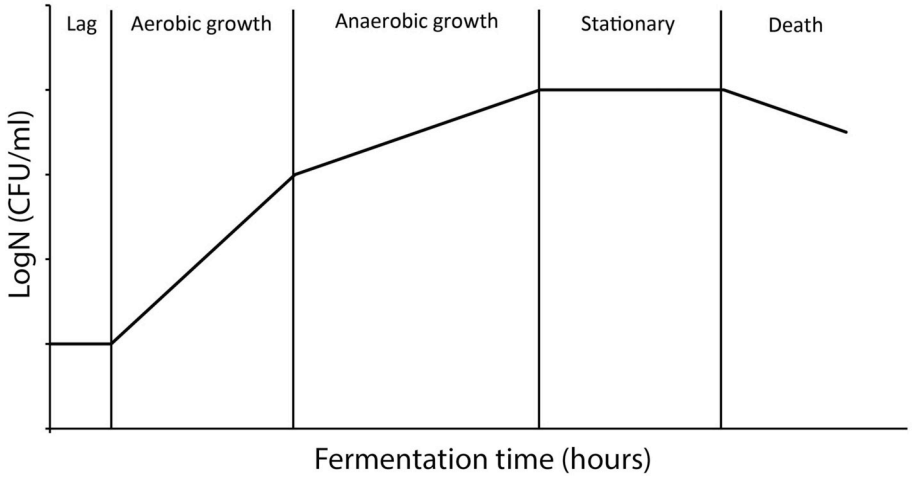


**Fig. 1.** Yeast growth phases considered in the model.

**Table 1**
Equations used to describe the model of brewers' yeast and *Cyberlindnera fabianii*.

| Brewers' yeast | (a) Lag | (b) Aerobic growth | (c) Anaerobic growth | (d) Stationary | (e) Death |
|---|---|---|---|---|---|
| (1) $\mu$ | 0 | $\mu_{max} * \frac{C_O}{C_O + K_O}$ | $\frac{(q_G + q_M + q_R) * Y_{ATP/S} - m_{ATP}}{Y_{ATP/X}}$ | 0 | $-K_d$ |
| (2) $\frac{dC_O}{dt}$ | $-q_O^{max} * C_X$ | $-\mu * C_X * Y_{O/X}$ | 0 | 0 | 0 |
| (3) $\frac{dC_E}{dt}$ | 0 | $\frac{\mu * C_X * Y_{O/X} * F_E}{Y_{O/E}}$ | $-\mu * C_X * Y_{E/X}$ | $-R_E$ | 0 |
| (4) $\frac{dC_N}{dt}$ | $\mu * C_N$ and $C_X = C_N * Y_{X/N}$ | | | | |
| (5) $\frac{dC_i}{dt}$ with i = G, M or R | $\frac{dC_O}{dt} * \frac{Y_{G/X(resp)}}{Y_{O/X}}$ | | $-q_i * C_X$ | | |
| (6) $q_G$ | 0 | | $q_G^{max} * \frac{C_G}{C_G + K_{m,G}}$ | | |
| (7) $q_M$ | 0 | | $q_M^{max} * \frac{C_M}{C_M + K_{m,M}} * \left(\frac{1}{1 + \frac{C_G}{K_{i,G}}}\right)$ | | |
| (8) $q_R$ | 0 | | $q_R^{max} * \frac{C_R}{C_R + K_{m,R}} * \left(\frac{1}{1 + \frac{C_G}{K_{i,G}}}\right) * \left(\frac{1}{1 + \frac{C_M}{K_{i,M}}}\right)$ | | |

| *C. fabianii* | (a) Lag | (b) Aerobic growth | (c) Anaerobic growth | (d)Stationary | (e) Death |
|---|---|---|---|---|---|
| (9) $\mu$ | 0 | $\mu_{max} * \frac{C_O}{C_O + K_O}$ | $\frac{q_G * Y_{ATP/S} - m_{ATP}}{Y_{ATP/X}}$ | 0 | 0 |
| (10) $\frac{dC_O}{dt}$ | $-q_O^{max} * C_X$ | $-\mu * C_X * Y_{O/X}$ | 0 | 0 | 0 |
| (11) $\frac{dC_X}{dt}$ | $\mu * C_N$ and $C_X = C_N * Y_{X/N}$ | | | | |
| (12) $\frac{dC_G}{dt}$ | $\frac{dC_O}{dt} * \frac{Y_{G/X(resp)}}{Y_{O/X}}$ | | $-q_G * C_X$ | | |
| (13) $q_G$ | 0 | | $q_G^{max} * \frac{C_G}{C_G + K_{m,G}}$ | | |

followed by maltose and maltotriose, respectively (Gancedo, 1998; Phillips, 1954). At the start of the fermentation, the DO was set at 100% and reached 0% in 15.5 h. Under anaerobic conditions, brewers' yeast needs ergosterol for cell membrane synthesis (Rosenfeld and Beauvoit, 2003; Snoek and Steensma, 2007). Under the circumstances used here, sufficient oxygen is present at the start of the fermentation to produce and store enough ergosterol to support growth (biomass production) throughout the experiment and to consume all fermentable sugars under anaerobic conditions.

A dynamic model as described was built and fitted to the experimental data. The parameters used to describe aerobic growth of brewers' yeast were maximum specific growth rate ($\mu_{max}$) and affinity for oxygen ($K_O$), and includes the oxygen concentration ($C_O$) as variable ( Equation (1b), Table I). Anaerobic growth of brewers' yeast was described using the yield of ATP per Cmol substrate ($Y_{ATP/S}$), the maintenance factor ($m_{ATP}$) and yield of ATP per Cmol biomass ($Y_{ATP/X}$) as parameters and the substrate consumption rates ($q_G$ (glucose), $q_M$ (maltose) and $q_R$ (maltotriose)) as variables (Table I, Equation (1c)). The growth of brewers' yeast was fitted to the experimental data (LogN (CFU/ml)) using equation (4) (Table I).

The model for oxygen consumption during the lag phase includes the maximum oxygen consumption rate ($q_O^{max}$) and biomass concentration ($C_X$). During aerobic growth, the model includes specific growth rate (μ) and biomass produced ($C_X$) as variables, and the amount of oxygen needed to produce the biomass ($Y_{O/X}$) as parameter (Equation 2a and b, Table I). The fraction of oxygen used for ergosterol production ($F_E$) could not be fitted to the mono-cultivation data and were therefore fitted using experimental data of both co-cultivations using the specific growth rate (μ), biomass ($C_X$), yield of oxygen consumed per Cmol biomass ($Y_{O/X}$) yield of ergosterol per mole oxygen ($Y_{E/O}$) (Equation (3b), Table I).

The carbohydrate concentrations were described using the biomass ($C_X$) and consumption rate for each substrate (Equation (5), Table I). This consumption rate was described by the carbohydrate concentration ($C_G$ for glucose, $C_M$ for maltose and $C_R$ for maltotriose), the affinity constant ($K_{m,G}$ for glucose, $K_{m,M}$ for maltose and $K_{m,R}$ for maltotriose) and the maximal consumption rate for the appropriate carbohydrate ($q_G^{max}$ for glucose, $q_M^{max}$ for maltose and $q_R^{max}$ for maltotriose). For maltose, an inhibition factor for glucose was included in the model and for maltotriose, inhibition factors for both glucose and maltose were included (catabolite repression) (Equations (7) and (8), Table I). The use of intracellularly stored ergosterol was described by the specific growth rate (μ), biomass ($C_X$) and the moles ergosterol needed to produce the biomass ($Y_{E/X}$) (Equation (3c), Table I). When the cells reach stationary phase, ergosterol will be used for cell membrane synthesis and maintenance for membrane integrity which was expressed in a constant ergosterol consumption rate ($R_E$) (Equation (3d), Table I) (Klug and Daum, 2014). Once ergosterol is depleted, the specific death rate ($K_d$) does result in a decline in cell numbers (Equation (1e), Table I). Under the circumstances used in this experiment, ergosterol was not depleted after 7 days of fermentation, thus no decline in cell numbers was predicted, which was in line with the experimental data (Fig. 2). All parameter settings were fitted to the experimental data of brewers' yeast in mono-cultivation or taken from literature (Table II).

### 3.2. *C. fabianii* mono-cultivation on wort

Identical wort composition and bioreactor settings were used for *C. fabianii* as for brewers' yeast mono-cultivation. The wort was pitched with $10^6$ CFU/ml *C. fabianii* and fermented for 7 days at ambient temperature (20 °C). *C. fabianii* grew up to ~$10^8$ CFU/ml, quickly consumed the oxygen (in 5.5 h), could only ferment the carbohydrate glucose and therefore only 0.67% (v/v) alcohol was produced (Fig. 2).

The model to describe the behaviour of *C. fabianii* in mono-cultivation on wort is a simplified version of the model of brewers' yeast, since *C. fabianii* can only grow and ferment glucose and utilize maltose when oxygen is available. No ergosterol production, storage and

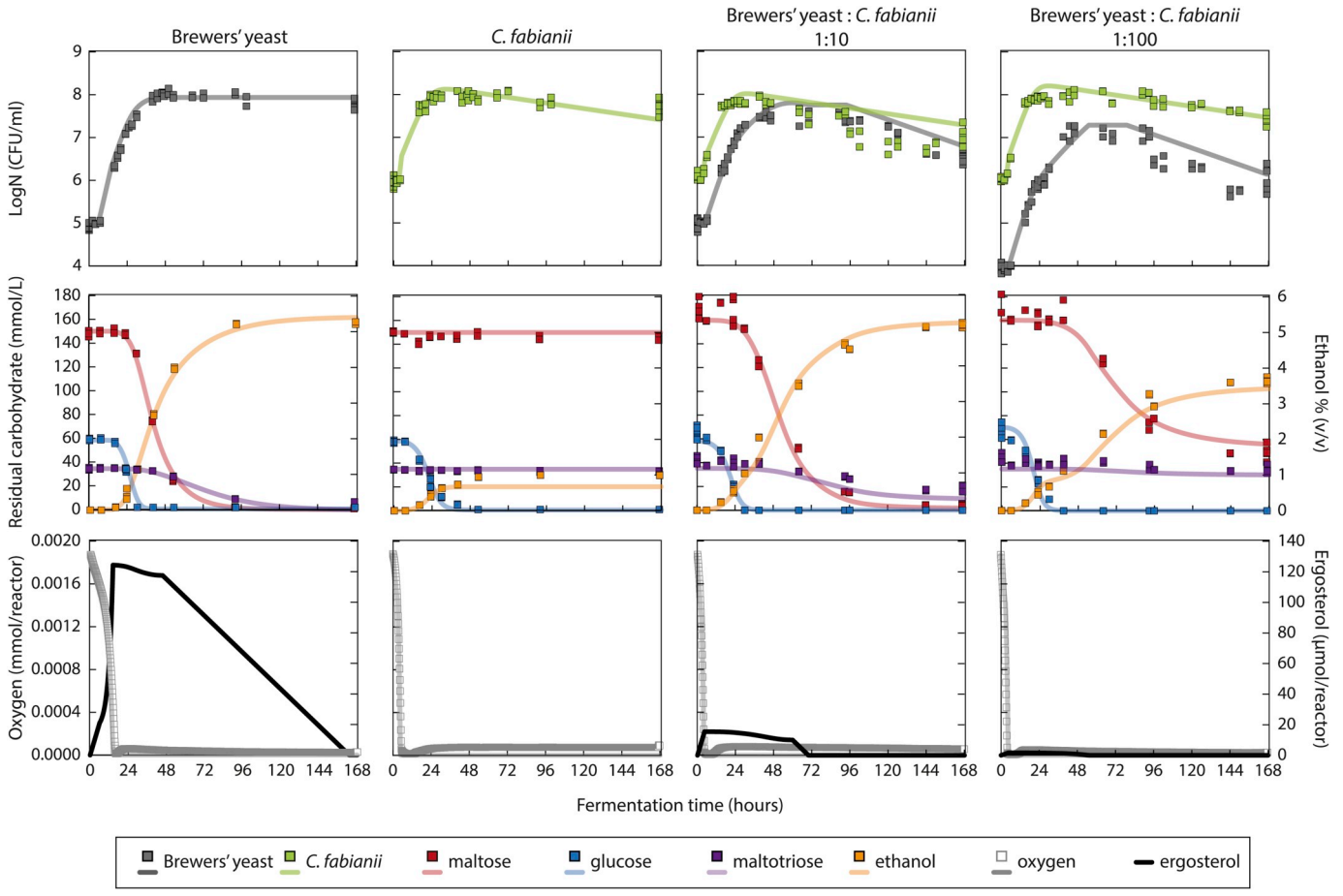


**Fig. 2.** Visualization of experimental data (dots) and model (line) of mono- and co-cultivations of brewers' yeast and *C. fabianii*. On the top, the amount of viable cells is expressed in log (CFU/ml) over time (grey (brewers' yeast) and green (*C. fabianii*) dots and lines). In the middle the residual sugars (red (maltose), blue (glucose) and purple (maltotriose) dots and lines) and ethanol percentage (orange dots and line) are shown over time. The bottom graphs show the oxygen (grey dots and line) over time and model for ergosterol production and consumption by brewers' yeast (black line). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

depletion was taken into account. The parameters and variables used to describe the growth rate for *C. fabianii* were; maximum specific growth rate ($\mu_{max}$), concentration of oxygen ($C_O$) and the affinity for oxygen ($K_O$) for aerobic growth. For anaerobic growth, the glucose consumption rate ($q_G$), yield ATP per Cmol substrate ($Y_{ATP/S}$), yield ATP per biomass ($Y_{ATP/X}$) and the maintenance factor ($m_{ATP}$) were used (Equation (9) b and c, Table I). Growth (LogN) of *C. fabianii* was described using equation (11) (Table I). The oxygen consumption during the lag phase was described by the maximum oxygen consumption rate ($q_O^{max}$) and biomass ($C_X$) (Equation (10a), Table I). In the aerobic growth phase, the oxygen consumption model uses the specific growth rate (μ) and biomass ($C_X$) as variable and the amount of oxygen needed for biomass production ($Y_{O/X}$) as parameter (Equation (10b), Table I).

The substrate consumption rate was fitted using the maximum consumption rate ($q_G^{max}$), substrate concentration ($C_G$ for glucose) and the affinity constant ($K_m$) (Equation (13), Table I). Since *C. fabianii* cannot ferment maltose and maltotriose, those concentrations remain constant and do not affect the specific growth rate. Once glucose is depleted the cells stop generating ATP, therefore the ATP used for maintenance of the cell ($m_{ATP}$) results in a decline in cell numbers which is in line with the experimental data. The ethanol production was calculated by the substrate consumption (2 mol of ethanol per mole glucose). All parameter settings were fitted to the experimental data of *C. fabianii* in mono-cultivation or taken from literature (Table II).

### 3.3. Model of brewers' yeast and C. fabianii co-culture on wort

The models for mono-cultivations of brewers' yeast and *C. fabianii* were combined to predict the outcome of two inoculation ratios; 1:10 and 1:100 brewers' yeast over *C. fabianii* using the same conditions as set for the mono-cultivations (n = 4) (Fig. 2). All dynamics of cell growth, ethanol production and substrate and oxygen consumption were surprisingly well predicted by using the model for both inoculation ratios. However, the fraction of oxygen used for ergosterol production ($F_E$) and the death rate ($K_d$) were fitted to the experimental data of the co-cultivations, since it was not possible to fit these parameters to the experimental data of brewers' yeast in mono-cultivation.

The model predicts maltose and glucose depletion for the ratio 1:10 within 7 days of fermentation and for the ratio 1:100 only glucose depletion was predicted within this timeframe. The amount of ethanol produced depends on the amount of carbohydrates consumed, therefore lower amounts of alcohol were predicted in the ratios 1:10 and 1:100 compared to brewers' yeast in mono-cultivation. Most oxygen was quickly consumed by *C. fabianii* and only a limited amount of oxygen was consumed by brewers' yeast. Thereby, insufficient levels of ergosterol were produced by brewers' yeast resulting in growth inhibition and limited fermentation providing a final product with lower alcohol levels.

Using this model, the outcome of 7 days of fermentation inoculated with decreasing ratios of brewers' yeast over *C. fabianii* was predicted (Fig. 3). It is clear that starting from a ratio of 1:10 brewers' yeast and *C.*

**Table 2**
Parameters used in the models.

| symbol | Explanation | BY | Cf | Reference |
|---|---|---|---|---|
| $Y_{O/X}$ | Mol oxygen needed per Cmol biomass (mol/Cmol) | 1 | 4 | Fitted |
| $Y_{G/X}$ | Mol glucose needed per Cmol biomass (Cmol/Cmol) | 0.74 | 0.74 | Fitted |
| $Y_{E/X}$ | mole ergosterol needed per Cmol biomass (mol/Cmol) | 0.000113 | / | Rosenfeld and Beauvoit (2003) |
| $Y_{X/N}$ | Cmol biomass per yeast cell (Cmol/cell) | 1.71E-12 | 4.57E-13 | Determined, (Dry weight/$MW_x$) |
| $MW_X$ | Molecular weight of biomass (g/Cmol) using $CH_{1.748}N_{0.148}O_{0.596}P_{0.009}S_{0.0019}M_{0.018}$ | 26.4 | 26.4 | Lange and Heijnen (2001) |
| $Y_{O/E}$ | Mole oxygen needed per mol ergosterol (mol/mol) | 0.083 | / | Snoek and Steensma (2007) |
| $Y_{ATP/S(resp)}$ | Yield ATP per Cmol substrate (ATP/Cmol) (respiration). 16 ATP from 6 Cmol substrate. | 2.67 | 2.67 | Verduyn et al. (1991) |
| $Y_{ATP/S(ferm)}$ | Yield ATP per Cmol substrate (ATP/Cmol) (fermentation). 2 ATP from 6 Cmol substrate. | 0.33 | 0.33 | |
| $Y_{ATP/X}$ | ATP needed per Cmol biomass (ATP/Cmol) | 1.97 | 2.29 | Calculated ($Y_{ATP/S(resp)}/Y_{X/S}$) |
| $Y_{X/S}$ | Cmol biomass per Cmol substrate (Cmol/Cmol) | 1.35 | 1.16 | Fitted |
| $Y_{EtOH/G}$ | Mol ethanol per mol substrate (mol/mol) | 2 | 2 | |
| $Y_{EtOH/M}$ | | 4 | 4 | |
| $Y_{EtOH/R}$ | | 6 | 6 | |
| $t_{lag}$ | Time of lag phase (hours) | 6.29 | 3.98 | Fitted |
| $m_{ATP}$ | Mol ATP needed per Cmol biomass per hour for maintenance (mol $Cmol^{-1}$ $h^{-1}$) | 0.124 | 0.029 | Fitted |
| $K_{m,O}$ | Concentration of oxygen where $\mu_{resp}$ is half the $\mu_{max}$ (mol/L) | 0.0004 | 0.0001 | Fitted |
| $K_{m,G}$ | Concentration of glucose where $q_G$ is half the $q_G^{max}$ (mol/L) | 1.5 | 1 | Fitted |
| $K_{m,M}$ | Concentration of maltose where $q_M$ is half the $q_M^{max}$ (mol/L) | 15 | / | Fitted |
| $K_{m,R}$ | Concentration of maltotriose where $q_R$ is half the $q_R^{max}$ (mol/L) | 1 | / | Fitted |
| $K_{i,G}$ | Inhibition factor by glucose (mol/L) | 0.6 | / | Fitted |
| $Ki_{,M}$ | Inhibition factor by maltose (mol/L) | 0.3 | / | Fitted |
| $q_G^{max}$ | Maximum specific consumption rate of glucose (Cmol $Cmol^{-1}$ $h^{-1}$) | 7 | 6 | Fitted |
| $q_M^{max}$ | Maximum specific consumption rate of maltose (Cmol $Cmol^{-1}$ $h^{-1}$) | 8 | / | Fitted |
| $q_R^{max}$ | Maximum specific consumption rate of maltotriose (Cmol $Cmol^{-1}$ $h^{-1}$) | 0.3 | / | Fitted |
| $q_O^{max}$ | Specific consumption rate of oxygen during the lag phase (mol $Cmol^{-1}$ $h^{-1}$) | 0.727 | 1.066 | Fitted |
| $R_E$ | Rate of ergosterol degradation when no growth and oxygen is present (mol $h^{-1}$ $L^{-1}$) | $2*10^{-7}$ | / | Fitted |
| $\mu_{max}$ | Maximum specific growth rate ($h^{-1}$) | 0.9 | 1.0 | Fitted |
| $K_d$ | Specific death rate when ergosterol is depleted for more than 24 h ($h^{-1}$) | 0.03 | / | Fitted |
| $F_E$ | Fraction of oxygen used for ergosterol production | 0.8 | / | Fitted |
| $V_l$ | Liquid volume in the bioreactor (L) | 0.45 | 0.45 | Measured |
| $\Delta t$ | Delta time (hours) | 0.2 | 0.2 | Chosen |
| Max ($dO_2$) | Maximum concentration of dissolved oxygen in water at 20 °C (mol/L) | 0.000279517 | 0.000279517 | Calculated |

*fabianii* and decreasing this ratio further resulted in significant inhibition of brewers' yeast performance resulting in higher levels of residual sugars and lower alcohol levels. Next to these product characteristics, the aroma formation is of major importance for the final product characteristics.

### 3.4. Aroma formation

HS-SPME GC-MS was used to determine the impact of co-cultivations with inoculum ratio 1:10 and 1:100 brewers' yeast to *C. fabianii* on aroma formation compared to mono-cultivations. Significant differences in volatile organic compounds (VOCs) profiles were observed (Fig. 4). The sum of peak areas is clearly higher for both co-cultivations compared to both mono-cultivations (Fig. 4, pie charts). Especially, the marked compounds represented by ethyl 9-hexanodecanoate, ethyl decanoate, ethyl dodecanoate, ethyl hexanoate, ethyl pentadecanoate, propyl decanoate, propyl octanoate, 2-methylpropyl hexanoate, 3-methylbutyl octanoate, 3-methylbutyl pentadecanoate and decanoic acid (indicated with ←) were found in higher levels in co-cultivation compared to the mono-cultivations (Fig. 4). Further information on the secondary metabolite production and cross-interactions is needed in order to model and predict the aroma formation in co-cultivations.

## 4. Discussion

The use of dynamic modelling to predict the outcome of a fermentation process and to describe the interaction between multiple organisms strengthens hypotheses made based on experimental data. In this study, a model was developed based on yeast performance in mono-cultivations taking growth, carbohydrate utilization, maintenance, oxygen consumption and ergosterol production/depletion (only for brewers' yeast) into account (Table I). Parameters were fitted to the experimental data or obtained from literature as indicated in Table II. Finally, mono-culture models of both brewers' yeast and *C. fabianii* were combined and the fraction of oxygen needed for ergosterol production and death rate were fitted to the experimental data (of the co-cultivations) to describe the interactions in co-cultivation.

It is well known that brewers' yeast is able to ferment all wort carbohydrates (glucose, maltose and maltotriose). As a result of catabolite repression, first glucose, then maltose and finally maltotriose was utilized, which was also confirmed by the experimental data displayed in Fig. 2. The oxygen consumption was fitted based on the known amount of oxygen in the bioreactor at t = 0 and the approximate time of oxygen depletion, measured using a dissolved oxygen probe. The yield of ergosterol per mole oxygen was based on literature (0.083 mol ergosterol per mole oxygen (Snoek and Steensma, 2007)), whereas the fraction of oxygen consumed used for ergosterol production was fitted based on the experimental data. Once oxygen was depleted, the use of ergosterol was based on the yield of biomass per mole ergosterol ($1.13*10^{-4}$ mol ergosterol per Cmol biomass) as described by Rosenfeld and Beauvoit (2003). It was assumed that brewers' yeast uses ergosterol for maintenance of the cell membrane functionality after carbohydrate depletion (Daum et al., 1998). The amount of oxygen present in the wort at the start of fermentation supports sufficient ergosterol production for anaerobic growth by brewers' yeast in mono-cultivation, therefore experimental data of the two co-cultivations were needed to fit the fraction of oxygen used for ergosterol production, since it was hypothesised that ergosterol was limiting as a result of competition for oxygen with *C. fabianii* (Smid and Lacroix, 2013). For brewers' yeast in co-cultivation, a decline in cell numbers was observed 24 h after ergosterol depletion, possibly caused by insufficient maintenance of the cell membrane due to ergosterol depletion (Daum et al., 1998). This has been incorporated in the model by introducing the specific death rate ($K_d$) (Maier, 2009), that signifies the decline in cell

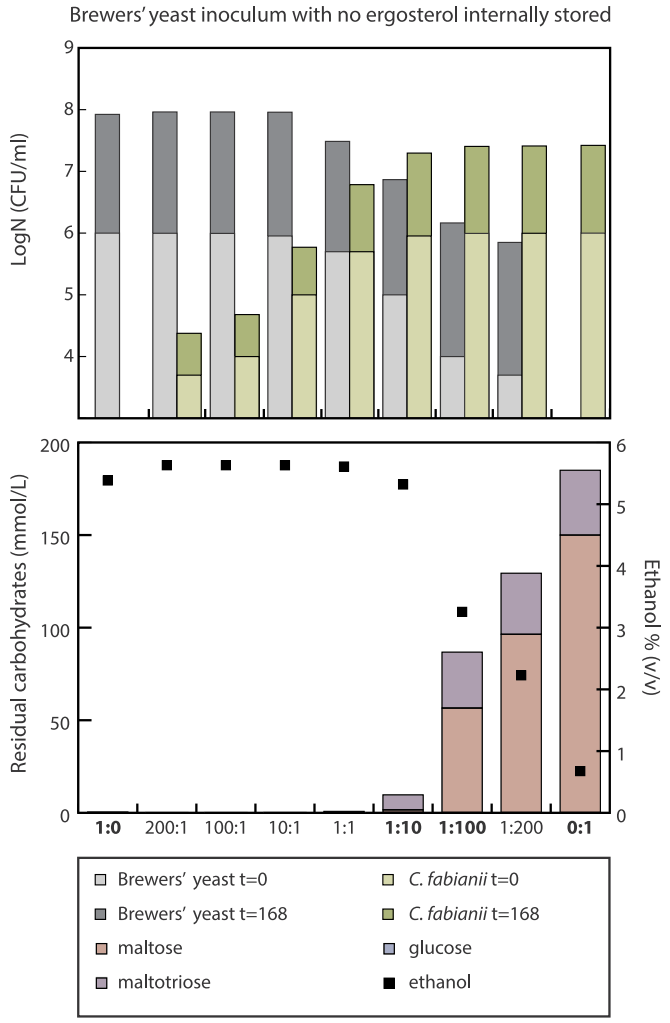


**Fig. 3.** Predicted outcome after 7 days of fermentation with various inoculum ratios of brewers' yeast (grey) over *C. fabianii* (green). The graph on the top displays the inoculum (light grey/green) and the predicted final number of cells after 7 days of fermentation (dark grey/green). The graph on the bottom displays the predicted ethanol % (v/v) (black square) and residual sugars (maltose (red), maltotriose (purple) and glucose (blue)) after 7 days of fermentation. The ratios 1:0, 1:10, 1:100 and 0:1 are experimentally validated (Fig. 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

numbers starting 24 h after depletion of ergosterol.

Using the model described in this paper, it is now possible to predict the outcome of various other brewers' yeast and *C. fabianii* inoculation ratios such as used in van Rijswijck et al. (2017) (Fig. 3). Indeed, a similar trend was observed, but slightly different yields of ethanol and residual sugars were obtained. This is conceivably caused by a different experimental set-up, where van Rijswijck et al. (2017) used culture flasks (with different volumes of liquid ($V_l$) and headspace ($V_g$)) whereas the model was based on data obtained from controlled bioreactors used in the current study (see materials and methods). The amount of oxygen at the start of fermentation is an important determinant of the fermentation outcome of the co-cultivation. The model describes the competition for oxygen, where *C. fabianii* has an advantage over brewers' yeast due to a higher specific oxygen consumption rate (1.066 and 0.727 $h_s^{-1}$ respectively). Reduced oxygen consumption by brewers' yeast will result in less ergosterol production and therefore an inhibition of fermentation performance (biomass production and maintenance). The model can be adapted for the use of other yeast species and for different fermentation and headspace volumes (oxygen availability) to predict and optimize the fermentation outcome (provided that no other than the described inhibitory microbial interactions occur).

The pre-culture conditions used consisted of two steps; the first pre-culture incubation was performed aerobically for 1 day, where the second pre-culture incubation was performed static for 3 days. During the static incubation brewers' yeast will conceivably consume all ergosterol produced in the first pre-cultivation. In the model it was assumed that the cells used for inoculation do not have intracellularly stored ergosterol (at t = 0, $C_E = 0$). If both first and second pre-culture incubations were performed aerobically it is conceivable that sufficient intracellularly stored ergosterol would be available to complete the wort fermentation without the need for oxygen. Since this parameter is an important determinant in the fermentation outcome, the intracellular ergosterol concentration at the start of fermentation (t = 0 $C_E > 0$) can be included in the model.

Obviously, modelling of production of secondary metabolites in mono-cultivation and in co-cultivation is challenging and more information is needed about the enzymatic/chemical reactions especially concerning metabolic interactions during co-cultivation. In the current study we investigated the volatile organic compounds produced in mono- and co-cultivations (Fig. 4). The sum of the peak areas of all esters produced by brewers' yeast and *C. fabianii* in mono-cultivation was smaller than the sum of peak area of all esters found in the tested co-cultivations (brewers' yeast + *C. fabianii* = $1.9 * 10^7$, ratio 1:10 = $3.4 * 10^7$ and ratio 1:100 = $3.6 * 10^7$). This indicates that in co-cultivation additional reactions took place by which esters were formed at higher levels (Fig. 4, marked with ←). Interestingly, among those compounds no acetate esters were found, but mainly medium chain fatty acid (MCFA) esters such as esters from decanoic acid and octanoic acid. These MCFAs can act as inhibitory compounds due to intracellular acidification, resulting in a sluggish fermentation (Legras et al., 2010; Viegas et al., 1989). It is suggested that esterification of these acids is a way for detoxification i.e. maintenance of intracellular pH homeostasis (Legras et al., 2010). Obviously, more insight is required in the extracellular and intracellular enzymatic and chemical reactions in such co-cultivations to enable adequate prediction of the fermentation outcome concerning the production of secondary metabolites.

In conclusion, our dynamic modelling approach predicts co-culture fermentation performance of different doses of brewers' yeast over *C. fabianii* resulting in lower alcohol beer with a more complex aroma bouquet in the co-cultures with a high C. fabianii inoculum. The use of predictive modelling delivered support for our hypothesis on the interaction between brewers' yeast and *C. fabianii* in co-cultivation and shows that brewers' yeast fermentation performance in mono- and co-cultivation can be predicted based on the availability of oxygen, carbohydrates and intracellularly stored ergosterol. According to the model, the addition of a fast oxygen consumer will result in less oxygen consumption by brewers' yeast, thus less ergosterol production and an inhibition of the fermentation performance. At the same time, *C. fabianii* contributes positively to the aroma formation, which masks the unfermented wort flavour. Notably, the described approach can also be used to predict the fermentation outcome of co-cultivations using other selected yeasts. Additionally, the outcome can be fine-tuned by changing the amounts and doses of the inoculation, the amount of oxygen present at the start of fermentation, by adding ergosterol or its precursors to the wort or by uploading the cells by aerobic pre-culturing. Our dynamic modelling approach may further support the implementation of co-cultivations as a novel approach for fermented product innovations.

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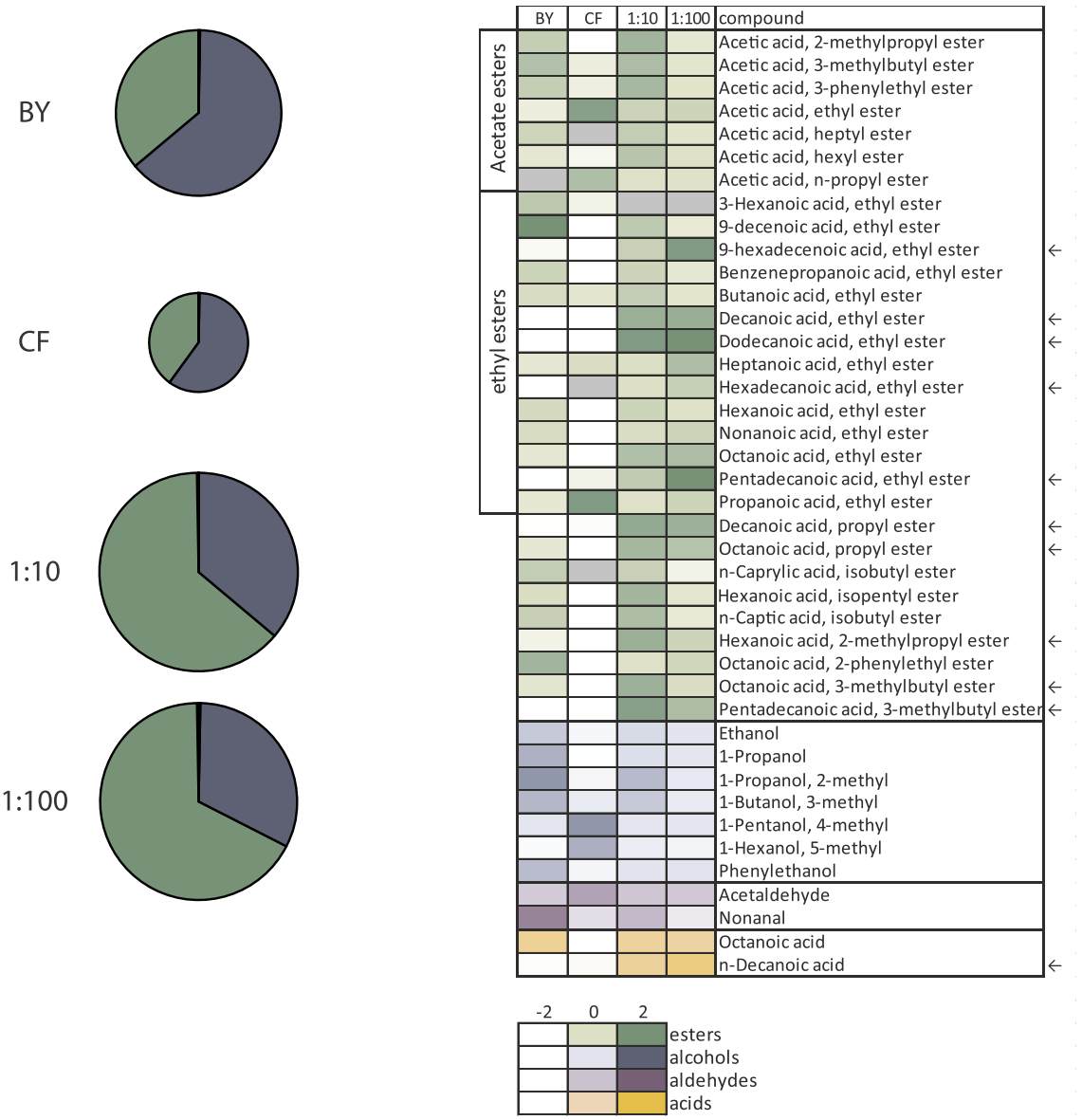


**Fig. 4.** Volatile organic compounds of brewers' yeast (BY) and *C. fabianii* (CF) in mono-cultivation and in co-cultivation in ratio 1:10 and 1:100. The pie charts on the left represent the total sum of all peak areas where the compound groups are indicated by colour (esters (green), alcohols (blue), aldehydes (purple) and acids (orange)). On the right the peak areas are normalized per compound (log2 (compound/median compound all samples) to indicate differences in intensity between the variants. A colour gradient is used to indicate the intensity of the compound (0 = median, −2 = below median and 2 = above median). The compounds are grouped as esters (green), alcohols (blue), aldehydes (purple) and acids (orange). Grey means not detected. Arrows indicate compounds found in higher levels in co-cultivation compared to the mono-cultivations. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2019.04.010.

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