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Production of non-alcoholic beer via cold contact fermentation with *Torulaspora delbrueckii*

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Use of non-conventional yeasts are increasingly seen as a option for the production of low and alcohol-free beers. In this study, the application of four non-conventional yeasts - *Kazachstania servazzii, Kluyevoromyces marxianus, Pichia fermentans* and *Torulaspora delbrueckii*, originally isolated from sourdough cultures, for cold contact fermentations was assessed by screening their ability to reduce wort aldehydes at a fermentation temperature of 1.0 ± 0.5°C. Of the evaluated yeasts, *Torulaspora delbrueckii* was found to be most promising, being capable of the removal of wort-derived aldehyde off-flavours, while being sufficiently sensitive to low temperatures to limit the formation of ethanol. Despite the different alcohol by volume (0.07% vs. 0.28%), the beers produced via cold contact fermentation at 10L scale with *T. delbrueckii* and a reference lager yeast strain were similar, with no major differences found after sensory analysis. The results suggest that *T. delbrueckii* could be used in cold contact fermentation to produce non-alcoholic beers with alcohol content at, or close to, 0%. © 2022 The Authors. *Journal of the Institute of Brewing* published by John Wiley & Sons Ltd on behalf of The Institute of Brewing & Distilling.

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Keywords: cold contact fermentation; non-alcoholic beer; aldehydes; brewing; *Torulaspora delbrueckii*

Introduction

The fast-growing non-alcoholic beer (NAB) segment has drawn a lot of attention in recent years *(1)*, and the production of NABs has been reviewed from multiple points of view. Brányik et al. *(2)* reviewed different production approaches, Bellut and Arendt *(1)* focused on non-*Saccharomyces* yeasts and Pilarski and Gerogiorgis *(3)* on the modelling of cold contact fermentation (CCF). Wild yeasts with their inability to utilise maltose have become a promising option to produce beer with ethanol content at or close to 0.5% *(1, 4, 5)*, which is required for NABs in many countries *(6)*. However, this does not meet the criteria for alcohol-free beers (AFB), where in the UK the ethanol content must be below 0.05% ABV *(6)*. A popular way to restrict ethanol formation is fermentation at near zero temperatures *(3, 7)* and then focusing on the removal of unpleasant wort-derived flavours that can compromise beer quality *(8, 9)*.

The unfermented wort flavours found in many NABs derive from a variety of aldehyde compounds with aroma descriptors ranging from cooked potato to cardboard and chocolate. They originate from malt, and are reduced to some extent during mashing *(10)*, but with low threshold values in beer - e.g. methional at 4.2 μg/L *(8)* – aldehydes must be further removed during fermentation. In conventional beer fermentations, this is not a problem. Brewing yeasts efficiently reduce aldehyde levels below threshold values early in fermentation *(11)*, and the flavour of residual aldehydes is masked by ethanol *(12)* and other volatile aroma compounds *(13)*. However, during NAB fermentations - as the fermentation times are often restricted - the aldehyde levels may remain above the threshold values, causing flavour defects described as raw, vegetal, grainy, or cereal-like *(9)*.

Control of aldehyde content in NABs is critical to producing acceptable beers. As noted by Filipowska et al. *(14)*, the initial concentrations of aldehydes in pale malts may vary significantly, highlighting the importance of careful malt selection. In the same study, it was shown that aldehyde levels can vary depending on malting conditions. Thus, it is not surprising that in the study of Gibson et al. *(11)*, the mash bill was found to play an important role in determining aldehyde concentrations in wort. Ditrych et al. *(10)* monitored the levels of aldehydes during mashing and found that, except for furfural, aldehyde levels were reduced during wort preparation, partly due to their low boiling points. The only exception was wort clarification in the whirlpool where the levels increased. In addition, the reduction of wort-derived flavours has been the subject of several patent applications. Recently, it was found, that the levels of aldehydes can also be reduced by optimising distillation conditions during ethanol removal (patent NL2023801), or by filtering the wort/beer through a specialised sieve (patent BE1026567).

Aldehyde reduction is mainly performed by yeast during fermentation. A well-established approach to produce non-alcoholic

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beers is 'cold contact fermentation' (CCF; also the cold contact process (CCP), first introduced by Schur *(7)*), in which yeast metabolic activity and the formation of ethanol is restricted using low, near 0°C temperatures and short contact times *(15, 16)*. This process is effective as aldehyde reduction is largely unaffected by low temperatures *(6)*. At the turn of the millennium, Perpète and Collin reported fundamental studies on the fate of wort aldehydes during cold contact fermentations. Here, the role of aldehydes, and especially methional, in the perception of 'worty' character of non-alcoholic beers was shown *(9, 12)*. It was also shown how the levels of aldehydes were reduced during the first hours of CCF with viable yeast cells *(17)*, and how reduction could be improved *(18)*. They also studied (for the first time) the application of different yeasts, including non-*Saccharomyces* strains, in the process and found differences in the reducing power of different strains *(9)*.

In a recent study by Johansson et al. *(4)*, the differing capacity of wild yeasts for aldehyde reduction was reported. In the early stage of fermentation, maltose-negative sourdough isolates yielded aldehyde levels that ranged from below those of a reference ale yeast, to above the initial wort levels, depending on the strain involved. The most efficient strains were those of *Torulaspora delbrueckii*, while the strains of *Kluyveromyces marxianus* and the reference *Saccharomycodes ludwigii* increased the net concentration of aldehydes in the beer, at least in the early stages of fermentation. These results suggest that some non-conventional yeasts may possess favourable traits for application in cold contact fermentations. However, it is unclear how the temperature tolerance of yeasts and lower fermentation yields will affect the outcomes at near 0°C temperatures.

Here, the suitability of four non-*Saccharomyces* strains - *Kazachstania servazzii, K. marxianus*, *Pichia fermentans* and *T. delbrueckii* - isolated from sourdoughs *(4)* were screened during laboratory scale, cold contact fermentations for aldehyde reducing power compared to reference strains *Saccharomycodes ludwigii* (VTT C-181010) and *Saccharomyces pastorianus* (VTT A-63015). We were interested to see (i) how low temperatures would affect the ethanol yields of maltose-negative strains, (ii) how temperature tolerance of the yeasts would affect performance at near zero temperatures, and (iii) would the beer produced in CCF with non-conventional yeasts be comparable to that produced with lager yeast. It was hypothesised that cold-sensitive and maltose-negative strains could be used in cold contact fermentation processes to minimise alcohol production. Further, that this would not be at the expense of beer quality given the apparent insensitivity of aldehyde reductase activity to low temperature *(9, 11)*.

Materials and methods

Yeast

The yeast strains used in this study are listed in Table 1. The sourdough derived strains were selected based on a recent study *(4)* and included both cold tolerant and cold sensitive strains. Reference strains were the lager yeast, *Saccharomyces pastorianus* (VTT A-63015) and *Saccharomycodes ludwigii* (VTT C-181010).

Wort

All-malt worts at an original gravity of 15° Plato were brewed at the VTT pilot brewery (1 hL). Pilsner malt (26 kg; Blend 10113, Viking Malt, Lahti, Finland) was mashed in an infusion process with temperature steps of 48°C (30 min) - 63°C (30 min) - 72°C (15 min), and filtered with a Meura filter (Meura, Belgium). Wort was boiled for 60 min with Magnum hop pellets (113 g, target of 45 IBUs). Hot wort (> 90°C) was transferred to sterile kegs and stored at 0°C. Prior to use, the 15°P wort was diluted to 12°P or 6°P with autoclaved Espoo City water. For the 10L scale fermentations, 100 g of aroma hops (Cascade pellets) was added to the whirlpool (130 L). Prior to fermentation, the pH of the wort was adjusted to pH 4.3 by the addition of 80% lactic acid (Vinoferm, Brouwland, Beverlo, Belgium).

Fermentation

Laboratory scale fermentations were performed in 500 mL Schott bottles capped with three outlet stoppers (airlock, CO2 feed and sampling) on a shaker (60 rpm) at 1.0 ± 0.5°C. 300 mL of 12°P wort was transferred to each bottle with CO_2 and purged for 30 secs with CO_2 (flow: 2 L/min). Yeast was propagated in 25 mL YPD (yeast extract 1%, peptone 2%, glucose 4%) for 24 hr in 100 mL Erlenmeyer flasks, followed by 48 hr in 200 mL YPD in 500 mL Erlenmeyer flasks, on a shaker at 120 rpm. Cultures were centrifuged (1,859 x g, 5 min, 1°C) twice, with washing (saline, 0.9% w/v NaCl) prior to preparation of 33% w/v slurries (330 mg fresh yeast/mL) in saline. The slurry was cooled to fermentation temperature (1.0 ± 0.5°C) and pitched into wort at a rate of 3.3 x 10^7 cells/mL. Outlets, excluding the airlock, were only opened at sampling times. All fermentations were in duplicate. For sampling, shaking was stopped, airlock line closed, and samples forced from the vessels via the sampling line by increasing pressure from the CO_2 feed line. The samples were collected in chilled, sterile centrifuge tubes, centrifuged (9,410 x g, 10 min, 1°C) and filtered (0.45 μm) prior to freezing (-23°C).

Table 1. Yeast strains and origin

Yeast	Code	Name	Source
Kazachstania servazzii	Kser	VTT C-191027	Sourdough
Kluyveromyces marxianus	Kmarx	VTT C-191030	Sourdough
Pichia fermentans	Pferm	VTT C-191033	Sourdough
Saccharomyces pastorianus	A15	VTT A-63015	Lager yeast strain
Saccharomycodes ludwigii	Slud	VTT C-181010	Fruit of *Viburnum* sp.
Torulaspora delbrueckii	Tdel5	VTT C-191035	Sourdough
Torulaspora delbrueckii	Tdel8	VTT C-191036	Sourdough
Torulaspora delbrueckii	Tdel14	-	Wild apple
Torulaspora delbrueckii	Tdel716	VTT C-05716T	Type strain of the species
Torulaspora delbrueckii	Tdel906	VTT C-12906	Grape must

Fermentations (10L) were conducted in cylindroconical steel tubes (0.11 x 1.43 m, cone 53°). Wort (9.75L, 6°P) was transferred into the fermenters from kegs and purged for 5 min with CO_2 (3 L/min) prior to capping with tri-clamp stoppers with a septum. The yeasts were propagated as described above, but with additional steps for increased fermentation volumes (final propagation volume of 2L for *T. delbrueckii* and additional propagation steps of 2 L and 4L for *S. pastorianus* strain A15). Washed 33% yeast slurries, at fermentation temperature (1.0 ± 0.5°C), were inoculated into the wort at a pitching rate of 3.3 x 10^7 cells/mL through the sampling septum of the vessel. The yeast/wort mixture was circulated from the bottom to the top of the fermenter (but underneath the surface) using an external pipe (3.2 mm bore x 1.6 mm wall thickness; Marprene, Watson-Marlow, Cornwall, UK) and a peristaltic pump at the circulation speed of 21 mL/min (equivalent to three times the fermentation volume per day). For a schematic, see supplementary Figure S1. Samples were taken through sampling septum and collected in sterile, pre-weighed and chilled centrifuge tubes on ice, centrifuged (9,410 x g, 10 min, 1°C) and sterile filtered. Samples for gas chromatography were stored frozen (-23°C).

Bottling

The circulation of the suspension in the fermenter with *T. delbrueckii* was ended after 72 h and the yeast was allowed to sediment for a further 72 h. 500 mL of green beer was cropped from the fermenter cone and discarded. The remaining green beer from replicate fermentations were combined in a sterile keg and filtered (Seitz EK, Pall Corporation, New York, NY, USA) into a second sterile keg. The filtered beer was kept 48 h at 0°C and the beer carbonated to 5 g/L. The beer was transferred to 330 mL brown bottles, pasteurised (2 PU) and stored at 0°C prior to sensory analysis. The circulation of wort in reference fermentations was ended after 48 h. The green beer was collected the following day and processed as above.

Chemical analyses

Specific gravity, alcohol level (% v/v) and pH of the degassed samples were measured with an Anton Paar Density Meter DMA 5000 M with Alcolyzer Beer ME and pH ME modules (Anton Paar GmbH, Graz, Austria). Ethanol content was determined by HPLC with a Waters 2695 Separation Module and Waters System Interphase Module liquid chromatograph coupled with a Waters 2414 differential refractometer (Waters Co., Milford, MA, USA). The Aminex HPX-87H Organic Acid Analysis Column (300mm x 7.8mm; Bio-Rad, Hercules, CA, USA) was equilibrated with mM H_2SO_4 (Titrisol, Merck, Germany) in water at 55°C, and samples eluted with 5 mM H_2SO_4 in water at 0.3 mL/min flow rate. Aldehydes were analysed as described previously by Gibson et al. *(11)*. The method used a headspace sampler (Agilent 7697 A) coupled with a gas chromatograph (Agilent 7890 B), used to analyse aldehyde as oximes. The compounds were detected using a Micro Electron Capture Detector (HS-GC-ECD). An HP-5 capillary column (50 m × 0.32 mm × 1.05 μm, J&W Scientific, Folsom, CA) was used to separate the compounds. Reported values are the average of two independent fermentations.

Sensory profiling

Bottled beers were analysed by nine assessors from VTT's trained food and beverage sensory panel using generic descriptive analysis. The panellists gave their assent to participate in the trial and all personal data related to assessors were collected and stored in accordance with the EU General Data Protection Regulation (GDPR) (2016/679). The vocabulary was established by four panellists in two consecutive pre-tasting sessions. In the first session, assessors compared four commercial, well known non-alcoholic beers available in Finland. In the second session they focused on attributes in a set of beers from this study. In panel training, all panellists participated in a discussion on the relevant attributes, and assessed the intensities of the standard samples (for a list of attributes and their intensities, see Supplementary material, Table S1). The sensory evaluation was performed in VTT's ISO-8589 sensory evaluation laboratory, using opaque beer glasses. The samples were coded with three-digit numbers and served in randomised order (Latin squares). 50 mL samples (5°C) were poured 15 min prior to evaluation and glasses were closed with plastic lids. The intensity of each attribute was evaluated on a 0-10 continuous line scale anchored with 0 = attribute not perceivable, and 10 = attribute perceived as very intense. The data were collected with Compusense five, version 5.6 (Compusense Inc., Guelph, ON, Canada). The panellists were instructed to cleanse their palates with water after each sample and to spit out the samples after tasting them.

Statistical analysis

Statistical analysis was performed on the fermentation data with a one-way ANOVA and Tukey's post hoc test using the 'agricolae' package in R (RStudio Inc, Boston MA, U.S.A.; R Core Team, r-project; http://www.r-project.org/). For the sensory data, a two-way mixed model ANOVA with Tukey's post hoc test was performed with IBM SPSS statistics version 26.0 (IBM Corp, Armonk, NY, USA). In this model, the samples were the fixed factor and assessors a random factor. Principal component analysis was performed with the Unscrambler X version 10.5.1 (CAMO Software AS, Oslo, Norway). All variables were mean-centred and auto-scaled prior to analysis.

Results

In the first set of cold contact laboratory fermentations, four different yeasts - Kazachstania *servazzii, K. marxianus*, *Pichia fermentans* and *T. delbrueckii* - were compared to each other and to two reference strains, *S. pastorianus* and *S. ludwigii*. The primary focus was on the ability of the yeasts to reduce aldehydes, and the levels of ethanol produced at 1 ± 0.5°C. The initial aldehyde concentrations of wort were high (5-10 times higher than in previous work *(4, 11)*). This was due to autoclaving, performed to ensure sterile conditions (Table 2). Aldehyde reduction was, however, efficient and most of the aldehydes were reduced below their flavour threshold value after 27 hours. The only exception was methional, which has a relatively low threshold value (4.2 μg/L) and none of the strains was able to achieve this limit. Of the above yeasts, a clear exception was *K. marxianus*, which was significantly less efficient at reducing most of the aldehydes, including 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, methional and phenylacetaldehyde, which were at concentrations above their threshold values. Beers from the other reference strain, *S. ludwigii*, had two aldehydes (2-methylpropanal and methional)

Table 2. Aldehyde levels after 27 hours of cold contact fermentation (1.0 ± 0.5°C) at laboratory scale (300 mL). The results are the mean of two independent fermentations. A15 - *S. pastorianus* lager yeast; Kmarx - *K. marxianus;* Kser - *K. servazzi*; Pferm - *P. fermentans*; Slud - *S. ludwigii*; Tdel8 - *T. delbrueckii*. Superscript letters (a-b) in rows represent post hoc groups. Threshold values from Gernat et al. *(8)*

μg/L	Wort (autoclaved)	A15	Kmarx	Kser	Pferm	Slud	Tdel8	Threshold
2-Methylpropanal	518.8 ± 35.2	5.6 ± 0.1 ^{c}	126.2 ± 12.8 ^{a}	31.3 ± 0.2 ^{c}	14.6 ± 1.8 ^{c}	86.7 ± 18.5 ^{b}	7.8 ± 3.7 ^{c}	86
2-Methylbutanal	271.6 ± 13.2	2.3 ± 0.1 ^{b}	53.4 ± 6.7 ^{a}	6.6 ± 1.1 ^{b}	1.8 ± 0.1 ^{b}	9.2 ± 4.2 ^{b}	2 ± 0.4 ^{b}	45
3-Methylbutanal	1296.9 ± 57.2	14.5 ± 0.2 ^{b}	355.5 ± 73.2 ^{a}	15.6 ± 3.2 ^{b}	18.3 ± 0.5 ^{b}	50.9 ± 22.9 ^{b}	16.6 ± 1 ^{b}	56
Hexanal	8.8 ± 1.3	0.7 ± 0 ^{a}	0.8 ± 0.1 ^{a}	0.7 ± 0 ^{a}	0.9 ± 0.3 ^{a}	0.9 ± 0.5 ^{a}	1 ± 0.3 ^{a}	88
Furfural	2243.1 ± 112.2	2.2 ± 0 ^{b}	25.2 ± 4.1 ^{a}	3.4 ± 0.7 ^{b}	2.1 ± 0.1 ^{b}	4.6 ± 1.7 ^{b}	2.8 ± 0.2 ^{b}	15000
Methional	1111.4 ± 224.4	5.4 ± 0 ^{b}	166.6 ± 51.4 ^{a}	6.8 ± 1.1 ^{b}	11.4 ± 0.6 ^{b}	91.6 ± 46 ^{ab}	11.3 ± 1.1 ^{b}	4.2
Benzaldehyde	7.3 ± 0.7	0.6 ± 0 ^{ab}	1.1 ± 0.1 ^{a}	0.6 ± 0 ^{ab}	0.5 ± 0.1 ^{b}	0.6 ± 0.1 ^{ab}	0.5 ± 0.2 ^{ab}	105
Phenylacetaldehyde	517.1 ± 71.8	10.3 ± 0 ^{b}	106.8 ± 39.5 ^{a}	11.4 ± 2.8 ^{b}	17 ± 0.4 ^{b}	38.3 ± 12 ^{b}	17.6 ± 1.2 ^{b}	0.03

at concentrations higher than beers from the other yeasts. The remaining yeasts, *K. servazzii, P. fermentans, T. delbrueckii*, and the reference lager strain A15, produced beers with similar aldehyde levels, with no statistically significant differences (Table 2), though some deviation in the PCA model was seen (Supplementary material, Figure S2).

The yeasts fell into two groups with respect to ethanol yield at 27 hours: the highest ethanol content was produced by lager yeast A15 with 0.14% ABV, followed by *P. fermentans* with 0.11% ABV (Figure 1, Supplementary Material Figure S2). With the remaining strains, ethanol yields were below 0.1% ABV, ranging from 0.02% (*T. delbrueckii*) to 0.06% (*K. servazzii*). Both yeasts were notable in that aldehydes were reduced to levels comparable to the reference lager yeast, while maintaining low levels of ethanol.

There was one fundamental difference between these two yeasts, with reduced growth of *T. delbrueckii* at 1.5°C, whilst *K. servazzii* showed robust growth (data not shown). Although the ethanol levels remained low after 27 hours with *K. servazzii*, the ethanol levels can be a concern with longer fermentations. In addition, the ABV levels with *K. servazzii* were over 0.05%, which is a maximum value for alcohol-free beers in some countries such as the UK *(6)*. The ethanol levels produced by *T. delbrueckii* remained below the limit for non-alcoholic beers, and the sensitivity of the yeast to cold suggests that it may be easier to control in cold contact fermentation. Thus, based on low alcohol production, efficiency of aldehyde reduction and low temperature sensitivity, *T. delbrueckii* was chosen for further study

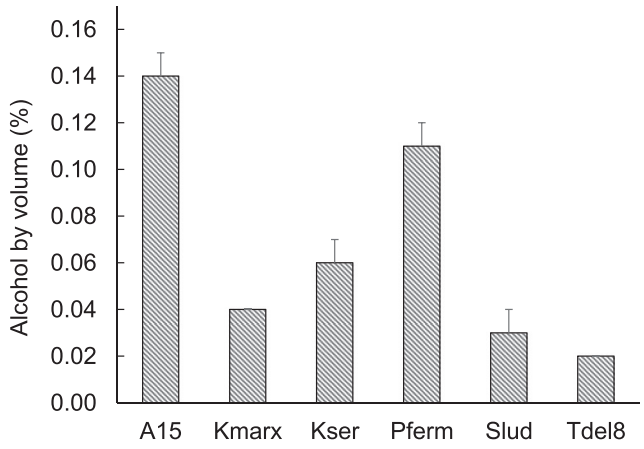

Figure 1. Alcohol by volume from laboratory-scale cold contact fermentations after 27 hrs. Values are the mean of two independent fermentations. Error bars - when visible - represent the deviation between duplicates.

In the next round of cold contact laboratory fermentations, the intraspecific aldehyde reduction capability of the selected yeast *T. delbrueckii* was determined (Table 3). In the first trial (A0), another strain from sourdough (Tdel5) and a strain isolated from apple (Tdel14) were compared to *T. delbrueckii* Tdel8. In a second trial (B0), the Tdel8 strain was further compared to the type strain (Tdel716) and one (Tdel906) isolated from grape must. At this occasion, the wort was not autoclaved prior to fermentation and consequently the initial concentration of many of the aldehydes was much lower, although this had no impact on the final values. The ethanol yields with Tdel8 after A0 were higher than after B0 (Table 3), suggesting either differences between wort batches or in oxygen availability. Despite this, aldehyde levels for Tdel8 were comparable for both trials. The main difference between trials was with methional. Tdel8 had a methional concentration of 12.4 μg/L, three times the threshold value in A0, and less than two times (7.8 μg/L) in B0. With regard to the other aldehydes, differences between A0 and B0 were negligible, and not of practical significance. The levels of 2- and 3-methylbutanal, – compounds which together with methional are considered as unpleasant flavours in NABs *(8)* – were in the range of 1.6-5.7 μg/L and 4.7-11.5 μg/L, respectively. The only statistically significant difference was found with the 2-methylbutanal level of strain Tdel14 (5.7 μg/L). These levels were markedly lower than the threshold values of 45 and 56 μg/L for 2-methylbutanal and 3-methylutanal, respectively. The only exception was *T. delbrueckii* Tdel906 with a 2-methylpropanal value of 61.6 μg/L, which with a threshold value of 86 μg/L might be detectable by sensitive individuals.

Amongst the strains of *T. delbrueckii*, Tdel14 had three aldehydes with significantly higher levels than the other strains in trial A0. In addition to 2-methylbutanal, the levels of 2-methylpropanal (32.1 μg/L) and benzaldehyde (1.1 μg/L) were high. As ethanol levels were similar to other strains, this suggests differences in reduction efficiency depending on the aldehyde. In trial B0, Tdel906 had levels of 2-methylbutanal, furfural and methional slightly higher than the other two strains, but only the level of 2-methylbutanal was significantly higher. The type strain of the species, *T. delbrueckii* Tdel716, reduced aldehydes efficiently, but had higher levels of ethanol (0.05% ABV). Overall, the strains isolated from sourdough, Tdel5 and Tdel8, performed best, with reduced aldehyde levels and without considerable alcohol production. Accordingly, subsequent studies were carried out with Tdel8.

Table 3. Comparative performance of three *T. delbrueckii* strains. ABV and aldehyde levels after 27 hours of cold contact fermentation at laboratory scale (300 mL) in two separate experiments. Values are the mean of two independent replicates. Superscript letters (a-b) in rows represent post hoc groups. Threshold values are listed in Table 2

	Wort A0	Tdel5	Tdel8	Tdel14
% ABV	-	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01
Aldehydes (μg/L)				
2-Methylpropanal	65.9 ± 1.3 ^{a}	15.6 ± 0.9 ^{c}	19.3 ± 0.6 ^{c}	32.1 ± 3.2 ^{b}
2-Methylbutanal	38.9 ± 0.8 ^{a}	1.6 ± 0 ^{c}	1.7 ± 0.1 ^{c}	5.7 ± 1.4 ^{b}
3-Methylbutanal	105.6 ± 2.7 ^{a}	4.7 ± 0.1 ^{b}	4.7 ± 0.1 ^{b}	11.5 ± 3.8 ^{b}
Hexanal	6.2 ± 0.3 ^{a}	0 ± 0 ^{b}	0 ± 0 ^{b}	0 ± 0 ^{b}
Furfural	270.7 ± 26.8 ^{a}	3.5 ± 0.2 ^{b}	3 ± 0 ^{b}	9.7 ± 2.9 ^{b}
Methional	243.2 ± 0.9 ^{a}	12.6 ± 1 ^{b}	12.4 ± 1.2 ^{b}	47.5 ± 20.3 ^{b}
Benzaldehyde	4 ± 0.1 ^{a}	0.7 ± 0 ^{c}	0.8 ± 0 ^{bc}	1.1 ± 0.1 ^{b}
Phenylacetaldehyde	49.5 ± 1 ^{a}	5.6 ± 0.3 ^{b}	5.4 ± 0 ^{b}	8.3 ± 1.6 ^{b}
	Wort B0	Tdel8	Tdel716	Tdel906
% ABV	-	0.02 ± 0.01	0.05 ± 0.01	0.01 ± 0.00
Aldehydes (μg/L)				
2-Methylpropanal	59.4 ± 1.4 ^{a}	20 ± 1.9 ^{b}	12.3 ± 0.7 ^{b}	61.6 ± 6.9 ^{a}
2-Methylbutanal	34.5 ± 1.2 ^{a}	2 ± 0 ^{b}	2.1 ± 0.4 ^{b}	3.1 ± 0.2 ^{b}
3-Methylbutanal	107.7 ± 4.5 ^{a}	5.5 ± 0.4 ^{b}	6.3 ± 0.5 ^{b}	7 ± 0.7 ^{b}
Hexanal	8.6 ± 0.3 ^{a}	0 ± 0 ^{b}	0 ± 0 ^{b}	0 ± 0 ^{b}
Furfural	290.3 ± 13.6 ^{a}	3.3 ± 0 ^{b}	2.8 ± 0.2 ^{b}	4.1 ± 0.4 ^{b}
Methional	275.9 ± 10.7 ^{a}	7.8 ± 0 ^{b}	7.4 ± 0 ^{b}	10.6 ± 1 ^{b}
Benzaldehyde	4.5 ± 0.1 ^{a}	0.9 ± 0 ^{b}	1.1 ± 0 ^{b}	1 ± 0 ^{b}
Phenylacetaldehyde	60.2 ± 1.4 ^{a}	6.8 ± 0 ^{b}	6.6 ± 0.3 ^{b}	6.9 ± 0.7 ^{b}

In 10L cold contact fermentations, extended fermentation times were employed. This highlighted the fundamental difference between the cold sensitive *T. delbrueckii* and cold tolerant lager yeast *S. pastorianus* A15. The latter yeast produced ethanol steadily throughout the fermentation reaching 0.28% ABV within 72 h, whereas the ethanol production of Tdel8 was sluggish, showing a small increase after 48 h (to 0.03% ABV) and reaching a final value of 0.07% ABV after six days of fermentation (Figure 2). In pH adjusted worts, the drop in pH was modest, from 4.32 to 4.28 with *S. pastorianus* A15 and from 4.32 to 4.24 with *T. delbrueckii* Tdel8.

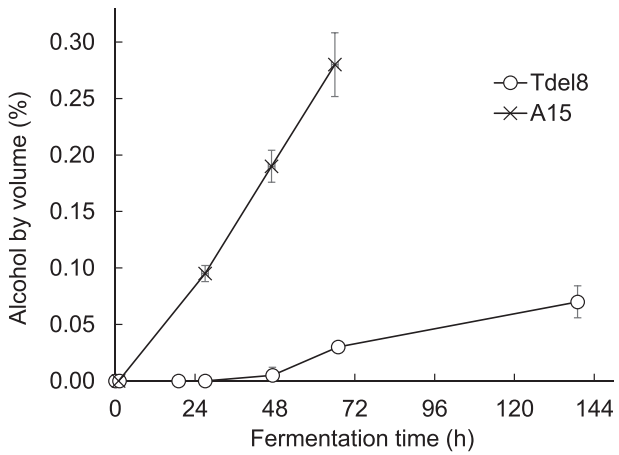

Figure 2. Increase in ABV by *T. delbrueckii* (Tdel8) and lager yeast (A15) in cold contact fermentations (1.0 ± 0.5°C) at 10L scale. Values are the mean from two independent fermentations, and error bars – where visible – represent the deviation between fermentations.

Aldehydes were efficiently reduced by both yeasts. *T. delbrueckii* Tdel8 was slightly more efficient, producing beers with 2-methylbutanal, 3-methylbutanal and phenylacetaldehyde concentration below *S. pastorianus* A15 levels, whereas with A15 only methional was more efficiently reduced. Excluding methional, the aldehyde levels in both yeasts in the experiment were comparable to a commercial non-alcoholic beer. It remains unclear why the methional levels remained high as in previous experiments at a laboratory scale both yeasts had levels 3-5 fold lower.

T. delbrueckii Tdel8 fermentations were extended beyond 72 hours as the ethanol levels at this point were still below 0.05% and it was of interest whether aldehyde reduction would continue. As shown in Table 4, the reduction continued steadily and after the extended fermentation period, all aldehyde levels - except methional - were similar, or less than, those observed with *S. pastorianus* A15

The bottled beers were evaluated via sensory analysis using descriptive profiling. Beers from *S. pastorianus* A15 and *T. delbrueckii* Tdel8 resembled each other and despite a small deviation in aroma, no significant differences were observed (Figure 3; for statistical key values, see supplementary material Table S2). Using the vocabulary, the sensory panel had created on tasting commercial non-alcoholic beers and the beers under evaluation, no statistically significant differences were found in the intensity of descriptors 'honey', 'worty', 'roasted' and 'stale'. Comparing the beers to the commercial beer, a statistically significant difference was found in terms of fruit. The commercial beer was found to be fruitier and less watery. However, the honey aroma was in the same range as with *T. delbrueckii* Tdel8. With regard to non-alcoholic beers, the worty off-flavour can be a major issue. Hence, as described by Ramsey et al *(19)*, the 'worty' character was evaluated using unfermented wort as a reference' The intensity of wort aroma was set at

Table 4. Ethanol and aldehyde levels after cold contact fermentations for 139 hours by *T. delbrueckii* (Tdel8) and 66 hours by *S. pastorianus* lager yeast (A15) at 10L scale. 'Commercial' is the analysis of a non-alcoholic beer available in Finland. Values from *T. delbrueckii* and *S. pastorianus* are the mean from two independent fermentations, while values of the commercial beer are the mean from two technical replicates. Superscript letters (a-b) in rows represent post hoc groups. Threshold values are listed in Table 2

	Tdel8 72h	Tdel8 Final	A15 Final	Commercial
% ABV	0.03 ± 0.00	0.07 ± 0.01	0.28 ± 0.02	0.0
Aldehydes (μg/L)				
2-Methylpropanal	4.2 ± 0.2 ^{a}	2.7 ± 0.1 ^{b}	2.6 ± 0.4 ^{b}	5.6 ± 1.1
2-Methylbutanal	1 ± 0 ^{ab}	0.7 ± 0 ^{b}	1.4 ± 0.2 ^{a}	1 ± 0.1
3-Methylbutanal	3.1 ± 0 ^{b}	2.7 ± 0.1 ^{b}	5.9 ± 0.6 ^{a}	5.4 ± 1
Hexanal	0.8 ± 0 ^{a}	0.7 ± 0.1 ^{a}	0.8 ± 0.1 ^{a}	1.1 ± 0
Furfural	2.7 ± 0.2 ^{a}	2.4 ± 0 ^{ab}	2.2 ± 0 ^{b}	2.6 ± 0.4
Methional	34.8 ± 0.2 ^{a}	31.9 ± 0.4 ^{a}	25.2 ± 1.8 ^{b}	3.9 ± 0.6
Benzaldehyde	0.8 ± 0.3 ^{a}	0.7 ± 0.2 ^{a}	1.2 ± 0.8 ^{a}	0.6 ± 0
Phenylacetaldehyde	7.4 ± 0.1 ^{ab}	6.6 ± 0.4 ^{b}	7.8 ± 0.3 ^{a}	8.2 ± 0.7

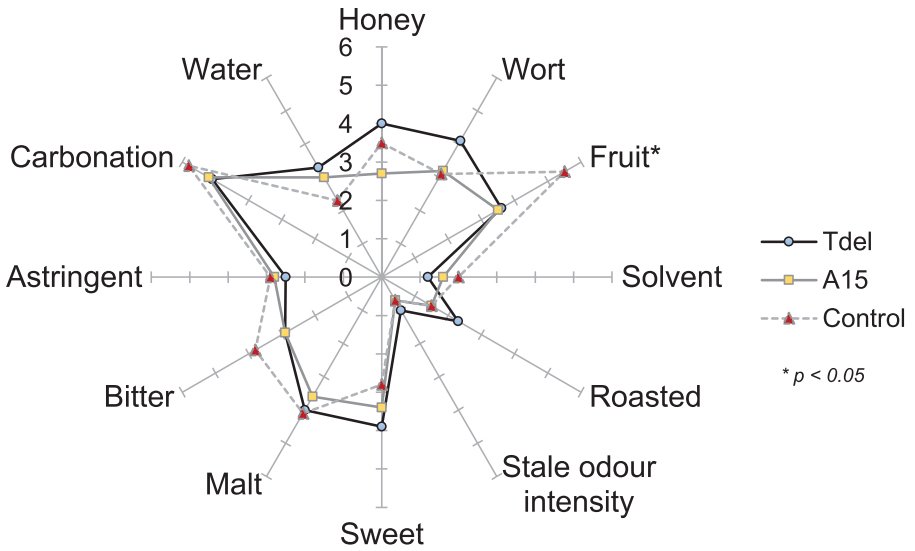

Figure 3. Sensory analysis results of bottled beers from cold contact fermentations at 10L scale (Tdel8 - *T. delbrueckii*, and A15 - *S. pastorianus*) and a commercial non-alcoholic beer ('Control') widely available in Finland. Statistically significant difference was only found for 'fruit'. For values containing averages, standard deviations, two-way mixed model ANOVA p-values and Tukey's HSD post hoc groups, see the supplementary material Table S2. [Colour figure can be viewed at wileyonlinelibrary.com]

8.0 with a clear difference between yeasts, with values of 4.1 for *T. delbrueckii* Tdel8 and and 3.2 for *S. pastorianus* A15.

Discussion

One of the challenges in producing non-alcoholic beers is to limit alcohol formation but have enough yeast activity to remove worty aromas, related to malt derived aldehydes. During the cold contact fermentation process, alcohol production is limited by low temperature, but lager yeasts are psychrotolerant and some activity occurs at the low temperatures. This necessitates careful monitoring throughout fermentation to maintain ethanol levels in the target range. This was seen here with the reference yeast *S. pastorianus* A15, which was capable of ethanol production throughout the fermentation at low temperature.

Substituting the conventional lager yeast with *T. delbrueckii* resulted in considerably lower ethanol yields. The low temperature did not limit the reduction of the aldehydes responsible for worty off-flavours, which were reduced as efficiently as with *S. pastorianus* A15. As the aldehyde results suggest, the total fermentation time of *T. delbrueckii* could be shortened without major impact on the final levels of aldehydes. At this point (after 72 hours of fermentation), the ethanol levels were low (0.03%) enabling the beer to be labelled as 'alcohol-free' in many countries.

The suitability of the *T. delbrueckii* strain for this type of fermentation can be explained by two traits. Firstly, the yeast has adequate aldehyde reductase activity and secondly, the cold sensitivity of the strain efficiently restricts fermentation. As suggested by Perpète and Collin *(9)*, the specificity of the reductases toward different aldehydes may vary between yeast species. This was apparent in the results reported here. For example, *K. marxianus* demonstrated relatively poor aldehyde reduction, and only the linear aldehyde hexanal was reduced efficiently by this strain. The Strecker aldehydes, which proved to be a challenge to reduce for *K. marxianus*, were also challenging for *S. ludwigii*. In contrast,

T. delbrueckii showed intraspecific capability to reduce both Strecker and linear aldehydes.

Based on the results, it was clear that aldehyde reduction capabilities were unrelated to fermentative power. *P. fermentans* produced ethanol almost as efficiently as the reference lager yeast, but with both yeasts, the aldehyde levels were still comparable to those from the *T. delbrueckii* strain. Aldehyde reduction at low temperature did not appear to be greatly affected by the temperature tolerance of the yeast strain. *K. servazzii*, a cold tolerant yeast, had aldehyde levels in a similar range to *T. delbrueckii*. In the study by Perpète and Collin *(17)*, fermentations at higher temperatures were found to be favourable for aldehyde reduction. Similarly, the aldehyde levels with *T. delbrueckii* after 24 hours of fermentation at 20°C *(4)* were slightly lower than the final values in the cold contact fermentation reported here. It is, however, hard to compare fermentation at 20°C to that at near 0°C. During the first hours of fermentation at 20°C, the yeast cells grow exponentially increasing the cell population. Accordingly, it is hard to separate the role of cell number from the effect of temperature. In the same study, Perpète and Collin *(17)* also found that a higher yeast pitching rate favourably affected aldehyde reduction. As in our study, neither cold tolerance nor fermentation activity had any major effect on aldehyde reduction. Accordingly, it seems plausible that reduction power was related to the number of cells in the fermentation as all the above yeasts had aldehydes in proportionate levels.

Of the bottled beers, the beer produced by *T. delbrueckii* had honey aroma notes which were more intense than the beer produced by lager yeast. This was the greatest difference between the beers found by the sensory panel. In a comprehensive study of aroma attributes and likeability of non-alcoholic beers among Californian consumers by Lafontaine et al. *(20)*, a positive correlation between honey notes and 2-furfural, methional, *trans*-2-nonenal and acetaldehyde levels was observed. From the aforementioned aldehydes, methional levels in the *T. delbrueckii* beer were higher and furfural levels slightly higher with *T. delbrueckii* than with *S. pastorianus* lager yeast. However, the methional levels of the commercial beer in the sensory analysis were only a fraction of the values of the strains assessed in this work. Yet, this commercial beer was found to have honey intensity higher than with *S. pastorianus* A15. Furfural levels were slightly higher, but as the difference was negligible, it is unlikely to have influenced the perception of honey aroma. There also is the possibility that honey notes were derived from another compound. For example, phenylethanol, has been described as having a honey aroma *(21)*. It is however unlikely that high levels of this compound or other higher alcohols would be produced by yeast at 1°C. Irrespective of this, the honey aroma was positively correlated with liking among Californian consumers *(20)*.

Methional is considered one of the major compounds contributing to the worty off-flavour *(9, 17)*. In particular, the degradation products of methional together with 2-methylbutanal and 3-methylbutanal are thought to be responsible for the unpleasant taste of some non-alcoholic beers *(8, 22)*. Although other aldehyde concentrations were reduced below 10 μg/L, the methional levels from lager yeast A15 and *T. delbrueckii* remained high, being many times greater than the threshold value after 10L scale cold contact fermentations. This result was unexpected as much lower methional levels were achieved in previous laboratory fermentations. As the levels were high with both strains, the outcome may be related to the fermentation set-up. Although the wort was purged with carbon dioxide, it is possible that not all oxygen was removed. The presence of low levels of residual oxygen may explain the different results between the two *T. delbrueckii* trials. Despite the higher methional levels, the beers from the 10L scale trial were not considered by the sensory panel to be more wort-like than the commercial beer which contained levels below the threshold value.

Based on results of this study, *T. delbrueckii* is recommended as a potential yeast strain in cold contact fermentations. The cold sensitivity of the yeast makes it easier to control during fermentation and enables the production of alcohol-free beer. The fermentative activity of lager yeast strains can be controlled by regulating carbonation levels (patent CZ2017509) but this requires a controllable feed of CO_2. With *T. delbrueckii*, the system requirements are restricted to a near 0°C temperature and to installation of vessel circulation. Many alcohol-free beers are produced using a distillation/separation technique [18] but these are costly in terms of capital expenditure and space. In comparison, the circulation system needed for the current process is simple and may already be used in cold contact processes involving brewing yeasts. The beer produced in this way can be used as is or blended with beer to produce a 0.5% ABV beer.

Future studies should focus on the initial gravity of wort. In this study, it was shown that aldehyde reduction was greater when autoclaved wort - with higher initial aldehyde levels - were used. Similarly, halving the initial strength of the wort (from 12°P to 6°P), did not halve the ethanol production. If confirmed, brewers could use high gravity wort in cold contact fermentation and dilute the final beer to the required original gravity, enabling more efficient use of fermentation capacity, but also lower ethanol and aldehyde levels.

Author Contributions

Jarkko Nikulin: Conceptualisation, methodology, validation and formal analysis, investigation, resources, writing (original draft, review and editing), visualisation.
Heikki Aisala: Methodology, validation and formal analysis, investigation, resources, writing (review and editing), visualisation, supervision
Brian Gibson: Conceptualisation, methodology, resources, writing (review and editing), supervision, project administration and funding acquisition.

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Conflict of interest

The authors declare there are no conflicts of interest.

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Supporting information

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