Aggregation of yeast cells in *Lactobacillus plantarum*: correlation with *msa* gene and description of a novel image analysis method

Parente, E.[1,2], Guidone, A.[1], Ricciardi, A.[1], Rea, M. C.[3,4], Stanton, C.[3,4], Ross, R. P.[3,4]

[1] Scuola di Scienze Agrarie, Forestali, Alimentari ed Ambientali, Università degli Studi della Basilicata, Potenza, Italy

[2] Istituto di Scienze dell'Alimentazione, Consiglio Nazionale delle Ricerche, Avellino, Italy

[3] Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

[4] Alimentary Pharmabiotics Centre, University College Cork, Ireland

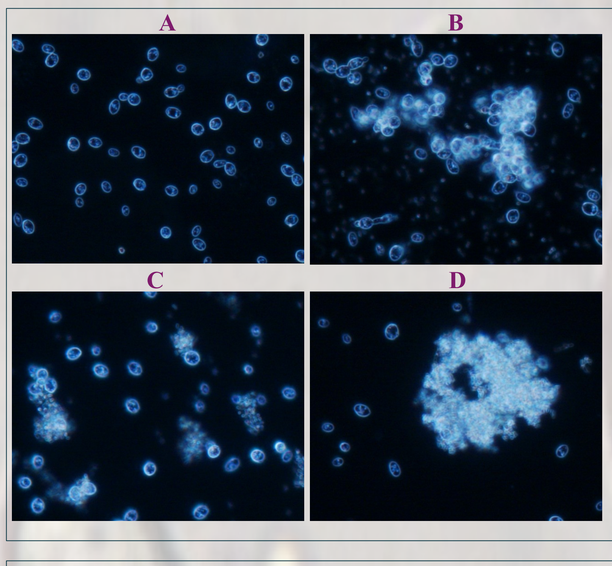
INTRODUCTION

Lactobacillus plantarum can colonize the human intestinal tract and the association with the epithelial surface or the mucus layer is essential for its persistence and for the competitive exclusion of intestinal pathogens which recognise the same adherence sites. One mechanism of adhesion is based on the binding to mannose-containing receptor sites on epithelial cells and the gene encoding the mannose-specific adhesin (Msa coded by gene *msa*) of *Lb. plantarum* has been identified (Pretzer et al., 2005). Mannose-dependent adhesion can be studied using an agglutination assay that is based on the presence of mannose-containing polysaccharides in the cell wall of *Saccharomyces cerevisiae.* Variations in quantitative levels of mannose-adhesion capacity in different strains of *Lb. plantarum* could suggest that additional factors besides Msa contribute to mannose-adhesion. Alternatively, such variations might be explained by genetic diversity of *msa* among *Lb. plantarum* strains, leading to different *msa* expression levels or distinct Msa protein sequences or domain compositions in individual strains of the species with consequently varying mannose-adhesion capacity (Gross et al., 2010). **AIM:** To develop an image analysis method to detect the ability of *Lb. plantarum* subsp. *plantarum* and *Lb. plantarum* subsp. *argentoratensis* strains to aggregate *S. cerevisiae* NCYC 1006 cells and to compare this method with visual analysis. To assess the correlations between occurrence of *msa* gene and the phenotype (mannose dependent or independent aggregation) in a collection of strains.

MATERIALS AND METHODS

***Strains*:** 29 strains of *Lb. plantarum* subsp. *plantarum* and 3 strains of *Lb. plantarum* subsp. *argentoratensis* were previously identified by Multiplex-PCR and PFGE. The presence of *msa* gene encoding for the mannose-specific adhesin (MSA) was evaluated by PCR. ***Agglutination assay*:** Bacterial strains were grown overnight, washed and suspended in phosphate buffered saline (PBS, 0.2 M, pH 7.0). 50 µL of the bacterial suspensions in PBS were transferred to microtiter plates. To each well, 50 µL buffer or buffer with methyl-α-D-mannopyranoside (final concentration 25 mM) and a 100 µL of *S. cerevisiae* NCYC1006 (SC) cell suspension in PBS were added. In a second experiment serial twofold dilutions of the bacterial suspensions were tested in the agglutination assay. In both experiments, the microtiter plates were shaken for 30 min at 28 °C and, 200x dark field images were captured using a Nikon Eclispe 80i microscope equipped with a 5 mega-pixels color cooled digital camera (DS-5Mc, Nikon). Images were processed using ImageJ macros (aggrcountsizev3_1.ijm and aggrcountsizev3_1_2.ijm) to obtain measurements of cells and aggregates. Aggregation was also visually evaluated visually and a panel of 24 subjects with experience in microbiology (Professors, researchers, PhD students), who were asked to identify aggregation or lack thereof in 16 images. ***Statistical analysis*:** Measurements were processed using Systat 13. The possibility of automatic recognition of yeast cells, yeast aggregates and bacterial aggregates was evaluated by discriminant analysis.

RESULTS



Agglutination: How many types?

Images:

A, *S. cerevisiae* NCYC1006 cells;

B, Yeast cells agglutination mediated by *Lb. plantarum* subsp. *plantarum* DPC2159 without methyl-α-D-mannopyranoside (MAN);

C, Yeast cells agglutination and *Lb. plantarum* subsp. *plantarum* P1.5 cells self-agglutinated without MAN;

D, *Lb. plantarum* subsp. *plantarum* DPC2120 self-agglutinated cells without MAN (note differences in texture and border).

Visual evaluation of aggregation

Exp. / True	Aggr+	Aggr-	Other	% wrong
Aggr+	83	10	3	13.5%
Aggr-	22	160	10	16.6%
Other	76	4	16	83.3%

❖ 16 images with agglutination of yeasts, self-aggregation of bacteria and yeasts alone were shown to a panel of 24 people with experience in microbiology.

❖ Images showing yeast agglutination and absence of agglutination were identified, with a relatively high error rate (13.5% and 16.6% of errors, respectively). The high error rate was due to the presence of clumps of budding yeast cells even in the absence of bacteria.

❖ Several people were unable to discriminate yeast-agglutination and *Lb. plantarum* cells with self-aggregation.

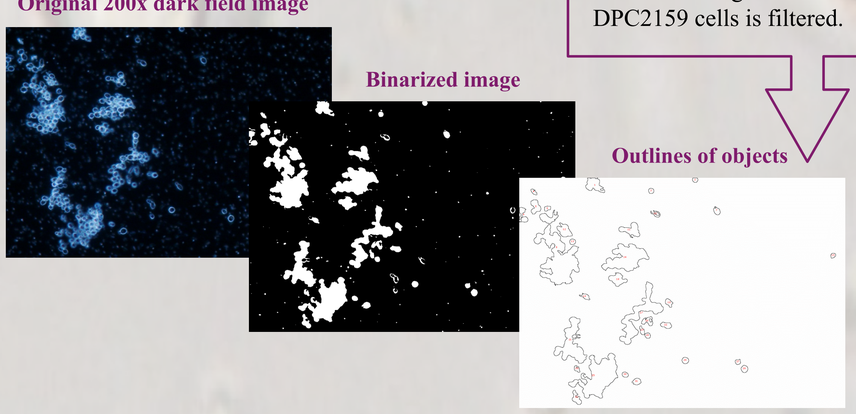
❖ Overall, for Aggr+ and Aggr- the sensitivity was 86.5% and the specificity was 83.4%.

Automated object identification in dark field images

An example of image processed by ImageJ:

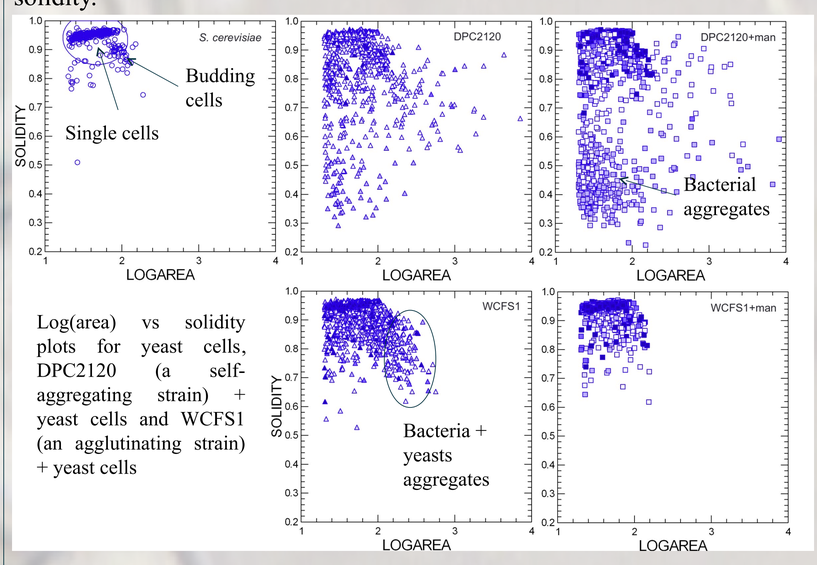
❖ Most objects are correctly identified and measured.

❖ The background of *Lb. plantarum* DPC2159 cells is filtered.



Automated image analysis

Automated image analysis allowed us to record several parameters on all objects detected in any given image. The two most useful parameters were the logarithm of the area of objects and solidity (area/convex area). In the image below different, relatively compact, subsets of yeast cells (single cells of different sizes, budding cells) are shown. Log(area) is usually between 1.3 and 2.1 and solidity is rarely <0.8. Bacterial aggregates (strain DPC2120) have low solidity (often <0.6). Aggregates of bacteria + yeasts have relatively large dimensions and intermediate solidity.



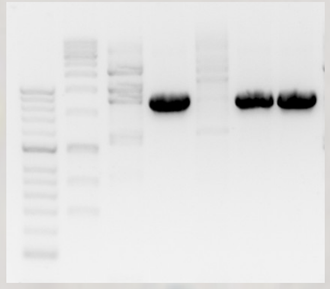
Log(area) vs solidity plots for yeast cells, DPC2120 (a self-aggregating strain) + yeast cells and WCFS1 (an agglutinating strain) + yeast cells

215 objects were randomly selected from the 27713 objects available in the experiment and were used to compare the effect of strain, mannose and dilution and were used for linear discriminant analysis using 6 properties: Feret, minimum feret, aspect ratio, roundness, solidity and log(area).

Although the discriminant function satisfactorily discriminated the aggregates from the yeast cells (only 4% of yeast cells were classified as aggregates, and only 5% of aggregates were classified as yeast cells), it was unable to discriminate between the two types of aggregates.

Correlation between msa *detection and mannose-specific aggregation*

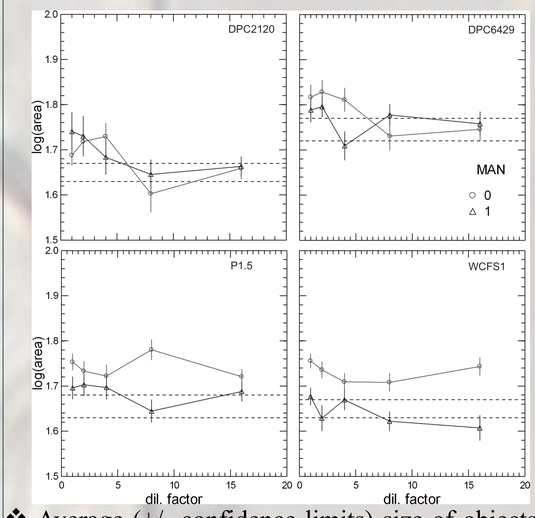
Agarose gel showing *msa* amplicons

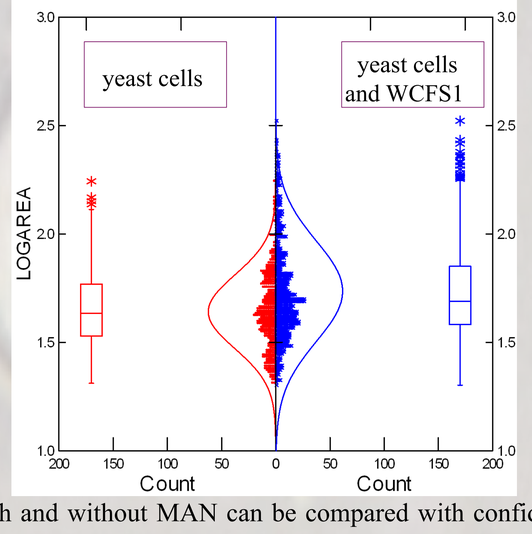


Lanes 1, 2: Hyper Ladder II and III, NEB; lane 3: 1740 bp *msa* band and some other aspecific bands; lanes 4, 6, 7: 1740 bp *msa* band; lane 5: No *msa* band detectable

- Overall 62.5% of the strains showed aggregation by image analysis; 40.6% of the strains showed mannose dependent aggregation.
- Only for 46.9% aggregation was scored positive by both visual examination and image analysis.
- 62.5% of strains showed the *msa* band (1740 bp); 33% of this showed aspecific bands.
- *msa* was never detected in *Lb. plantarum* subsp. *argentoratensis* but all three strains showed scant mannose dependent aggregation.
- No significant relationship was found between detection of the gene and mannose-specific aggregation. This is in agreement with what has been find by Gross et al. (2010).
- Only 46% of the strains with the *msa* gene showed a mannose dependent aggregation phenotype was found; 64% of the 11 strains which were *msa*- showed mannose-dependent aggregation: other gene products may be involved in mannose dependent and independent aggregation.

Statistical analysis of objects recovered by image analysis





❖ Average (+/- confidence limits) size of objects with and without MAN can be compared with confidence limits for yeast cells alone (left).

❖ Two sample t-tests and non parametric Kolmogorov-Smirnov tests can be used to compare the distribution of objects in yeast cells alone or with bacterial cells, or yeast + bacteria suspensions with and without MAN.

CONCLUSIONS

The traditional way to define the presence of yeast aggregates is based on the visual evaluation but this was subject to a subjective interpretation of results and only expert microbiologists were able to do a correct evaluation. We developed ImageJ macros to process a large number of images quickly and to obtain parameters to use in further statistical analysis to correctly identify aggregation and the dependence or not from the presence of methyl-α-D-mannopyranoside.

There is little correlation between presence of *msa* gene and mannose- dependent yeast aggregation. We were unable to amplify *msa* from *Lb. plantarum* subsp. *argentoratensis* strains.

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