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# A new method for the determination of *Leuconostoc mesenteroides* cell number

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## Abstract

Dilution needed for plate counts (CFU/ml) is known to disrupt streptococci chains, and therefore the validity of this method is controversial. A novel apparatus was designed to disrupt *Leuconostoc mesenteroides* chains in pairs, by forcing them to pass through a narrow capillary tube. When *Leuconostoc mesenteroides* occurs in pairs it appears that the real cell concentration can be simply deduced by doubling the CFU/ml.

**Keywords:** *Leuconostoc mesenteroides*; Chain disruption; Plate count; Cell concentration

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## 1. Introduction

*Leuconostoc mesenteroides* are commonly used in the dairy industry. For example, in Roquefort cheese manufacture, they are used to create holes to implant *Penicillium roqueforti*. *Leuconostoc mesenteroides* cells occur in pairs and chains of various lengths. The chain length depends on the media, the stage of growth and the growth temperature [1,2]. Furthermore, shaking, which is indispensable for plate-counting, is responsible for the chain disruption [3]. Thus, because of the variation in chain length, some investigators contest the validity of the conventional plate count [4–6]. The accurate enumeration of streptococci by plate-counting is difficult because only one colony is formed from

either a single cell, or a chain or a clump. So the number of micro-organisms cannot be obtained from the number of colonies.

In this paper, we describe a method that is able to disrupt streptococci chains in order to obtain the real cell concentration by plate-counting.

## 2. Materials and methods

### 2.1. Organisms

*Leuconostoc mesenteroides* subsp. *mesenteroides* (*L. mesenteroides*) was obtained from a industrial laboratory. The strain was maintained in MRS medium (Biokar Diagnostics) with an admixture of glycerol (10% w/w), at  $-18^{\circ}\text{C}$ . Liquid cultures of 150 ml on MRS media were incubated in 250 ml Erlenmeyer flasks at  $30^{\circ}\text{C}$

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with magnetic stirring at 250 rpm. The cell growth was monitored by measurement of optical density at 600 nm, by using 2 mm light path glass cells.

## 2.2. Plate count technique

In each sterile Petri dish 1 ml of cellular suspension was spread and covered with melted agar (MRS from Biokar Diagnostics) that was maintained at 45°C [7]. For each sample, three Petri dishes were incubated at 30°C and colonies were counted after 72 h. Dilutions were made in sterile water in order to obtain an ideal count from 30 to 300 colonies [8]. For each sample, the average number of colonies for the three Petri dishes was taken.

## 2.3. Determination of viability of bacteria

Viability of the bacterial population was measured by counting dead and viable cells under an epifluorescent microscope after staining. The 8-anilino-1-naphthalene sulfonic acid magnesium salt (Mg-ANS) was used to stain dead cells. 0.3 g Mg-ANS were dissolved in 100 ml of sterile Ringer solution (17.1 mM NaCl, 13.4 mM KCl, 2.2 mM CaCl<sub>2</sub>·6H<sub>2</sub>O, 11.9 mM NaHCO<sub>3</sub>, 55.6 mM glucose) [9,10].

Mg-ANS solution was added in equal volume to the bacterial sample. Epifluorescence examination was made after a staining time of 5 min. Dead cells fluoresced green while viable cells remained colourless.

Counts were carried out using a transmitted light microscope (Olympus system microscope, model BHS). To detect Mg-ANS staining, a fluorescent system (Olympus, model BH2-RFC) and a deuterium illuminator were added. The fluor cluster used was that designed for use with Mg-ANS, so the exciting filter was at 490 nm and the barrier filter was at 515 nm. The ratio of dead cells to total cells was determined by first enumerating the fluorescing cells in a microscope field under epifluorescence, then by counting all cells in the same field in the brightfield mode at the 1000× magnification [11]. Cells should be exposed to ultraviolet light for as short a time as

possible because if they are exposed for more than one minute they will burst and the cytoplasm flows out [12].

## 2.4. *L. mesenteroides* chain concentrations

The concentration of *L. mesenteroides* chains was obtained by counting the chains on a Petit Salumbeni hemacytometer (Cofralab, 31770 Colomiers France) [13], under a microscope at 400× magnification.

## 2.5. Chain length determination

The average number of cells per chain was calculated by counting cells of about 200 chains at 1000× magnification.

## 2.6. Disruption of *L. mesenteroides* chains

*L. mesenteroides* suspension were submitted to shear stress which was generated by successive passes through a capillary tube of diameter 190 μm. 14 ml of *L. mesenteroides* suspension mixed in an Erlenmeyer flask were sucked up by an HPLC pump (Gilson : 100-SC type for the piston pump head and 805 type for the manometric module) (Fig. 1) and then forced to pass through the capillary tube. At the exit of this tube, the suspension return to the Erlenmeyer flask and continuous runs were then performed at 7 ml/

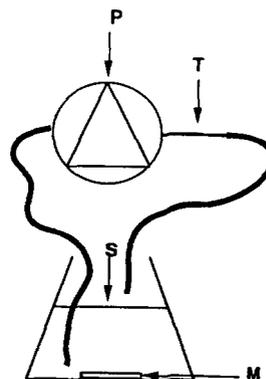


Fig. 1. Generator for chain disruption: a HPLC pump (P) sucks up the *Leuconostoc mesenteroides* suspension (S) and forces it through a capillary tube (T). M, magnetic stirrer.

min. The average number of passes in the tube is defined as follows:

$$n = t \times (Q/V)$$

where  $t$  is the circulation time,  $Q$  the volumetric flow and  $V$  the volume of the suspension.

### 3. Results and discussion

#### 3.1. The *L. mesenteroides* suspension

The *L. mesenteroides* suspension used for our experiment was a 4 h old culture whose optical density was equal to 0.2. The repartition of chains was then the one presented in Fig. 2. This graph shows that the chain length varies not only with the media, the stage of growth and the growth temperature [1,2], but is also not uniform within one sample. Indeed, in our suspension, *L. mesenteroides* occurs in chains of 4 to 32 cells.

#### 3.2. The disruption of *L. mesenteroides* chains

Our apparatus was designed to disrupt the chains in order to obtain a uniform suspension in terms of chain length. At the beginning, to confirm the real effect of the capillary tube, 14 ml of the suspension was treated by the apparatus and then another 14 ml was treated by the apparatus without the capillary tube. The run carried out with the tube was done twice in order

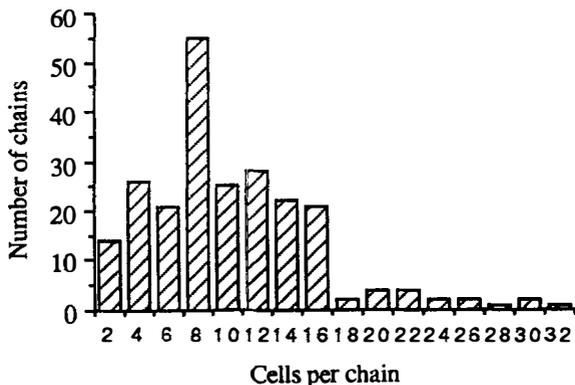


Fig. 2. Distribution of chain lengths in a *Leuconostoc mesenteroides* culture of 4 h old.

to test if the results were reproducible. The generation time of *L. mesenteroides* is about 2 h and during each run the non-treated suspensions stand at 4°C, so they do not grow. During the three runs, samples were collected and the average chain length was calculated after microscopic observations. Results are shown in Table 1. They clearly show that the capillary tube was useful in disrupting the *Leuconostoc mesenteroides* chains and the disruption with this tube resulted in a homogenous suspension of leuconostocs in pairs. Our new design for chain disruption gave reproducible results between test 1 and test 2 (Table 1). The disruption of leuconostoc chains has been studied by shaking with or without glass beads [3] and it was shown that this type of agitation did not lead to a homogenous suspension of pairs; the chains were just broken up in two-, four-, or six-cell frag-

Table 1  
Average chain lengths in *L. mesenteroides* suspensions treated by an HPLC pump with or without a capillary tube

Average number of passes	Average chain length (cells/chain)		
	Pump	Pump + capillary tube	
		Test 1	Test 2
0	9.8	9.8	9.8
1	9.0	5.2	6.3
1.4	9.6		
1.5			5.1
2.6	9.5		
2.7		4.9	
4.3		4.3	
4.7	8.6		
5.2			4.1
5.5		3.9	
5.6			3.9
6.6	8.9		
6.7		3.6	
10.1		2.6	
11.3			2.7
12.4		2.1	
12.6			2.2
13.6	8.8		
14.1			2.1
14.9		2.0	
17.3			2.0
19.2	8.9		
21.6		2.0	

ments. So with our design, it is now possible to obtain a homogenous suspension of known length.

### 3.3. Effect of chain length on plate-counting

For all samples collected during the treatment by our apparatus with the capillary tube, the chain concentration and the viability were determined by microscopic examination. They were all plated on Petri dishes. Results are presented in Table 2. The chain concentration determined by microscopic examination on the Petit Salumbeni hemacytometer could be considered to be the real chain concentration. Furthermore, this method of counting gives reproducible results [11]. The plate count method gives a concentration of colony-forming units (CFU). The viability observed after 13.2 passes in our apparatus indicated that *L. mesenteroides* were not altered by passing through the capillary tube.

The coefficient *E* indicates that for chains longer than 2 cells, it is wrong to assimilate the CFU concentration to chains concentration. The accurate enumeration of streptococci by plate-counting has always been difficult because they are chain-forming organisms [2].

The count of leuconostocs expressed as cells

per ml could be determined by multiplying the count of CFU obtained from pour plating by the average number of cells per chain determined microscopically. This should be right when chains do not disrupt during the fold-dilutions needed before plating. With our novel design, chains obtained after treatment occur in pairs and it is then possible to determine the concentration of cells per ml which is simply twice the CFU concentration.

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Table 2  
Treatment of *Leuconostoc mesenteroides* by the shear stress apparatus: effect on different parameters

Average number of passes	Microscopic observations			Colonies ( $\times 10^6$ CFU/m) (X2)	<i>E</i> <sup>a</sup> (%)
	Chain length (cells/chain)	Chain concentration ( $\times 10^6$ chain/ml) (X1)	Viability (%)		
0	9.8	122	95	363	197
2	5.2	511	96	750	46
2.7	4.9	523	88	1000	91
4.3	4.3	792	89	1280	62
5.5	3.9	971	90	1640	69
6.7	3.6	1173	90	1340	14
9.6	3.5	1365	89	1300	5
10.1	2.6	1430	92	1610	13
12.4	2.1	1803	94	1896	5
13.2	2.0	2084	91	1996	4

<sup>a</sup> With  $E = (X1 - X2) \times 100 / X1$ .

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